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ISOLATION AND CHARACTERIZATION
OF PECTIC POLYSACCHARIDES
FROM Lemna minor

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By

Liang Cheng

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF PECTIC POLYSACCHARIDES FROM *Lemna minor*

By

Liang Cheng

Pectic polysaccharides were purified from cell walls of *Lemna minor* and their homogeneity was examined by column chromatography on DEAE-Sephadex A-25 and Sephacryl S-400. The relative molecular size of the samples was determined.

Sugar composition of seven purified pectic polysaccharides were determined by gas chromatography—mass spectrometry. Galacturonic acid (20.8 to 67.7%) and apiose (23.0 to 39.8%) were the two most abundant components , but rhamnose, fucose, arabinose, xylose, mannose, glucose and were also found in all samples. Three types of pectic polysaccharides were found: Samples extracted in 15 min and eluted with 0.25 M NaCl from DEAE-Sephadex columns have the largest molecular size ($\overline{M}_w$ of standard dextrans with the same K_{av} are 139,000 to 205,000), and are the most acidic (44.8 to 67.7% galacturonic acid), while those eluted with 0.2 M NaCl have a much smaller molecular size ($\overline{M}_w$ of standard dextrans with the same K_{av} are 15,500 to 26,000), high in neutral sugars (69.8 to 79.2%) and low in galacturonic acid (20.8 to 30.2%). Samples extracted in a 4.75 h period following the 15 min extraction have the smallest molecular size ($\overline{M}_w$ of standard dextrans with the same K_{av} are 6,120 to 12,000) and an intermediate content of galacturonic acid (34.3 to 38.8%).

To my parents and wife

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LIST OF ABBREVIATIONS

CMC —1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate

DE —Degree of methylesterification

EI —Electron impact ionization

GC —Gas chromatography

IDT —Isodityrosine

MS —Mass spectrometry

RG-I —Rhamnogalacturonan I

RG-II —Rhamnogalacturonan II

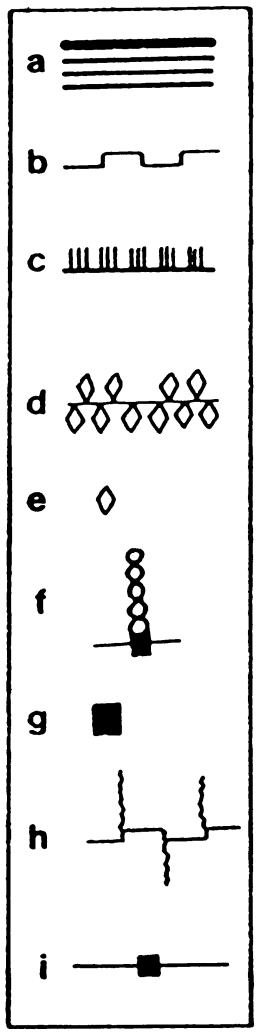
TFA —Trifluoroacetic acid

INTRODUCTION

Primary Plant Cell Wall, Models

The walls of growing plant cells are called primary cell walls. Northcote considered the primary cell wall to be a growing, constantly changing, composite material consisting of a dispersed phase of microfibrils within a complex, continuous matrix (57). Lamport (44), in 1965, considered the primary cell wall to be a single, bag-shaped macromolecule having a coherent, cross-linked structure, with bonds both between the hydroxyproline-rich wall protein "extensin" and wall polysaccharides. Fry (26) gave a simple description of the primary cell wall, "a biphasic structure, consisting of a rigid skeleton of cellulose microfibrils held together (and apart) by a gel-like matrix". After investigating the primary cell wall of suspension-cultured sycamore cells and a number of other plants (7, 16, 19, 49, 72, 76, 80), Albersheim's group constructed a model for the primary cell wall of higher plants (37). This model, which is shown in Figure 1, proposes that the cellulose microfibrils are linked to each other through xyloglucans, rhamnogalacturonans (with arabinan and galactan side chains), arabinogalactans and cell wall proteins. In this model, hemicellulose xyloglucans are tightly held on the surface of the cellulose microfibrils by hydrogen bonds, the reducing ends of xyloglucans are covalently linked to galactan side chains of rhamnogalacturonans, which, in turn, are linked to serine residues of the hydroxyproline-rich cell wall protein through 3,6-linked arabinogalactans. They suggested that each of the rhamnogalacturonan molecules had a number of galactan side chains, and that these side chains were connected to a number of different cellulose

Figure 1. Tentative Structure of Sycamore Primary Cell Wall (37). [This model is not intended to be quantitative, but the wall components are presented in approximately proper proportions, although the distance between cellulose microfibrils is expanded, in order to allow room to present the interconnecting structures. The key in the Figure depicts regions representing the various wall components. (a) cellulose microfibrils, (b) rhamnogalacturonan (pectic backbone), (c) xyloglucan, (d) wall protein with arabinosyl tetrasaccharides (e) attached to hydroxyl-L-proline residues, (f) (3→6)-linked arabinogalactan attached to serine (g) of the wall protein, (h) total pectic polysaccharide, showing L-arabinan and 4-linked D-galactan side-chains attached to rhamnogalacturonan backbone, and (i) unsubstituted L-serine residues of wall protein.] Figure and legend taken from reference (21).


a
b
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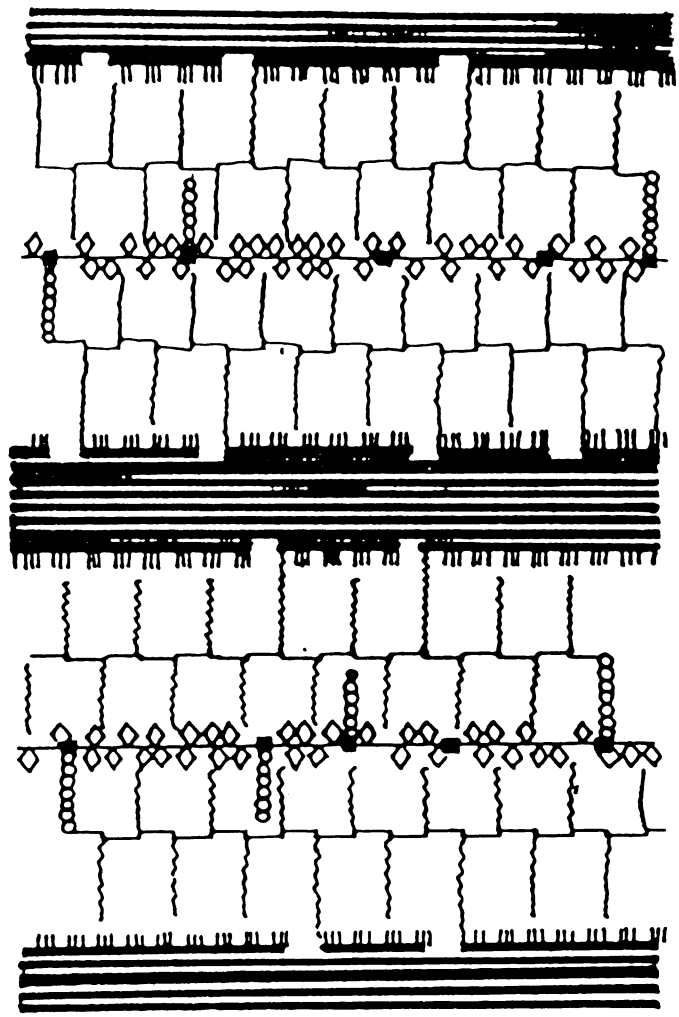
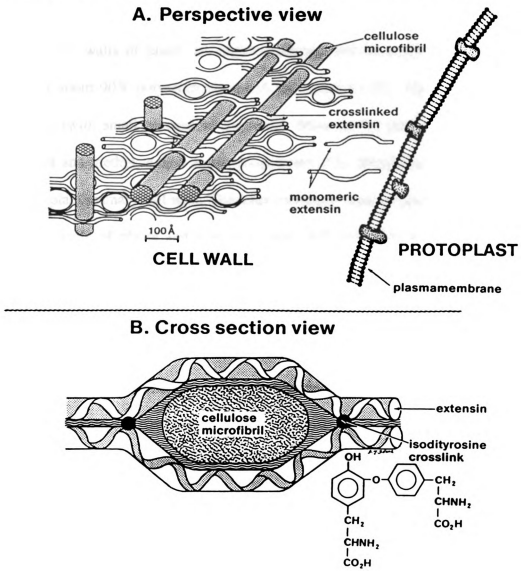


microfibrils through xyloglucans. Each cellulose microfibril, in turn, was connected to several different rhamnogalacturonans through the same type of connection, and in this way, cellulose microfibrils were extensively cross-linked (37). However, this model and some other models including Lamport's early model (42, 45) (which suggests extensin is linked to cellulose microfibrils by an arabinosyl hydroxy-L-proline-linked galactan, and intrapeptide and interpeptide linkages of extensin are formed by disulphide bridges) are now considered to be incorrect because they do not explain why, after substantial cleavage of glycosidic linkages, the extraction of the extensin protein is still difficult. Based on the fact that after removal of wall polysaccharides through treatment with hydrogen fluoride the extensin protein remained insoluble (55), and that the isodityrosyl unit of extensins is found to be an intrapolypeptide bridge and may be an interpeptide bridges also (25, 27), Lamport proposed a "warp-weft" cell wall model (44, 46). This model, which was based on his earlier dual network hypothesis (43), suggested that the cross-linked extensin formed a glycoprotein network of defined porosity and that the cellulose microfibrils penetrated the extensin network through those pores, and connected with other polysaccharides to form a cellulose based network (Figure 2). A "pH-induced pectin-extensin switch" hypothesis was suggested based on this model (44). The hypothesis states that since at low pH the ionization of the carboxyl groups of pectin is suppressed, the pectin tends to self-associate through carboxyl-carboxyl and carboxyl-carboxylate hydrogen bonding. Increasing the pH increases the pectin anionic charge, leading to ion pair formation ("salt bridges") between pectin carboxylate groups and the protonated ϵ-amino groups of the lysine residues of extensin. In this way a pH change can alter the rheology of the cell wall as the cell grows. This cell wall model did not

Figure 2. A Hypothetical Extensin-cellulose Network. The perspective view (A) represents an extensin-rich, i.e. tightly woven primary cell wall of high tensile strength. A loosely woven wall would contain correspondingly less extensin and would have a lower tensile strength. The view in cross section (B) shows how two IDT-crosslinked strands of extensin might define the size and shape of a cellulose microfibril. Figure and legend taken from reference (46).

EXTENSIN-CELLULOSE NETWORK



describe how polysaccharides are connected with each other. Due to the great technical difficulties in isolating and purifying "intact" polysaccharides from cell walls, the biochemistry of plant cell walls is still at the stage of identifying and elucidating the covalent structures of the macromolecular components of the primary cell wall.

Components of the Plant Primary Cell Wall

Primary cell walls of plants are highly hydrated, but on a dry weight basis, are composed of about 90% polysaccharides and 10% proteins (21, 52) which are mostly glycoproteins (with small amount of enzymes). Some of the polysaccharides contain methyl ethers and methyl, acetyl and feruloyl esters (52). Small amounts lipids is also found in the plant primary cell wall. There are numerous types of polysaccharides in the primary cell walls of plants and they vary with different plant sources, organs and different stages of cell growth. Classically, they are separated into three major groups (21): The pectic polysaccharides, which can be extracted by hot water, weak acids and ammonium oxalate solution and other chelating agents; the hemicelluloses, which can be extracted by relatively strong alkali; and the residue remaining, which is mainly cellulose and extensin proteins.

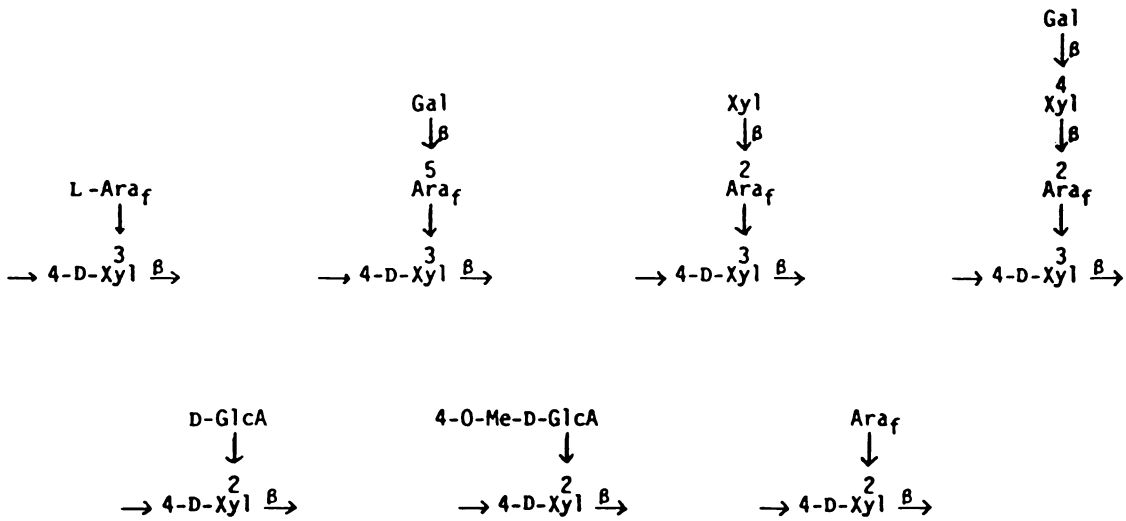
Cellulose, a polymer of glucose units linked β-1,4, constitutes approximately 20-30% of the dry weight of the primary cell wall (15, 52, 61). Frequently, small amounts of other neutral sugars such as xylose and mannose are also found with the cellulose but whether their presence is the result of contamination remains unclear. Microfibrils, which are formed by aggregation of chains of cellulose, are the major structural component of the rigid skeleton of cell walls.

Extensin is an insoluble hydroxyproline-rich (20%) glycoprotein of the primary cell wall (42, 45, 61, 72). There are about 9 half-isodityrosyl residues per extensin molecule, and they can form intrapolypeptide and possibly interpolypeptide isodityrosyl bridges resulting in a cross-linked extensin molecule network. The cellulose with attached xyloglucans could be held by the network.

Two major groups of polysaccharides are present in the matrix of the primary cell walls—hemicellulose and pectic polysaccharides. A typical primary wall of a dicotyledon contains 15-25% hemicellulose on a dry weight basis (15). Xyloglucan is a type of hemicellulose that consists of a backbone of β-1,4-linked glucosyl residues with α-1,6-linked xylosyl side chains. Some of the xylosyl side chains are extended by the addition of β-D-Gal→ or α-L-Fuc→2-β-D-Gal→ to O-2 of the xylosyl residues (7, 20). About 20% of the primary cell wall of dicots is xyloglucan, only 2% for monocots (52). Another type of hemicellulose is xylan which is the major hemicellulose found in primary cell wall of monocots, but present only in smaller amounts in dicots. Structurally xylan consists of a β-1,4-linked xylan backbone with various types of side chains attached to the O-2 or O-3 position of the xylosyl residues (3, 52). The most common side chain is a single arabinofuranosyl residue attached to O-3 of the xylosyl residues in the backbone, but different xylans have different side chains and some other examples are shown in Figure 3. Ferulic and *p*-coumaric acids have been also found attached to arabinosyl side chains of xylan through ester bonds (29, 71, 73, 79).

Pectic polysaccharides, or pectic substances, are polysaccharides with a backbone of galacturonosyl and rhamnosyl residues and having side chains of neutral sugars. The carboxyl groups of the galacturonosyl residues may be free or partially or fully methyl

Figure 3. A Summary of the Side Chains Attached to β-4-Linked D-Xylopyranosyl Residues of the Backbone of Arabinoxylans. From reference (52).


L-Ara$_f$
3
→ 4-D-Xyl β→
Gal
β
5
Ara$_f$
3
→ 4-D-Xyl β→
Xyl
β
2
Ara$_f$
3
→ 4-D-Xyl β→
Gal
β
4
Xyl
β
2
Ara$_f$
3
→ 4-D-Xyl β→
D-GlcA
2
→ 4-D-Xyl β→
4-O-Me-D-GlcA
2
→ 4-D-Xyl β→
Ara$_f$
2
→ 4-D-Xyl β→

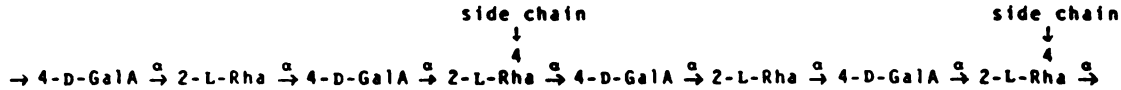
esterified. They are the most abundant matrix component of the primary cell wall and the middle lamella of most higher plants. They are a complex family of polysaccharides and make up to 35% of the dry weight of the dicot primary cell wall and (15, 20, 21, 35). Pectic polysaccharides are also widespread in many monocot cell walls (36), although their contents are usually lower than that in dicots. Traditionally, several neutral polysaccharides (arabinans, galactans, and arabinogalactans) extracted from cell walls with the acidic pectic polysaccharides are also called pectic polysaccharides. They are believed to be attached covalently to the backbone primarily through the rhamnosyl residues (4).

Homogalacturonans are defined as polymers consisting solely or "predominantly" (52) of α-1,4-linked-D-galacturonosyl residues. No pure homogalacturonan has been isolated from primary cell walls without using treatments that are likely to cleave covalent bonds. However, there is some evidence that they are present in primary cell walls (18). They have been found in suspension-cultured *Rosa* cell walls with a degree of polymerization greater than 100 (17). A polymer containing 91% galacturonosyl residues was also purified from rice endosperm cells (70), which may suggest the possible presence of homogalacturonans in monocots. Homogalacturonans have been also found in the media of tobacco (87% galacturonic acid) (82) and sycamore (95% galacturonic acid) (52) cell suspension cultures.

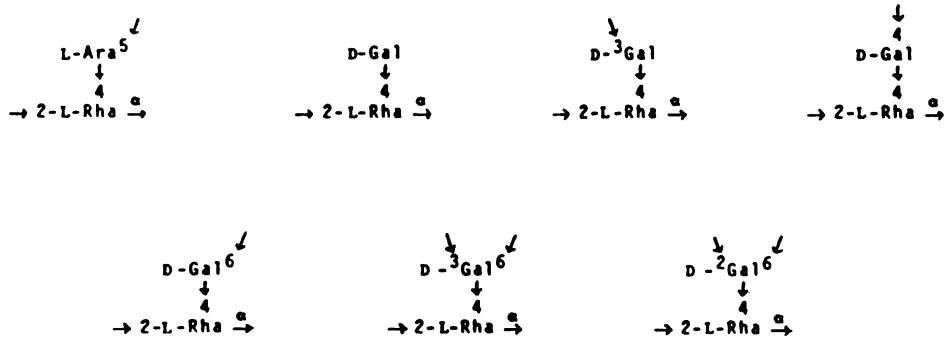
Rhamnogalacturonan I (RG-I), the pectic polysaccharide first isolated from the walls of suspension-cultured sycamore cells, has been studied in some detail (47, 48, 50, 51, 54). With a degree of polymerization of about 2000, RG-I has a backbone composed of alternating 2-linked rhamnosyl and 4-linked galacturonosyl residues (Figure 4A).

Figure 4. Structure of Rhamnogalacturonan I. (A) The backbone glycosyl-residue sequence of rhamnogalacturonan I. From reference (52). (B) A summary of the different kinds of linking glycosyl residues of the side chains that are attached to O-4 of 2,4-linked rhamnosyl residues of the backbone. From reference (51).

A


side chain
side chain
→ 4-D-GalA α→ 2-L-Rha α→ 4-D-GalA α→ 2-L-Rha α→ 4-D-GalA α→ 2-L-Rha α→ 4-D-GalA α→ 2-L-Rha α→

B


L-Ara5
D-Gal
D-3Gal
D-Gal
D-Gal6
D-3Gal6
D-2Gal6
→ 2-L-Rha α→

Approximately half of the 2-linked rhamnosyl residues are branched and at least 30 different side chains, averaging 6-7 residues in length, have been found attached to its O-4 (50, 52). The first glycosyl residue (attached to O-4 of the rhamnosyl residues of the backbone) of several different side chains was determined (51) and these are shown in Figure 4B. The side chains are rich in arabinosyl and galactosyl residues, although small amounts of fucosyl residues have also been found. Pectic polysaccharides that are similar to RG-I have been found in other plants, both monocots and dicots, such as tobacco (24), potato (33), onion (34), maize and rice (75).

