This is to certify that the

dissertation entitled

Isolation and characterization of 22°C chelator-soluble pectic polysaccharides of *Lemna minor*

presented by

LIANG CHENG

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Biochemistry

Paul K. Kindel
Major professor

Date December 5, 1996

ISOLATION AND CHARACTERIZATION OF 22°C CHELATOR-SOLUBLE PECTIC POLYSACCHARIDES OF *Lemna minor*

By

Liang Cheng

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1996

ABSTRACT

ISOLATION AND CHARARCTERIZATION OF 22°C CHELATOR-SOLUBLE PECTIC POLYSACCHARIDES OF *Lemna minor*

By

Liang Cheng

The methods used to isolate, purify and characterize pectic polysaccharides have significant limitations. Procedures were developed for the isolation of cell walls and for the extraction, purification and characterization of pectic polysaccharides. The chelator-soluble pectic polysaccharides were isolated from cell walls of *Lemna minor* and characterized.

Procedure were developed for preparing purified cell walls. Chelator-soluble pectic polysaccharides were solubilized under non-degradative conditions and accounted for 31.7% (by weight) of the total pectic polysaccharides of the cell walls.

The recovery of pectic polysaccharides from diethylaminoethyl (DEAE) anion-exchange columns was found to be greatly influenced by cations. Six pectic polysaccharides from citrus, apple, duckweed, and celery were quantitatively (or nearly so) eluted from DEAE-Trisacryl columns by 0.5 M NH_4Cl in ammonium oxalate buffer. Li^+ and Cs^+ are as effective as NH_4^+ but the presence of Na^+ or K^+ resulted low sample recovery. A procedure for purifying pectic polysaccharides from cell walls was developed.

Homogeneity of polysaccharides with respect to sugar composition, molecular size and methylester content was established by analyzing the polysaccharide material in individual DEAE column fractions with micro-scale methods. Laser light scattering was used with high

performance anion exchange chromatography to determine true $\overline{M}_w$. Two chemically homogeneous pectic polysaccharides, PS-IIb and PS-IVb, were isolated from *L. minor*.

PS-IIb is an apiogalacturonan with a peak $\overline{M}_w$ range of 28,100 - 99,700. It consists of a backbone of α-1,4-linked D-galacturonic acid units with 1,3'-linked apiobiose as the predominant side chains attached to the *O*-2 positions of approximately every other galacturonosyl residues. Terminal-linked rhamnose (2-3%), xylose (2.5%), arabinose (1.4%) and trace amounts (<0.5%) of fucose, mannose, and glucose were detected in PS-IIb.

PS-IVb is as an α-1,4-linked homogalacturonan with a peak $\overline{M}_w$ range of 53,300 to ≥163,000. PS-IVb consists of 96.3% non-esterified galacturonic acid units, apiose (2.1%), and xylose (1.6%). Trace amounts of rhamnose, fucose, arabinose, mannose, and glucose were detected in PS-IVb. PS-IVb was virtually completely degraded by poly-[1,4-α-D-galacturonide] glycanohydrolase (EC 3.2.1.15).

A micro-scale method for depolymerizing pectic polysaccharides was developed.

To my parents, my wife and my son

ACKNOWLEDGMENTS

I am truly grateful to my graduate advisor and mentor, Dr. Paul K. Kindel, for offering me an opportunity to work with him, for his guidance, support, and patience, and for being always willing to help me whenever I needed. I was benefited tremendously from his advice, both in science and in general, over the years.

I thank the members of my committee, Dr. Rawle I. Hollingsworth, Dr. Estelle McGroarty, Dr. Jack Preiss and Dr. Robert Bandurski, for their guidance and valuable suggestions.

A special thanks goes to Dr. Hollingsworth for allowing us to have weekly joint-group meetings in the past three years and for his generous advice on carbohydrate chemistry. Thanks also go to all members in Dr. Hollingsworth's group for their friendship and helpful discussions.

I thank Dr. Douglas Gage and Ms. Bev Chamberlin of Mass Spectrometry Facility for their assistance. I am grateful for Dr. Gage's participation in my final examination in defense of the dissertation. I also owe thanks to Dr. Long D. Le of NMR Facility for his technical assistance.

Thanks to all my Chinese friends in MSU, especially in Biochemistry department, who shared wonderful times with me when cooking and enjoying Chinese food, playing with computer and chatting together. You made the years in Lansing full of fun.

Finally, I am most grateful to my parents for their unconditional love, consistent support and faith in me, and to my wife and son, whose love and full cooperation are the endless source of my energy.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Api	Apiose
Ara	Arabinose
AUA	Anhydrogalacturonic acid
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CDTA	1,2-Cyclohexanediaminetetraacetic acid
DE	Degree of esterification
DEAE	Diethylaminoethyl
DM	Degree of methylesterification
DMSO	Dimethyl sulfoxide
EDAC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
FID	Flame ionization detector
f	Furanose
Fuc	Fucose
Gal	Galactose
GalA	Galacturonic acid
GC	Gas chromatography
GC-MS	Gas chromatography-mass spectrometry
Glc	Glucose
HPAEC-PAD	High performance anion exchange chromatography - pulse amperometric

	detection
HPLC	High performance liquid chromatography
HPSEC	High pressure size exclusion chromatography
MALLS	Multi-angle laser light scattering
Man	Mannose
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
PC	Personal computer
PGUA	Polygalacturonic acid
PMAA	Partially methylated alditol acetate
p	Pyranose
Rha	Rhamnose
RG-I	Rhamnogalacturonan-I
RG-II	Rhamnogalacturonan-II
RRI	Relative refractive index
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
t-	Terminal-
TFA	Trifluoroacetic acid
Xyl	Xylose

CHAPTER I

LITERATURE REVIEW

INTRODUCTION

Cell walls of growing plants provide mechanical support and a protective barrier for cells. They are also dynamic in their shape, composition and properties in response to growth, stage of differentiation, environment, pathogen invasion, and their activities including signal conducting and regulating enzyme activity. If we can alter the composition and the structure of plant cell walls, it may be possible to make them more resistant to pathogenic and environmental stress. It also may be possible to improve them as a clean, economical, and renewable source of biomass for fuel and other chemicals. But before we can alter the plant cell walls through molecular biological techniques, we have to know how the cell wall polysaccharides are synthesized and this in turn requires that their structure be known. Our knowledge about plant cell walls and their constituent polysaccharides lags behind that of proteins and nucleic acids due to: (i) technical difficulties in isolating "intact" polysaccharides from cell walls, (ii) the heterogeneous nature (both in size and composition) of the polysaccharides, and (iii) the fact that the sugar sequence of cell wall polysaccharide is not directly specified by a template. Much of the current research on plant cell walls is still based on identifying and elucidating the covalent structures of the macromolecular components of the walls.

Pectic polysaccharides are present in all higher plants. They are found in the primary cell wall, usually as the most abundant component. Pectic polysaccharides are extracted from the middle lamella and primary cell wall with hot water, chelating agents, dilute alkali, weak acids and enzymes such as endo-1,4-α-polygalacturonase (EC 3.2.1.15) (1-7). Three

types of pectic polysaccharides or pectic polysaccharide units, homogalacturonan, RG-I and RG-II, have been isolated from a number of different plants and characterized (8,9). Pectic polysaccharides of the cell wall of *Lemna minor* have a somewhat different sugar composition than the pectic polysaccharides from other plants in that apiose is the main neutral sugar (10-13). Isolation and characterization of pectic polysaccharides from other plants with apiose as a major component has not been reported, although apiose is widely distributed in higher plants (14) and results suggest it is present in polysaccharides (15,16).

In this review, recent models of primary cell walls of plants are discussed. The major types of known pectic polysaccharides are reviewed including the partially characterized, apiose-containing pectic polysaccharides of *L. minor*. Physical and chemical properties of pectic polysaccharides related to their solubilization, purification and function in cell walls are examined. Current problems in fractionation, purification and homogeneity of pectic polysaccharides are discussed. Literature reviewed in this chapter will provide us with a general understanding of cell wall pectic polysaccharides. Issues related to each specific chapter are reviewed in the Introduction of that chapter.

LITERATURE REVIEW

Primary cell wall of plants

Primary cell walls of plants are highly hydrated. On a dry weight basis, they consist of ~90% polysaccharides and ~10% proteins (9,17). Recently, models for the primary cell walls of flowering plants (type I cell wall) and the Poaceae (type II cell wall) were proposed by Carpita and Gibeaut (18) based on the structures of known polysaccharides and the physical properties of cell walls. In their model, the type I cell wall has a skeleton of cellulose microfibrils (Figure 1.1a) which is interlaced and interlocked by xyloglucan polymers (Figure 1.1b) through hydrogen bounding and the cellulose microfibrils-xyloglucan framework (about 50% of the wall mass) is embedded in a matrix of pectic polysaccharides (about 30% of the wall mass). The type II cell wall is also composed of a cellulose microfibrils network (Figure 1.2a) but it is interlocked by glucuronoarabinoxylans (Figure 1.2b) instead of xyloglucans. A smaller amount of pectic polysaccharide is found in the type II primary cell wall (19) and large portions of the non-cellulosic polymers are "wired on" (18) the microfibrils by phenolic cross-links (Figure 1.2a). In both type I and type II cell walls, the major pectic polysaccharides proposed are polygalacturonic acid and rhamnogalacturonan (RG); the latter are substituted with polysaccharide side chains of arabinan, galactan and arabinogalactan. Adjacent pectic polysaccharide chains can associate to form junction zones through their free carboxyl groups and Ca^{2+} bridges (20-22). Pores are formed when the junction zones are separated and spaced by the degree of esterification and the side chain-bearing regions of the pectic polysaccharides. It has been suggested that

Figure 1.1. Proposed primary cell wall of most flowering plants, designated type I. Taken from reference (18). (a) Representation of a single stratum of the cell wall just after formation in dividing cells of a meristem. Several strata such as this coalesce to form a wall (see text for a further description of the wall). (b) A unit structure of xyloglucan in type I cell walls. The basic heptasaccharide repeating unit structure may bear additional substitutions as indicated.

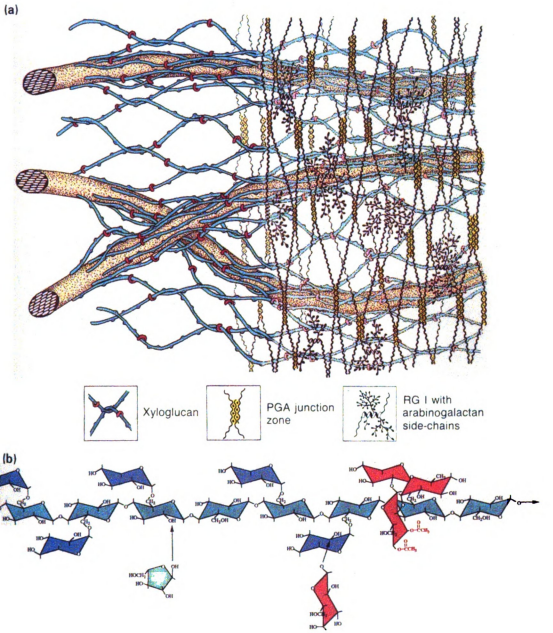
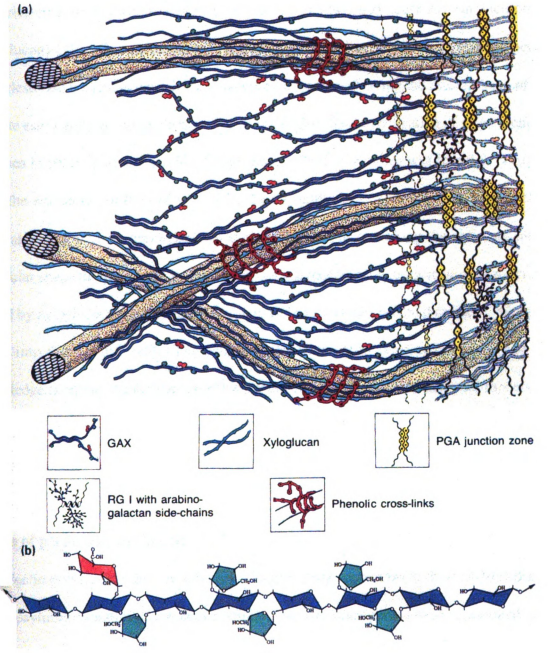

(a)
Xyloglucan
PGA junction zone
RG I with arabinogalactan side-chains
(b)

Figure 1.2. The proposed type II cell wall. Taken from reference (18). (a) Representation of a single stratum of microfibrils just after cell division. The microfibrils are interlocked by glucuronoarabinoxylans (GAX). A substantial portion of the non-cellulosic polymers are 'wired on' the microfibrils by alkali-resistent phenolic linkages. (b) A unit structure of the highly substituted glucuronoarabinoxylan.


(a)
GAX
Xyloglucan
PGA junction zone
RG I with arabino-galactan side-chains
Phenolic cross-links
(b)

the expansion of the cell wall during growth is modulated by pectic polysaccharides through pore size (barrier or opening to enzymes) (23), which, in turn, is affected by degree of methylesterification (18), availability of Ca^{2+} ions (24), and local pH (in vicinity of xyloglucan) (18). One mechanism proposed for controlling growth was that the pectin methylesterase located in the cell wall controlled the pH by cleaving the ester linkage of the uronate esters and converting them to free acids (25,26). The esterification of galacturonosyl residues in pectic polysaccharides of maize embryonal coleoptiles was found to correlate with the elongation of the cell wall (27). Extensin, the hydroxyproline-rich glycoprotein (28), also may play an important role in the mechanism of locking the cell wall into a particular shape after elongation; the microfibrils arranged in the transverse axis are cross-linked by xyloglucans in the longitudinal axis and by extensins oriented radially, "like pins stuck into thick cloth" (28). The details of the structure of primary cell walls and our knowledge about its metabolism are still far from complete. Since the cell wall models are based heavily on the structure of known polysaccharides, the isolation and characterization of additional polysaccharides will greatly improve our knowledge about plant cell walls.

Types of pectic polysaccharides

Pectic polysaccharides are a family of acidic polysaccharides with a 1,4-linked α-D-galactosyluronic acid-rich backbone which may be interspersed with 1,2-linked α-L-rhamnosyl residues, and have neutral sugar side chains attached. The carboxyl groups of the galacturonosyl residues may be free or partially or fully methyl esterified (8). Pectic polysaccharides without or with only a negligible content of methyl ester groups are pectic

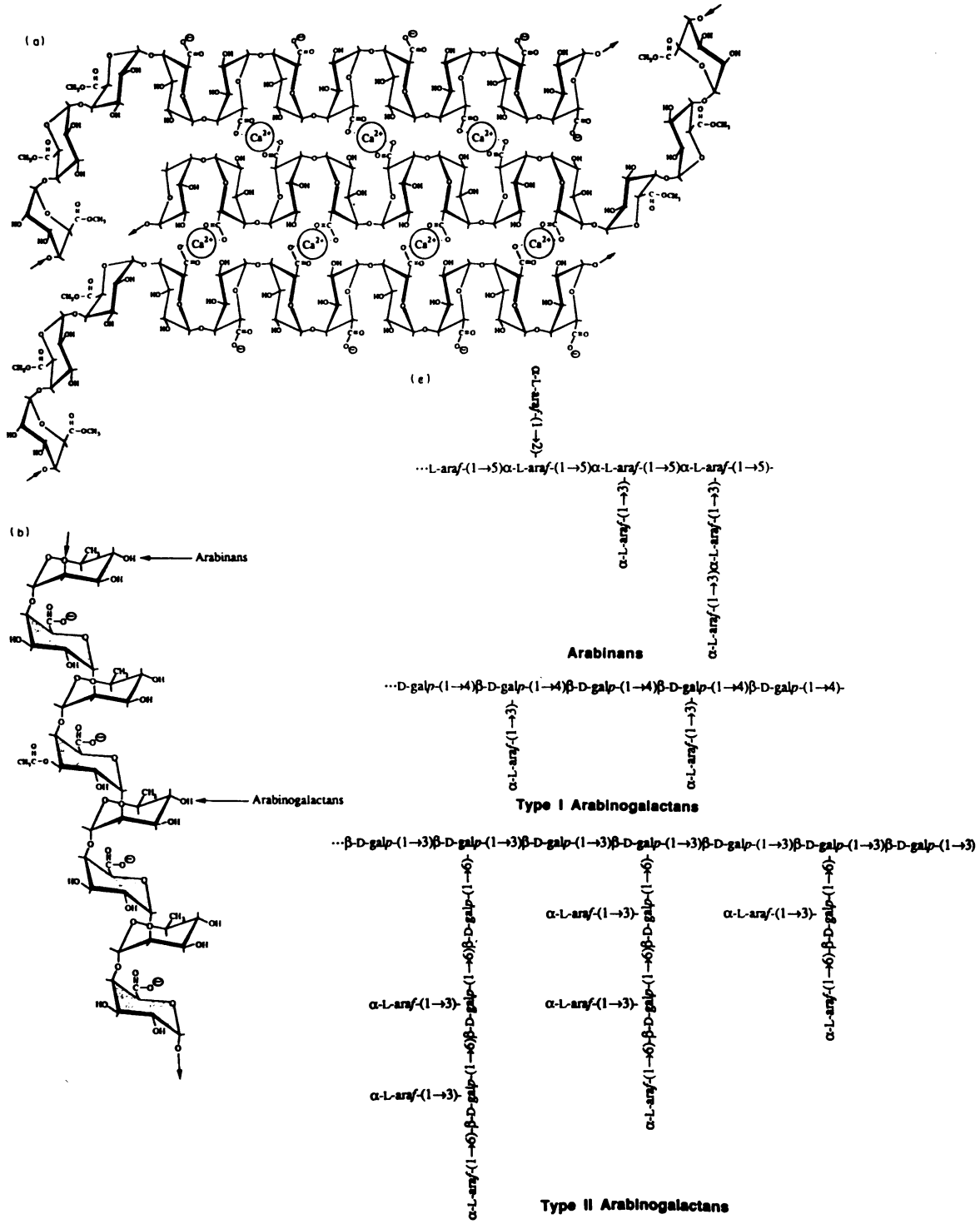
acids, and those with "various, but greater than negligible, contents of methyl ester groups" are pectins (29). Plant cell wall pectic polysaccharides have been classified as one of three types: homogalacturonan, rhaminogalacturonan I and rhaminogalacturonan II.

Homogalacturonan. Homogalacturonans are defined as polymers consisting solely or "predominantly" (9) (i.e. containing ~90% or higher GalA) of α-1,4-linked-D-galacturonosyl residues. A homogalacturonan with 3% galactose was isolated from suspension-cultured *Rosa* cell walls by sequential treatment with 2.5 M NaOH, 1.0 M H_2SO_4 at 120°C, and 0.15 M NaOH (30). This polymer was not purified by column chromatography (homogeneity was not determined) and the molecular size and degree of esterification are unknown. Two homogalacturonans containing 93.9% and 92.7% galacturonic acid were solubilized by hot water from *Zea* shoots (31) but their degree of esterification was not determined. A pectic polysaccharide containing 91% galacturonosyl residues and with a relative $\overline{M}_w$<10,000 (relative to dextran) was purified from rice endosperm cell walls that were treated with glucoamylase and protease and extracted with 0.25% ammonium oxalate at 90°C for 24 h (32); the degree of esterification was not determined. This suggests the possible presence of homogalacturonans in monocots. Homogalacturonans were also found in the media of tobacco (87% galacturonic acid) (33) and sycamore (95% galacturonic acid) cell suspension cultures (9). Recently, homogalacturonan fractions with > 98% galacturonic acid residues and degrees of polymerization of 72-100 were isolated from de-esterified pectic substances of citrus (DM = 72), apple (DM = 75) and sugar beet (DM = 54) with 0.1 M HCl at 80°C for 72 h (34). However, no pure homogalacturonan has been isolated from primary cell walls without using

treatments that are likely to cleave covalent bonds. In addition, the size homogeneity of isolated homogalacturonans has not been established.

Rhamnogalacturonan-I. RG-I, the pectic polysaccharide first isolated from the cell walls of suspension-cultured sycamore cells after the treatment of cells with endo-α-1,4-polygalacturonase, has been studied in some detail (35-39). With a molecular weight of about 200,000 (estimated by comparing the mobility of RG-I with that of globular proteins and dextrans on an agarose A-5m column), the backbone of RG-I consists of chains of α-1,4 linked D-galacturonosyl residues interspersed with α-1,2-linked L-rhamnosyl residues (Figure 1.3b). Approximately half of the 1,2-linked rhamnosyl residues are branched and at least 30 different side chains have been found attached to O-4 of the rhamnosyl residues (9). The side chains are rich in L-arabinosyl and D-galactosyl residues, although small amounts of other sugars, such as D-xylose and L-fucose, were also found (35,40). Three major types of side chains are arabinans, type I arabinogalactans and type II arabinogalactans (Figure 1.3c) (18). Glucuronic acid and 4-*O*-methyl-glucuronic acid residues were found to be components of the galactosyl-containing side chains of RG-I (39). The distribution of the rhamnosyl residues (with attached side chains) in the backbone is not even. Both regions free of rhamnosyl residues and side chains, "smooth regions", and the regions with a high frequency of side chain-attached rhamnosyl residues, "hairy regions", were found. The backbone of "smooth regions", corresponding to 25 and >72-100 galacturonosyl residues, were isolated from cell walls of citrus, apple, sunflower (41) and sugar-beet (34) with mild acid hydrolysis. Fragments of "hairy regions" of the RG-I backbone, with molar ratios of rhamnosyl to galacturonosyl residues of 1-2.3:6.7 (total neutral sugar composition is 79-

Figure 1.3. Diverse structures of the pectic polysaccharides. From reference (18). (a) An example of 'eggbox' junction zone. Anti-parallel chains of polygalacturonic acid cross-linked at unestrified regions by Ca^{2+} bridges. (b) A section of RG-I backbone. Galacturonic acid residues (grey shadded) are interspersed with 1,2-linked rhamnosyl residues. Arabinan and arabinogalactan side chains are attached to the 4-O position of the rhamnose. (c)Three types of side chains that attached to about half of the rhamnosyl residues of RG-I.


(a)
(b)
Arabinans
Arabinogalactans
(c)
α-L-araf-(1→2)-
···L-araf-(1→5)α-L-araf-(1→5)α-L-araf-(1→5)α-L-araf-(1→5)-
α-L-araf-(1→3)-
α-L-araf-(1→3)α-L-araf-(1→3)-
Arabinans
···D-galp-(1→4)β-D-galp-(1→4)β-D-galp-(1→4)β-D-galp-(1→4)β-D-galp-(1→4)-
α-L-araf-(1→3)-
α-L-araf-(1→3)-
Type I Arabinogalactans
···β-D-galp-(1→3)β-D-galp-(1→3)β-D-galp-(1→3)β-D-galp-(1→3)β-D-galp-(1→3)β-D-galp-(1→3)β-D-galp-(1→3)
α-L-araf-(1→3)-
α-L-araf-(1→3)-
α-L-araf-(1→3)-
α-L-araf-(1→3)-
α-L-araf-(1→6)-β-D-galp-(1→6)-
α-L-araf-(1→6)-β-D-galp-(1→6)-β-D-galp-(1→6)-
α-L-araf-(1→6)-β-D-galp-(1→6)-β-D-galp-(1→6)-
Type II Arabinogalactans

83%)(42), 1:1.5-2.9 (total neutral sugar composition is 30-60%) (34) and 1:3.6-4.8 (total neutral sugar composition is 72-82%) (43) were isolated from cell walls of sugar-beet, apple, citrus and lemon peel with endopolygalacturonase (42), HCl hydrolysis (34) and "Rapidase C600" (Gist Brocades, Deft, The Netherlands) (43,44). The "hairy region" of RG-I was found to be degraded by rhamnogalacturonase (44,45). Pectic polysaccharides of the RG-I type have been found widely distributed in plants, both monocots and dicots, such as tobacco (46), potato (47), onion (48), alfalfa (49), pea (50), maize and rice (51,52).

Rhamnogalacturonan II. RG-II is another pectic polysaccharide originally isolated from suspension-cultured sycamore cells after the treatment of cells with endo-α-1,4-polygalacturonase (6). RG-II is much smaller than RG-I (molecular weight ~11,000), 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*-methylxylosyl, apiosyl and 3-*C*-carboxyl-5-deoxy-L-xylosyl (aceric acid) residues (53,54). Pectic polysaccharides of the RG-II type are found in the primary cell walls of other plants such as pea, pinto bean, tomato (6), potato (47), onion (48), and rice (51,55) which suggests that RG-II type pectic polysaccharides are common in plant cell walls. Since RG-II is solubilized from primary cell walls with either endo-α-1,4-polygalacturonase or strong base and is usually present in small quantities, it may be covalently linked as a large side chain to a larger cell wall polysaccharide.

Apiogalacturonan. Pectic polysaccharides isolated from cell walls of *L. minor* with 0.5% ammonium oxalate were characterized as apiogalacturonan (10-12). Apiose accounted for 25.2-27.9% (10) and 7.9-38.1% (11) of the apiogalacturonan and was the major neutral

sugar component. The results showed that apiogalacuronan are composed of a linear galacturonan backbone with D-apiose and apiobiose side chains (11,12). Homogeneity with respect to molecular size and sugar composition was not determined for apiogalacturonans. Apiose is present in small amounts in RG-II (6,54) and appears to be present in more substantial amounts in polysaccharides from *Zosteraceae* (15,16) and *Lemna gibba* (56). Evidence has been presented that apiose is also a constituent of polysaccharides in *Posidonia australis* and *Tilia* sp. (57). Duff (14) 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 apiose in polysaccharides, his findings showed that apiose is relatively widely distributed in the plant kingdom. Also, the apiose content of samples possibly is being underestimated by researchers who determine sugar composition by GC-MS analysis of alditol acetates, since apiitol and xylitol peracetates have almost identical GC retention times and mass spectra (6,58).

When apiogalacturonans were isolated from *L. minor* a number years ago (10-12), paper chromatography was used to identify apiose. Results from GC-MS reported later showed small amounts of rhamnose, xylose and several other neutral sugars are present in these apiogalacturonans (13). In the earlier work, the homogeneity of these polysaccharides was not determined. Also the molecular size of the polysaccharides and the position of attachment of the side chains to the backbone were not determined.