Rhamnogalacturonan II, or RG-II, is another pectic polysaccharide originally found in suspension cultured sycamore cells (19). It is structurally very different from RG-I. RG-II is much smaller than RG-I (degree of polymerization $\approx$ 60), and has a higher proportion of rhamnosyl residues which are 3, 3,4, 2,3,4 and terminally linked instead of 2 and 2,4 linked as in RG-I. In addition, RG-II has many unusual glycosyl residues including 2-*O*-methylfucosyl, 2-*O*-methyl-xylosyl, apiosyl and 3-*C*-carboxyl-5-deoxy-L-xylosyl (aceric acid) residues. RG-II was also found in the primary cell walls of other dicots such as pea, pinto bean and tomato (19) which may suggest that RG-II or similar pectic polysaccharides are common in dicot plant cell walls.

Apiogalacturonans are pectic polysaccharides isolated from cell wall of *Lemna minor* (8, 30, 31) and contain mainly galacturonosyl and apiosyl residues. The apiose content in different fractions varied from 7.9% to 38.1% (30). In one case in addition to the apiosyl residues, which composed 25-28% of the apiogalacturonans, small amounts of xylosyl and galactosyl residues were also reported to be present (8). Apiogalacturonans are considered as "unconventional pectins" (35), "apparently rare polymer" (21), and

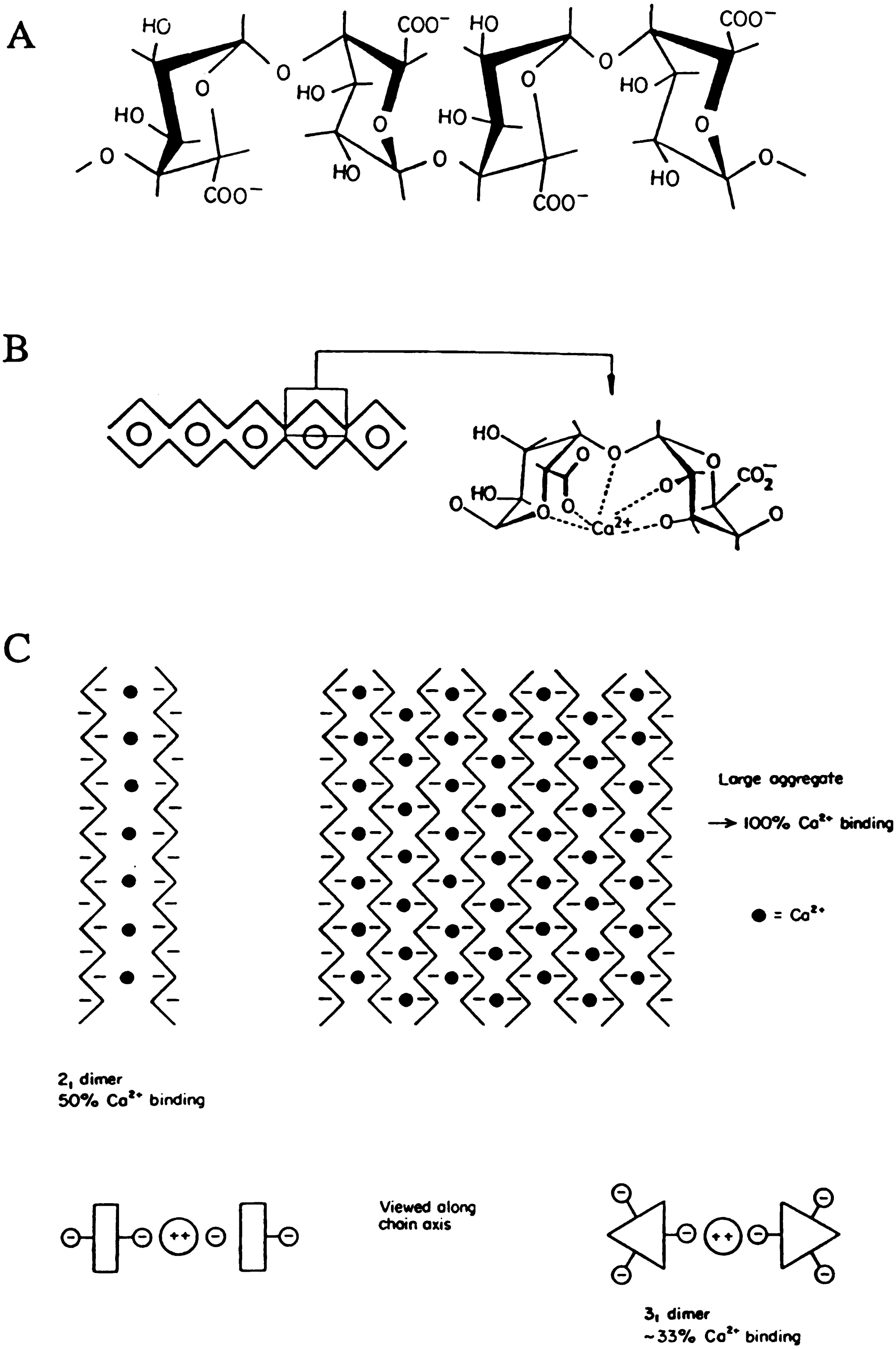
"pectins of unusual composition" (36) because the apiosyl residue is one of their main components and because the rhamnosyl residue a common residue of RG-I and RG-II, has not been detected in them. Apiose, however, has been found to be a component of polysaccharides in a number of different plants such as *Posidonia australis* (10), *Tilia* sp. (5), *Zostera marina* (5, 58, 81), *Zostera nana* (23), *Zostera pacifica*, *Phyllospadix* (58), *Lemna gibba* (9) and *Acer pseudoplatanus* (suspension-cultured sycamore) (19). In addition, Duff (23) reported that of 175 plant species examined, 31 showed "traces" amounts, 51 had "moderate" amounts and 17 were "good sources" of apiose. Although Duff did not distinguish between apiose in glycosides and that in polysaccharides, his findings indicated that apiose must be relatively widely distributed in the plant kingdom. However, the apiose content in polysaccharides could be underestimated or the sugar may not even be detected by researchers who determine sugar composition by using GC-MS analysis of alditol acetates, since apiitol acetate has a very similar retention time (on the common GC columns used to separate alditol acetates) and mass spectrum to xylitol acetate (19, 38). When apiogalactcuronans were partially characterized a number years ago, paper chromatography was used to determine their glycosyl compositions (8, 30, 31). In one case they were characterized as unesterified α-1,4 linked polygalacturonic acids to which monomeric side groups (75% apiose and 25% xylose residues) were attached (8). In another case both D-apiose and apiobiose (31) are attached to the galacturonan with only 1.0-3.5% of the galacturonosyl residues esterified. Rhamnose was not reported to be present. Pectic polysaccharides of high apiose content are resistent to pectinase-catalyzed cleavage (30, 31). Since a lack of rhamnose in these polysaccharides would make them exceptions to the more common pectic polysaccharides and since a

number of techniques for characterizing polysaccharides have been improved or developed since then, a re-examination of their structure was undertaken.

Physical and Chemical Properties of Pectic Polysaccharides

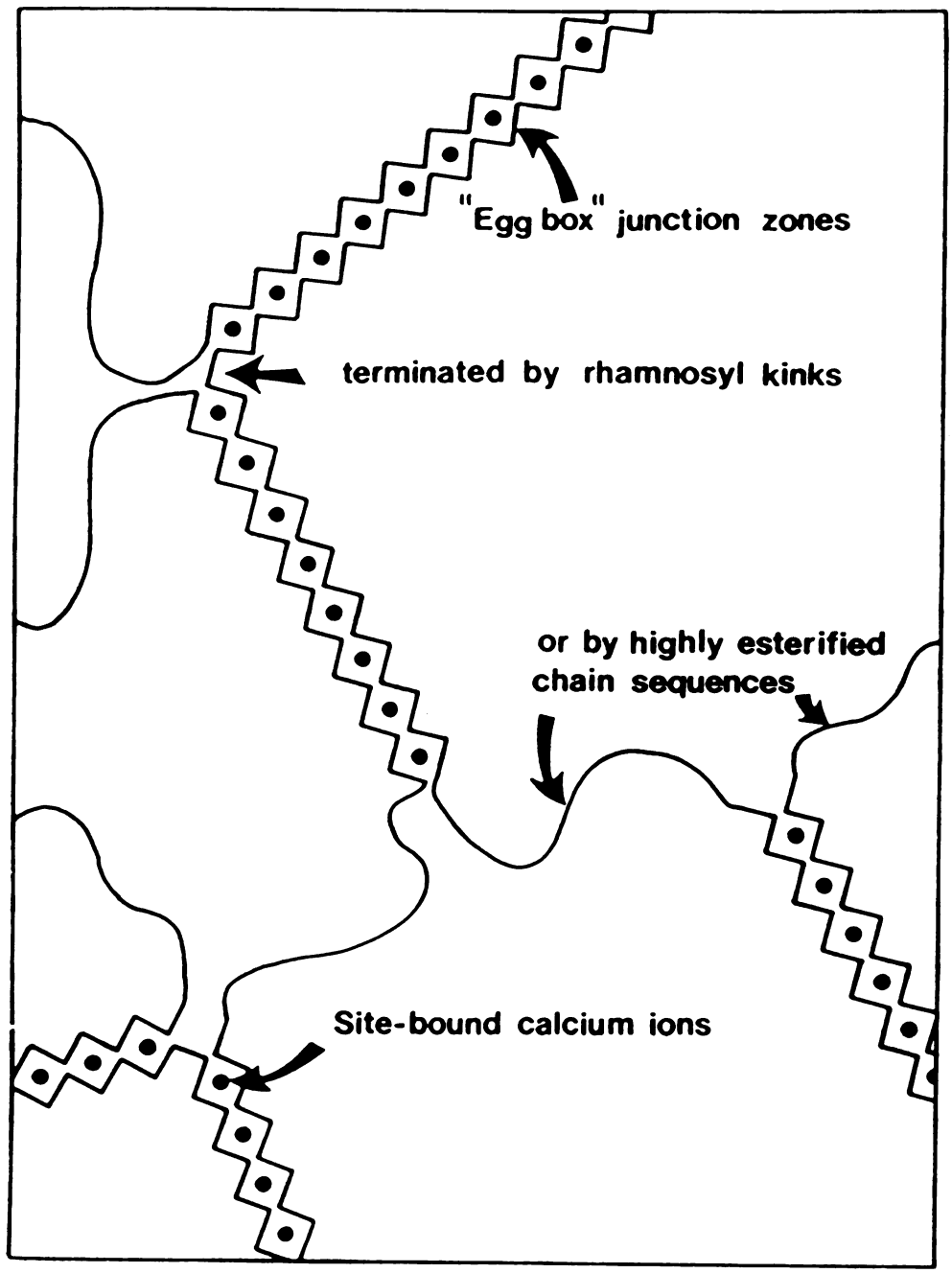
Pectic polysaccharides are polyanionic molecules at most pH's. When their main chains interact noncovalently to form a three dimensional network that entraps solvent and solute molecules, gels are formed. The property of the gel is dependent upon the formation of "junction zones". These are zones where two or more chains or segments of chains are in association through intermolecular hydrogen or ionic bonding (62-64, 66, 67). The greater the number and length of the junction zones, the more rigid is the gel. If too many junction zones exist, precipitation rather than gel formation occurs. The size and number of the junction zone depends on the structure of the pectic polysaccharide, its concentration, types of cations present and their concentration, pH of the solution and temperature. Pectic polysaccharides are soluble in pure water. In aqueous solutions, the polygalacturonic acid portion of the backbone folds into a 2_1-helix (2 stands for two galacturonosyl residues per conformational repeat unit; the subscript, 1 stands for one turn of the helix per repeat) conformation which has a zig-zag type of arrangement involving an oxygen bridge between neighboring galacturonosyl residues (53, 65) (Figure 5A). Pectic polysaccharides form two types of gels, the calcium gel and the acid gel (11, 35). For the Ca^{2+} gel, the level of bound Ca^{2+} decreases with increasing concentration of competing monovalent ions, until it reaches a constant value of ~50% of the total stoichiometric equivalent of carboxyl groups (28) (Figure 5C, top left). Ca^{2+} in the solution causes the formation of "egg-box"-like dimers (65) (Figure 5B) among

Figure 5. Proposed Structures of the Polygalacturonate Portion of the Pectic Polysaccharide Backbone. (A) Structure of the polygalacturonate portion of the pectic polysaccharide backbone. From reference (53). (B) Structure of the "egg-box" dimer formed by two polygalacturonate chains and associated Ca^{2+} ions. Left, schematic representation of two buckled chains (each by a zig-zag line) with Ca^{2+} ions represented by circles. Right, detailed conformational representation of a possible mode of cation coordination by one chain. From reference (65). (C) Orientation and stoichiometry of Ca^{2+} binding within ordered interchain junctions. From reference (53).


A
HO
COO⁻
B
HO
CO_2^-
Ca^{2+}
C
Large aggregate
→ 100% Ca^{2+} binding
● = Ca^{2+}
2_1 dimer
50% Ca^{2+} binding
Viewed along
chain axis
3_1 dimer
~33% Ca^{2+} binding

unbranched, unesterified segments of the polygalacturonosyl chains, and the univalent cation competitors such as Na^+ prevent the dimers from aggregating further. The dimers consist of two 2_1-helix chains or segments linked by calcium bridges with the carboxyl groups on one side of one 2_1-helix sharing calcium ions with the carboxyl groups on the opposite side of the other 2_1-helix through ionic bonds (53, 65). The carboxyl groups on the dimer on the side away from the Ca^{2+} ionic bounds are free to associate with the univalent cation competitors. If calcium is the sole or principal cation in solution, the dimers can aggregate further without rearrangement by using the free carboxyl groups on the opposite side of each helix chain to form Ca^{2+} bridges with other dimers. This would eventually lead to an approximate doubling of the amount of Ca^{2+} bound cooperatively and to the formation of sheetlike aggregates (53) (Figure 5C, top right). If the aggregates are dried to a solid, the conformation of the polygalacturonosyl chains changes to a 3_1-helix due to a polymorphic phase transition (53, 77) (Figure 5C, bottom right). Side chains of neutral glycosyl residues affect the formation of the junction zones (11). Rhamnosyl residues found in the backbone of pectic polysaccharides interrupt junction zones through their alteration of conformation i.e. their 1,2-links form kinks in the chains (35, 60). Many of the rhamnosyl residues have a variety of side chains attached to them and these are considered to interfere with formation of junction zones, although they may also interact noncovalently with each other to enhance gelling (4, 35). The formation of a Ca^{2+} bridge junction zone requires a sequence of at least seven consecutive free carboxyl groups along the participating face of each chain (60). The esterification of the carboxyl groups interferes with formation of Ca^{2+} junction zones (Fig. 6) and makes the gel network more flexible. At constant pH, the gel strength of a

Figure 6. Schematic Illustration of the Structure of the Ca^{2+} Gel Network. From reference (67).


"Egg box" junction zones
terminated by rhamnosyl kinks
or by highly esterified chain sequences
Site-bound calcium ions

Ca^{2+} gel increases as the content of methyl esterified groups decreases (67), although it has been reported that some degree of methyl esterification can be accommodated within the junction zones of such gels if one of the two chains in the junction zone is not esterified (60). The acetyl substituents on the hydroxyl groups of galacturonosyl residues can also affect the binding of Ca^{2+} by the carboxyl group presumably through steric hinderance (41).

Pectic polysaccharides can form another type of gel, the "acid gel". This gel is formed at low pH without the participation of Ca^{2+}. Pectins are highly ionized in solution at neutral and alkaline pH, and the charges along the molecules keep them free from aggregation by columbic repulsion. At a sufficiently low pH, the number of charges is reduced enough so that sufficient interchain hydrogen bonds are formed between two hydroxyl groups, between a hydroxyl and an uncharged carboxyl group or between two uncharged carboxyl groups or some combination to produce junction zones and an acid gel (11). Pectins with higher degrees of methylesterification have less charge so they can form an acid gel at a higher pH. At constant pH, the gel strength of an acid gel increases with increasing DE of the pectin. The apparent pK of the carboxyl group of pectin with a DE of 65% is 3.55 while that of pectic polysaccharides with no methyl esterification is 4.10 (59). Increasing the concentration of a monovalent cation ,such as Na^{+} and K^{+}, will result in decreasing hydrogen bonding in junction zones thereby decreasing gel strength and reducing precipitation, and as a result a "salting in" occurs, although too high a concentration of the monovalent cations may cause precipitation (32).

In solution, pectins are most stable at a pH of about 4. Both higher and lower pH can cause deesterification and depolymerization (including hydrolysis) (1, 2, 11, 12, 40, 78),

however the present of solutes (i.e. cations) lowers water activity so that the rates of both reactions are reduced (11). At pH values of 5~6, pectins are stable only at room temperature. As the temperature is raised, pectin chains with methyl esters can be cleaved by β-elimination (2, 40). Some of the glycoside bonds in pectic polysaccharides are also quite labile although the glycoside bond of the uronosyl residues is particularly stable to acid hydrolysis. For example, hydrolysis of the side chains of apiogalacturonans is substantially completed in 3 h at pH 4.5 and 100°C (31).

Since the stability and solubility of pectic polysaccharides in solution change with changes in concentration, pH, and concentration and type of cation, great care must be taken to avoid degradation, precipitation and gel-formation through the entire isolation, purification and characterization process.

PART 1

ISOLATION AND HOMOGENEITY OF THE PECTIC POLYSACCHARIDES FROM *L. minor*

MATERIALS AND METHODS

Materials: *L. minor* (duckweed) was grown as described elsewhere (39). DEAE Sephadex A-25 and Dextrans T-10, T-40, T-70 and T-500 were obtained from Pharmacia Inc. Sephacryl S-400 was obtained from Sigma Chemical Co. 3-Phenylphenol (3-hydroxydiphenyl) was obtained from Aldrich Chemical Company.

General Methods: DEAE-Sephadex A-25 was used for all ion exchange chromatography. Column fractions were assayed for uronic acid or total carbohydrates or both by the method of Blumenkrantz and Asboe-Hansen (14) and Dubois *et al.* (22), respectively. Appropriate column fractions were combined, and dialyzed against double distilled water for about 36 h with seven water changes. Dialyzed samples were concentrated *in vacuo* at less than 35°C.

Isolation of Pectic Polysaccharides: Cell walls were prepared from fresh *L. minor* (164.2 g, wet weight) following the procedure of Kindel (unpublished). Basically the procedure involved homogenizing the plants with a Waring Blendor in 1.0 M NaCl at 4°C, centrifuging, washing the plants with 1.0 M NaCl, centrifuging, and washing a final time with water. The residue was called washed cell walls, and was extracted with

0.05 M ammonium oxalate (pH 6.5) at 22°C for 15 min and centrifuged. The supernatant solution was collected, dialyzed, and the dialyzed solution was concentrated and called sample A-15m. The residue was resuspended in 0.05 M ammonium oxalate, stirred at 22°C for 4.75h, centrifuged, and the supernatant solution was treated as above and called A-4.75h. A complete second isolation was performed and the samples were called B-15m and B-4.75h.

Column chromatography of the above four samples was performed on DEAE-Sephadex with 0.067M sodium phosphate (pH 7.7) as the column buffer as described by Hart and Kindel (30) except for the following changes: For the A-15m and B-15m samples, the column was 1.9 cm (i.d.) × 14 cm (h) and the flow rate was 0.43 ml/min. For the A-4.75h and B-4.75h samples the column was 1.7 cm (i.d.) × 14 cm (h) and the flow rate was 0.3 ml/min. Before application to a column, each sample was filtered through a membrane filter with a 1.2 μ pore size. Samples and column fractions were assayed for uronic acid. Recovery of material from the columns was based on the uronic acid test. The following major samples were obtained: sample A-15m yielded samples A-15m-II and A-15m-III, sample A-4.75h gave sample A-4.75h-II, sample B-15m gave samples B-15m-II and B-15m-III, and sample B-4.75h gave sample B-4.75h-II.