Properties of pectic polysaccharides

Interaction of pectic polysaccharides — formation of junction zones, egg box structures and gels. The apparent pK_a of the carboxyl groups of polygalacturonic acid is 4.10 (59). Esterification of carboxyl groups in the polysaccharide lowers the pK_a; the apparent pK_a of a pectin with a DE of 65% is 3.55 (59). In living plant cells most of the unesterified carboxyl groups of the uronic acid residues of pectic polysaccharides are deprotonated and usually interact with cations such as Ca^{2+}. In aqueous solution, the polygalacturonic acid portion of the backbone folds into a 2_1-helix conformation (2 stands for two galacturonosyl residues per conformational repeat unit; the subscript 1 stands for one turn of the helix per repeat unit)(22,60,61). With formation of ionic and hydrogen bonding, the helical chains of the polygalacturonic acid portion of the backbones can interact with each other intermolecularly to form "junction zones"(62-65). A junction zone is the complex formed when the portions of two or more anti-parallel helical chains are cross-linked by Ca^{2+}. Their structure has been postulated to be like a "egg box" (Figure 1.3 a) (21,41, 61). The number of contiguous unesterified uronic acid residues needed to form stable junction zones and the extent to which several chains can stack together remain unknown both *in vivo* or *in vitro*. Results of statistical calculations suggest that at low Ca^{2+} concentration, a minimum of 14 galacturonic acid units from each chain are needed to from a stable junction zone (21,41). If sufficient Ca^{2+} is present, some interrupting methyl esterified galacturonic acid residues can be tolerated in the stable junction zone. When excess Ca^{2+} is available, multiple polygalacturonic acid chains can stack together to form a calcium pectate gel (21,66). The 1,2-linked rhamnosyl residues in RG-I interrupt the junction zones by forming "kinks" in the

polysaccharide backbone (21,41). Side chains attached to the backbone also prevent formation of the junction zones. It was suggested that pectic polysaccharides in some species can be cross-linked to each other and to other non-cellulosic polysaccharides by ester linkages involving dihydroxylcinnamic acid derivative such as diferulic acid (67).

At sufficiently low pH and in the presence of sufficient sugar, pectic polysaccharides can form another type of gel, the "acid gel", without participation of Ca^{2+}. The junction zones in the acid gel form when the number of charges on the polygalacturonic acid backbone are reduced enough so that sufficient interchain hydrogen bonds are formed (29). Pectins with higher DE have less charge so they can form a gel at a higher pH. At constant pH, the gel strength of an acid gel increases with increasing DE of the pectin. Increasing the concentration of a monovalent cation, such as Na^+ and K^+, will result in decreasing hydrogen bonding in junction zones and thereby decreasing gel strength and reducing precipitation, and as a result a "salting in" occurs, although too high a concentration of monovalent cations may cause precipitation (68).

Stability of pectic polysaccharides. The glycosidic linkage of unesterified uronic acid residues in pectic polysaccharides is quite resistant to acid hydrolysis (69). Esterified or partial esterified polysaccharides are most stable at about pH 4 (29). Lower pH causes hydrolysis of some acid-labile glycosidic bonds such as as those involving L-rhamnopyranosyl (29,70), L-arabinofuranosyl (69) and D-apiosyl residues (12) in pectic polysaccharides. At pH 4.5, apiose and apiobiose side chains can be completely released from apiogalacturonans in 3 h at 100°C (12). At pH values of 5-6, pectins are stable only at room temperature. At high pH, β-elimination causes depolymerization of pectin (71,72).

Pectin breaks down rapidly at pH 6.8 with elevated temperature (73,74) through β-elimination (74). Since the stability or solubility or both of pectic polysaccharides in solution change with changes in concentration of polysaccharide, pH, and concentration and type of cation, great care must be taken to avoid degradation, precipitation and gel-formation throughout the isolation, purification and characterization processes.

Fractionation and purification of plant cell wall pectic polysaccharides and determination of homogeneity

Generally, a portion of the pectic polysaccharides of plant cell walls are extracted by hot water, dimethyl sulfoxide (DMSO) (51), enzymes such as endo-α-1,4-polygalacturonase (37), chelating agents such as ammonium oxalate, EDTA, and CDTA and weak base such as Na_2CO_3 (51). Pectic polysaccharide fractions that were solubilized from cell walls by sequential extraction with different reagents, such as hot water, ammonium oxalate, dilute acid and alkali solution, in some cases were directly characterized (sugar composition and glycosidic linkage) without further purification and without establishing homogeneity (4,42,43,75). In these cases, there is a possibility that different polysaccharides could be extracted together in a single extraction and then would contaminate each other. Usually pectic polysaccharides with different sugar compositions and sizes are co-solubilized with an individual extractant (4,6,27,35,46,49,76-84) and they need to be purified further before characterization. In addition, other polysaccharides such as arabinans (85), galactans (86) and arabinogalactans (87) can be co-solubilized with pectic polysaccharides even when mild non-degradative conditions (e.g. chelating agents) are used. It is important to establish the

homogeneity of the polysaccharides to be characterized in order to obtain accurate structural information. Commonly, purification of extracted cell wall polysaccharide involves the use of anion-exchange chromatography or size exclusion chromatography or both. In some cases, extracted polysaccharides were pre-fractionationed by precipitation with $(NH_4)_2SO_4$ (31), ethanol or Cetavlon (cetyltrimethylammonium bromide) (82,83) before being applied to columns. Pectic polysaccharides have been purified with anion exchange material such as DEAE-cellulose (4,15,32,46,50,88-90), DEAE-Sephacel (76,77), DEAE-Sephadex (6,11,27,31,35,76,78,82,91,92), DEAE-Sepharose CL-6B (83,84,93), DEAE-Spectra/Gel M (49), and DEAE-Trisacryl (5,94,95). Selective elution of pectic polysaccharides from the column is effected by stepwise or linear gradient elution with salt solutions or buffers. Usually, the eluted fractions of pectic polysaccharides were tested for uronic acid or total sugar or both, and fractions were pooled according to the uronic acid and total sugar peaks. The pooled fractions were directly characterized (such as sugar composition analysis) (4,5,27,43,49,82) or characterized after further purification by size exclusion chromatography with Agarose A-5 (32,35), Sephadex 4B (32), Bio-Gel A 5 m (46), Sepharose CL-2B (4), Sepharose CL-4B (46), Sepharose CL-6B (4,31,83), Bio-Gel P-10 (6), and Sephacryl S-500 (84). In some cases rechromatographed by anion exchange column (31,77) was performed. Pectic polysaccharides extracted from plant cell walls were also directly purified with size exclusion chromatography (43,50,51,81). However, in all the above, except one report by Yamada's group (83), fractions were pooled based on the elution profiles of total sugar or uronic acid after either ion exchange or size exclusion chromatography. But the homogeneity of polysaccharides in the fractions was not established before they were pooled.

A single uronic acid or total sugar peak on either ion-exchange or size exclusion chromatography does not prove the polysaccharide material is chemically or physically homogeneous. For example, a pectic polysaccharide fraction, GR-2IIa, solubilized from the root cell walls of *Glycyrrhiza uralensis* and purified by DEAE-Sepharose CL-6B showed a single, nearly symmetrical peak of uronic acid (as well as total sugar) on size exclusion chromatography (Sepharose CL-6B) (83). But analysis of the individual fraction across the peak with size exclusion HPLC (Asahi-pak GS-510 + Gs 320) resulted in detection of five polysaccharides whose molecular size varied from 2.0×10^4 to 1.9×10^5 and galacturonic acid contents varied from 41.8% to 85.2% (83). In another example, a pectic polysaccharide fraction extracted from purified sugar beet cell walls showed a single uronic acid and total sugar peak on ion-exchange chromatography (DEAE-Sepharose CL-6B, linear gradient of sodium succinate 0.05-1 M, pH 4.8), but three peaks were observed when it was fractionated by Sephacryl-S500 size exclusion chromatography (84). With the development of an improved micro-scale method of sugar composition analysis (Chapter IV), the development of a procedure for determining $\overline{M}_w$ on small quantities of polysaccharide by using HPSEC with MALLS (Chapter V), and the use of a micro-scale methyl ester method (96), the homogeneity with respect to sugar composition, degree of methyl esterification and $\overline{M}_w$ can be established for polysaccharide material present in individual fractions from ion-exchange or size exclusion chromatography. This can undoubtedly help us to decide how to pool fractions with same type of polysaccharide and obtain pure samples for characterization.

Since a mixture of polysaccharides is often extracted with a single extractant (e.g. a chelating agent), incomplete recovery of the polysaccharides from the purification procedure

can result in biased information about the character of the polysaccharides in the cell wall. Poor recovery of pectic polysaccharides has been observed from many types of ion-exchange columns (95). Sample recovery of pectic polysaccharide of 51%, 82 to 85%, 0 to 87%, 32 to 88% and 24 to 51% have been reported with columns of DEAE-Trisacryl (1), DEAE-Cellulose (88,89), DEAE-Sephadex (31,76,91), DEAE-Sephacel (77,78,97-100), DEAE-Sepharose CL-6B (4,101), respectively. Some pectic polysaccharides adsorb strongly onto the cellulose resin and require the use of strong reagents, such as urea or 0.5 M NaOH, to release them (88). Chromatography procedures with high recovery of pectic polysaccharide using non-degradative conditions need to be developed.

OBJECTIVE

The overall goal of this work is the isolation and characterization of the 22°C chelator-soluble pectic polysaccharides of cell walls of *L. minor*. Chapter II describes a simple method for the isolation of cell walls and an improved method for polysaccharide extraction. Chapter III discusses the effect of cations on the elution of pectic polysaccharides from anion exchange resins. A quantitative method has been developed for fractionation and elution of pectic polysaccharides from anion exchange columns. In Chapter IV, an improved micro-scale method of depolymerization of pectic polysaccharide samples for GC-MS and HPLC analysis is described. The homogeneity of the purified pectic polysaccharides, with respect to molecular size, sugar composition and degree of methyl esterification, are examined in Chapter V. Chapter VI provides information on the questions: How are the sugar residues

in two purified pectic polysaccharides, PS-IIb and PS-IVb, linked? What is the primary structure of these two polysaccharides? Results of methylation analysis, partial hydrolysis and enzyme degradation are discussed. In the last chapter, Chapter VII, general conclusions are made for knowledge gained in the previous chapters and directions for future research are suggested.

REFERENCES

1. Selvendran, R. R. (1985) *J. Cell Sci. Suppl.*, **2**, 51-88.
2. Redgwell, R. J. and Selvendran, R. R. (1986) *Carbohyr. Res.*, **157**, 183-199.
3. Selvendran, R. R., Stevens, B. J. H. and O'Neill, M. A. (1985) in *Biochemistry of Plant Cell Walls*, SEB Seminar Series, Vol. 28 (Brett, C. T. and Hillman, J. R., Eds.) Cambridge University Press, pp. 39-78.
4. Rombouts, F. M. and Thibault, J.-F. (1986) *Carbohydr. Res.*, **154**, 177-187.
5. Selvendran, R. R. and O'Neill, M. A. (1987) *Methods Biochemical Analysis*, **32**, 25-153.
6. Darvill, A. G., McNeil, M. and Albersheim, P. (1978) *Plant Physiol.* **62**, 418-422.
7. English, P. D., Maglothin, A., Keegstra, K. and Albersheim, P. (1972) *Plant Physiol.*, **49**, 293-297.
8. O'Neill, M., Albersheim, P. and Darvill, A. *Methods in Plant Biochemistry*, Dey, P. M. (Ed.), Academic Press, 1990, Vol. **2**, 415-441
9. McNeil, M., Darvill, A. G., Fry S. C., and Albersheim, P. (1984) *Ann. Rev. Biochem.* **53**, 625-663
10. Beck, E. (1967) *Z. Pflanzenphysiol. Bd.* **57**, S. 444-461.
11. Hart, D. A. and Kindel, P. K. (1970) *Biochem. J.* **116**, 569-579
12. Hart, D. A. and Kindel, P. K. (1970) *Biochemistry* **9**, 2190-2196
13. Cheng, L. *Isolation and Characterization of Pectic polysaccharides from Lemna minor*, Thesis for M. S., Michigan State University, 1991.
14. Duff, R. B. (1965) *Biochem. J.* **94**, 768-772.

15. Ovodova, R. G., Vaskovsky, V. E. and Ovodov, Y. S. (1968). *Carbhyd. Res.* **6**, 328-332

16. Ovodov, Y. S. Ovodova, R. G., Shibaeva, V. I. and Mikheyskaya, L. V. (1975) *Carbohydr. Res.* **42**, 197-199.

17. Dey, P. M. and Brinson, K. (1984) *Advan. Crbohydr. Chem. Biochem.*, **42** , 265-382

18. Carpita, N. C. and Gibeaut, D. M. (1993) *The Plant Journal*, **3**, 1-30.

19. Carpita, N. C. (1989) *Phytochemistry*, **28**, 121-125.

20. Rees, D. A. in *Polysaccharide Shapes*, Chapman and Hall Ltd, London, 1977, 41-61.

21. Jarvis, M. C. (1984) *Plant Cell Environ.*, **7**, 153-164.

22. Powell, D. A., Morris, E. R., Gidley, M. J. and Rees, D. A. (1982) *J. Mol. Biol.*, **155**, 517-531.

23. Baron-Eple, O., Gharyl, P. K. and Schindler, M. (1988) *Planta*, **175**, 389-395.

24. Nakajima, N., Morikawa, H., Igarashi, S. and Senda, M. (1981) *Plant Cell Physiol.*, **22**, 1305-1315.

25. Moustacas, A. M., Nari, J., Diamantidis, G., Noat, G., Grasnier M., Borel, M. and Ricard, J. (1986) *Eur. J. Biochem.*, **155**, 191-197

26. Nari, J. Noat, G. Diamantidis, G., Woudstra, M. and Ricard, J. (1986) *Eur. J. Biochem.*, **155**, 199-202

27. Kim, J.-B. and Carpita, N. C. (1992) *Plant Physiol.*, **98**, 646-653.

28. Lamport, D. T. A. (1986) *Cellulose: Structure, Modificatio and Hydrolysis* (Young, R. A. and Rowell, R. M., Eds.) New York: John Wiley, pp. 70-90.

29. BeMiller, J. N. (1986) in *Chemistry and Function of Pectins*, (Fishman, M. L. and

Jen, J. J., Ed.), American Chemical Society, 1986, 1-21.

30. Chambat, G. and Joseleau, J. (1980) *Carbohydr. Res.*, **85**, C10-C12.

31. Kato, Y. and Nevins, D. J. (1989) *Plant Physiol.* **89**, 792-797.

32. Shibuya, N. and Iwasaki, T. (1978) *Agric. Biol. Chem.*, **42**, 2259-2266.

33. Yamaoka, T. and Sato, S. (1977) *Bot. Mag.* Tokyo, **90**, 153-163.

34. Thibault, J.-F, Renard, C. M.G.C., Axelos, M. A. V., Roger, P. and Crépeau, M-J (1993) *Carbohydr. Res.*, **238**, 271-286

35. McNeil, M., Darvill, A. G. and Albersheim, P. (1980). *PLant Physiol.* **66**, 1128-1134

36. McNeil, M., Darvill, A. G. and Albersheim, P. (1982). *Plant Physiol.* **70**, 1586-1591 37. Lau, J. M., McNeil, M., Darvill, A. G. and Albersheim, P. (1985). *Carbohydr. Res.* **137**, 111-125.

38. Lau, J. M., McNeil, M., Darvill, A. G. and Albersheim, P. (1987) *Carbohydr. Res.* **168**, 245-247.

39. An, J., O'Neill, M. A., Albersheim, P. and Darvill, A. G. (1993) *Carbohydr. Res.*, **252**, 235-243.

40. Aspinall, G. O. (1980) in *The Biochemistry of Plants, A comprehensive Treatise*, (Preiss, J. Ed.), Vol. 3, Academic Press, New York, pp. 473-500.

41. Powell, D. A. Morris, E. R. Gidley, M. J. and Rees, D. A. (1982) *J. Mol. Biol.*, **155**, 517-531.

42. Rombouts, F. M. and Thibault, J.-F. (1986) *Carbohydr. Res.*, **154**, 189-203.

43. Ros, J. M., Schols, H. A. and Voragen, A. G. J. (1996) *Carbohydr. Res.* **282**, 271-284.

44. Schols, H. A. and Voragen, A. G. J. (1994) *Carbohydr. Res.* **256**,83-95.

45. Schols, H. A., Geraeds, C. C. J. M., Leeuwen, M. F. S., Kormelink, F. J. M. and Voragen, A. G. J. (1990) *Carbohydr. Res.* **206**, 105-115.

46. Eda, S. and Kato, K. (1980). *Agric. Biol. Chem.* **44**, 2793-2801.

47. Ishii, S. (1981). *Phytochemistry* **20**, 2329-2333.

48. Ishii, S. (1982). *Phytochemistry* **21**, 778-780.

49. Hatfield, R. D. (1991) *Carbohydr. Res.*, **212**, 177-186.

50. Talbott, L. D. and Ray, P. M. (1992) *Plant Physiol.*, **98**, 357-368.

51. Thomas, J. R., Darvill, A. G. and Albersheim, P. (1989) *Carbohydr. Res.* **185**, 279-305.

52. Shibuya, N. and Nakane, R. (1984) *Phytochemistry*, **23**, 1425-1429.

53. Spellman, M. W., McNeil, M., Darvill, A. G., Albersheim, P. and Henrick, K. (1983) *Carbohydr. Res.* **122**, 115-129.

54. Spellman, M. W., McNeil, M., Darvill, A. G., Albersheim, P. and Dell A. (1983) *Carbohydr. Res.* **122**, 131-153.

55. Thomas, J. R., Darvill, A. G. and Albersheim, P. (1989) *Carbohydr. Res.* **185**, 261-277.

56. Beck, E. and Kandler, O. (1965). *Z. Naturf.* **20 B**, 62-67.

57. Bacon, J. S. D. (1963). *Biochem. J.* **89**, 103P-104P.

58. Kindel, P. K. and Cheng, L. (1990) *Carbohyd. Res.* **199**, 55-65.

59. Plaschina, I. G., Braudo, E. E. and Tolstoguzov, V. B. (1978) *Carbohyd. Res.* **60**, 1-8.

60. Rees, D. A., Morris, E. R., Thom, D. and Madden, J. K. (1982) *The Polysaccharides,* **Vol. 1**, (Aspinall, G. O., Ed.) Academic Press, pp. 195-290.

61. Morris, E. R., Powell, D. A., Gidley, M. J. and Rees, D. A. (1982) *J. Mol. Biol.*, **155**, 507-516.

62. Rees, D. A. (1969) *Adv. Crbohydr. Chem.*, **24**, 267-332.

63. Rees, D. A. (1972) *Biochem. J.* **126**, 257-273.

64. Rees, D. A. (1975) *MTP Int. Rev. Sci. Biochem. Ser. One,* Vol. **5**, 1-42.

65. Rees, D. A. and Welsh, E. J. (1977) *Angew. Chem. Int. Ed. Engl.*, **16**, 214-224.

66. Walkinshaw, M. D. and Arnott, S. (1981) *J. Mol. Biol.*, **153**, 1075-1085.

67. Fry, S. C. (1986) *Ann. Rev. Plant Physiol.*, **37**, 165-186.

68. Haug, A. and Smidsrod, O. (1968) in *Solution Properties of Natural Polymers*, Special Publication No. **23**, by The Chemical Society, Alden & Mowbray Ldt in London, Great Britain, 273-282.

69. BeMiller, J. N. (1967) *Adv. Carbohydr. Chem. Biochem.*, **22**, 25-108.

70. Tuerena, C. E., Taylor, A. J. and Michell, J. R. (1984) *J. Sci. Food. Agric.*, **35**, 797-804.

71. Kiss, J. (1974) *Adv. Carbohydr. Chem. Biochem.*, **29**, 229-303.

72. Whistler, R. L. and Bemiller, J. N. (1958) *Advan. Carbohydr. Chem.*, **13**, 289-329.

73. Albersheim, P. (1959) *Biochem. Biophys. Res. Commun.*, **1**, 253-256.

74. Albersheim, P., Neukom, H. and Deuel, H. (1960) *Arch. Biochem. Biophys.*, **90**, 46-51.

75. Stevens, B. J. H. and Selvendran, R. R. (1980) *J. Sci. Food Agric.*, **31**, 1257-1267.

76. Stevens, B. J. H. and Selvendran, R. R. (1984) *Phytochemistry*, **23**, 107-115.

77. Stevens, B. J. H. and Selvendran, R. R. (1984) *Carbohydr. Res.*, **128**, 321-333.

78. Stevens, B. J. H. and Selvendran, R. R. (1984) *Carbohydr. Res.*, **135**, 155-166.

79. Yamada, H., Sun, X. -B., Matsumoto, T., Ra, K. -S., Hirano, M. and Kiyohara, H. (1991) *Planta Med.*, **57**, 555-559.

80. Yamada, H., Ra, K. -S., Kiyohara, H., Cyong, J. -C. and Otsuka, Y. (1989) *Carbohydr. Res.* **189**, 209-226.

81. Hirano, M., Kiyohara, H., Matsumoyo, T. and Yamada, H. (1994) *Carbohydr. Res.*, **251**, 145-162.

82. Kiyohara, H., Cyong, J. -C. and Yamada, H. (1988) *Carbohydr. Res.*, **182**, 259-275.

83. Zhao, J. -F., Kiyohara, H. and Yamada, H. (1991) *Carbohydr. Res.*, **219**, 149-172.

84. Renard, C. M. G. C. and Thibault, J. -F. (1993) *Carbohydr. Res.*, **244**, 99-114.

85. Rees, D. A. and Richardson, N. G. (1966) *Biochemistry*, **5**, 3099-3107.

86. Wood, P. J. and Siddiqui, I. R. (1972) *Carbohydr. Res.*, **22**, 212-220.

87. Aspinall, G. O. and Cottrell, I. W. (1971) *Can. J. Chem.*, **49**, 1019-1022.

88. Neukom, H., Deuel, H, Heri, W. J. and Kundig, W. (1960) *Helv. Chim. Acta,* **43**, 64-71.

89. Siddiqui, I. R. and Wood, P. J. (1974) *Carbohydr. Res.*, **36**, 35-44.

90. Knee, M. (1970) *J. Exp. Botany*, **21**, No. 68, 651-662.

91. Aspinall, G. O. and Jiang, K. -S. (1974) *Carbohydr. Res.*, **38**, 247-255.

92. York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T. and Albersheim, P. (1985) *Methods Enzymol.*, **118**, 3-40.

93. Thibault, J. -F. (1983) *Phytochemistry*, **22**, 1567-1571.

94. Joseleau, J. -P., Chambat, G. and Lanvers, M. (1983), *Carbohydr. Res.*, **122**, 107-113.

95. Cheng, L. and Kindel, P. K. (1995) *Analy. Biochem.*, *288*, 109-114.

96. Wood, P. J. and Siddiqui, I. R. (1971) *Anal. Biochem.*, **39**, 418-428.

97. Aspinall, G. O. and Fanous, H. K. (1984) *Carbohydr. Polymers*, **4**, 193-214.

98. Saulnier, L. and Thibault, J. -F. (1984) *Carbohydr. Polymers*, **7**, 329-343.

99. Massiot, P., Rouau, X. and Thibault, J. -F. (1988) *Carbohydr. Res.*, **172**, 229-242.

100. Marcelin, O., Saulnier, L. and Brillouet, J. -M. (1991) *Carbohydr. Res.*, **212**, 159-167.

101. Renard, C. M. G. C., Voragen, A. G. J., Thibault, J. -F. and Pilnik, W. (1990) *Carbohydr. Polymers*, **12**, 9-25.

CHAPTER II

ISOLATION OF CELL WALLS AND SOLUBILIZATION OF PECTIC POLYSACCHARIDES FROM *Lemna minor*

[In part from Kindel, P. K., Cheng, L., and Ade, B. R. (1996) *Phytochemistry*, **41**, 719-723.]

ABSTRACT

Improved procedures for isolation and purification of cell walls of *L. minor* and extraction of pectic polysaccharides from the cell walls were developed. Plants were homogenized with a Waring blender and the homogenate was treated with a French press. This treatment resulted in a much more complete breakage of cell walls and less contamination by other cell components, such as chloroplasts, than cell walls homogenized only with a Waring blender. Fifty-six percent (56%) more pectic polysaccharide was extracted by ammonium oxalate from the cell walls prepared with both a Waring blender and a French press than from those prepared only with a Waring blender. Pectic polysaccharides extracted from the cell walls that were prepared by homogenizing plants with salt (1 M NaCl) or detergent (1% sodium deoxycholate) solution were similar in molecular size and protein content. Pectic polysaccharides, which accounted for 26.4-31.7% of the total anhydrogalacturonic acid in cell walls of *L. minor*, were solubilized with 0.05 M ammonium oxalate at 22°C in 30 min. Further extracting the cell wall residues with 0.05 M ammonium oxalate for 6 h solubilized only 6.4% more pectic polysaccharides. Extraction of the cell wall residue, following ammonium oxalate extraction, with 0.05 M Na_2CO_3/0.02 M $NaBH_4$ at 4°C for 17 h followed by 0.05 M Na_2CO_3 at 23°C for 3 h resulted in solubilization of less than 0.5% of the total anhydrogalacturonic acid of the cell walls. A fast and effective method of cell wall isolation was established.

INTRODUCTION

Methods for cell wall preparation and polysaccharide isolation vary with different plants and cell types. Methods considered to be generally suitable for isolation of pectic polysaccharides from parenchymatous tissue of vegetables and fruits, lignified tissues, and cereals as well as special techniques used for starch- and protein-rich tissues have been described (1). The quantity of extractable material varies with different methods, type of plant tissue and the stage of development of the cell wall but it has not been possible to completely extract all pectic polysaccharides from any plant material by any method without causing degradation of the polysaccharides (1,2). However, effort should be made to minimize degradation caused by enzyme activity and to avoid contamination by intracellular compounds, such as starch and cytoplasmic proteins, when cell walls are prepared.

A number of different methods have been used to break cell walls of parenchymatous tissues, such as homogenizing with a Waring blender (3), ball-milling (4), grinding with a pestle and mortar followed by blending with a Ultra-Turrax (5), vibrating with a cell mill containing glass beads (6,7) and using a French pressure cell (8-10). A Parr bomb (11), and a French pressure cell plus sonication (12) were used to break suspension cultured single plant cells successfully. When pectic polysaccharides were isolated from *L. minor* a number of years ago (3) the cell walls were prepared by homogenizing the plants with a Waring blender. The purity of the cell walls was not established. Re-examination of such preparations microscopically showed that disruption of cells was incomplete (see Results).

In this study, the cell walls were prepared by homogenizing the plants with a Waring blender followed by treatment with a French press. The effectiveness of using a French press was evaluated.

Homogenization of cell walls in solutions of SDS and SDC is considered to be an effective way of removing cytoplasmic proteins (4,5). SDC (0.5 and 1%w/v) is preferred by some investigators because homogenization in SDS results in excessive foaming (13-16). Since SDS interferes with color development in the uronic acid assay (17) and SDC solubilizes the glycoprotein cell walls of an alga (18), the necessity of using detergent in the homogenization of *L. minor* was examined in this work.

Recent research on the kinetics of solubilization of pectic polysaccharides from cell walls of *L. minor* showed that ~30% of the total AUA of the cell walls was solubilized by ammonium oxalate in 15 min and that extending the time of extraction to 5 h only resulted in 4% more total AUA being solubilized (17). Jarvis *et al.* (16) reported that 0.1 M Na_2CO_3 was able to extract the remaining galactan-rich pectic polysaccharide effectively after the cell walls of potato were extracted with chelating agents. After extraction with CDTA, extraction with 0.05 M Na_2CO_3 solubilized an additional 12.6% and 8.1%, respectively, of the pectic polysaccharides from the cell wall residues of onions and apples (4). Selvendran *et al.* (4) proposed a general procedure for sequential extraction of cell wall polysaccharides from parenchymatous tissues which involved the use of 0.05 M Na_2CO_3/0.02 M $NaBH_4$ at 1 °C and 0.05 M Na_2CO_3 at 22 °C after the chelator extraction. The effectiveness of this procedure with *L. minor* was studied in this work.