Samples from the first column were rechromatographed individually on a second column of DEAE-Sephadex. The columns (1.35 cm, i.d. × ~14.2 cm, h) were developed first with above column buffer and then with a linear gradient of NaCl in column buffer. For samples A-15m-II, A-4.75h-II, B-15m-II and B-4.75h-II the NaCl gradients were 0.075 M to 0.35 M; for samples A-15m-III and B-15m-III, the NaCl gradients were 0.1 M to 0.4 M. Column fractions (10 ml) were collected and analyzed

for uronic acid and total sugar. Fractions were combined and dialyzed. Polysaccharides obtained from this re-chromatography were examined for homogeneity by chromatographing each on DEAE-Sephadex and Sephacryl S-400.

Homogeneity of Polysaccharides: Chromatography of the above samples on DEAE-Sephadex with 0.067 M sodium phosphate pH 7.7 as the column buffer, was performed as described above except for the following changes: Each column was 0.95 cm (i.d.) × ~12 cm (h); fractions of ~2.5 ml were collected at a flow rate of ~0.1 ml/min, and the linear gradient of NaCl varied from starting concentrations of 0.05 to 0.1 M to final concentrations of 0.3 to 0.4 M, depending on the individual sample (see Results).

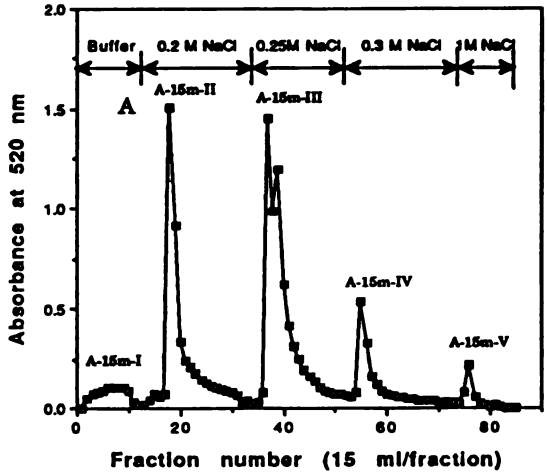
Polysaccharide samples were also examined by column chromatography on Sephacryl S-400 (1.0 cm, i.d. × 61.2 cm, h). Polysaccharide samples were concentrated to between 0.2 and 0.4 ml (the range of the polysaccharide concentrations in the concentrated samples based on the uronic acid test was 0.3 to 1.6 mg/ml) before being applied to the column. The column was developed with 0.01 M sodium phosphate (pH 6.5) at a flow rate of approximately 0.1 ml/min. Fractions of 1.5 ml were collected, and the appropriate fractions were combined, dialyzed and stored at -20°C. Blue dextran and $K_3Fe(CN)_6$ were used to obtain the void (V_o) and total volumes (V_t). Samples (0.2 ml, 2.5 mg/ml) of Dextran T-10, T-40 and T-70 and T-500 (3.75 mg/ml) were chromatographed individually on the column and the column fractions were tested for total sugar.

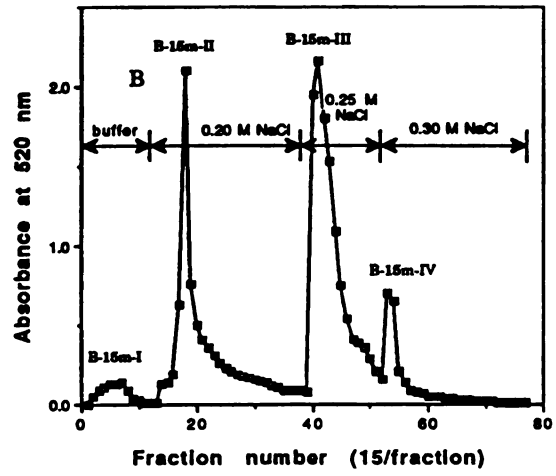
RESULTS

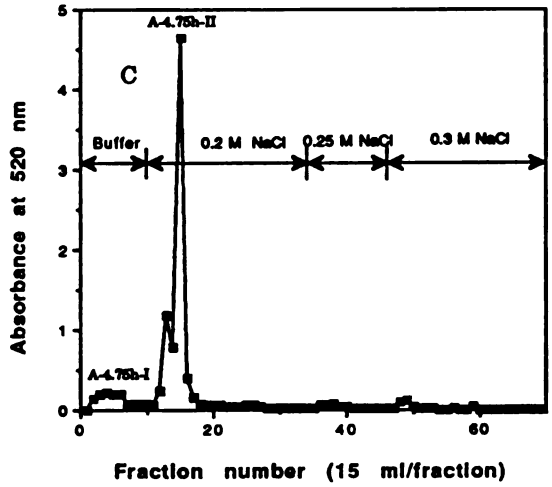
Chromatography on DEAE-Sephadex — Step Gradient: The column chromatograms of samples A-15m, B-15m, A-4.75h and B-4.75h are shown in Figure 7, A - D, respectively. For the 15m samples, the majority of the material eluted with 0.2 and 0.25 M NaCl (Figure 7, A and B). Sample A-15m yielded two major fractions, A-15m-II and A-15m-III, that contained 28.2% and 41.8%, respectively, of the material recovered. Sample B-15m also yielded two major fractions, B-15m-II and B-15m-III, that contained 26.8% and 50.5%, respectively, of the material recovered. For the 4.75h samples, most of the material was eluted with 0.2 M NaCl. Fractions A-4.75h-II and B-4.75h-II contained 72.8% and 75.9%, respectively, of the material recovered. The average recovery of material from the columns (based on the uronic acid test) was 57.8% (range was 30.3% to 93.2%).

Second Chromatography on DEAE-Sephadex — Linear Gradient: The column chromatograms of samples A-15m-II, A-15m-III, A-4.75h-II, B-15m-II, B-15m-III and B-4.75h-II on DEAE-Sephadex are shown in Figure 8, A - F, respectively. The average recovery of sample from the columns was 92.1%. Sample A-15m-II (Figure 8A) and B-15m-II (Figure 8D) both yielded two incompletely separated fractions on rechromatography. They were treated separately and combined as A-15m-IIa and A-15m-IIb, and B-15m-IIa and B-15m-IIb. Rechromatography of sample A-15m-III showed a single peak with a visible shoulder (Figure 8B). Fractions on the shoulder were opaque and had a slight precipitate, and were combined separately as sample A-15m-IIIb. Sample

Figure 7. Column Chromatograms of Cell Wall Pectic Polysaccharide Samples. Chromatography was on DEAE-Sephadex as described in the Materials and Methods. Panels A, B, C and D are chromatograms of samples A-15m, B-15m, A-4.75h, and B-4.75h, respectively. Fraction were tested for uronic acid (▫). The samples obtained are named as indicated on the graphs.


A
Buffer
0.2 M NaCl
0.25M NaCl
0.3 M NaCl
A-15m-I
A-15m-II
A-15m-III
A-15m-IV
A-15m-V
Absorbance at 520 nm
Fraction number (15 ml/fraction)


B
buffer
0.20 M NaCl
0.25 M NaCl
0.30 M NaCl
B-15m-I
B-15m-II
B-15m-III
B-15m-IV
Absorbance at 520 nm
Fraction number (15/fraction)


C
Buffer
0.2 M NaCl
0.25 M NaCl
0.3 M NaCl
A-4.75h-I
A-4.75h-II
Absorbance at 520 nm
Fraction number (15 ml/fraction)

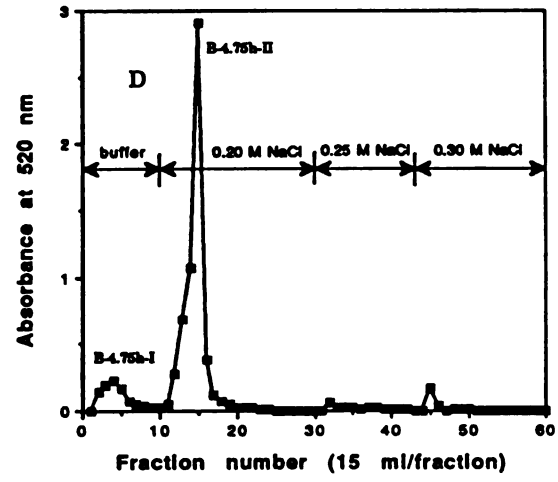
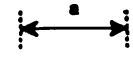
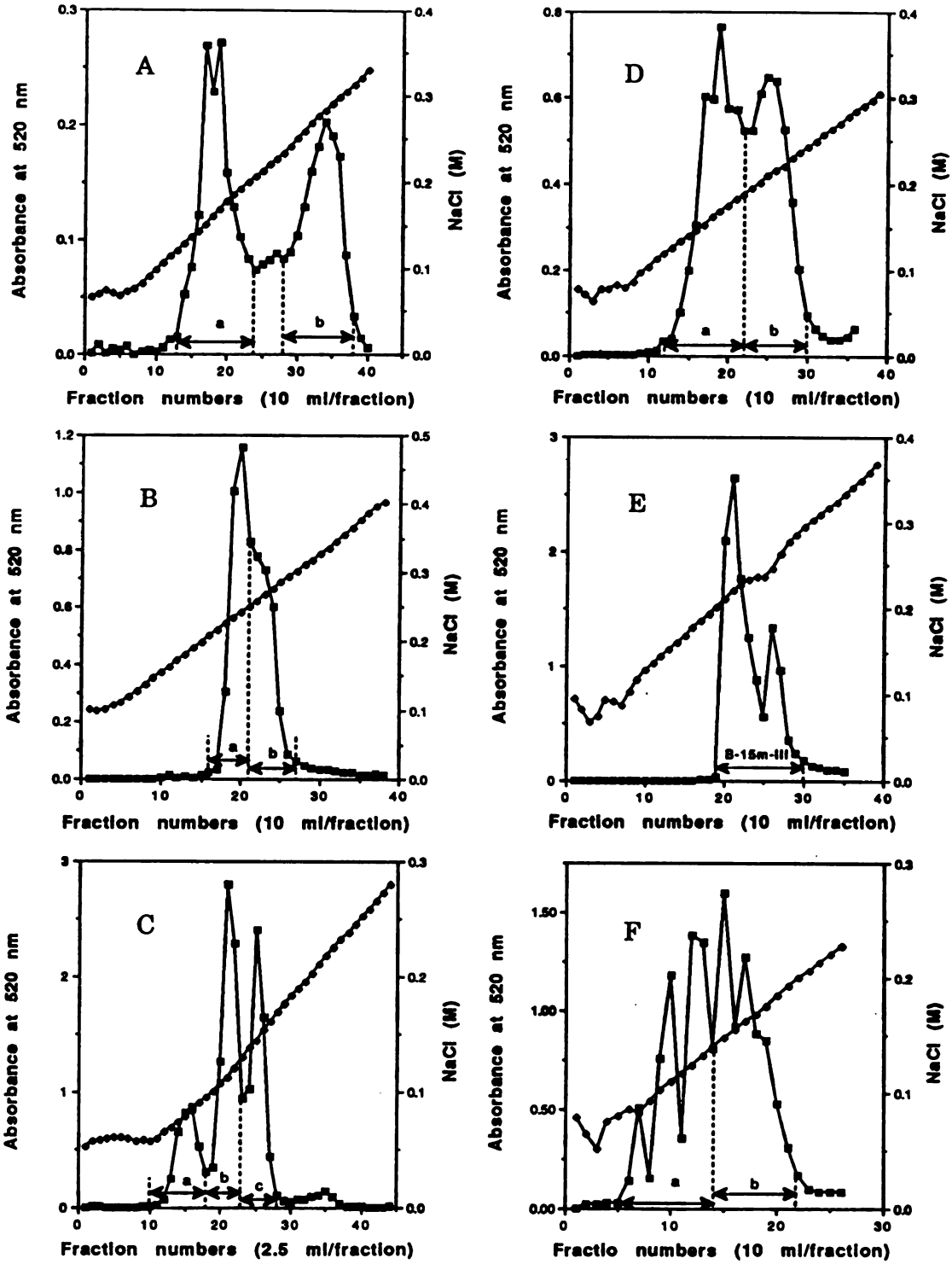

D
buffer
0.20 M NaCl
0.25 M NaCl
0.30 M NaCl
B-4.75h-I
B-4.75h-II
Absorbance at 520 nm
Fraction number (15 ml/fraction)

Figure 8. Column Chromatograms of Rechromatograpned Pectic Polysaccharide Samples. Rechromatography was on DEAE-Sephadex as described in the Materials and Methods. Panels A, B, C, D, E, and F are chromatograms of samples A-15m-II, A-15m-III, A-4.75h-II, B-15m-II, B-15m-III, and B-4.75h-II, respectively. Fractions were tested for uronic acid (▫) and conductivity (•). Fractions between arrows (e.g. ) were combined as individual samples.


A
B
C
D
E
F
Absorbance at 520 nm
NaCl (M)
Fraction numbers (10 ml/fraction)
Fraction numbers (2.5 ml/fraction)
Fractio numbers (10 ml/fraction)
a
b
c
S-15m-III

A-4.75h-II was separated into three fractions: A-4.75h-IIa, A-4.75h-IIb and A-4.75h-IIc (Figure 8C). Sample B-15m-III showed a major peak with a incompletely separated minor peak (Figure 8E). Because there was a drop in the rate of increase of the NaCl gradient followed by a relatively sharp increase as the minor peak was eluting, the small peak was considered an artifact and was combined with the larger peak as sample B-15m-III. Rechromatography results presented later showed sample B-15m-III eluted as a single band from a DEAE-Sephadex column. Five incompletely separated peaks were found on rechromatography of sample B-4.75h-II (Fig. 8 F). They were divided into samples B-4.75h-IIa and B-4.75h-IIb.

Homogeneity of polysaccharides: DEAE Sephadex column chromatograms of samples A-15m-IIa, A-15m-IIb, A-4.75h-IIa, A-4.75h-IIb and A-4.75h-IIc are shown in Figure 9, A - E, respectively, and those of samples B-15m-IIa, B-15m-IIb, B-15m-III, B-4,75h-IIa and B-4.75h-IIb are in Figure 10, A - E, respectively. Column chromatograms of samples A-15m-IIa, A-15m-IIb, B-15m-IIa, B-15m-IIb, B-15m-III, B-15m-4.75h-IIa and B-4.75h-IIb were also obtained by testing for total sugar and a comparison of these results and those obtained with the uronic acid test (Figure 9, A and B and Figure 10, A - E) is shown in Figure 11, A, B, E, F, G, H, and I, respectively. The results showed that each sample eluted from DEAE-Sephadex as a single, major peak and that the total sugar and uronic acid chromatograms were basically coincident. However, for samples A-15m-IIa and A-15m-IIb, some small neutral sugar peaks were presented in early fractions (Figure 11, A and B). Samples A-15m-IIIa and b were not chromatographed a third time on DEAE-Sephadex because when together as sample A-15m-III (Figure

Figure 9. Column Chromatograms of Pectic Polysaccharides Samples Obtained from Sample A and Chromatographed Twice Previously on DEAE-Sephadex. Chromatography was on DEAE-Sephadex as described in the Materials and Methods. Panels A, B, C, D, and E are chromatograms of samples A-15m-IIa, A-15m-IIb, A-4.75h-IIa, A-4.75h-IIb, and A-4.75h-IIc respectively. Fractions were tested for uronic acid (■) and conductivity (◆).

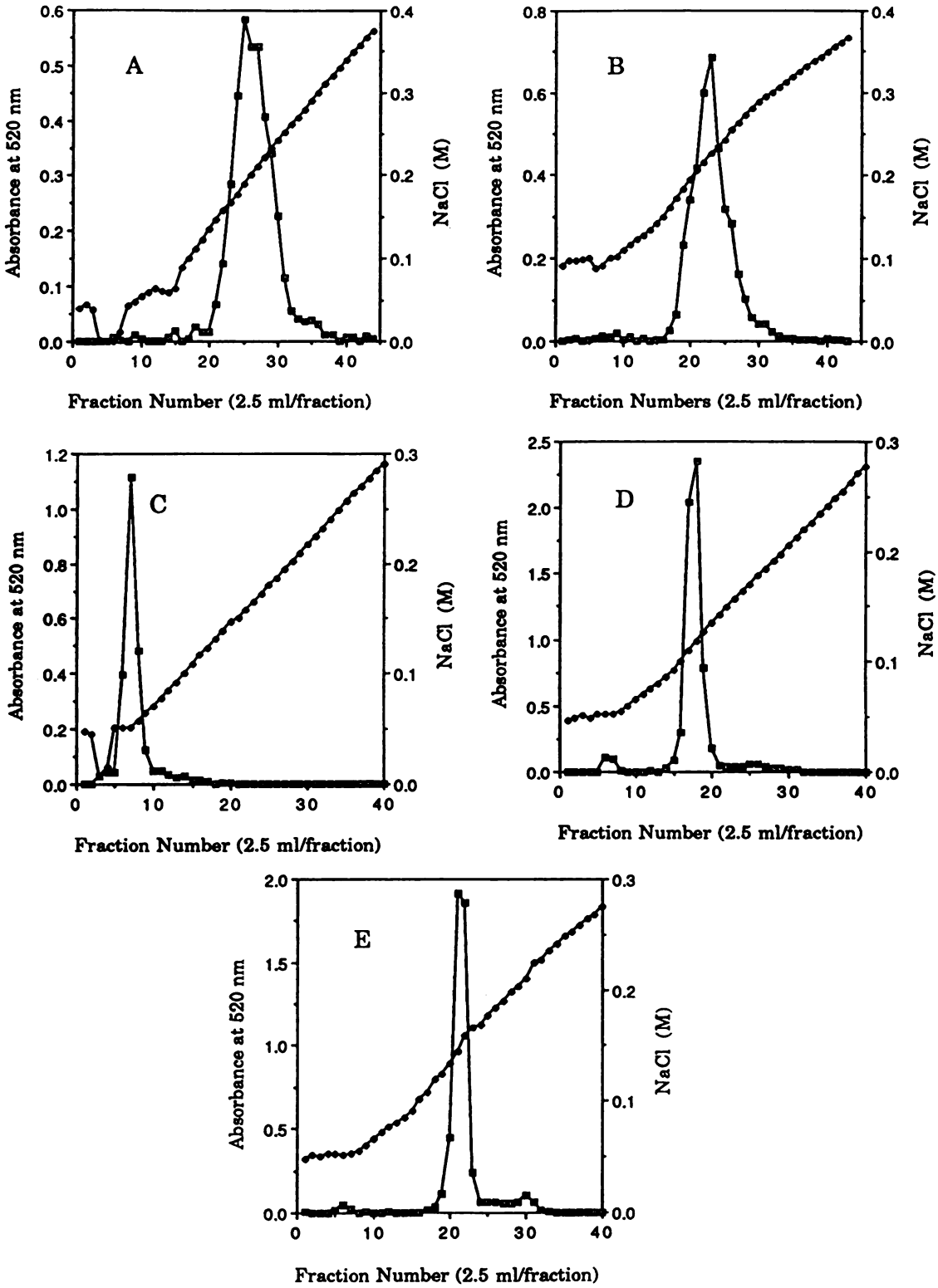

A
Absorbance at 520 nm
NaCl (M)
Fraction Number (2.5 ml/fraction)
B
Absorbance at 520 nm
NaCl (M)
Fraction Numbers (2.5 ml/fraction)
C
Absorbance at 520 nm
NaCl (M)
Fraction Number (2.5 ml/fraction)
D
Absorbance at 520 nm
NaCl (M)
Fraction Number (2.5 ml/fraction)
E
Absorbance at 520 nm
NaCl (M)
Fraction Number (2.5 ml/fraction)

Figure 10. Column Chromatograms of Pectic Polysaccharide Samples Obtained from Sample B and Chromatographed Twice Previously on DEAE-Sephadex. Chromatography was on DEAE-Sephadex as described in the Materials and Methods. Panels A, B, C, D, and E are chromatograms of samples B-15m-IIa, B-15m-IIb, B-15m-III, B-4.75h-IIa, and B-4.75h-IIb respectively. Fractions were tested for uronic acid (▫) and conductivity (●).