MATERIALS AND METHODS

Materials and general methods. DEAE-Sephadex A-25 [particle (bead) size: 40-120 μm, dry; 80-242 μm, wet], Sephacryl S-400, and MF-Millipore filters were purchased from Pharmacia Biotech, Inc., Sigma Chemical Co., and Millipore Corp., respectively. BCA reagent was obtain from Pierce. Total protein in purified cell walls was determined with BCA reagent and BSA was used as the protein standard (19). Uronic acid and total sugar were determined with 3-hydroxydiphenyl (20) and phenol-sulfuric acid (21), respectively. Total AUA in cell walls was determined (17). The determinations of uronic acid and neutral sugar in filtrates were corrected for mutual interference as described (17). In the preparation of cell walls and solubilization of pectic polysaccharides from the cell walls, all flitrations were done with 15 μm Nylon mesh (3-15/6, Tetko, Inc.).

Preparation of cell walls. *L. minor* was grown as described elsewhere (22). The cell walls were prepared from whole plants of *L. minor* at 4°C, basically with the procedure of Kindel *et al.*(Appendix A and Ref. 17) except for the following changes: Plants (245.3 g, wet wt) were homogenized with 600 ml of 1.0 M NaCl in a Waring blender. The homogenate was filtered, and the cell walls resuspended in 500 ml of water and filtered. Two weighed portions of cell walls, each 0.0632 $\pm$ 0.0043 g, wet wt, were removed from the residue (97.9 g, wet wt) and dried to constant weight *in vacuo* and over P_2O_5. The rest was divided into two equal weight parts: one part was called Cell Wall-W (cell walls prepared by homogenizing with a Waring blender only) and saved for extraction of pectic polysaccharides. The other part was suspended in 300 mL water, passed through

a French pressure cell (at 16000-18000 psi), filtered, resuspended in 250 mL of water, filtered and the residue was called Cell Wall-F. Two weighed portions of Cell Wall-F (34.05 g, wet wt), each 0.042 ± 0.002 g, wet wt, were dried as described above and the rest was saved for extraction of pectic polysaccharides. In a separate experiment, cell walls were prepared from 70 and 50 g of plants as described for the preparation of Cell Wall-F but the homogenization was conducted in 170 ml of 1% (w/v) SDC and 120 mL of 1 M NaCl, respectively, and the cell walls were called Cell Wall-SDC and Cell Wall-NaCl, respectively. The solutions of the cell wall preparations following filtration were combined and analyzed for uronic acid and total sugar. Total protein was determined for Cell Wall-SDC and Cell Wall-NaCl.

Solubilization of pectic polysaccharides from cell walls. Cell Wall-W and Cell Wall-F were extracted with 585 mL and 500 mL, respectively, of 0.05 M ammonium oxalate (pH 6.0) at 22°C for 20 min, filtered, resuspended in 100 mL water and filtered. The residues and combined filtrates were tested for uronic acid. In a separate experiment, 31.9 g of fresh cell walls that were prepared by the same procedure used to prepare Cell Wall-F were extracted with 530 mL of 0.05 M ammonium oxalate for 30 min, filtered, resuspended in 300 mL water and filtered. The ammonium oxalate extraction of the residue was repeated once, this time for 6 h. Two further extractions of the ammonium oxalate residue were conducted with 180 mL of 0.05 M Na_2CO_3/0.02 M $NaBH_4$ at 4°C for 17 h, and with 0.05 M Na_2CO_3 at 23°C for 3 h, respectively. Cell wall residues and filtrates were tested for uronic acid.

Cell Wall-SDC and Cell Wall-NaCl were extracted with 190 mL and 150 mL, of 0.05

M ammonium oxalate at 22°C for 30 min and centrifuged. The supernatant solution was collected, dialyzed against distilled water for 38.5 h and called P-SDC and P-NaCl, respectively, for polysaccharides extracted from Cell Wall-SDC and Cell Wall-NaCl. P-SDC (345 mL) and P-NaCl (262 mL) were tested for total sugar and uronic acid and the remainder of each was concentrated and fractionated by chromatography with DEAE-Sephadex.

In a large scale experiment, cell walls prepared from 597 g (wet wt) of plants (as the preparation of Cell Wall-F) was extracted with 1070 mL 0.05 M ammonium oxalate (pH 5.5), filtered, resuspended in 850 mL of 0.05 M ammonium oxalate and filtered. The filtered solutions were combined and tested for uronic acid. Purification and characterization of this pectic polysaccharide fraction are described in Chapters V and VI of this thesis.

Fractionation of P-SDC and P-NaCl on DEAE-Sephadex. Solutions of polysaccharides P-SDC and P-NaCl were concentrated to 114 mL and 74 mL, respectively, and filtered with a 5 μm membrane (MF-Millipore, Millipore Corp.). The filtered samples were adjusted to pH 7.7 and 0.067 M sodium phosphate with 0.67 M sodium phosphate buffer (pH 7.6), and each was applied to a column of DEAE-Sephadex [1.3 cm (i.d.) × 13.9 cm (h), and 1.05 cm (i.d.) × 12.4 cm (h), respectively]. The columns were developed with 580 and 340 mL, respectively, of 0.067 M sodium phosphate buffer, pH 7.7, containing a linear gradient of NaCl from 0 to 0.4 M. Fractions of 4.3 and 2.5 mL were collected at rate of 0.21 and 0.13 mL/min, respectively. Each fraction were tested for total sugar and uronic acid. Appropriate fractions were combined as P-SDC-I, P-SDC-II, P-NaCl-I and P-NaCl-II

(these are defined in Figure 2.2). They were dialyzed, concentrated and analyzed by size exclusion column chromatography.

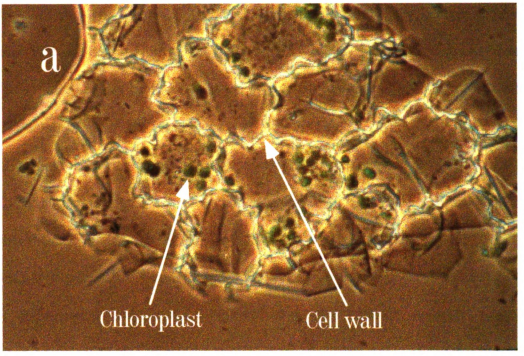
Size exclusion column chromatography. P-SDC-I, P-SDC-II, P-NaCl-I and P-NaCl-II were concentrated at 35°C to 1-3 mL and applied to a Sephacryl S-400 column (1.05 cm, i.d. × 83 cm, h). The column was developed with 0.1 M NaCl/0.05 M sodium phosphate (pH 6.5) at a flow rate of 0.11 mL/min. Fractions of 1.5 mL were collected and analyzed for uronic acid. The relative molecular size of each polysaccharide was determined by comparing their mobilities with those of Dextran T-500, T-70 and T-10.

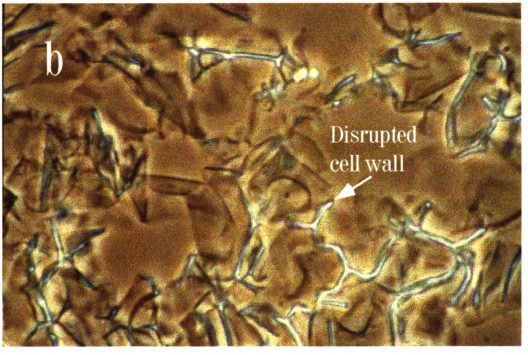
RESULTS

Comparison of cell walls disrupted with the Waring blender and French press. The color of Cell Wall-W was green. Under 330× magnification, intact cells (with chloroplasts) and multicellular pieces of tissue were observed (Figure 2.1a). Cell Wall-F was cream-colored and appeared to be free of chloroplast and other cell organelles. Few intact cells remained in Cell Wall-F (Figure 2.1b). The pectic polysaccharides solubilized (determined as AUA) from Cell Wall-W and Cell Wall-F with 0.05 M ammonium oxalate in 20 min were 13.1% and 20.4% (w/w), respectively, of the total AUA found in cell walls, which means that treatment of the cell walls with both the Waring blender and the French resulted in a 56% increase of pectic polysaccharides released from the cell wall.

Media used for cell wall homogenization. In the 30 min extraction of Cell Wall-SDC and Cell Wall-NaCl (after dialysis) with 0.05 M ammonium oxalate, pectic polysaccharides

Figure 2.1. Light microscopic view of cell walls of *L. minor*. (a) Cell Wall-W (330×) , the cell walls prepared by homogenizing the plant with a Waring blender. (b) Cell Wall-F (530×), the cell walls prepared by homogenizing the plants with a Waring blender plus a French press.


a
Chloroplast
Cell wall


b
Disrupted
cell wall

that are 24.4% and 22.6%, respectively, of the total AUA in the cell walls were solubilized while pectic substances (including mono-, oligo- and polysaccharides) present in the filtrates obtained during preparation of cell walls were 5.7% and 10.1%, respectively, of the total AUA in the cell walls (Table 2.1). On the dry weight basis, Cell Wall-SDC and Cell Wall-NaCl contained nearly the same amount of protein (Table 2.1).

The elution profiles from DEAE-Sephadex column chromatography of P-SDC and P-NaCl are shown in Figure 2.2. Two polysaccharide fractions were obtained from P-SDC, named P-SDC-I and P-SDC-II (Figure 2.2a), and two from P-NaCl, named P-NaCl-I and P-NaCl-II (Figure 2.2b). Fractions under each peak were combined as indicated (Figure 2.2). Size exclusion chromatography showed that the relative molecular weights of polysaccharides P-SDC-I, P-NaCl-I, P-SDC-II, and P-NaCl-I are 11,600, 12,000, 18,700 and 18,200, respectively (Table 2.1).

Sequential extraction of pectic polysaccharides from cell walls. The total AUA found in cell walls of *L. minor* (Cell Wall-F) is 18.7% (w/w) on a dry weight basis (Table 2.2). The pectic polysaccharides solubilized from Cell Wall-F and the cell walls of large scale experiment by 0.05 M ammonium oxalate in 30 min were 26.4% and 31.7%, respectively, of total AUA in the cell walls, which were acceptably close. Further extraction of the residue with ammonium oxalate for up to 6 h only resulted in 6.4% more pectic substance being solubilized (Table 2.2). The extraction of additional pectic substances from the cell wall residue of the ammonium oxalate extraction with weak base is difficult; only 0.20% and 0.24% more pectic substance were solubilized with the sequential treatments of 0.05 M Na_2CO_3/0.02 M $NaBH_4$ at 4 °C for 17 h followed by 0.05 M at 23 °C for 3 h (Table 2.2).

Table 2.1. Pectic substances solubilized, total cell wall protein and relative molecular weight of the pectic polysaccharides from cell wall prepared in different homogenizing media[a].

Medium for homogenizing	Pectic substances solubilized (% of total AUA in cell wall)			Protein/dry wt of cell wall (%, w/w)	Molecular size of the polysaccharides relative to standard Dextrans[b]	
	Filtrate of cell wall preparation	0.05 M ammonium oxalate, 30 min	Total		polysaccharide I[c]	polysaccharide II[d]
1% SDC	5.7	24.4	30.1	3.34	11,600	18,700
1M NaCl	10.1	22.6	32.7	3.37	12,000	18,200

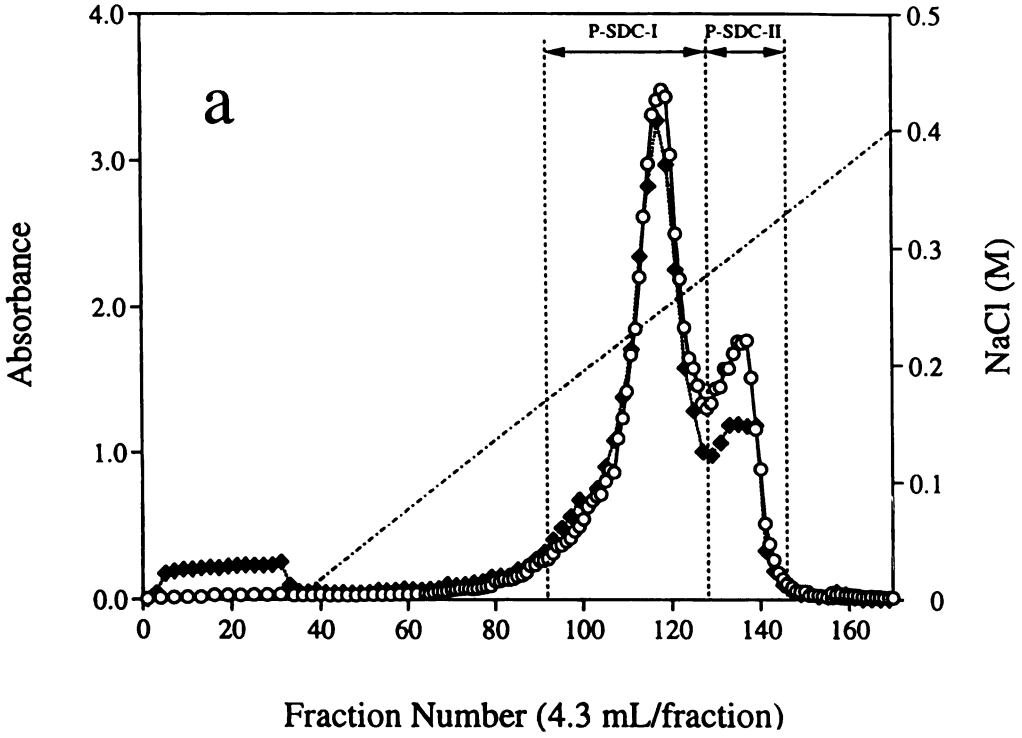
[a] Cell walls were processed by the procedure similar to that used to prepare Cell Wall-F (see Materials and Methods) except the medium of homogenization was varied as indicated.