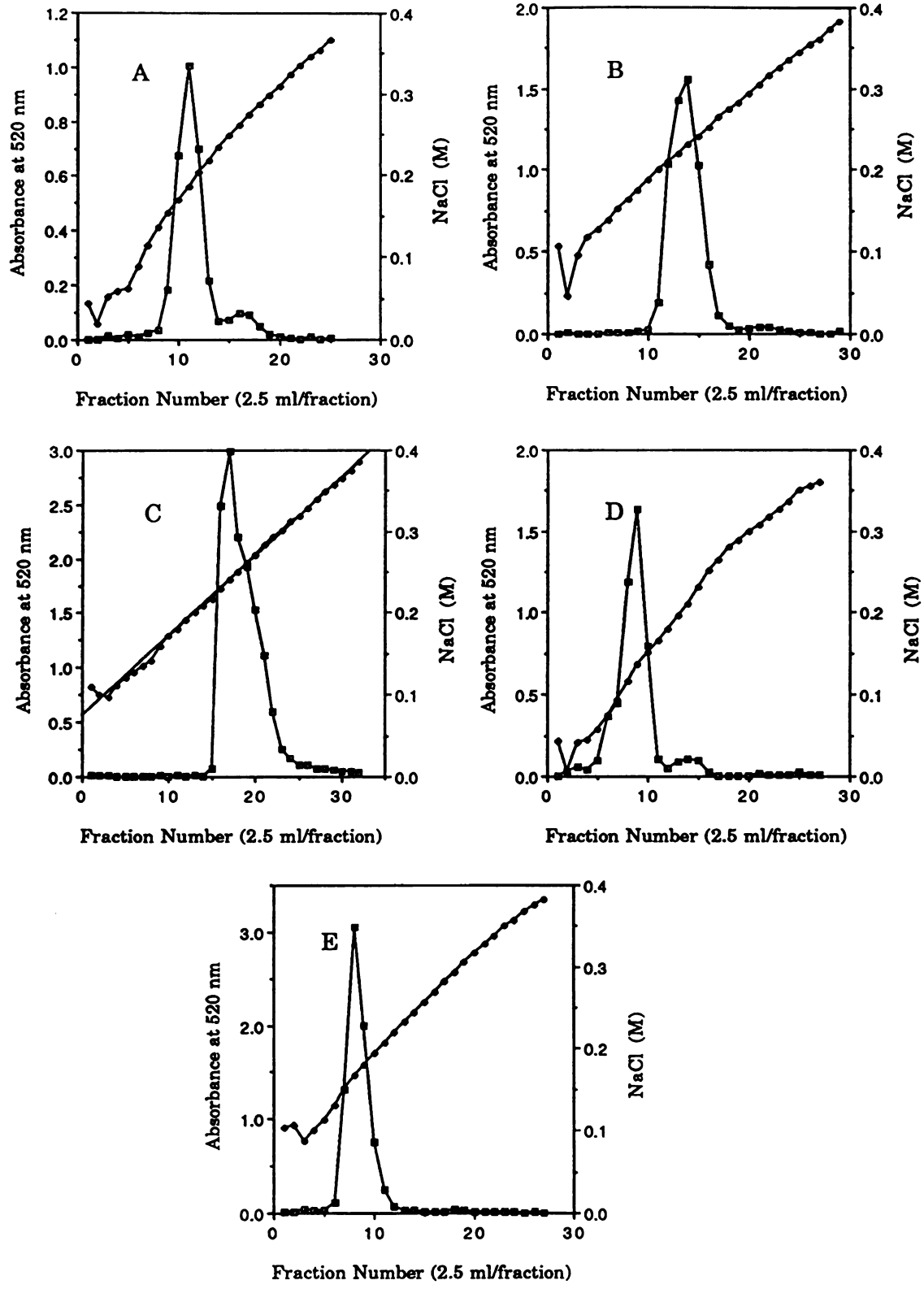
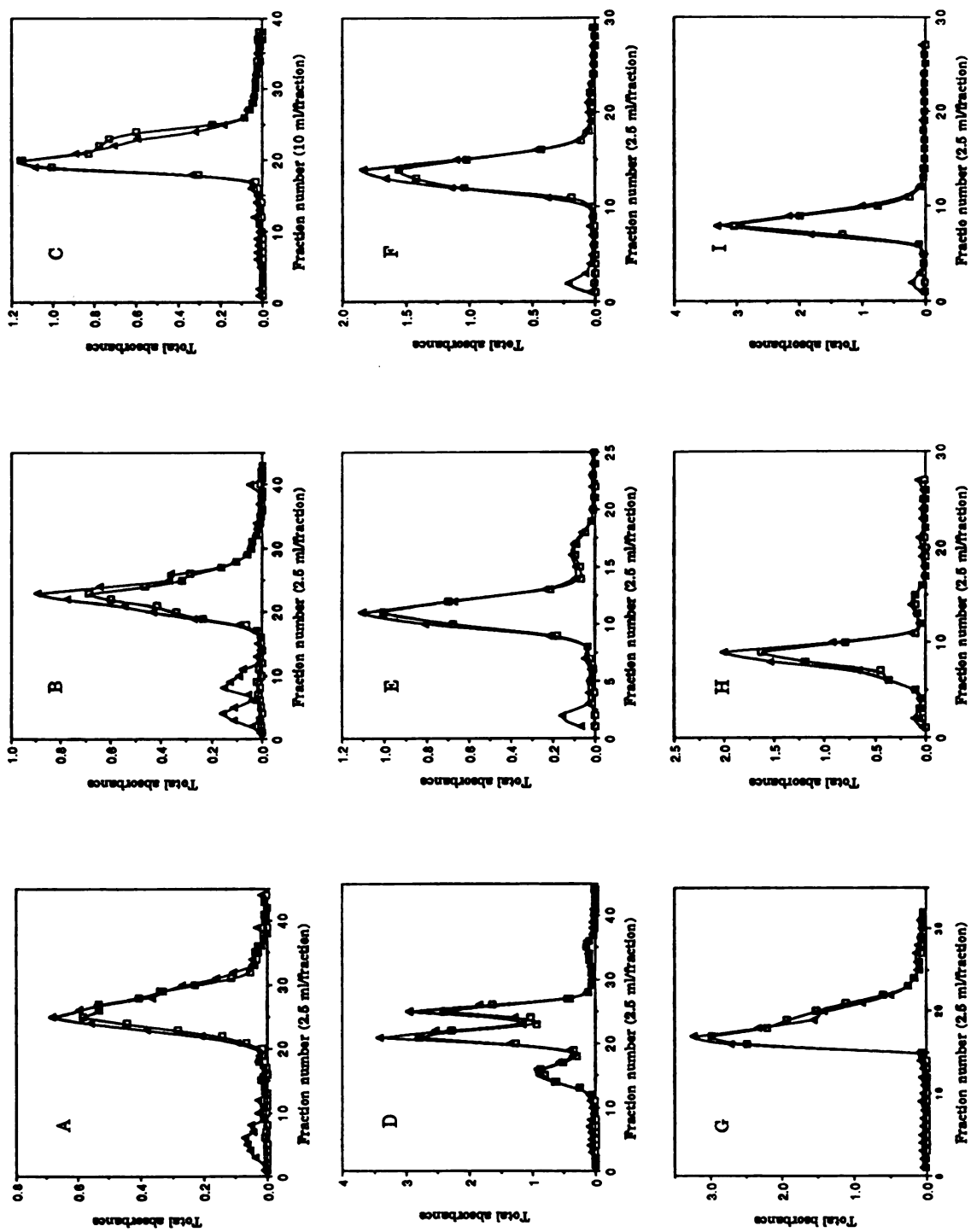

A
Absorbance at 520 nm
NaCl (M)
Fraction Number (2.5 ml/fraction)
B
Absorbance at 520 nm
NaCl (M)
Fraction Number (2.5 ml/fraction)
C
Absorbance at 520 nm
NaCl (M)
Fraction Number (2.5 ml/fraction)
D
Absorbance at 520 nm
NaCl (M)
Fraction Number (2.5 ml/fraction)
E
Absorbance at 520 nm
NaCl (M)
Fraction Number (2.5 ml/fraction)

Figure 11. Comparison of Column Chromatograms Obtained by the Uronic Acid and Total Sugar Tests. Panels A, B, C, D, E, F, G, H, and I are chromatograms of samples A-15m-IIa (Figure 9A), A-15m-IIb (Figure 9B), A-15m-III (Figure 8B), A-4.75h-II (Figure 8C), B-15m-II-a (Figure 10A), B-15m-IIb (Figure 10B), B-15m-III (Figure 10C), B-4.75h-IIa (Figure 10D), and B-4.75-IIb (Figure 10E) respectively. Fraction were tested for uronic acid (▫) and total sugar (▲).


A
B
C
D
E
F
G
H
I
Total absorbance
Fraction number (2.5 ml/fraction)
Fraction number (10 ml/fraction)

8C), they basically chromatographed as a single band and the chromatograms from both uronic acid and total sugar tests were coincident (Figure 11C). In further work they were treated separately. Samples A-4.75h-IIa, A-4.75h-IIb and A-4.75-IIc (Figure 9, C - E) were also not tested for total sugar since sample A-4.75h-II (Figure 8C), the sample from which they were derived, had already been tested for both uronic acid and total sugar and the chromatograms were coincident (Figure 11D).

Purified samples were chromatographed on Sephacryl S-400, and each showed a single band (Figure 12, A - L) except samples A-15m-IIb (Figure 12B), A-15m-IIa (Figure 12C) and A-15m-IIIb (Figure 12D) which showed either a small peak just before or after the major peak. For samples A-15m-IIIa and A-15m-IIIb (Figure 12, C and D), it is possible that the small peaks are due to cross contamination of each by the other (Figure 13). Sample A-15m-IIIb was a shoulder in the parent peak (Figure 8B)

K_{av} values were calculated for all samples and standard dextrans as follows:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where K_{av} = the partition coefficient of the sample; V_o = void volume;

V_e = elution volume of the sample; V_t = total volume of the gel bed

K_{av} values of the standard dextran samples were plotted against the log_{10} of their weight average molecular weight, $\overline{M}_w$ (Figure 14). The K_{av} value of each pectic polysaccharide, the weight average molecular weight that a standard dextran with the same K_{av} would have, and the NaCl concentration at the mid-point at which each pectic polysaccharide peak eluted from DEAE- Sephadex columns 2 and 3, are given in Table 1.

Figure 12. Column Chromatograms of Purified Pectic Polysaccharide Samples Obtained with Sephacryl S-400. Chromatography was performed as described in the Materials and Methods. Panels A, B, C, D, E, F, G, H, I, J, K, and L are chromatograms of samples A-15m-IIa, A-15m-IIb, A-15m-IIIa, A-15m-IIIb, A-4.75h-IIa, A-4.75h-IIb, A-4.75h-IIc, B-15m-IIa, B-15m-IIb, B-15m-III, B-4.75h-IIa, and B-4.75h-IIb, respectively. Fraction were tested for uronic acid (▫).

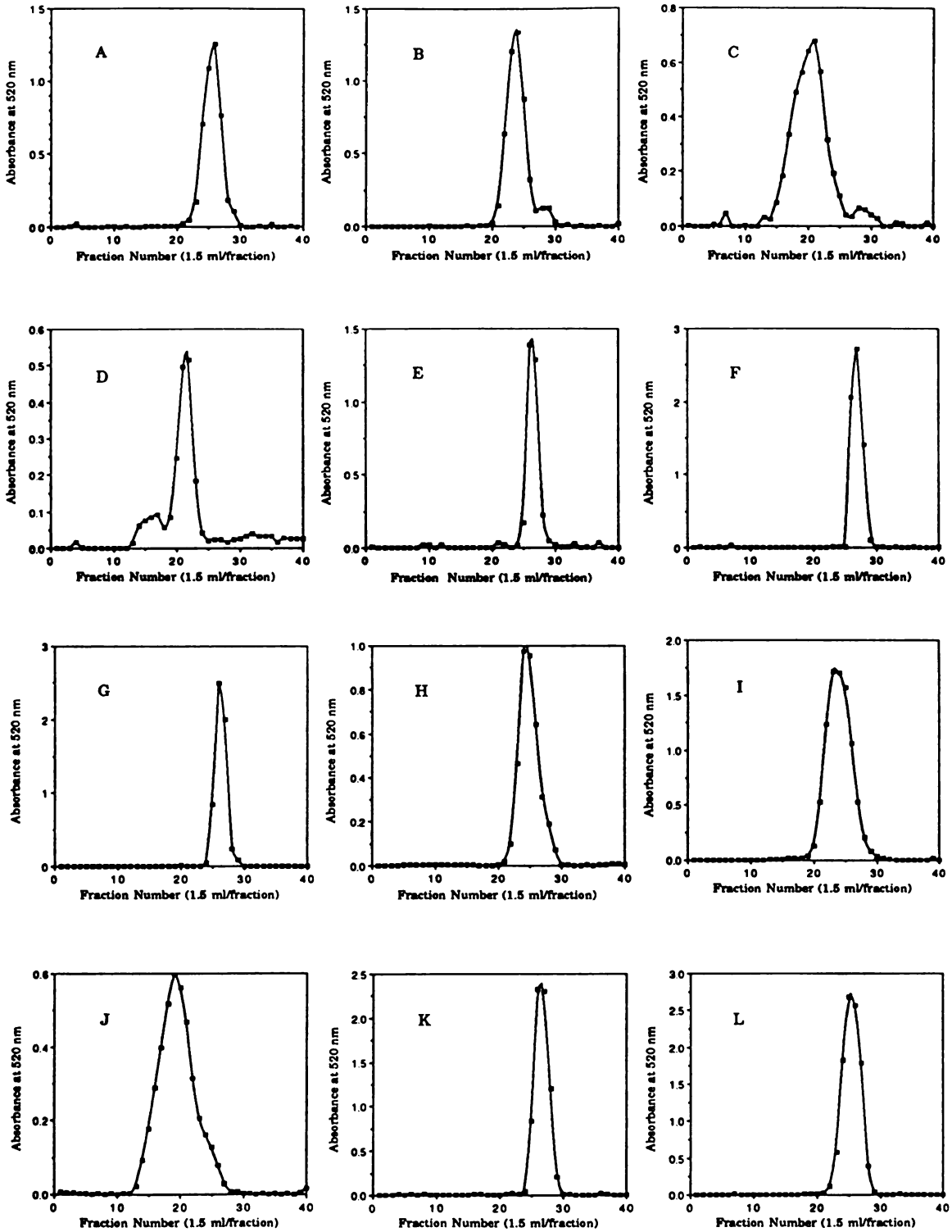

A
B
C
D
E
F
G
H
I
J
K
L
Absorbance at 520 nm
Fraction Number (1.5 ml/fraction)

Figure 13. Comparison of Column Chromatograms of Samples A-15m-IIIa and A-15m-IIIb. The column chromatograms of samples A-15m-IIIa (▪) and A-15m-IIIb (●) obtained with Sephacryl S-400. Fractions were analyzed for uronic acid as described in the Materials and Methods.

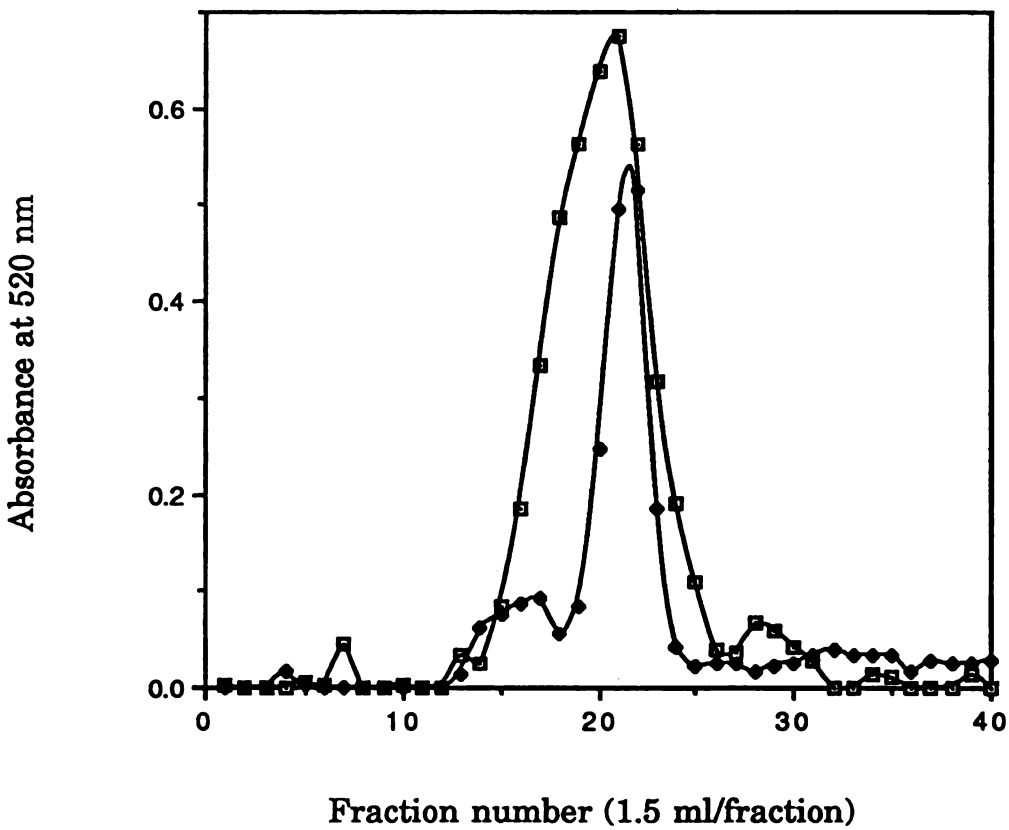

Absorbance at 520 nm
0.6
0.4
0.2
0.0
0
10
20
30
40
Fraction number (1.5 ml/fraction)

Figure 14. Relationship between $\bar{M}_w$ of Standard Dextrans and K_{av}. The equation for the line is: $y = 1.71 - 0.254\,x$, where $y = K_{av}$ and $x = \log_{10}\bar{M}_w$.

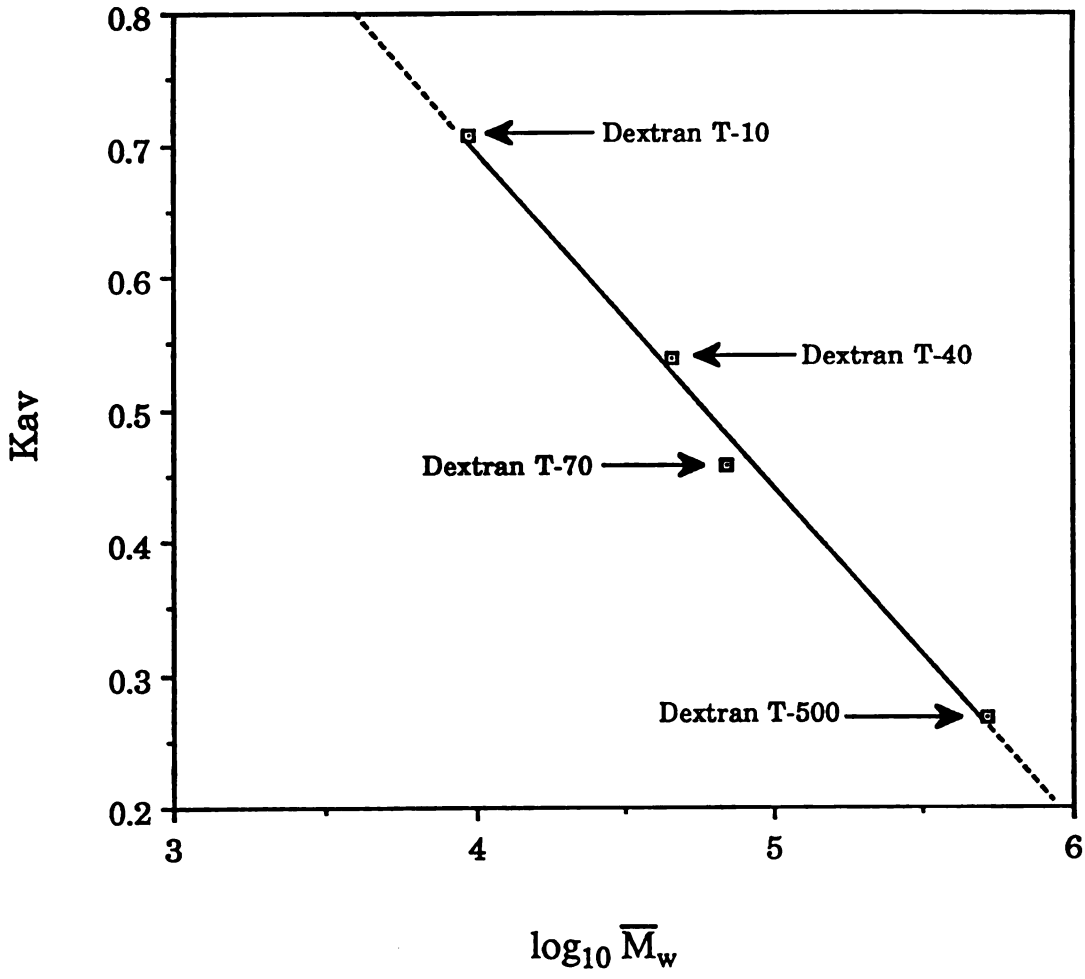

0.8
0.7
0.6
0.5
0.4
0.3
0.2
Kav
Dextran T-10
Dextran T-40
Dextran T-70
Dextran T-500
3
4
5
6
log10 Mw

Table 1. Comparison of NaCl concentrations needed to elute polysaccharide samples from DEAE-Sephadex, relative mobilities, as K_{av}, of samples on Sephacryl S-400, and the expected weight average molecular weight of a standard dextran with the same K_{av}.

Pectic polysaccharide sample		DEAE Sephadex chromatography, NaCl molarity at mid-point of elution		Sephacryl S-400 chromatography	
		Column 2	Column 3	K_{av}	Expected $\overline{M}_w$ of standard dextran with this K_{av}
First Isolation (Sample A)	A-15m-IIa	0.160	0.189	0.650	14,900
	A-15m-IIb	0.282	0.226	0.527	45,500
	A-15m-IIIa	0.242	Not deter.	0.361	205,000
	A-15m-IIIb	0.259	Not deter.	0.404	139,000
	A-4.75h-IIa	0.083	0.051	0.673	12,100
	A-4.75h-IIb	0.112	0.119	0.711	8,650
	A-4.75h-IIc	0.145	0.150	0.656	14,200
Second Isolation (Sample B)	B-15m-IIa	0.168	0.187	0.553	15,500
	B-15m-IIb	0.209	0.231	0.500	26,000
	B-15m-III	0.220	0.240	0.316	159,000
	B-4.75h-IIa	0.117	0.136	0.647	6,120
	B-4.75h-IIb	0.155	0.168	0.579	12,000

DISCUSSION

All cell wall pectic polysaccharide samples of *L. minor* isolated proved to be homogenous in terms of charge content, since each eluted as a single, reasonably narrow and symmetrical band from a DEAE-Sephadex column, and the chromatogram profiles from the uronic and total sugar tests were largely coincident. There were no significant neutral sugar peaks detected. The small peak of neutral sugar found in the early column fractions of samples A-15m-IIa and A-15m-IIb (Fig. 9, A and B) may indicate a loss of side chains. In addition, all the samples analyzed by gel filtration column chromatography with Sephacryl S-400 also showed a single, reasonably symmetrical pectic polysaccharide band except for samples A-15m-IIIa and A-15m- IIIb, which probably are cross contaminated with the each other, and sample A-15m-IIb, which has a small shoulder. This indicates they all are homogenous or reasonable so with respect to size. However, each sample very likely consists of a population of molecules in which there is a range of molecular sizes that has a Gaussian destribution. However, it is within this context that they are considered to be homogenous.

Generally similar results were obtained from ion exchange and gel filtration chromatography of cell wall pectic polysaccharides from both isolations. For samples A-15m-IIa, A-15m-IIb, A-4.75h-IIb and A-4.75h-IIc, the molarity of NaCl at the mid-point of elution from the DEAE-Sephadex columns and the K_{av} value obtained from chromatography on Sephacryl S-400 are both similar to the values for samples B-15m-IIa, B-15m-IIb, B-4.75h-IIa and B-4.75h-IIb, respectively (Table 1), although one more small sample peak, A-4.75h-IIa, was separated from sample A-15m-II (at low NaCl

concetration, 0.083 M) than from B-15m-II. The NaCl molarities for elution and the K_{av} of samples A-15m-IIIa and A-15m-IIIb were also similar to that of sample B-15m-III.

Comparison of the pectic polysaccharide samples extracted in 15 min with those extracted in the next 4.75 h showed the latter generally have a smaller molecular size and eluted from a DEAE-Sephadex column at a lower NaCl concentration. A examination of the purified samples isolated from a single cell wall preparation shows that the larger the molecular size (smaller K_{av} value) of the purified sample, the higher the concentration of NaCl needed to elute it from the column. The exception is sample A-15m-IIIb, whose molecular size is smaller than A-15m-IIIa but it eluted at a higher salt concentration.

PART 2

SUGAR COMPOSITION OF PECTIC POLYSACCHARIDES

MATERIALS AND METHODS

Materials: CMC, $NaBD_4$ (98 atom % D), $NaBH_4$, TFA and 3-phenylphenol (3-hydroxydiphenyl) were obtained from Aldrich Chemical Company, Inc.; 1-methylimidazole and polygalacturonic acid were obtained from Sigma Chemical Company; D-xylose, L-arabinose, L-fucose, L-rhamnose, D-galactose, D-mannose, and myo-inositol were obtained from Pfanstiehl Laboratories, Inc.; D-glucose was from the U.S. Department of Commerce, National Bureau of Standards. D-Apiose was isolated from *L. minor* by method of Neal and Kindel (56). Capillary columns SP-2380 and DB-225 were from Supelco, Inc. and J & W Scientific, Inc., respectively.

Reduction of Pectic Polysaccharides: The carboxyl group of the galacturonosyl residues of pectic polysaccharides was reduced basically as described by Taylor and Conrad (74). Dialyzed sample solutions of A-15m-IIIa, A-15m-IIIb, B-15m-IIa, B-15m-IIb, B-15m-III, B-4.75h-IIa and B-4.75h-IIb, each containing 1.2 - 4.7 mg of sample, were evaporated to about 10 - 15 ml. After the CMC was added to the sample solution, the pH was maintained at 4.75 for about 2.5 h by addition of 0.05 N HCl with a Sargent-Welch Recording pH-STAT. After esterification with CMC, 250 - 400 mg of $NaBD_4$ in 6 - 10 ml water was added dropwise over 1.5 h, and the sample was maintained at pH 7 with 5 N HCl and the pH-STAT. The solution was transferred to dialysis tubing that retained

molecules with a molecular weight of 6000-8000 or larger and dialyzed against water for 36 h. All samples were reduced twice except sample B-15m-III which was reduced three times.

Before and after reduction, a portion of the sample solution was used for determination of uronic acid and total carbohydrates by the method of Blumenkrantz and Asboe-Hansen (14) and Dubois *et al.* (22), respectively. The amount of uronic acid and neutral sugar in the pectic polysaccharides was calculated by using the following equation (derivation in Appendix):

$$c_n = \frac{A_{490_T} - \frac{m_3 \cdot D_2}{m_2 \cdot D_1}(A_{520_T} - b_2 - b_7 - b_8) - b_5 - b_6 - b_3}{1 - \frac{m_3[m_7 x + m_8(1-x)]}{m_2[m_5 x + m_6(1-x)]}} \cdot \frac{D_1}{m_5 x + m_6(1-x)}$$

where c_n = concentration of the neutral sugar in the sample, units depend on units used in standard curves

$A_{490\,T}$ = absorbance of sample (including neutral sugar and uronic acid residues) by the total sugar test

$A_{520\,T}$ = absorbance of the sample by the uronic acid test

b_2 and m_2 = intercept and slope of the standard curve for uronic acid residue in the uronic acid test

b_3 and m_3 = intercept and slope of the standard curve for uronic acid residue in the total sugar test.

b_5 and m_5 = intercept and slope of the standard curve for apiosyl residue in the total sugar test

b_6 and m_6 = intercept and slope of the standard curve for galactosyl residue in the total

sugar test

b_7 and m_7 = intercept and slope of the standard curve for apiosyl residue in the uronic acid test

b_8 and m_8 = intercept and slope of the standard curve for galactosyl residue in the uronic acid test

D_1 = —fold dilution of the sample for the total sugar test

D_2 = —fold dilution of the sample for the uronic acid test

x = proportion of the total neutral sugar residues that is apiosyl residue

Amount of uronic acid in the sample was calculated by using the following:

$$c_a = \frac{A_{520_T} - b_7 - b_8 - [m_7 x + m_8 (1-x)] \cdot \frac{c_a}{D_2} - b_2}{m_2} \cdot D_2$$

where c_a = concentration of the uronic acid residue in the sample

A software program (written with BASIC) was developed to perform the necessary calculations. Based on the results for the uronic acid and total sugar determinations, the total recovery of material from each reduction was calculated.

Analysis of Reduced Pectic Polysaccharides: Reduced samples were evaporate to about 1 to 2 ml and blown to dryness with N_2. Myo-inositol (0.15 mg) was added to each sample and the sample was hydrolyzed with 2 M TFA at 110°C for 1.5 h. A time course of hydrolysis of a typical reduced pectic polysaccharide sample was performed. The sample was treated with 2 M TFA at 110°C for 0.5, 1, 2, 3 and 4 h and after hydrolysis, each portion was blown to dryness with N_2 at 22°C.

The procedure of Blakeney *et al.* (13) as modified by Kindel and Liang (38) was

used to prepare alditol acetates, except after acetylation, the sample in 1.5 ml of chloroform was washed five times with water. After the last extraction, the solution was centrifuged and the organic phase was transferred to a 1 ml vial, brought to dryness with N_2 and the sample dissolved in 0.2 ml of chloroform and used for GC. A standard sample mixture, containing rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, myo-inositol (each 150 μg) and apiose (153.5 μg), was acetylated by the procedure described above and washed with water 1, 2 and 5 times, and gas chromatography was used to determine the effect of washing. The sample extracted 5 times served as the standard sample to obtain the gas chromatography response factor for each sugar. Myo-inositol served as the internal standard.

Gas chromatography of alditol acetates was performed with a Hewlett Packard gas chromatograph, Model 5480A, equipped with a flame-ionization detector, a 5840A data terminal and a splitter from J & W Scientific, Inc. Samples were chromatographed on a DB-225 fused-silica capillary column (30 m $\times$ 0.25 mm i. d., film thickness 0.25 μm) isothermally at 230°C, with helium as the carrier gas at a flow rate of $\sim$0.8 ml/min. N_2 was the makeup gas and had a flow rate of 45 ml/min. Samples of 0.5 - 1.5 μl were injected and the split ratio was 1:45.

EI mass spectrometry were performed with a JEOL JMS-AX505H mass spectrometer interfaced to a Hewlett Packard gas chromatograph, Model 5890 A, which was equipped with a splitless injector and the same type of DB-225 capillary column as above. Helium was the carrier gas and the flow rate was $\sim$1 ml/min. The ion source temperature was 200°C and the ionizing voltage was 70 eV. Alditol acetate samples were diluted with chloroform, and about 0.5 μl was injected with a on-column injection system.

Alditol acetates were identified by retention time and ion patterns. The proportion of galactitol acetate derived from galactosyl residues and that from galacturonosyl residues was calculated from the equation (derivation in Appendix),

$$R=\frac{2\times(I_{x+2}-I_x\times F_{x+2})}{I_x\times(1-F_{x+2})+I_{x+2}}\times\frac{100}{98}$$

where R = the ratio of galactitol acetate molecules that are labeled with deuterium to total galactitol acetate molecules. When the ion pair 217/219 m/z was chosen to represent galactitol acetate/galactitol acetate-D_2, I_x and I_{x+2} were the intensities of ion 217 m/z (the nominal ion, x) and 219 m/z (ion x containing two natural ^{13}C, a ^{18}O or two deuterium atoms from $NaBD_4$), respectively. F_{x+2} is the theoretical abundance ratio of natural isotope contributed to the x+2 ion which has a value of 0.0169 (the probability of two ^{13}C in nine carbon atoms is $(9)^2 \times 0.006\%$ or 0.00486, and of one ^{18}O in six oxygen atoms is $6 \times 0.2\%$ or 0.012).

When the ion pair 259/261 m/z was chosen to represent galactitol acetate/galactitol acetate D_2, I_x and I_{x+2} were the intensities of ion 259 m/z (the nominal ion, x) and 261 m/z (ion x containing two natural ^{13}C, a ^{18}O or two deuterium atoms from $NaBD_4$), respectively. F_{x+2} is the theoretical abundance ratio of natural isotope contributed to the x+2 ion which has a value of 0.0213 (the probability of two ^{13}C in 11 carbon atoms is $(11)^2 \times 0.006\%$ or 0.00726, and of one ^{18}O in 7 oxygen atoms is $7 \times 0.2\% = 0.014$).

RESULTS

The unreduced uronic acid remaining after each reduction and the total recovery of material from the reductions are shown in Table 2. After reduction was performed once, the average uronic content was approximately 13% (w/w) of the reduced pectic polysaccharide samples (range from 5.6% to 23.4%, excluding B-15m-IIa). A second reduction decreased this to 8.6%. All samples were reduced twice, except sample B-15m-III, which was reduce three times. Although reduction of pectic polysaccharides a third time resulted in a further decrease in the uronosyl residue content (e.g. the third reduction of sample B-15m-III brought the uronosyl residue content from 11.1% to 5.5%), each reduction resulted in loss of sample material and therefore, because of limited sample quantities, a third reduction was not performed. The average amount of sample material left after two reductions was 44% of the starting material (Table 2).

The time course of TFA hydrolysis (Figure 15) showed that the yield of arabinose reached maximum within a half hour of hydrolysis, the apiose yield was highest after 1 hour of hydrolysis, for galactose, rhamnose and glucose 2 hours of hydrolysis were needed to obtain the maximum yield, and the yield of mannose was highest after 3 hour.

Gas chromatograms of the standard alditol acetate sample in chloroform after being washed with water 1, 2, and 5 times are compared in Figure 16, A - C, respectively. The tailing after the solvent peak for the sample washed twice (Figure 16B) is significantly less than for the sample washed once (Figure 16A). After 5 washings, the interference from tailing is negligible (Figure 16C).

Rhamnose, fucose, arabinose, xylose, apiose, mannose, glucose, and galacturonic

Table 2. Reduction of cell wall pectic polysaccharides samples. The neutral sugar (%) and uronic acid (%) are weight/weight percentages of each residue in the reduced sample. The total recovery is also a weight/weight percentage. These were determined and calculated as described in Materials and Methods. Only sample B-15m-III was reduced three times.

Sample	After first reduction		After second reduction		After third reduction		Total recovery of neutral sugar and uronic acid (%)
	Neutral sugar (%)	Uronic acid (%)	Neutral sugar (%)	Uronic acid (%)	Neutral sugar (%)	Uronic acid (%)	
A-15m-IIIa	87.7	12.3	95.7	4.3			40.4
A-15m-IIIb	86.2	13.8	86.4	13.6			62.1
B-15m-IIa	61.2*	38.8*	86.0	14.0			43.8
B-15m-IIb	91.1	8.9	94.2	5.8			27.3
B-15m-III	84.9	15.1	88.9	11.1	94.5	5.5	73.3
B-4.75h-IIa	76.6	23.4	93.3	6.7			27.2
B-4.75h-IIb	94.4	5.6	95.6	4.4			34.1
Average	86.8**	13.2**	91.4	8.6			44.0

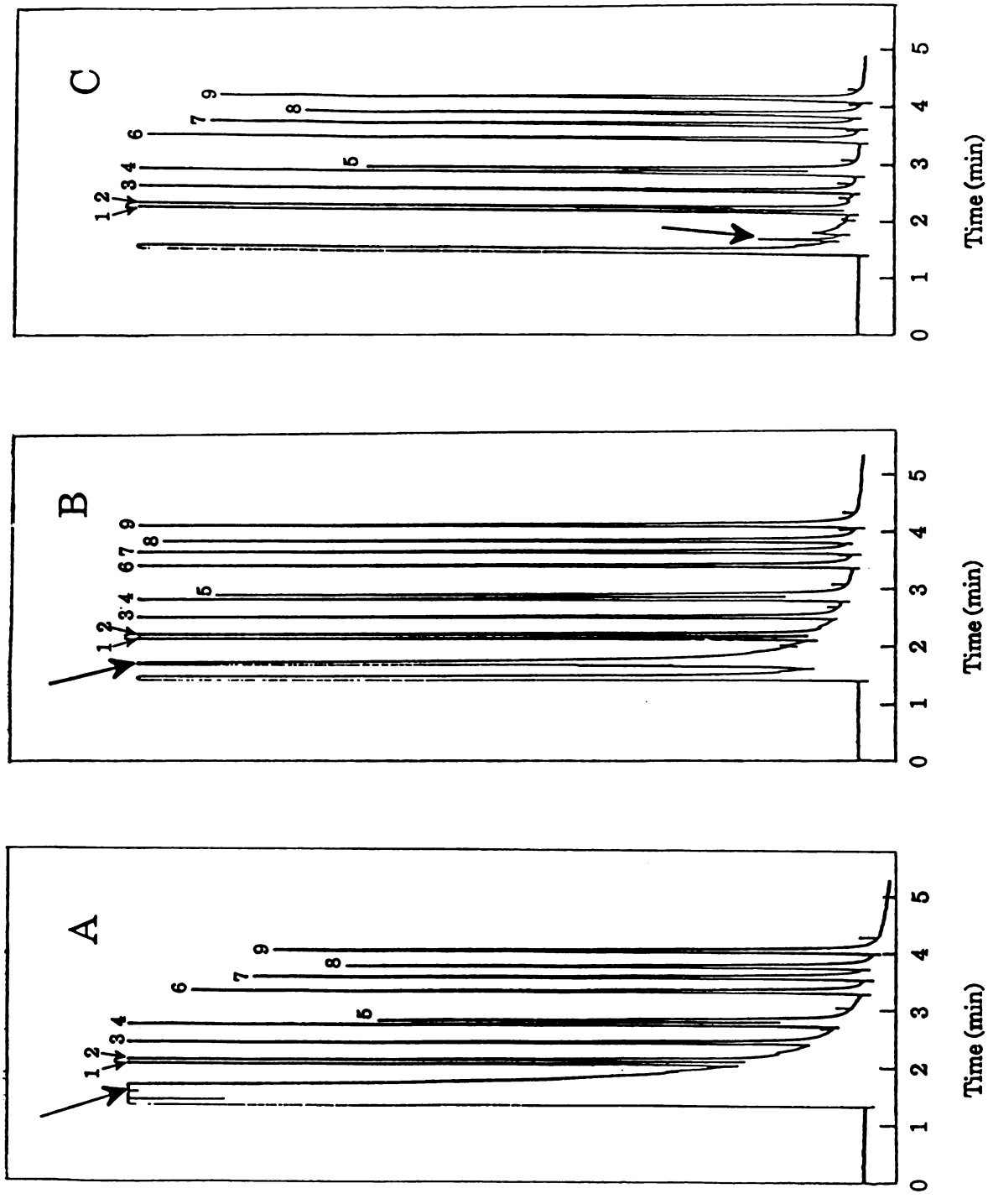
* Abnormal color (it was yellowish-green instead of the normal pink color of uronic acid) was observed in the uronic acid test. Sample had a high starting neutral sugar content (Table 4).

** For the reason stated above, the data from the first reduction of sample B-15m-IIa were not used for the calculation of average percentage of neutral sugar and uronic acid.

Figure 15. Time Course of Hydrolysis of a Carboxyl-reduced Pectic Polysaccharide Sample. Reduction and TFA hydrolysis of the cell wall pectic polysaccharide sample and preparation of alditol acetates and GC were performed as described in the Materials and Methods.

Yield of Sugars (ug)
160
140
120
100
80
60
40
20
Gal
Api
Rha
Glc
Man
Ara
Sugars
0.5 h
1 h
2 h
3 h
4 h
Time of Hydrolysis

Figure 16. Treatment of an Alditol Acetate Sample with Water. Panels A, B and C are gas chromatograms of a standard mixture of alditol acetates prepared as described in the Materials and Methods and washed with water 1, 2, and 5 times, respectively. Samples (2 μl) were chromatographed on a SP-2380 fused-silica capillary column isothermally at 260°C. The split ratio was 1:50. Other conditions were as described in the Materials and Methods. The tailing caused by contaminants in the samples is indicated by the arrow. Peaks 1 through 8 represent the alditol acetates of rhamnose (1), fucose (2), arabinose (3), xylose (4), apiose (5), mannose (6), galactose (7), and glucose (8) while peak 9 is myo-inositol hexaacetate.