[b] Relative molecular size is based on the comparison to mobilities of dextran standard samples on a Sephacyl S-400 column (see Materials and Methods).

[c] Polysaccharide I of Figs. 2a and 2b.

[b] Polysaccharide II of Figs. 2a and 2b.

Figure 2.2. Column chromatograms of *L. minor* cell wall pectic polysaccharide fractions P-SDC (a) and P-NaCl (b). Columns were developed with 0.067 M sodium phosphate buffer, pH 7.7, and buffer containing a linear gradient of NaCl (·-·-·-·). Uronic acid ($A_{520\,nm}$, —○—) and total sugar ($A_{490\,nm}$, ·····◆·····) were determined as described in the Materials and Methods.


a
P-SDC-I
P-SDC-II
Absorbance
NaCl (M)
Fraction Number (4.3 mL/fraction)

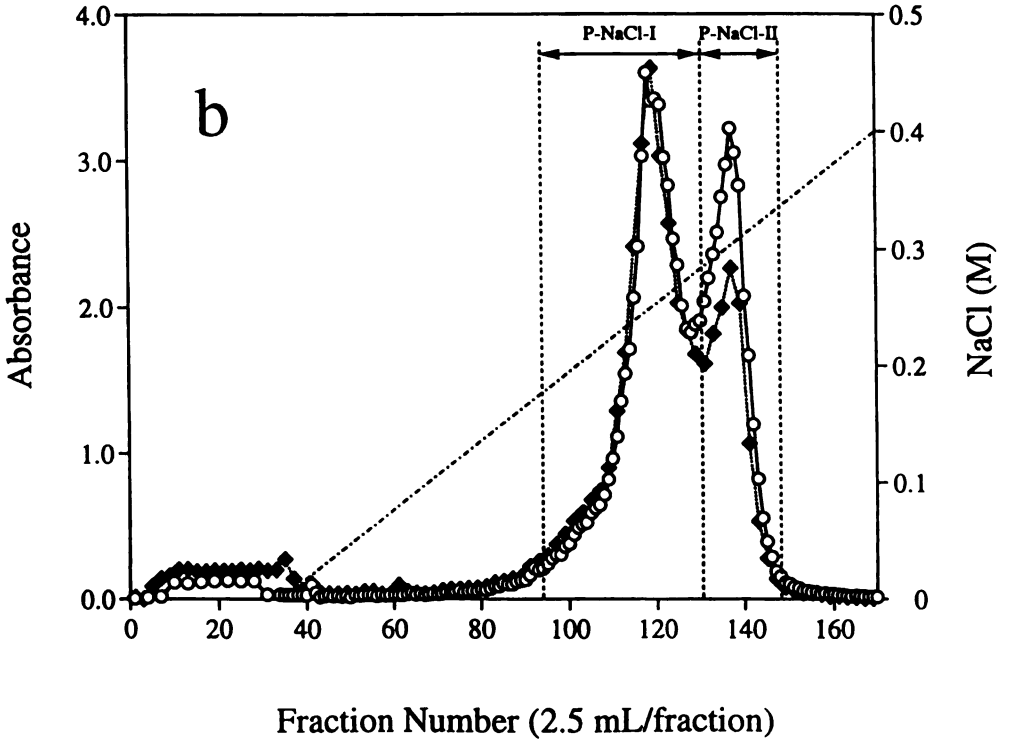

b
P-NaCl-I
P-NaCl-II
Absorbance
NaCl (M)
Fraction Number (2.5 mL/fraction)

Table 2.2. Solubilization of pectic polysaccharide from purified cell walls of *L. minor*.

Ratio	Cell wall	Water soluble (in filtrate)	Solubilized by 0.05 M ammonium oxalate, 22°C			Solubilized by 0.05 M Na_2CO_3/ 0.02 M $NaBH_4$, 17 h, 1°C	Solubilized by 0.05 M Na_2CO_3, 3 h, 22°C	Residue from extractions
			1st/0.5 h	2nd/6 h	Sum			
AUA/dry cell wall	18.7%	1.9%	4.9%	1.2%	6.1%	0.038%	0.044%	10.6%
AUA in fraction	100%	10.1%	26.4%[a]	6.4%	32.8%	0.20%	0.24%	56.6%

[a] For the large scale experiment (described in Materials and Methods), 31.7% of total AUA was solubilized with 0.05 M ammonium oxalate in 30 min from the cell walls.

DISCUSSION

Homogenizing *L. minor* with a Waring blender (3) resulted incomplete disruption of cells since many intact cells with chloroplasts and multicellular pieces of tissue were observed in Cell Wall-W. However, after homogenization with a Waring blender, the cell wall suspension can be processed readily with a French pressure cell and this resulted in almost complete disruption of cells. It is not surprising to find that 56% more pectic polysaccharides were extracted by ammonium oxalate form Cell Wall-F than from Cell Wall-W. With the more thoroughly disrupted cell walls, the chelating reagent has a greater chance to interact with cell wall Ca^{2+} ions and release pectic polysaccharides held by Ca^{2+} bridges. When the French pressure cell was used the cell walls (Cell Wall-F) were cream-white and appeared to be free of chloroplast and other cell organelles under microscope while Cell Wall-W was green.

Cell walls isolated by homogenization with 1 M NaCl or 1% SDC were basically the same since: (i) the protein content of each type of cell wall is basically the same, (ii) the percentage of pectic substances solubilized with 0.05 M ammonium oxalate in 30 min from Cell Wall-NaCl, 22.6%, was fairly close to that solubilized from Cell Wall-SDC, 24.4%, (iii) no degradation of the polysaccharides resulted from using 1% SDC for 1 M NaCl as the homogenization media, based on the finding that the relative molecular sizes of the two pectic polysaccharides isolated from Cell Wall-SDC and Cell Wall-NaCl are basically the same (Table 2.1). Detergent, such as SDS, was interferes with color formation in the uronic acid test (17). Some foaming was observed when using 1% SDC as the homogenization

medium. Consequently NaCl solution is preferred as the homogenization medium.

Although the amount of pectic polysaccharides solubilized from Cell Wall-F (26.4%) with 0.05 M ammonium oxalate in 30 min was somewhat lower than that of the large scale experiment (31.7%) and that reported (30%) by Kindel *et al.* (17), it is clear that the release of ammonium oxalate soluble pectic polysaccharides from *L. minor* is not a continuum and most of these molecules were solubilized within 30 min with 0.05 M ammonium oxalate. The determination of the kinetics of solubilization showed this conclusively (17). Further extraction of the cell wall residue for 6 h (these results) and 5 h (17) resulted in 6.4% and 4%, respectively, more pectic polysaccharides being extracted. The pectic substances in the cell wall residue from ammonium oxalate extraction were resistant to extraction with weak base –only 0.4% of the total AUA in the cell walls was solubilized by the two weak base extractions. If the remaining AUA represents pectic polysaccharides of the cell walls, these pectic polysaccharides should be different than the chelator soluble ones, at least their bonging in the cell wall does not depend on Ca^{2+} bridge since they are not solubilized by the chelator. Also their attachment to the cell wall appears not to involve an ester linkage, since they are resist to weak base extraction. Since the solubilization process is fast (no significant additional release of AUA after 30 min) and the conditions of solubilization are non-destructive, the breakage of acid or base labile glycosidic linkages under extraction conditions should be minimal.

REFERENCES

1. Selvendran, R. R. and O'Neill, M. A., (1987) *Methods Biochemical Analysis* **32**, 25-153.
2. Selvendran, R. R. (1985) *J. Cell Sci. Suppl.* **2**, 51-88.
3. Hart, D. A. and Kindel, P. K. (1970) *Biochem. J.* **116**, 569-579.
4. Selvendran, R. R., Stevens, B. J. H. and O'Neill, M. A. (1985) *Biochemistry of Plant Cell Walls*, Society for Experimental Biology, Seminar seriese: **28**, Cambridge University Press, 39-78.
5. Selvendran, R. R. (1975) *Phytochemistry*, **14**, 1011-1017.
6. Northcote, D. H., Goulding, K. J. and Horne, R. W. (1958) *Biochem. J.*, **70**, 391-397.
7. Lamport, D. T. A. (1965) in *Advances in Botanical Research*, (R. D. Preston, Ed.) Vol **2**, pp. 151-218, Academic Press, New York and London.
8. Bacon, S.D., Gordon, A. H. and Morris, E. J. (1975) *Biochem. J.*, **149**, 485-487.
9. Nakamura, N. and Suzuki, H. (1981) *Phytochemistry*, **20**, 981-984.
10. Takeda, H. and Hirokawa, T. (1978) *Plant Cell Physiol.*, **19**, 591-598.
11. York, W. S., Darvill, A. G., McNeil, M., Stevenson, T. T. and Albersheim, P. (1986) *Methods Enzymology*, **118**, 3-40.
12. Harris, P. J. (1983) in *Isolation of Membranes and Organelles from Plant Cells*, (Hall, J. L. and Moore, A. L., Ed.), pp. 25-53, Academic Press, New York and London.

13. Klis, F. M. and Eeltink, H. (1979) *Planta*, **144**, 479-484.

14. Mankarios, A. T., Hall, M. A., Jarvis, M. C., Threlfall, D. R. and Friend, J. (1980) *Phytochemistry*, **19**, 1731-1733.

15. Van Host, G. J., Klis, F. M., Bouman, F. and Stegwee, D. (1980) *Planta*, **149**, 209-212.

16. Jarvis, M. C., Hall, M. A., Threlfall, D. R. and Friend, J. (1981) *Planta*, **152**, 93-100.

17. Kindel, P. K., Cheng, L. and Ade, B. R. (1995)*Phytochemistry*, Vol. **41**, No. 3, 719-723.

18. Roberts, K. (1974) *Philos. Trans. R. Soc. London, Ser B.*, **268**, 129-146.

19. Redinbaugh, M. G. and Turley, R. B. (1986) *Anal. Biochem.*, **153**, 267-271.

20. Blumenkrantz, N. and Asboe-Hansen, G. (1973) *Anal. Biochem.* **54**, 484-489.

21. DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal.Chem.* **28**, 350-356.

22. Kindel, P. K. and Watson, R. R. (1973) *Biochem. J.* **133**, 227-241.

CHAPTER III

EFFECT OF CATIONS ON THE ELUTION OF PECTIC POLYSACCHARIDES FROM ANION-EXCHANGE RESINS

[Adapted from Cheng, L. and Kindel, P. K. (1995) *Analy. Biochem.*, **228**, 109-114]

ABSTRACT

Conditions for quantitatively eluting six plant pectic polysaccharides from diethylaminoethyl(DEAE)-columns were established. Surprisingly, the cation greatly affected the elution of pectic polysaccharides from these anion exchange columns. Elution of apple pectic acid from DEAE-Sephadex A-25 and DEAE-Trisacryl Plus-M columns was quantitative with 0.5 M of NH_4Cl, LiCl, or CsCl in 0.05 M acetate buffer (pH 5.5). In contrast, sample recovery from the two columns was only 6.3% and 8.6%, respectively, with up to 1 M NaCl in buffer, and 54% and 32%, respectively, with 1 M KCl in buffer. In each case, the retained apple pectic acid was eluted quantitatively by 0.5 M NH_4Cl in buffer. The elution of four plant pectic polysaccharides —one from *Apium graveolens* (celery), two from *L. minor* (duckweed) and a commercial citrus polygalacturonic acid —from columns of DEAE-Trisacryl was incomplete when the columns were developed with 0.5 M NaCl or 0.5 M KCl in buffer; the recovery of the samples from the columns ranged from 0 to 89%. However, again, the retained portion of each sample was eluted quantitatively or almost so from the columns with 0.5 M NH_4Cl in buffer. Elution of a commercial citrus pectin from DEAE-Trisacryl columns was accomplished equally well and almost quantitatively by 0.5 M NaCl, KCl, and NH_4Cl in buffer. On a preparative DEAE-Trisacryl Plus M column, 99% of the pectic polysaccharides extracted from cell walls of *L. minor* was eluted by a gradient of 0 to 0.5 M NH_4Cl in buffer, while, in a basically identical experiment only 55% of the sample was eluted when NH_4Cl was replaced by NaCl in buffer. The discovery that NH_4^+ ion in the eluent brings about the essentially quantitative elution of pectic polysaccharides

from anion exchange resins solves the long-standing problem of incomplete recovery of pectic polysaccharides from DEAE-columns. Li^+ and Cs^+ ions are similar to NH_4^+ ion in elution effectiveness.

INTRODUCTION

The separation and purification of pectic polysaccharides solubilized from plant cell walls typically involves anion exchange column chromatography. A common problem in the purification of cell wall polysaccharides is that the elution of the polysaccharide from the anion exchange column is incomplete. Since (i) pectic polysaccharides extracted from cell walls are usually both chemically and physically heterogeneous, (ii) polysaccharide matreials retarded in the column can be structurally different from the eluted ones, analyzing the sample that is incompletely recovered from the column can result in biased structural information. Incomplete elution has been observed with a variety of pectic polysaccharides from a number of different plants with all the different ion exchange resins commonly used for purification of pectic polysaccharides. When DEAE-cellulose (1,2), DEAE-Sephadex (both A-25 and A-50) (3-6), DEAE-Sephacel (7-12) and DEAE-Sepharose CL-6B (13,14) were used, the recoveries of pectic polysaccharides from columns where elution was incomplete ranged from 82 to 85%, 0 to 87%, 32 to 88%, and 24 to 51%, respectively. When DEAE-Trisacryl M was used, the recoveries of pectic polysaccharides ranged from 69 to 88% (15,16). Recoveries of 94 and 100% were obtained with the soluble portion of two freeze-dried pectic polysaccharide fractions of runner bean pods when DEAE-Trisacryl was

used (17); however, the portion of the freeze-dried fraction that was soluble was only 85 and 27%, respectively, of the total fraction. Redgwell and Selvendran compared the recoveries of an onion pectic polysaccharide fraction from four different anion exchange resins at either pH 4.5 or 6.3 or both (15). The recoveries from DEAE-Sephadex, DEAE-Sephacel, and DEAE-Sepharose ranged from 44 to 60%. The recovery was 88% when DEAE-Trisacryl was used and 0.05 M phosphate (pH 6.3) and 0.5 M NaCl in buffer were the eluents, but this dropped to 51% when DEAE-Trisacryl, 0.05 M acetate buffer (pH 4.5), and 0.5 M NaCl were used. When a pectic polysaccharide fraction from purified cell walls of *L. minor* was applied to columns of DEAE-Sephadex A-25 and DEAE-Trisacryl Plus-M and the sodium form of the eluents was used, the recovery of pectic polysaccharides ranged between 48 and 57% (Results).

We have found that the type of cation present in the eluent profoundly affects the elution of pectic polysaccharides from anion exchange resins.

MATERIALS AND METHODS

Materials and general methods. DEAE-Sephadex A-25 [particle (bead) size: 40-120 μm, dry; 80-242 μm, wet], DEAE-Trisacryl Plus-M (bead size: 40-80 μm, wet), and MF-Millipore filters were purchased from Pharmacia Biotech, Inc., Sigma Chemical Co., and Millipore Corp., respectively. Uronic acid was determined colorimetrically (18) and is expressed as AUA (molecular weight: 176.12). Apple pectic acid was purchased from Aldrich Chemical Co. Polygalacturonic acid and citrus pectin were obtained from Sigma

Chemical Co. The suppliers provided the following information: (i) apple pectic acid — $\overline{M}_w$ 228,000; $\overline{M}_n$, 88,000; AUA content, approximately 75%; degree of methyl esterification, less than 5%, (ii) citrus polygalacturonic acid — approximate molecular weight range, 4000 to 6000; AUA content, approximately 100%; degree of methyl esterification, approximately 5%, and (iii) citrus pectin — approximate molecular weight range, 23,000 to 71,000; AUA content, approximately 76%; degree of methyl esterification, approximately 50%. Pectic polysaccharides of *L. minor* were prepared from highly purified cell walls. Plants were grown as described previously (19). Cell walls of *L. minor* were prepared as described (Appendix A, Ref. 20) and were extracted with 0.05 M ammonium oxalate (pH 5.5) at 22°C for 30 min. The pectic polysaccharides in the extract were chromatographed on DEAE-Trisacyl and column fractions were tested for uronic acid. Two pectic polysaccharides were isolated; polysaccharide F-129, which consisted of 54% neutral sugars and 46% galacturonic acid and had a relative molecular weight (relative to standard dextrans) of 480,000, and polysaccharide F-167, which consisted of 7% neutral sugars and 93% galacturonic acid and had a relative molecular weight of 780,000 (Cheng and Kindel, 1996, submitted). A pectic polysaccharide fraction from *Apium graveolens* (celery) was prepared by extracting purified cell walls of celery with 0.05 M ammonium oxalate (pH 5.5) at 22°C for 1 h (20).

Analytical anion exchange column chromatography of pectic polysaccharides. Apple pectic acid, polygalacturonic acid, and citrus pectin were dissolved by stirring each in water and adding 5% (w/v) NaOH to bring the pH to 5.5. The solutions were filtered through a 0.8 μm MF-Millipore filter. Polysaccharide concentrations up to 2 mg/mL were prepared.

Apple pectic acid (6 mg in 3 mL of water at pH 5.5) was applied to columns of DEAE-

Sephadex A-25 and DEAE-Trisacryl Plus M. The columns [0.7 cm (diameter) x 8.5 cm (height)] were developed with 20 mL of column buffer [0.05 M sodium acetate, potassium acetate, ammonium acetate, lithium acetate, or cesium acetate (pH 5.5)], 40 mL of 0.5 M salt (NaCl, KCl, NH_4Cl, LiCl, or CsCl) in 0.05 M of the corresponding column buffer, and 25 mL of 1 M salt (NaCl, KCl, NH_4Cl, LiCl, or CsCl) in column buffer. The columns developed with NaCl or KCl in column buffer were further developed with 40 mL of 0.5 M NH_4Cl and 20 to 25 mL of 1 M NH_4Cl each in 0.05 M ammonium acetate (pH 5.5). The columns developed with LiCl or CsCl in column buffer were further developed with 20 to 25 mL of 1 M NH_4Cl in 0.05 M ammonium acetate (pH 5.5).

A celery pectic polysaccharide fraction (approximately 0.8 mg AUA in 20 mL of 0.05 M ammonium oxalate at pH 5.5), citrus polygalacturonic acid (6 mg in 6 mL of water at pH 5.5), citrus pectin (6 mg in 6 mL of water at pH 5.5), pectic polysaccharide F-129 from *L. minor* (0.93 mg AGalU in 6 mL of water), and pectic polysaccharide F-167 from *L. minor* (1.1 mg AUA in 3 mL of water) were applied to individual DEAE-Trisacryl Plus M columns and eluted with the salts stated in Table 3.2. For citrus polygalacturonic acid and citrus pectin, the columns were the same size as above and developed as described above. The final treatment for these six columns was with 0.5 M NH_4Cl in water at pH 9.5 (adjusted with NH_4OH; final [NH_4^+] was 0.64 M). For the celery pectic polysaccharide fraction and polysaccharides F-129 and F-167, the columns were 0.59 cm (diameter) x 6 cm (height). The columns were also developed basically as described above through treatment with 0.5 M NH_4Cl in buffer, with a proportional reduction in solution volumes. With these three samples, all salts were dissolved in 0.05 M sodium acetate (pH 5.5). Polysaccharide F-167

was also directly eluted from a DEAE-Trisacryl Plus M column with 0.5 M LiCl in buffer.

Column eluates were tested for AUA colorimetrically (18). The yield of pectic polysaccharide from the columns was based on recovery of AUA in the eluates.

Preparative anion exchange column chromatography of a pectic polysaccharide fraction from L. minor. Cell walls and ammonium oxalate extracts of *L. minor* were prepared as described above. The extracted pectic polysaccharides (178 mg of AUA equivalents) in 0.03 M sodium oxalate (pH 5.0) (column buffer) were applied to a column of DEAE-Sephadex, 1.75 cm (diameter) x 18 cm (height). The column was washed with 0.05 M sodium acetate (pH 5.0, column buffer) and developed sequentially with 1600 mL of column buffer containing a linear gradient of NaCl that increased from 0 to 0.4 M, 168 mL of 1M NaCl in column buffer, 240 mL of 1 M NaCl at pH 2.0 and 156 mL of 1 M NaCl at pH 1.2.

In another large scale experiment, the extracted pectic polysaccharides, 192 mg (AUA equivalents), in 0.05 M sodium oxalate buffer (pH 5.0, column buffer) were fractionationed with a DEAE-Trisacryl column (2.8 cm, i.d., x 28 cm, height). The column was sequentially developed with 2550 mL of column buffer containing a linear gradient of 0 to 0.5 M NaCl, 191 mL of 1 M NaCl in column buffer and 229 mL of 1 M NaCl in 0.05 M sodium phosphate (pH 8.2). In an experiment with an identical column, 210 mg (AUA equivalents) of extracted pectic polysaccharides from cell walls of *L. minor* were fractionated with 2800 mL of ammonium oxalate (pH 5.5, column buffer) and a linear gradient of 0 to 0.5 M NH_4Cl followed by 520 mL of 1M ammonium oxalate in the column buffer. Column fractions were assayed for uronic acid.

Effect of salts on the solubility of apple pectic acid. Solutions in test tubes, 1.03 cm

(i.d.) x 7.5 cm (length), contained: (i) apple pectic acid at 0.5 or 1.0 mg/mL and (ii) NaCl, KCl, NH_4Cl, LiCl, or CsCl at 0.5, 1.0, 1.5 or 2 M in a final volume of 4.0 mL. The solutions were prepared from stock solutions and after mixing stood at 22°C. Precipitation and gel formation were detected by measuring absorbance at 600 nm. Measurements were taken at 0.5, 6, 16, 28, 52, 75 and 102 h after mixing.

RESULTS

The effect of cations on the recovery of apple pectic acid from DEAE-Sephadex and DEAE-Trisacryl columns is summarized in Table 3.1. When DEAE-Sephadex and DEAE-Trisacryl columns were developed with 0.5 M NH_4Cl in column buffer, apple pectic acid was eluted immediately and quantitatively (97%, Table 3.1). When the columns were developed with 0.5 M LiCl or CsCl in column buffer, the recovery of apple pectic acid was almost quantitative, ranging from 88 to 96% (Table 3.1). The remainder was recovered by eluting with the corresponding 1 M salts and 1 M NH_4Cl in buffer. In contrast, when 0.5 M NaCl in column buffer was the eluent, the recovery of apple pectic acid from DEAE-Sephadex and DEAE-Trisacryl columns was 8.6 and 6.3%, respectively (Table 3.1). When 0.5 M KCl in column buffer was used for column development, somewhat better recoveries were obtained from both DEAE-Sephadex and DEAE-Trisacryl (54 and 32%, respectively). Further development of the columns with 1 M NaCl or KCl in column buffer did not result in the elution of any additional pectic acid. However, development of the columns with 0.5 M NH_4Cl in buffer eluted the remaining apple pectic acid from both columns basically

Table 3.1. Effect of Cations on the Recovery of Apple Pectic Acid from DEAE-Trisacryl Plus M and DEAE-Sephadex A-25[a].

Column material	Column buffer	Eluent 1		Eluent 2		Eluent 3		Recovery of pectic acid (%)[c]
		Salt	Pectic acid (%)[b]	Salt	Pectic acid (%)[b]	Salt	Pectic acid (%)[b]	
DEAE-Sephadex	0.05 M NaOAc, pH 5.5	NaCl, 0.5 M	8.6	NaCl, 1 M	0	NH_4Cl, 0.5 M	90	99[c]
	0.05 M KOAc, pH 5.5	KCl, 0.5 M	54	KCl, 1 M	0	NH_4Cl, 0.5 M	45	100[c]
	0.05 M NH_4OAc, pH5.5	NH_4Cl, 0.5 M	97	NH_4Cl, 1 M	1.5	—	—	98
	0.05 M LiOAc, pH 5.5	LiCl, 0.5 M	96	LiCl, 1 M	2.9	NH_4Cl, 1 M	0.9	100
	0.05 M CsOAc, pH 5.5	CsCl, 0.5 M	88	CsCl, 1 M	6.8	NH_4Cl, 1 M	1.9	97
DEAE-Trisacryl	0.05 M NaOAc, pH 5.5	NaCl, 0.5 M	6.3	NaCl, 1 M	0	NH_4Cl, 0.5 M	88	95[d]
	0.05 M KOAc, pH 5.5	KCl, 0.5 M	32	KCl, 1 M	0	NH_4Cl, 0.5 M	63	96[d]
	0.05 M NH_4OAc, pH5.5	NH_4Cl, 0.5 M	97	NH_4Cl, 1 M	1.2	—	—	98
	0.05 M LiOAc, pH 5.5	LiCl, 0.5 M	91	CsCl, 1 M	5.4	NH_4Cl, 1 M	1.7	98
	0.05 M CsOAc, pH 5.5	CsCl, 0.5 M	91	CsCl, 1 M	5.2	NH_4Cl, 1 M	1.1	97

[a] Procedures are in Materials and Methods.
[b] Percentage of AUA applied to the column in eluate.
[c] Percentage of AUA in eluates 1 through 3. AUA in the fractions collected during sample loading and column washing before eluent 1 was started was less than 0.2% of that applied and was not considered in the calculation of total recovery of pectic polysaccharide.
[d] These columns were also treated with 1 M NH_4Cl in buffer and an additional 0.6 to 0.7% AUA was eluted.

quantitatively.

When apple pectic acid was applied to DEAE-Trisacryl columns and the columns were developed with either 0.5 M NaCl or 0.5 M KCl in column buffer, a liquid-filled gap of approximately 1 cm developed in the column bed between a top layer of approximately 1.5 cm and the rest of the column bed. The top layer did not shrink or collapse during the elution with 0.5 M and 1 M NaCl or KCl. When the columns were treated with 0.5 M NH_4Cl in column buffer, the top layer collapsed. The collapse corresponded with the quantitative elution of the remainder of the apple pectic acid from the columns. When DEAE-Trisacryl columns were developed only with 0.5 M NH_4Cl, 0.5 M LiCl or 0.5 M CsCl in column buffer, there was no separation of the resin bed as the resin shrunk. Also no gap in the resin bed developed when DEAE-Sephadex was used in any of the above experiments including those with NaCl and KCl.