A
B
C
1 2 3 4 5 6 7 8 9
0 1 2 3 4 5
Time (min)

acid were detected in all seven cell wall pectic polysaccharides samples (Table 3). Small amounts (4.1 to 11.3 mole %) of galactose was found in samples B-15m-IIa, B-15m-IIb, B-4.75h-IIa and B-4.75h-IIb but not in the others. Each alditol acetate was identified by matching its retention time to a known in the standard sample chromatographed under the same conditions, and by comparison of its mass spectrum with that of the known. The amount of each sugar in the sample was determined by the internal standard method and the molar percentage of each sugar in the cell wall pectic polysaccharide samples is shown in Table 3. Since samples A-15m-IIIa and A-15m-IIIb were obtained by dividing sample A-15m-III during the isolation procedure and were analyzed separately , a weighted average sugar composition was calculated to represent sample A-15m-III (Table 3). The A-15m-III (ave.) and B-15m-III samples are similar except that the apiose content of the B sample is considerably higher than that of the A (ave.) sample.

A comparison of selected properties of the pectic polysaccharides samples to the rhamnose/galacturonic acid ratio of these samples is shown in Table 4.

The ion clusters around the two ion pairs, 217/219 m/z and 259/261 m/z, were analyzed to find the ratio, galactitol acetate/galactitol acetate-D_2, which represents the ratio of galactosyl/galacturonosyl residues in the pectic polysaccharide samples. The results (Table 5) showed that samples A-15m-IIIa, A-15m-IIIb and B-15m-III had 100% of galactitol acetate as galactitol acetate-D_2 (values greater than 100% were counted as 100%, since this is maximum possible), while samples B-15m-IIa and B-15m-IIb had low values (galactitol acetate-D_2 was 49 and 69%). The percent galactitol acetate-D_2 values of samples extracted in the 4.75 h period were intermediate.

Table 3. Sugar composition of the cell wall pectic polysaccharide samples. The values in the table are mole percents. The weight of each sugar detected by GC (internal standard method) was divided by the molecular weight of the sugar to calculate the mole percent. The mole percent of galacturonic acid (GalUA) and galactose were calculated from the mole percent of galactose obtained from GC and the R value (shown in Table 4) of the sample. The amount of uronic acid remaining after reduction of the carboxyl groups (calculated from the colometric tests) was assumed to be all galacturonosyl residue and was used to adjust the mole percent of galacturonic acid.

Sugar	A-15m -IIIa	A-15m -IIIb	Average* of A-15m-IIIa & A-15m-IIIb	B-15m -IIa	B-15m -IIb	B-15m -III	B-4.75h -IIa	B-4.75h -IIb
Rhamnose	4.6	2.8	3.8	3.0	4.5	3.0	4.0	7.2
Fucose	1.3	0.80	1.1	1.5	1.5	1.5	2.2	2.5
Arabinose	2.5	1.5	2.1	4.0	3.5	1.4	2.8	4.7
Xylose	3.3	2.7	3.0	14.5	6.8	0.68	3.6	4.3
Apiose	32.5	16.3	25.6	23.7	23.0	39.8	39.0	34.9
Mannose	2.1	1.7	1.9	11.5	6.0	0.68	2.3	2.7
GalUA	44.8	67.7	54.6	20.8	30.2	51.3	38.8	34.3
Galactose	0	0	0	9.0	11.3	0	4.1	6.3
Glucose	9.0	6.5	7.9	11.9	13.4	1.6	3.2	3.2

* The average mole percent values for combined sample A-15m-IIIa and A-15m-IIb was calculated as follows:

$$\overline{M} = \frac{M_a \times wt_a + M_b \times wt_b}{wt_a + wt_b} \times 100$$

where $\overline{M}$ = average mole percent of individual sugar residues in combined sample A-15m-IIIa and A-15m-IIb

M_a and M_b = mole percent of individual sugar residues in samples A-15m-IIIa and A-15m-IIIb, respectively.

wt_a and wt_b = amount of samples A-15m-IIIa and A-15m-IIIb, respectively isolated from the cell wall (see Figure 8B).

Table 4. Comparison of selected properties of the seven pectic polysaccharide samples with the ratio of rhamnose/galacturonic acid (Rha/GalUA, mole/mole) for these samples.

Sample	Time of Extraction	Elution Concentration of NaCl (M)	Relative Molecular Size*	Ratio of Rha/GalUA
A-15m-IIIa	15 min	0.25	205,000	1/10
A-15m-III-b	15 min	0.25	139,000	1/24
Average of A-15m-IIIa & A-15m-IIIb**	15 min	0.25	177,000	1/16
B-15m-III	15 min	0.25	159,000	1/17
B-15m-IIa	15 min	0.20	15,500	1/7
B-15m-IIb	15 min	0.20	26,000	1/7
B-4.75h-IIa	4.75 h	0.20	6,000	1/10
B-4.75h-IIb	4.75 h	0.20	12,000	1/5

* The relative molecular size of each sample is represented by the $\overline{M}_w$ of a standard dextran whose mobility is the same as that of the sample on a Sephacryl S-400 column.

** The average relative molecular size and ratio of Rha/GalUA of samples A-15m-IIIa and A-15m-IIIb is weighted average (see Table 3).

Table 5. Intensity of ions in two major ion clusters from the mass spectrums of galactitol acetate/galactitol acetate-D_2 prepared from the pectic polysaccharides samples. The ratio of galactitol acetate-D_2/galactitol acetatea + galactitol acetate-D_2 (R) was calculated as described in the Materials and Methods.

Sample		A-15m-IIIa	A-15m-IIIb	B-15m-IIa	B-15m-IIb	B-15m-III	B-4.75h-IIa	B-4.75h-IIb
Ion Intensity	217 m/z	10.3609	13.3649	16.2231	56.0119	56.9824	24.5813	53.3447
	219 m/z	10.3311	14.3796	5.3405	28.3447	53.6743	18.4599	37.023
R		101.4	105.0	48.6	67.0	98.1	86.4	82.4
Ion Intensity	259 m/z	8.7105	10.4002	13.8702	40.5700	42.8466	15.3357	39.3615
	261 m/z	8.2411	11.9290	4.7439	22.0947	44.7692	12.9675	27.6367
R		98.1	108.1	49.6	70.1	103.2	92.2	82.7
Average R		100	107	49	69	101	89	83

DISCUSSION

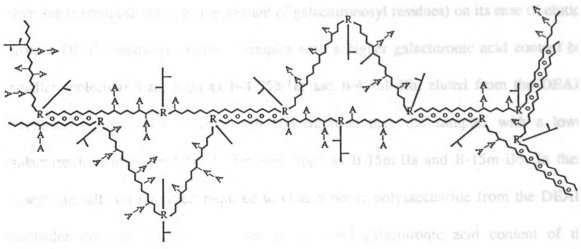
Rhamnose, fucose, arabinose, apiose, xylose, mannose, glucose and galacturonic acid were found in the all pectic polysaccharide samples isolated from the cell wall of *L. minor*. Galacturonic acid (20.8 to 67.7%) and apiose (23.0 to 39.8%) were the two most abundant components in the samples. Galactose was found in four of the seven samples: B-15m-IIa, B-15m-IIb, B-4.75h-IIa and B-4.75h-IIb. Results of GC-MS, size exclusion chromatography and ion exchange chromatography suggested that three types of pectic polysaccharides were isolated. They differ in sugar composition and molecular size. The pectic polysaccharides extracted in 15 min are of two types. Samples of the first type, B-15m-IIa and B-15m-IIb, were eluted with 0.2 M NaCl and samples of the second type, A-15m-IIIa, A-15m-IIIb and B-15m-III, with 0.25 M NaCl. The former have a relatively small molecular size (the molecular weights of standard dextrans with the same mobilities would be 15,500 and 26,000), a high neutral sugar (79.2 and 69.8%) and low galacturonic acid content (20.8 and 30.2%), and a relatively high ratio of rhamnose/galacturonic acid (1/7) while the latter have a much larger molecular size (the molecular weight of standard dextrans with the same mobility would be 205,000, 139,000 and 159,000, respectively), are more acidic (galacturonic acid content is 44.8, 67.7 and 51.3%, respectively) and contain no galactose, and have a lower ratio of rhamnose/ galacturonic acid (the ratio for sample B-15m-III is 1/17 and for the weighted average of sample A-15m-IIIa and A-15m-IIIb is 1/16). The third type of pectic polysaccharide, samples B-4.75h-IIa and B-4.75h-IIb, was extracted in the 4.75 h period. They have the smallest molecular size (the molecular weights of standard dextrans with the same

mobilities as those of samples of B-4.75h-IIa and B-4.75h-IIb are 6,120 and 12,000, respectively), a high apiose (39.0 and 34.9%, respectively) and intermediate galacturonic acid content (38.8 and 34.3%, respectively) and a high ratio of rhamnose/galacturonic acid (1/10 and 1/5, respectively).

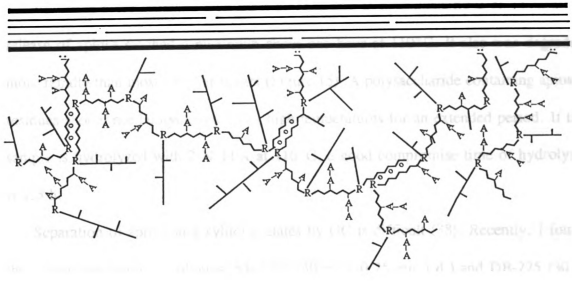
The above results indicate that both relatively large "smooth" (assuming less rhamnose and other neutral sugars indicates less side chains) polysaccharides and somewhat smaller, "hairy" pectic polysaccharides are extracted from the cell wall of *L. minor* with ammonium oxalate in 15 min. However, since it has been suggested that pectic polysaccharides extracted with chelating reagent (such as EDTA and CDTA) in short times are mainly from the middle lamella (68, 69) and have lower ratio of rhamnose/galacturonic acid than those from cell walls (68), I suggest that the pectic polysaccharides, A-15m-IIIa, A-15m-IIIb and B-15m-III, were isolated from the middle lamella (Figure 17A), while pectic polysaccharides B-4.75h-IIa and B-4.75h-IIb are present in cell wall proper (Figure 17B). Although there are some differences, the molecular size and rhamnose/galacturonic acid ratio of sample B-15m-IIa and B-15m-IIb, are closer to those of samples B-4.75h-IIa and B-4.75h-IIb than those of samples A-15m-IIIa, A-15m-IIIb and B-15m-III (Table 5), so that the samples B-15m-IIa and B-15m-IIb may also come from cell wall. Since no large size pectic polysaccharide fraction was detected in the samples isolated with 0.05 M ammonium oxalate in the 4.75 h period, it is possible that the time needed to extract only the pectic polysaccharides of the middle lamella, uncontaminated with pectic polysaccharides from the cell wall proper, could be shorter than 15 min. A proposed model for cell wall of *L. minor* is shown in Figure 17. This model shows that the pectic polysaccharides of the middle lamella are different from

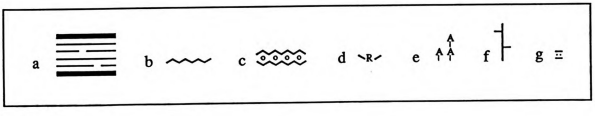
Figure 17. Proposed Model for the Primary Cell Wall of *L. minor*. The proposed model of primary cell wall of *L. minor* shows the general structure of pectic polysaccharides and cellulose microfibril and the linkage between them. Hemicelluloses and cell wall proteins are not shown in the model. The pectic polysaccharides of the middle lamella (A) are larger in molecular size, have longer segments of undisrupted polygalacturonosyl chains (associated with Ca^{2+}) and less and shorter neutral sugar side chains than the pectic polysaccharides of the cell wall (B). The key in the Figure depicts the following: (a) cellulose microfibril, (b) polygalacturonosyl backbone, (c) two segments of polygalacturonosyl chains associated with Ca^{2+} to form an "egg box", (d) rhamnosyl residue in the pectic polysaccharide backbones, (e) apiose and apiobiose side chains, (f) neutral sugar side chain and (g) ester bond between pectic polysaccharides and cellulose microfibril.

A



B





those of the cell wall proper.

The results show that the molecular size of a pectic polysaccharide has a greater effect than sugar composition (e.g. the amount of galacturonosyl residues) on its ease of elution from a DEAE-Sephadex column. Samples with a higher galacturonic acid content but smaller molecular size, such as B-4.75h-IIa and B-4.75h-IIb, eluted from the DEAE-Sephadex column with a lower salt concentration than did samples with a lower galacturonic acid content but larger size, such as B-15m-IIa and B-15m-IIb. In these cases, the salt concentration required to elute a pectic polysaccharide from the DEAE-Sephadex column is directly related to the total galacturonic acid content of the polysaccharide.

Apiose is more sensitive than most other sugars to hydrolysis by 2 N TFA, since release of apiose reached a maximum about one hour at 110°C. It also was degraded more rapidly than most of other sugars (Figure 15). A polysaccharide containing apiosyl residues should not be hydrolyzed at a high temperatures for an extended period. If the sample is hydrolyzed with 2 N TFA at 110°C, a good compromise time of hydrolysis is 1.5 h.

Separation of apiitol and xylitol acetates by GC is difficult (38). Recently, I found that when two capillary columns, SP-2380 (30 m × 0.25 mm i.d.) and DB-225 (30 m × 0.32 mm i.d.), were used close to their maximum operating temperature (265°C and 235°C, respectively), the apiitol and xylitol acetates were eluted separately in 9 and 18 min, respectively. Interestingly, their order of elution was reversed compared to elution at 175°C. The separation of alditol acetates at high column temperature also has the advantage that the quantification of the sugars by the integrator/computer is more precise,

since peaks are sharper. However, at a high column temperature, the alditol acetates with short retention times are more likely to appear on the tail following the solvent peak and this could interfere with quantification. Tailing, however, can be greatly reduced by washing the chloroform solution of the sample 5 times with water. A second benefit of sample washing is that contaminants that could reduce column life are removed. A disadvantage of the high column temperature is that the GC columns deteriorate more rapidly.

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APPENDIX A

DERIVATION OF EQUATIONS FOR CALCULATION OF THE AMOUNT OF NEUTRAL SUGARS AND URONIC ACID IN SAMPLES

I. BASIC EQUATION:

If the concentration of a substance in solution has a linear relationship with absorption, the linear equation for the standard curve is:

$$A = b + m \cdot c \qquad \text{or} \qquad c = \frac{A-b}{m} \tag{1}$$

where c = the concentration of the substance
A = absorbance
b = intercept of the standard curve
m = slope of the standard curve

II. CONCENTRATION OF NEUTRAL SUGAR IN A SAMPLE MIXTURE WITH BOTH NEUTRAL SUGAR AND URONIC ACID (e. g. pectic polysaccharide sample)

For a sample solution containing only neutral sugar, the concentration of the sugar can be calculated by the equation:

$$c_n = \frac{A_{490_n} - b_1}{m_1} \cdot D_1 \tag{2}$$

where c_n = concentration of the neutral sugar in the undiluted sample.
$A_{490\,n}$ = absorbance of the neutral sugar in the total sugar (phenol-sulfuric acid) test at 490 nm.
b_1 and m_1 = intercept and slope of the standard curve of neutral sugar concentration vs. absorbance for total sugar test*
D_1 =—fold dilution of the sample for the total sugar test

However, when both neutral sugar and uronic acid are present in the same solution, equation (2) must be modified as follows:

$$c_n = \frac{(A_{490_t} - A_{490_u}) - b_1}{m_1} \cdot D_1 \tag{3}$$

where $A_{490\,t}$ = absorbance of total sugar in sample in total sugar test
$A_{490\,u}$ = absorbance of uronic acid in total sugar test
$A_{490\,u}$ cannot be measured directly in the sample. But it can be calculated from:

$$A_{490_n} = b_2 + m_2 \cdot \frac{c_u}{D_1} \tag{4}$$

where b_2 and m_2 = intercept and slope of the standard curve of uronic acid concentration vs. absorbance in the total sugar test.
c_u = concentration of uronic acid in the sample.

The concentration of uronic acid in the sample is:

$$c_u = \frac{(A_{520_t} - A_{520_n}) - b_3}{m_3} \cdot D_2 \tag{5}$$

where $A_{520\,t}$ and $A_{520\,n}$ = absorbance of total and neutral sugar, respectively, in the sample in the uronic acid test
b_3 and m_3 = intercept and slope of the standard curve of uronic acid concentration vs. absorbance in the uronic acid test
D_2 = —fold dilution of the sample for the uronic acid test

$A_{520\,n}$ cannot be determined directly from the sample. But it can be calculated from:

$$A_{520_n} = b_4 + m_4 \cdot \frac{c_n}{D_2} \tag{6}$$

where b_4 and m_4 = intercept and slope of the standard curve of neutral sugar vs. absorbance in the uronic acid test

The expression for $A_{520\,n}$ in (6) is substituted into (5) to give:

$$c_u = \frac{A_{520_t} - b_4 - m_4 \cdot \frac{c_n}{D_2} - b_3}{m_3} \cdot D_2 \tag{7}$$

The expression for c_u in (4) is substituted into (7) to give:

$$A_{490_n} = b_2 + m_2 \cdot \frac{A_{520_t} - b_4 - m_4 \cdot \frac{c_n}{D_2} - b_3}{m_3} \cdot \frac{D_2}{D_1} \tag{8}$$

The expression for $A_{490\,n}$ in (3) is substituted into (8) to give:

$$c_n = \frac{A_{490_t} - b_2 - m_2 \cdot \frac{A_{520_t} - b_4 - m_4 \cdot \frac{c_n}{D_2} - b_3}{m_3} \cdot \frac{D_2}{D_1} - b_1}{m_1} \cdot D_1 \tag{9}$$

After being simplified, the equation (9) becomes:

$$C_n = \frac{A_{490_t} - \frac{m_2 \cdot D_2}{m_3 \cdot D_1}(A_{520_t} - b_3 - b_4) - b_1 - b_2}{1 - \frac{m_2 \cdot m_4}{m_1 \cdot m_3}} \cdot \frac{D_1}{m_1} \qquad (10)$$

*If the neutral sugar component in a pectic polysaccharide sample is mostly galactose, then b_1 and m_1 can be consider to be the intercept and slope of the standard curve of galactose concentration vs. absorbance in the total sugar test, and the concentration of the galactose can be obtained by equation (10). Since the apiosyl residue in a reduced pectic polysaccharides of *L. minor* is also a significant neutral sugar component (together with the galactosyl residue) and it has an absorbance response in the total sugar test quite different from that of galactose, b_1 and m_1 must be modified as follows:

If x is the portion of the neutral sugar component of a sample that is apiose, then

$$c_n x = \frac{A_{490_{api}} - b_5}{m_5} \cdot D_1 \qquad (11)$$

where c_n and D_1 are the same as in equation (2) above

$A_{490\ api}$ = absorbance of apiose of the polysaccharide sample in the total sugar test

b_5 and m_5 = intercept and slope of the standard curve of apiose concentration vs. absorbance in the total sugar test

Similarly, the concentration of galactosyl residue in total neutral sugar components can be calculated by equation:

$$c_n (1-x) = \frac{A_{490_{gal}} - b_6}{m_6} \cdot D_1 \qquad (12)$$

where $A_{490\ gal}$ = absorbance of galactose of the polysaccharide sample by the total sugar test

b_6 and m_6 = intercept and slope of the standard curve of galactose concentration vs. its absorbance in the total sugar test

Equation (11) and (12) can be re-written as:

$$A_{490_{api}} = \frac{c_n}{D_1} m_5 x + b_5 \qquad \text{and} \qquad A_{490_{gal}} = \frac{c_n}{D_1} m_6 (1-x) + b_6$$

They are combined to give:

$$A_{490_{api}} + A_{490_{gal}} = \frac{c_n m_5 x + c_n m_6 (1-x)}{D_1} + b_5 + b_6$$

Rearranging gives:

$$c_n = \frac{(A_{490_{api}} + A_{490_{gal}}) - (b_5 + b_6)}{m_5 x + m_6 (1-x)} \cdot D_1 \qquad (13)$$

Compare equation (13) with equation (2). Since $A_{490\,n} = A_{490\,api} + A_{490\,gal}$ then:

$$b_1 = b_5 + b_6 \qquad (14)$$
$$m_1 = m_5 x + m_6 (1-x) \qquad (15)$$

Similarly, in equation (6) it follows that:

$$b_4 = b_7 + b_8 \qquad (16)$$
$$m_4 = m_7 x + m_8 (1-x) \qquad (17)$$

where b_4 and m_4 are the same as in equation (6),
b_7 and m_7 = intercept and slope of the standard curve of apiosyl residue concentration vs. absorbance in the uronic acid test
b_8 and m_8 = intercept and slope of the standard curve of galactosyl residue concentration vs. absorbance in the uronic acid test

By using the equations (14), (15), (16) and (17) to calculate b_1, m_1, b_4 and m_4 and substituting these in the equation (10), the concentration of neutral sugar in a sample (including apiosyl and galactosyl residues) can be obtained.

III. CALCULATION OF THE CONCENTRATION OF URONIC ACID IN A SAMPLE MIXTURE WITH BOTH NEUTRAL SUGAR AND URONIC ACID

The equation (7) above is:

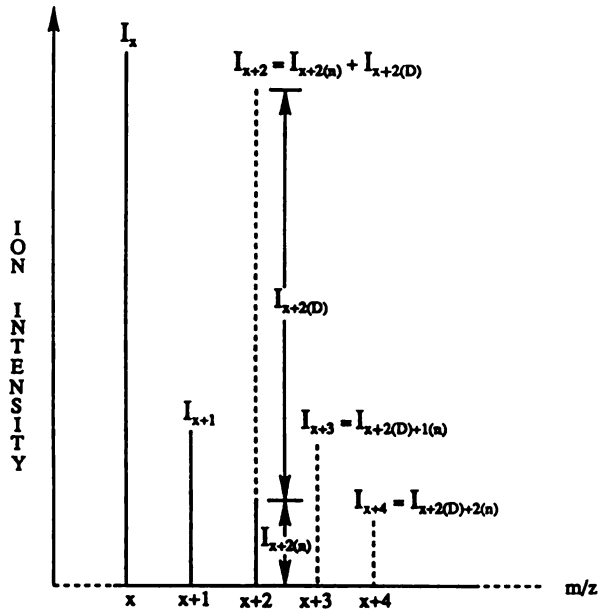
$$c_u = \frac{A_{520_t} - b_4 - m_4 \cdot \frac{c_n}{D_2} - b_3}{m_3} \cdot D_2 \qquad (7)$$

where $A_{520\,t}$ is the experimentally measured absorbance (used in equation(5)) and a_4, b_4, a_2, b_2 and D_2 are as described above. When the concentration of neutral sugar, c_n, is obtained from equation (10), it can be used in equation (7) to calculate the concentration of uronic acid, c_u, in the sample.

APPENDIX B

DERIVATION OF THE EQUATION USED TO CALCULATE THE GALACTITOL ACETATE-D_2 IN GALACTITOL ACETATE

For a nominal ion x in the mass spectrum of galactitol acetate-D_2, ions with five different masses should be found in the typical ion cluster of related ions. These are shown in the diagram:



where x and I_x = the nominal ion x and its intensity, respectively.

x+1 and I_{x+1} = the ion with one mass unit larger than ion x and the intensity of this ion, respectively. The additional one mass unit is mainly due to the presence of natural ^2H, ^{13}C or ^{17}O in x.

x+2 and I_{x+2} = the ion with two mass units larger than ion x and the intensity of this ion, respectively. The intensity of the ion is due in part to the presence of two ^{13}C atoms or an ^{18}O atom in ion x (labeled $I_{x+2(n)}$,

where 2(n) means the two mass units contributed by natural isotopes), and in part to two introduced deuterium atoms in ion x ($I_{x+2(D)}$).

x+3 and I_{x+3} = the ion with three mass units larger than ion x and the intensity of this ion, respectively. The three mass units are mainly contributed by two introduced deuterium atoms and one ^{2}H, ^{13}C or ^{17}O atom ($I_{x+3} = I_{x+2(D)+1(n)}$).

x+4 and I_{x+4} = the ion with four mass units larger than ion x and the intensity of this ion, respectively. The additional four mass units are mainly contributed by two introduced deuterium atoms and two ^{13}C atoms or an ^{18}O atom ($I_{x+4} = I_{x+2(D)+2(n)}$).

The sum of the intensities from all ions with two introduced deuterium atoms is:

$$(I_{x+2(D)} + I_{x+2(D)+1(n)} + I_{x+2(D)+2(n)}) = (I_{x+2(D)} + I_{x+3} + I_{x+4})$$

Since galactitol acetate-D_2 has a symmetrical structure (not considering the deuterium atoms), then for any type of ion with two introduced deuterium atoms there must be a corresponding ion with the same structure except without the introduced deuterium atoms. Therefore, in above ion cluster, the total intensity of the ions from galactitol acetate-D_2 is: $2 \times (I_{x+2(D)} + I_{x+3} + I_{x+4})$.

When the ratio (R) of galactitol acetate-D_2/(galactitol acetate-D_2 + galactitol acetate) is 1 then the galactose content of the sample is zero. R can be calculated as follows:

$$R = \frac{2\times(I_{x+2(D)}+I_{x+3}+I_{x+4})}{I_x+I_{x+1}+I_{x+2}+I_{x+3}+I_{x+4}} \qquad (1)$$

where, $I_{x+2(D)} = I_{x+2} - I_{x+2(n)}$. $I_{x+2(n)}$ can not be obtained directly but can be calculated

from: $I_{x+2(n)} = I_x \times F_{x+2}$ (2)

where F_{x+2} is the expected abundance ratio of ion x+2 ion due to the occurrence of two natural ^{13}C atoms or an ^{18}O atom in ion x. Therefore, $I_{x+2(D)}$ can be calculated as follows:

$$I_{x+2(D)} = I_{x+2} - (I_x \times F_{x+2}) \quad (3)$$

Equation (1) can now be re-written as:

$$R = \frac{2\times[(I_{x+2} - I_x \times F_{x+2}) + I_{x+3} + I_{x+4}]}{I_x + I_{x+1} + I_{x+2} + I_{x+3} + I_{x+4}} \times \frac{100}{98} \quad (4)$$

where 100/98 accounts for $NaBD_4$ being 98% deuterium. The values for I_x and I_{x+2} were taken from the mass spectrum while I_{x+1}, I_{x+3} and I_{x+4} were calculated by using the expected abundance ratios, F_{x+1} and F_{x+2} rather than taking the values from the mass spectrum:

$$I_{x+1} = I_x \times F_{x+1}$$

$$I_{x+3} = I_{x+2(D)} \times F_{x+1} = (I_{x+2} - I_x \times F_{x+2}) \times F_{x+1}$$

$$I_{x+4} = I_{x+2(D)} \times F_{x+2} = (I_{x+2} - I_x \times F_{x+2}) \times F_{x+2}$$

Substituting, equation (4) becomes:

$$R = \frac{2\times[(I_{x+2} - I_x \times F_{x+2}) + (I_{x+2} - I_x \times F_{x+2}) \times F_{x+1} + (I_{x+2} - I_x \times F_{x+2}) \times F_{x+2}]}{I_x + (I_x \times F_{x+1}) + I_{x+2} + (I_{x+2} - I_x \times F_{x+2}) \times F_{x+1} + (I_{x+2} - I_x \times F_{x+2}) \times F_{x+2}} \times \frac{100}{98}$$

which can be simplified to:

$$R = \frac{2\times(I_{x+2} - I_x \times F_{x+2})}{I_x \times (1 - F_{x+2}) + I_{x+2}} \times \frac{100}{98} \quad (5)$$

When the ion pair 217/219 m/z was chosen to represent galactitol acetate/galactitol acetate-D_2, I_x = intensity of ion 217 m/z and I_{x+2} = intensity of ion 219 m/z. Ion 217 contains 13 H, 9 C and 6 O, so abundance ratios, F_{x+1} and F_{x+2}, can be calculated as follows:

Ion	x	x+1	x+2
Natural D in 13 H		0.01×13 = 0.13	
^{13}C in 9 C		1.1×9 = 9.9	0.006×(9)^2 = 0.486
^{17}O in 6 O		0.04×6 = 0.24	
^{18}O in 6 O			0.2×6 = 1.2
A (abundance)*	100	A_{x+1}=0.13+9.9+0.24=10.27	A_{x+2}=0.486+1.2=1.686
F**	1	F_{x+1}=0.1027	F_{x+2}=0.01686

* A represents the relative abundance of an ion.

** F (factor) represents the abundance ratio of an ion. $F_{ion} = A_{ion}/100$ when the abundance of ion x is set equal to 100.

Similarly, when the ion pair 259/261 m/z was chosen to represent galactitol acetate/galactitol acetate-D_2, I_x = intensity of ion 259 m/z and I_{x+2} = intensity of ion 261 m/z. Ion 259 contains 15 H, 11 C and 7 O, so abundance ratios, F_{x+1} and F_{x+2} can be calculated as follows:

Ion	x	x+1	x+2
Natural D in 15 H		0.01×15 = 0.15	
^{13}C in 11 C		1.1×11 = 12.1	0.006×(11)^2 = 0.726
^{17}O in 7 O		0.04×7 = 0.28	
^{18}O in 7 O			0.2×7 = 1.4
A	100	A_{x+1}=0.15+12.1+0.28=12.53	A_{x+2}=0.486+1.2=2.126
F	1	F_{x+1}=0.1253	F_{x+2}=0.02126

APPENDIX C

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The acetylation of apiitol in the determination of apiose

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ABSTRACT

The complete acetylation of apiitol required 9 h when acetic anhydride at 120° was used and sodium acetate was the catalyst. Both apiitol pentaacetate and apiitol tetraacetate were detected before acetylation was complete. When the reaction was done in dimethyl sulfoxide, with 1-methylimidazole as the catalyst, a third compound was observed, and identified as 1,2,4-tri-*O*-acetyl-3-*C*-(acetoxymethyl)-3-*O*-(methylthiomethyl)-D-*glycero*-tetritol [3-*O*-(methylthiomethyl)apiitol tetraacetate] by gas-liquid chromatography and mass spectrometry. In *N*,*N*-dimethylformamide, with 1-methylimidazole as catalyst, the acetylation of apiitol was essentially complete in 4 h at 85°, and the formation of methylthiomethyl ether was avoided. A method for preparing alditol acetates using 1-methylimidazole as the catalyst, and suitable for samples containing apiose as well as ordinary sugars, is described. The separation of apiitol pentaacetate from xylitol pentaacetate by gas-liquid chromatography proved difficult. However, a virtually complete separation of the peracetates of apiitol and xylitol as well as complete separation of those of rhamnitol, fucitol, arabinitol, mannitol, galactitol, glucitol, and *myo*-inositol, plus apiitol tetraacetate and 3-*O*-(methylthiomethyl)apiitol tetraacetate, was accomplished with a 30 m × 0.53 mm (i.d.) SP-2380 column in 49 min, and on a 30 m × 0.75 mm (i.d.) SP-2330 column in 82 min. A complete separation of apiitol and xylitol pentaacetates as well as four other alditol peracetates was obtained with a 60 m DB-1 column in 15.2 min, however this column did not resolve the acetates of fucitol and arabinitol. A variety of other columns and column conditions were ineffective.

INTRODUCTION

Apiose [3-*C*-(hydroxymethyl)-D-*glycero*-aldotetrose] is widely distributed in the plant kingdom[1]. It is present both in cell-wall polysaccharides and in non-cell-wall compounds[2]. It is therefore of interest when the sugar composition of plant cell walls and cell-wall fractions is determined, typically by the preparation of alditol acetates after acid hydrolysis of the sample. All of the known sugars present in plant cell-wall material, except apiose and aceric acid[3], have only primary and secondary hydroxyl groups, and consequently their alditols are acetylated relatively easily. Apiose and aceric acid, on the other hand, have tertiary hydroxyl groups, which past experience has shown are difficult to acetylate when the more common catalysts such as pyridine are used[4]. The conditions for complete acetylation of apiitol have not been established, although the assumption may have been made that the published conditions for the acetylation of other alditols also gave complete acetylation of apiitol. We investigated the acetylation of apiitol by two methods currently used to acetylate alditols[5-7], and found that complete acetylation in both cases required considerably more rigorous

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conditions than those needed for sugar alcohols having only primary and secondary hydroxyl groups. We also found that when the procedure of Blakeney *et al.*[7] was used under conditions that gave complete conversion of apiitol, substantial amounts of 1,2,4-tri-*O*-acetyl-3-*C*-(acetoxymethyl)-3-*O*-(methylthiomethyl)-D-*glycero*-tetritol [herein called 3-*O*-(methylthiomethyl)apiitol tetraacetate] were formed as a side-product.

EXPERIMENTAL

Materials. — Apiose was isolated as described previously[8]. Other sugars and *myo*-inositol were obtained from Pfanstiehl Laboratories, Inc. 1-Methylimidazole, 4-dimethylaminopyridine, dimethyl sulfoxide, and fully deuterated acetic anhydride were obtained from Aldrich Chemical Co., Inc. *N,N*-Dimethylformamide was purchased from Sigma Chemical Co. *myo*-Inositol hexaacetate (m.p. 213–214° was prepared by a modification of the procedure described by Wachowiak and Conners[6] for glucose pentaacetate. Columns packed with SP-2330, SP-2380, and DB-1 were purchased from Supelco, Inc. and J & W Scientific, Inc., respectively.

Preparation of alditol acetates. — Alditol acetates were prepared by the following four procedures: (1) Albersheim *et al.*[5], (2) Blakeney *et al.*[6,7], (3) Steglich and Höfle[9], and (4) a modification of the procedure of Blakeney *et al.*[6,7] The particular procedure used, along with any changes, is stated when the results of individual experiments are described. In the preparation of individual samples, 0.89–1.78 μmol of apiose and 0.82–1.0 μmol of rhamnose, fucose, arabinose, xylose, galactose, glucose, and mannose were used*. Except where indicated otherwise, *myo*-inositol was added to the samples as an internal standard.

The procedure of Blakeney *et al.*[7] was modified as follows so that complete acetylation of apiitol was achieved without formation of any side-product (acetylation procedure IV). The sample of apiose was in 0.2 mL of M ammonium hydroxide, and after 1 mL of 2% (w/v) $NaBH_4$, in DMF was added it was kept for 60 min at 40°, cooled to 22°, acidified with 0.2 mL of glacial acetic acid, and mixed. For acetylation, 0.2 mL of 1-methylimidazole and 2 mL of acetic anhydride were added and the sample was mixed and heated for 4 h at 85°. Five mL of water was added to the solution at 22° and after mixing and cooling to 22°, 1 mL of dichloromethane was added. The sample was mixed, then centrifuged, and the organic phase used for gas chromatography. The modified procedure was also tested with rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, and *myo*-inositol.

Gas chromatography. — Gas-liquid chromatography of alditol acetates was performed initially with a Varian Aerograph, Series 2100, and more recently with a Varian Model 3700 instrument, each equipped with a flame-ionization detector. A

*Abbreviations: e.i., electron impact; c.i., chemical ionization; M^+, molecular ion; DMSO, dimethyl sulfoxide; DMF, *N,N*-dimethylformamide; all sugars have the D configuration except for L-rhamnose, L-fucose, and L-arabinose.

capillary glass column (30 m × 0.75 mm, i.d.; film thickness 0.2 μm), coated with SP-2330 and a fused-silica column (30 m × 0.53 mm, i.d.; film thickness 0.2 μm), coated with SP-2380, were used. Samples were injected directly into the SP-2330 column. When the SP-2380 column was used the Model 3700 chromatograph was equipped with a splitter, with the split ratio ranging from 1:7 to 1:15. With the SP-2330 column, nitrogen was used as both the carrier and makeup gas, usually at flow rates of 5 and 40 mL. min^{-1}, respectively, while with the SP-2380 column helium was the carrier gas and the flow rate normally was 5 $mL.min^{-1}$. Data from these two columns were collected with a Hewlett–Packard Reporting Integrator, Model 3390A. Alditol acetates were also separated on a DB-1 fused-silica capillary column (60 m × 0.25 mm, i.d.; film thickness 0.1 μm) attached to a Hewlett-Packard gas chromatograph, Model 5840A, equipped with a splitter, a flame-ionization detector, and a 5840A data terminal. Helium was the carrier gas and the flow rate was 0.8 $mL.min^{-1}$. The split ratio usually was 1:14.

Samples injected into gas chromatographs ranged in size from 0.1 to 1.0 μL when no splitter was used and from 1 to 2.5 μL with a splitter.

Mass spectrometry. — E.i. and c.i. mass spectrometry were performed with a JEOL HX110-HF, double-focusing mass spectrometer interfaced to a Hewlett–Packard gas chromatograph, Model 5890A, which was equipped with a splitless injector and a DB-1 fused silica column (15 m × 0.53 mm, i.d.; film thickness 1.5 μm). Helium was the carrier and makeup gas; the flow rates were 10 and 20 $mL.min^{-1}$, respectively. The ion source temperature was 200° and the ionizing voltage was 70 eV. For c.i. mass spectrometry, ammonia was the reagent gas.

RESULTS

Acetylation with sodium acetate as catalyst. — Complete acetylation of apiitol required 9 h when acetic anhydride at 120° was used and sodium acetate was the catalyst[5]. Samples prepared from apiose by the procedure of Albersheim *et al.*[5] were analyzed with the 30 m SP-2330 column at 200° (see Experimental) at acetylation times of 20 min, 40 min, 1, 1.5, 2, 3, 4, 5, 7, and 9 h. The observed values for apiitol pentaacetate and apiitol tetraacetate were 10.7, 41.4, 59.5, 69.3, 72.3, 85.7, 98.0. 97.0, 99.2, and 100% and 89.3, 58.6, 40.5, 30.7, 27.7, 14.3, 2.0, 2.9, 0.8, and 0%, respectively, calculated from the area under each chromatographic peak, with the sum of each pair of areas set equal to 100%. The acetates were identified by gas–liquid chromatography and mass spectrometry (Figs. 1a and 1b). Based on the fragmentations proposed, ions at *m/z* 289, 303, 319, 347, and 362 would be unique to the pentaacetate. Of these only *m/z* 289 was present in substantial amounts; it had a relative intensity of 16.8 with respect to the base peak* at *m/z* 187. The other ions were not present except for *m/z* 303, having intensity 0.82. In the spectrum of the tetraacetate derivative all these ions were un-

*In this paper: (1) the base ion (peak) was assigned a value of 100, (2) relative intensities are with respect to the intensity of the base ion, and (3) ions with *m/z* of 50 or less were not considered.

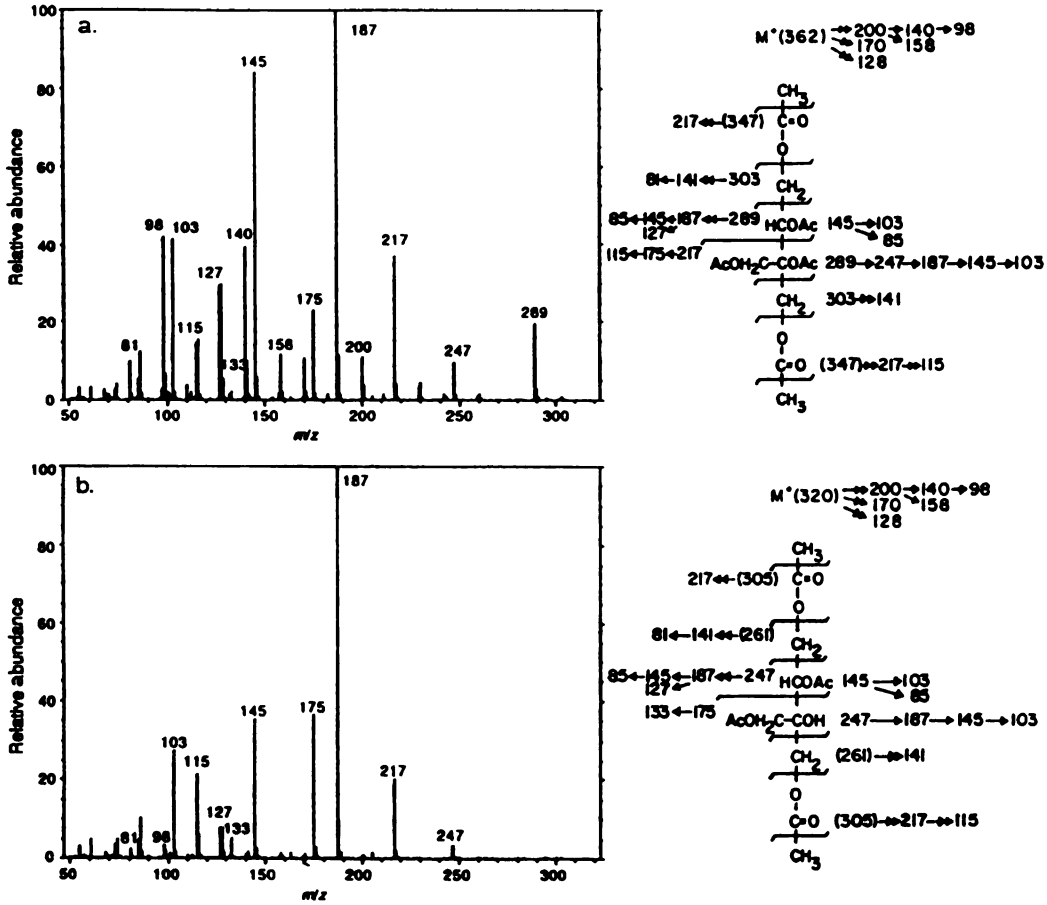

Fig. 1. E.i. mass spectra of (a) apiitol pentaacetate, and (b) apiitol tetraacetate. See Experimental for procedure. Parentheses mean the ion was not detected. Double-headed arrows represent two or more steps. Sequences to minor ions are not shown.

detectable or scarcely apparent. Ion 289 therefore can be used to distinguish between apiitol pentaacetate and apiitol tetraacetate.

According to the fragmentations proposed, the ions at *m/z* 133, 159, 218, 261, 277, 305, and 320 are unique to the tetraacetate. Of these only 133 and 218 were present in the spectrum of the tetraacetate at intensities greater than 0.85; their peak heights were 5.3 and 2.3, respectively. In the spectrum of the pentaacetate, the ions at *m/z* 133 and 218 had relative intensities of 2.8 and 3.8; the remaining ions were of intensity 0.38 or less except for *m/z* 159 at 2.2. The relative intensity of ion 133 was always 2–3 times greater in the spectra of the tetraacetate than in the spectra of the pentaacetate. The reactions proposed to account for the spectra of the two derivatives accounted for 92 and 91%, respectively, of the total ions detected (background ions included, $\leq 0.05\%$ of the total ion current).

Acetylation with 1-methylimidazole as catalyst — When apiitol and acetic anhydride were heated at 35° in the presence of 1-methylimidazole and DMSO for various

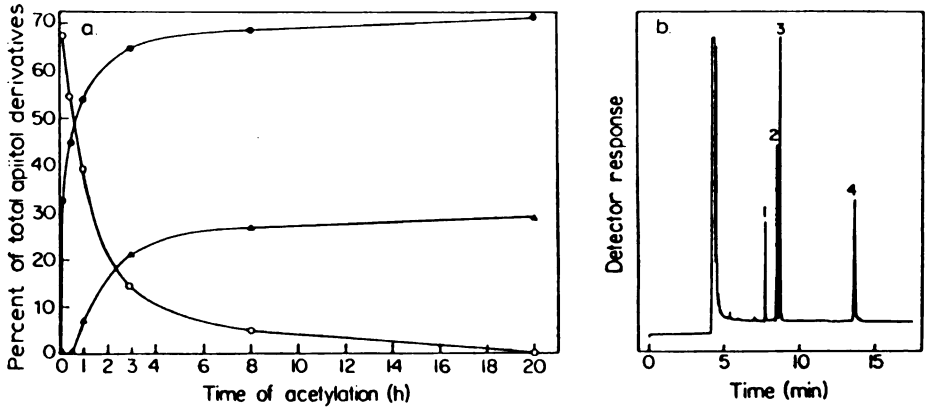

Fig. 2. Acetylation of apiose by the procedure of Blakeney *et al.*[7]. Samples were prepared from apiose as described[7], except that acetylation was performed at 35° for the times indicated. A single extraction with dichloromethane was performed, resulting in underestimation of the proportions of apiitol tetraacetate (see Results). Samples were analyzed with the 60 m DB-1 column as described in the Experimental part. Column operation was isothermal at 200°. (a) Time course of formation of products, showing apiitol tetraacetate (o), apiitol pentaacetate (•), and 3-*O*-(methylthiomethyl)apiitol tetraacetate (▲) as percentages of the total dichloromethane-extracted material at each time point. (b) Gas-liquid chromatogram of the products. Acetylation time for this sample was 5 h, and xylitol pentaacetate in dichloromethane was added to the mixture before g.l.c. Compounds 1, 2, 3, and 4 are apiitol tetraacetate, xylitol pentaacetate, apiitol pentaacetate, and 3-*O*-(methylthiomethyl)apiitol tetraacetate, respectively.

time periods the results shown in Fig. 2a were obtained. A typical separation of the three compounds detected is shown in Fig. 2b. Two of the compounds were identified as apiitol pentaacetate and apiitol tetraacetate by their e.i. mass spectra. These were as shown in Figs. 1a and 1b, with the ions at *m/z* 289 and 133 having the same relative intensities in the respective spectra. The c.i. mass spectrum of the compound considered to be apiitol pentaacetate showed ions at *m/z* 380 ($M + NH_4^+$), 363 ($M + H^+$), and 303; their relative intensities were 100, 0.31, and 55, respectively. When fully deuterated acetic anhydride was used, ions at *m/z* 395 ($M + NH_4^+$), 378 ($M + H^+$), and 315 had relative intensities 28.5, 0.2, and 100, respectively. The c.i. mass spectrum of the compound considered to be apiitol tetraacetate showed ions at *m/z* 338 ($M + NH_4^+$), 321 ($M + H^+$), and 303 at relative intensities 62, 2.7, and 100, respectively. Fully deuterated acetic anhydride gave ions at *m/z* 350 ($M + NH_4^+$), 333 ($M + H^+$), and 315 with intensities 7.5, 0.84, and 100. These data confirm the identity of two of the products (Fig. 2a) as apiitol pentaacetate and apiitol tetraacetate, as well as provide confirmation of the identity of the two compounds formed from apiose when the procedure of Albersheim *et al.*[5] was used.

The third compound in Fig. 2a was identified as 3-*O*-(methylthiomethyl)apiitol tetraacetate by mass spectrometry. The base ion in the e.i. spectrum (Fig. 3) had *m/z* of 61 and probably was mainly $CH_3SCH_2^+$. The fragmentations proposed accounted for 87.5% of the total ions in the spectrum (background included). The c.i. mass spectrum of the compound showed ions at *m/z* 398 ($M + NH_4^+$), 381 ($M + H^+$), and 303 at relative intensities of 100, 0.38, and 55, respectively. Fully deuterated acetic anhydride

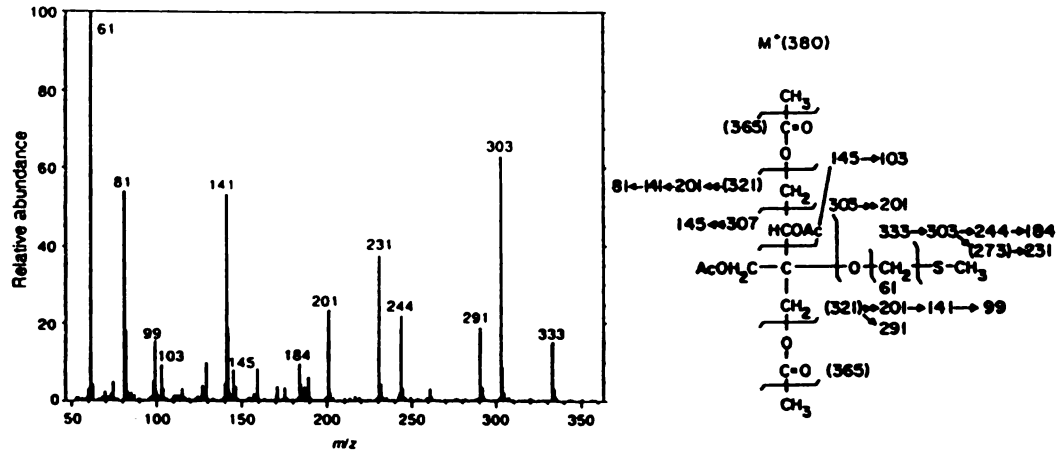

Fig. 3. E.i. mass spectrum of 3-*O*-(methylthiomethyl)apiitol tetraacetate. See legend to Fig. 1.

gave ions at *m/z* 410 ($M + NH_4^+$), 393 ($M + H^+$), and 315, having intensities 24.8, 0.47, and 76.2, respectively. Ion 61 was now the base ion. In addition, the following data further support the identification of the third compound: (1) the compound was not formed when apiitol was acetylated by procedure IV (information presented later), (2) the data in Fig. 2a show that formation of the compound ceased when apiitol tetraacetate was no longer present, (3) the compound was not formed when the other tested acetylation procedures were used with apiitol, and (4) it was not formed when alditol peracetates of rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose were prepared by the procedure of Blakeney *et al.*[7], either as described in the reference or by acetylation for 4 h at 85°.

Acetylation with 4-dimethylaminopyridine as catalyst. — When apiitol was acetylated by acetic anhydride with 4-dimethylaminopyridine as the catalyst[9], only apiitol pentaacetate and apiitol tetraacetate were observed (data not presented). Under our best conditions (9.5 h, 22°, no pyridine added), 96% of the material detected by the gas chromatograph was apiitol pentaacetate, and 4% was apiitol tetraacetate. The yield of apiitol pentaacetate was not increased by acetylating at 40°, increasing the acetylation time to 24 h, adding pyridine together with 4-dimethylaminopyridine, or various combinations of these.

Acetylation procedure for apiose and other sugars — The modification of the procedure of Blakeney *et al.*[7] permitting its use with apiose consisted of replacing DMSO with DMF and increasing the time and temperature of acetylation to 4 h and 85°, respectively. With the new procedure (acetylation procedure IV), greater than 99% of the apiitol recovered was fully acetylated and no side-products were observed. The time course is given in Fig. 4. A small amount of apiitol tetraacetate was still present after 4 h of acetylation at 85° (0.56%) and some was present even after 18 h at 85° (0.17%). However, since these amounts are less than 1%, acetylation for 4 h at 85° should be acceptable for virtually all work.

The degree of reduction of sugars treated with $NaBH_4$ in DMF was examined

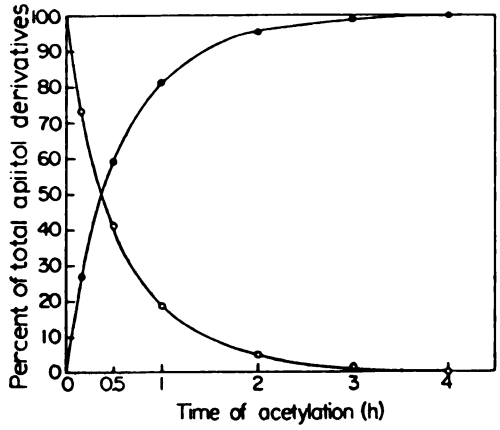

Fig. 4. Formation of apiitol pentaacetate from apiitol and acetic anhydride with acetylation procedure IV. Samples were prepared as described in the Experimental part, except that acetylation times were varied. A single extraction with dichloromethane was performed, resulting in underestimation of the apiitol tetraacetate. Samples were analyzed with the SP-2380 column operating isothermally at 200°. The curves show apiitol tetraacetate (o) and apiitol pentaacetate (•).

after 15, 30, 60, and 90 min at 40°. A sample was prepared that contained the seven sugars listed in the Experimental but no apiose; a second contained only apiose. Sugars were reduced and subjected to acetylation procedure IV, except that the reduction time was varied as indicated above and the sample containing the seven sugars was acetylated for 10 min at 22°. *myo*-Inositol hexaacetate (0.15 mg) was added with the dichloromethane. The relative peak areas of the peracetates of apiitol, xylitol, mannitol, galactitol, and glucitol with respect to *myo*-inositol hexaacetate were maximal at 15 min of reduction and did not change more than ±4% between 15 and 90 min. The relative peak areas of the peracetates of rhamnitol, fucitol, and arabinitol at 15 min were 93, 94, and 93%, respectively, of those obtained at 90 min. The relative peak areas of the peracetates of rhamnitol, fucitol, arabinitol, and the other alditols were not different by more than ±4% at 60 and 90 min. These results showed reduction in DMF proceeded more rapidly than in DMSO[7].

When the acetylation mixture of Blakeney *et al.*[7] was held at 22° for extended periods or heated at 85°, the solution darkened[10] owing to a reaction between 1-methylimidazole and acetic anhydride. When 0.2 mL of 1-methylimidazole, 2 mL of acetic anhydride, and 1 mL of DMF were heated for 4 h at 85° with 0.1, 0.15, or 0.2 mL of M NH_4OH neutralized with glacial acetic acid, the absorbance of the solutions at 420 nm was 1.85, 0.47, and 0.29, respectively. Color formation also decreased when decreasing amounts of 1-methylimidazole were used. Addition of mannitol (a representative alditol) and *myo*-inositol together to the acetylating solution or substituting water for the neutralized ammonium hydroxide solution had no significant effect on color formation.

Completeness of extraction by dichloromethane was examined. Apiose was treated by acetylation procedure IV, except that the sample was heated only 15 min at

85°. A mixture of rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose was subjected to acetylation procedure IV as described. Samples were prepared in duplicate. *myo*-Inositol hexaacetate (0.15 mg) was added with the first 1.0 mL of dichloromethane. Each sample was extracted successively four times, each time with 1.0 mL of dichloromethane, and an aliquot of each organic phase was analyzed by gas chromatography. The recoveries, based on total amount extracted, of compounds present in organic phases 1 through 4, respectively, were: (1) apiitol tetraacetate, 38, 40, 16, and 5.8%, (2) apiitol pentaacetate, 81, 19, 0, and 0%, and (3) *myo*-inositol hexaacetate, 86, 13, 1.0, and 0.2%. The recoveries of the peracetates of rhamnitol, fucitol, arabinitol, xylitol, mannitol, galactitol, and glucitol in organic phases 1 through 3 ranged from 78 to 87, 13 to 21, and 0 to 3%, respectively, and were zero in organic phase 4. Clearly, if apiose is incompletely acetylated and a single extraction with dichloromethane is performed, the amount of apiose originally present in the sample will be seriously underestimated.

When acetylation procedure IV was used with rhamnose, fucose, arabinose, xylose, mannose, galactose, and glucose, the yields of alditol acetates ranged from 104 to 119% of those obtained with the original procedure[7]. When acetylation procedure IV was used, but with a reaction time of 10 min at 22°, the yields of the individual alditol acetates were basically the same as from the original procedure[7].

Separation of alditol acetates. — Complete separation of apiitol and xylitol pentaacetates was achieved on a 60 m DB-1 capillary column (Fig. 2b). When the DB-1 column was used with a temperature program designed for rapid elution, apiitol pentaacetate, apiitol tetraacetate, *myo*-inositol hexaacetate, and the alditol peracetates of rhamnose, xylose, mannose, glucose, and galactose were separated in 15.15 min. The acetates of fucitol and arabinitol were separated from the others but not from each other, and mannitol hexaacetate was not separated from 3-*O*-(methylthiomethyl)apiitol

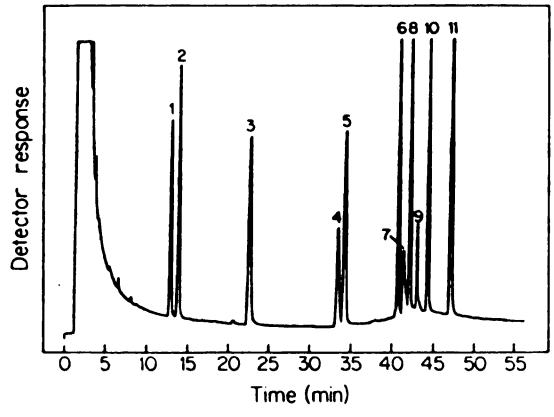

Fig. 5. Gas-liquid chromatogram showing the separation of alditol peracetates and related compounds. The sample was prepared by the procedure of Blakeney *et al.*[7], except that acetylation was for 15 min at 85°. The sample was analyzed with the SP-2380 column programmed to hold at 170° for 32 min, then increase 4°. min^{-1} to 210°, and hold at 210°. Peaks represent the peracetates of the listed alditols, and other compounds as noted: 1, rhamnitol; 2, fucitol; 3, arabinitol; 4, apiitol; 5, xylitol; 6, mannitol; 7, apiitol tetraacetate; 8, galactitol; 9, 3-*O*-(methylthiomethyl)apiitol tetraacetate; 10, glucitol; 11, *myo*-inositol.

tetraacetate. Virtually complete separation of apiitol and xylitol pentaacetates as well as separation of the alditol peracetates of the other six sugars listed above, apiitol tetraacetate, 3-*O*-(methylthiomethyl)apiitol tetraacetate, and *myo*-inositol hexaacetate was achieved in 49 min with the SP-2380 column (Fig. 5). A similar separation of these compounds was achieved in 80 min with the SP-2330 column. The order of elution of the compounds from the latter column was the same as that shown in Fig. 5, except that apiitol tetraacetate eluted immediately before mannitol hexaacetate rather than after. The use of a variety of other carrier-gas flow rates and temperatures with these two columns did not improve the separation of apiitol and xylitol pentaacetates. A number of other columns, both packed and capillary, were tested but gave either no or only partial separation of apiitol and xylitol pentaacetates.

DISCUSSION

Complete acetylation of apiitol is considerably more difficult to achieve than complete acetylation of alditols having only primary and secondary hydroxyl groups. This is shown by the requirement for a 9-hour reaction period at 120° when the procedure of Albersheim *et al.*[5] is applied to apiitol.

The procedure described by Blakeney *et al.*[7] is not suitable for the acetylation of either apiitol or presumably any other alditol possessing a tertiary hydroxyl group because of formation of a methylthiomethyl-ether derivative as a side-product. This might be expected, because the acetylation mixture is analogous to reaction mixtures containing DSMO and acetic anhydride that are used in organic synthesis to oxidize primary and secondary alcohols to the corresponding carbonyl compounds[11,12]. A common side-product in these oxidations is the methylthiomethyl ether of the alcohol[11,12]. In the usual applications of the Blakeney *et al.* procedure acetylation evidently proceeds much more rapidly than oxidation, consequently oxidized sugars have not been observed. When tertiary alcohols are subjected to these conditions oxidation cannot occur, allowing methylthiomethyl ether formation to proceed, often in high yield[13,14]. Moreover, the formation of the methylthiomethyl ether is facilitated by acetic acid[13], which is present in substantial amounts in the Blakeney *et al.*[7] acetylation mixture. We found that heating the acetylation mixture for 4 h at 85° resulted in an organic phase 3-fold larger in volume than those obtained from samples kept at 22° for 10 min or 4 h, perhaps because of the formation of acetoxymethyl methyl sulfide by Pummerer rearrangement[15] of acetoxydimethylsulfonium acetate derived from the DMSO and acetic anhydride present.

Color formation in the acetylation solution can be diminished by increasing the volume of the aqueous sample solution or by decreasing the amount of 1-methylimidazole used, or both. However, both changes have potential for decreasing the yield of alditol acetates. The quantities specified in acetylation procedure IV provide for a full yield of alditol acetates while minimizing color formation. Moreover, this procedure should be suitable for alditols possessing tertiary hydroxyl groups.

The mass spectra presented in Figs. 1a, 1b, and 3 can be explained in large part by

reactions reported in the literature. Particular relevant are processes resulting in the loss of acetic acid, ketene, formaldehyde, acetic anhydride, and water[16,17], but other reactions are also involved. In this connection it is of interest to consider the ion of *m/z* 244, found in the spectrum of 3-*O*-(methylthiomethyl)apiitol tetraacetate, which we suggest must be formed from *m/z* 303. The change involves conversion of a cation having an even number of electrons (*m/z* 303) into an odd-electron cation-radical (*m/z* 244) and a radical (*m* 59), a process that is usually considered energetically unfavorable. However it is not always unfavorable; a recent survey revealed a large number of examples of this type of conversion[18]. The e.i. mass spectrum of apiitol pentaacetate is similar to those of the common alditol acetates, in particular xylitol and arabinitol pentaacetates. The interpretation of these spectra has been extensively discussed[16,17,19].

Separation of apiitol pentaacetate from xylitol pentaacetate by gas chromatography is difficult, but complete or almost complete resolution of the two was achieved with three columns. On the polar columns, SP-2330 and SP-2380, apiitol tetraacetate overlapped other compounds because of tailing (Fig. 5). However, when acetylation procedure IV is used apiitol tetraacetate will not be present.

In previous analyses of natural materials apiose may not have been detected either because the acetylation of apiitol was incomplete and the resulting apiitol tetraacetate was not detected (or not identified), or because apiitol pentaacetate was not separated from xylitol pentaacetate, or both. With the methods described here, the separation, identification, and quantitative determination of apiose is possible.

ACKNOWLEDGMENTS

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