The effect of cations on the recovery of five different pectic polysaccharide samples from DEAE-Trisacryl columns is summarized in Table 3.2. The recoveries of four were incomplete, ranging from 0 to 89%, when elution was with 0.5 M NaCl or KCl in buffer. With one polysaccharide, citrus pectin, elution was near quantitative and it made no difference whether Na^+, K^+, or NH_4^+ ion was present in the eluent. The retained portion of the celery pectic polysaccharide fraction and of pectic polysaccharide F-129 from *L. minor* following column chromatography with NaCl and KCl was eluted quantitatively with 0.5 M NH_4Cl in buffer. Pectic polysaccharide F-167 from *L. minor*, which was completely retained when NaCl or KCl was the eluent, was also eluted by 0.5 M NH_4Cl in buffer, almost quantitatively. Most, but not all, of the retained portion of citrus polygalacturonic acid was

Table 3.2. Effect of Cations on the Recovery of Different Pectic Polysaccharides from Analytical Columns of DEAE-Trisacryl Plus M[a].

Type of polysaccharide	Eluent 1		Eluent 2		Eluent 3		Recovery of pectic polysaccharide (%)[c]
	Salt	Polysaccharide eluted (%)[b]	Salt	Polysaccharide eluted (%)[b]	Salt	Polysaccharide eluted (%)[b]	
Celery pectic polysaccharide	NaCl, 0.5 M	67	NaCl, 1 M	0	NH_4Cl, 0.5 M	27	94
	KCl, 0.5 M	74	KCl, 1 M	0.4	NH_4Cl, 0.5 M	20	94
Citrus polygalacturonic acid	NaCl, 0.5 M	39	NaCl, 1 M	0	NH_4Cl, 0.5 M	43	82[d]
	KCl, 0.5 M	66	KCl, 1 M	0.4	NH_4Cl, 0.5 M	19	85[d]
	NH_4Cl, 0.5 M	85	NH_4Cl, 1 M	2.6	—	—	88[d]
Citrus pectin	NaCl, 0.5 M	91	NaCl, 1 M	0.7	NH_4Cl, 0.5 M	1.0	93[d]
	KCl, 0.5 M	91	KCl, 1 M	0.7	NH_4Cl, 0.5 M	1.1	93[d]
	NH_4Cl, 0.5 M	91	NH_4Cl, 1 M	0.9	—	—	92[d]
Pectic polysaccharide F-129 from *L. minor*	NaCl, 0.5 M	89	NaCl, 1 M	0.8	NH_4Cl, 0.5 M	9.2	99
	KCl, 0.5 M	89	KCl, 1 M	3.3	NH_4Cl, 0.5 M	9.7	102
Pectic polysaccharide F-167 from *L. minor*	NaCl, 0.5 M	0	NaCl, 1 M	0	NH_4Cl, 0.5 M	92	92
	KCl, 0.5 M	0.8	KCl, 1 M	0	NH_4Cl, 0.5 M	87	88
	LiCl, 0.5 M	83	LiCl, 1 M	14	NH_4Cl, 1 M	1.9	99

[a] Procedures are in Materials and Methods.

[b] Percentage of AUA applied to the column in eluate.

[c] Percentage of AUA applied to the column in eluates 1 through 3. AUA in the fractions collected during sample loading and column washing before eluent 1 was started was 0.7% or less of that applied and was not considered in the calculation of total recovery of pectic polysaccharide.

[d] Further treatment of the columns with 1 M NH_4Cl and LiCl or 1 M LiCl alone eluted an additional 0.7 to 1.7 % of the starting AUA. The remainder was eluted by 0.5 M NH_4Cl in water at pH 9.5. The total recoveries from the columns ranged from 97 to 100%.

eluted by 0.5 M NH_4Cl in buffer. Although only a small portion of citrus pectin was retained, the retained material was also not eluted by 0.5 M NH_4Cl in buffer. The retained portion of citrus polygalacturonic acid and of citrus pectin following chromatography with 0.5 M NH_4Cl was completely eluted by 0.5 M NH_4Cl in water at pH 9.5 (Table 3.2, footnote). An experiment was performed to determine the pH required to elute the retained portion of citrus polygalacturonic acid. Citrus polygalacturonic acid was applied to three analytical columns of DEAE-Trisacryl. The columns were developed in order with 0.5 M (83 to 86% of the AUA applied was eluted) and 1 M (2% was eluted) NH_4Cl in buffer at pH 5.5. Treatment of the columns with 0.5 M NH_4Cl in water at pH 6.5, 7.5, or 8.5 (pH adjusted with NH_4OH) eluted 0.4, 0.7, and 7.1% additional sample. The remainder was eluted from the columns with 0.5 M NH_4Cl in water at pH 9.5. The total recovery of sample from the columns ranged from 96 to 99%. Pectic polysaccharide F-167 was very effectively eluted with 0.5 M and 1.0 M LiCl (Table 3.2).

Similar results were obtained on a preparative scale. Recovery of AUA was 47.4% when the DEAE-Sephadex column was treated with 0 to 0.4 M NaCl in column buffer (Table 3.3). Additional washing of the column with 1 M NaCl in column buffer and at pH 2.0 and 1.2 eluted only an additional 1.5% AUA (Table 3.3). Similarly, when the pectic polysaccharides were fractionationed on a DEAE-Trisacryl column and eluted with 0 to 0.5 M NaCl in column buffer, 55% of the sample was eluted and washing the column with 1 M NaCl in pH 5.0 column buffer and pH 8.2 buffer only resulted in the recovery of an additional 0.4% of the sample (Table 3.3). However, when a linear gradient of 0 to 0.5 M NH_4Cl in column buffer was used to develope an identical colunm, 99% of the pectic

Table 3.3. Effect of salt and resin types on the elution of the 22°C ammonium oxalate-soluble pectic polysaccharide fraction of *L. minor* from ion-exchange columns.

Column material	Eluent 1		Eluent 2		Eluent 3		Eluent 4		Total recovery of pectic polysaccharide (%)[b]
	Salt and buffer	AUA recovered (%)[a]	Salt and buffer	AUA recovered (%)[a]	Salt and buffer	AUA recovered (%)[a]	Salt and buffer	AUA recovered (%)[a]	
DEAE-Sephadex	0-0.4 M NaCl/ 0.05 M sodium oxalate, pH 5.0	47.4	1 M NaCl/0.05 M sodium oxalate pH 5.0	0.7	1 M NaCl, pH 2	0.6	1 M NaCl, pH 1.2	0.2	48.9
DEAE-Trisacryl	0-0.5 M NaCl/ 0.05 M sodium oxalate, pH 5.0	55.1	1 M NaCl/0.05 M sodium oxalate pH 5.0	0.1	1 M NaCl/0.05 M sodium phosphate pH 8.2	0.3			55.5
DEAE-Trisacryl	0-0.5 M NH_4Cl/ 0.05 M ammonium acetate, pH 5.5	99.4	1 M NH_4Cl/0.05 M ammonium acetate, pH 5.5	0.4					99.8

[a] AUA was determined as described in the Materials and Methods. AUA recovered (%) is the percentage of AUA found in the eluate of that applied to the column.

[b] The sum of AUA found in eluates 1, 2, 3 and 4 was the total recovery of the pectic polysaccharide. AUA in the sample-loading fractions and in the column-washing fractions (before eluent 1 started) was less than 0.2% and was not considered in the calculation of total recovery of pectic polysaccharide.

polysaccharide material applied was eluted (Table 3.3).

Gel formation was observed when apple pectic acid at concentrations of 0.5 and 1 mg/mL was incubated with four different concentrations of NaCl and KCl (Figures 3.1a and b). For apple pectic acid samples that contained both 0.5 mg/mL (Figure 3.1a) and 1 mg/mL (Figure 3.1b), the opacity of the gel, as measured by absorbance at 600 nm, did not change significantly between 0.5 h and 102 h after NaCl or KCl was mixed with the pectic acid, although the absorbance of the samples that contained 1 mg/mL apple pectic acid is 1.9-4.0 times as high as the absorbance of the samples that contained 0.5 mg/mL. For samples that contained apple pectic acid at 1 mg/mL, the highest absorbance of the gel was at 1 M NaCl (absorbance ranged from 0.115 to 0.121 over the 102 h), while for samples that contained 0.5 mg/mL apple pectic acid the highest absorbance was observed at 0.5 M NaCl (absorbance ranged from 0.037 to 0.039). Lower gel formation was observed with samples that contained KCl; for samples containing 0.5 and 1 mg/mL of apple pectic acid the highest absorbance was at 0.5 M KCl, and it ranged from 0.015 to 0.017 (Figure 3.1a) and from 0.028 to 0.032 (Figure 3.1b), respectively, over the 102 h. No detectable gel or precipitate was observed with apple pectic acid samples of 0.5 and 1.0 mg/mL when 0.5 M and 1 M NH_4Cl ($A \leq 0.003$) or 0.5, 1, 1.5 and 2 M LiCl ($A \leq 0.004$) or CsCl ($A \leq 0.005$) was present. However, a precipitate was observed in all samples containing 1.5 M and 2 M NH_4Cl and, in contrast to the gels obtained with NaCl and KCl, the precipitate increased with time (Figures 3.1a and b). Samples containing 1 mg/mL of apple pectic acid and 2 M NH_4Cl produced a precipitate that was more opaque and formed more rapidly than those containing 0.5 mg/mL pectic acid and 1.5 M NH_4Cl.

Figure 3.1. Precipitation of apple pectic acid with NH_4Cl, NaCl, KCl, CsCl and LiCl. Precepitation was measured as absorbance at 600 nm after 0.5, 1, 1.5 and 2 M salts were mixed with 0.5 mg/mL (a) and 1 mg/mL (b) of apple pectic acid and solutions stood at 22°C for 0.5, 6, 16, 28, 52, 75 and 102 h.

A 600 nm
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
NH4Cl, 2 M
NH4Cl, 1.5 M
NH4Cl, 1 M
NH4Cl, 0.5 M
NaCl, 2 M
NaCl, 1.5 M
NaCl, 1 M
NaCl, 0.5 M
KCl, 2 M
KCl, 1.5 M
KCl, 1 M
KCl, 0.5 M
CsCl, 2 M
CsCl, 1.5 M
CsCl, 1 M
CsCl, 0.5 M
LiCl, 2 M
LiCl, 1.5 M
LiCl, 1 M
LiCl, 0.5 M
0.5 h
6 h
16 h
28 h
52 h
75 h
102 h

A 600 nm
0.32
0.28
0.24
0.20
0.16
0.12
0.08
0.04
NH4Cl, 2 M
NH4Cl, 1.5 M
NH4Cl, 1 M
NH4Cl, 0.5 M
NaCl, 2 M
NaCl, 1.5 M
NaCl, 1 M
NaCl, 0.5 M
KCl, 2 M
KCl, 1.5 M
KCl, 1 M
KCl, 0.5 M
CsCl, 2 M
CsCl, 1.5 M
CsCl, 1 M
CsCl, 0.5 M
LiCl, 2 M
LiCl, 1.5 M
LiCl, 1 M
LiCl, 0.5 M
0.5 h
6 h
16 h
28 h
52 h
75 h
102 h

The five salts were tested for their effect on color formation in the uronic acid test. The uronic content of standard galacturonic acid samples containing 1 M NaCl, KCl, NH_4Cl, LiCl, and CsCl was the same, within ± 3%, as the control, which contained no salt.

DISCUSSION

The type of cation present in the eluent dramatically affected the recovery of pectic polysaccharides from columns of DEAE-Sephadex A-25 and DEAE-Trisacryl Plus M. Quantitative or near quantitative recovery of all pectic polysaccharides from DEAE-resins was achieved when up to 1 M NH_4^+ ion was used in the eluent. Similar high recoveries were achieved with Li^+ or Cs^+ ion. The recovery of apple pectic acid from the columns was lowest when Na^+ ion was in the eluent and next lowest with K^+ ion. The discovery that the presence of NH_4^+, Li^+, or Cs^+ ion in the eluent brings about the essentially quantitative elution of pectic polysaccharides from anion exchange resins solves the long-standing problem of incomplete recovery of pectic polysaccharides from DEAE-columns.

We suggest that pectic polysaccharide samples retained on DEAE-columns when 0.5 M NaCl or KCl is the eluent form a gel around the resin beads at the top of the column. This is supported by the observation that both 0.5 M and 1.0 M NaCl and KCl caused formation of a gel when mixed with solutions of apple pectic acid and by the positive correlation between the formation of a thicker, heavier gel with NaCl than with KCl and the lower recovery of three of the polysaccharide samples from columns developed with NaCl compared to those developed with KCl. This is also supported by the observation that a gap

formed in DEAE-Trisacryl columns when apple pectic acid was applied and the columns were developed with 0.5 M NaCl or KCl. Gel formation presumably occurred when polysaccharide collecting at the top of the column during loading reached a sufficiently high concentration. Increasing the concentration of NaCl or KCl or changing the anion (data not presented) or the pH of the eluent did not dissolve the gel on the column. We suggest the gel was dissolved when the cation in the eluent was changed to NH_4^+ ion. The collapse of the upper, separated layer of DEAE-Trisacryl columns on the lower layer in experiments with apple pectic acid and NaCl and KCl plus the immediate, basically quantitative elution of all polysaccharides when the cation was changed from 0.5 M Na^+ or K^+ ion to 0.5 M NH_4^+ ion both indicated the gel was dissolved by NH_4^+ ion. Presumably Li^+ and Cs^+ ions would also be effective in dissolving the gel on the column since their elution effectiveness is similar to that of NH_4^+ ion. The formation of the gel is also dependent upon structure of pectic polysccharides. With citrus pectin, gel formation apparently does not occur and with pectic polysaccharide F-129 of *L. minor* little occurs as indicated by the near quantitative recoveries of these polysaccharides from the columns when NaCl and KCl are the eluting salts. Citrus pectin may not form a gel because of its high degree of methyl esterification and pectic polysaccharide F-129 from *L. minor* may not because the neutral sugars in the polysaccharide may be present as a large number of small side-chains distributed relatively equally along the main chain. F-129 has a neutral sugar content of 54%.

When solutions of apple pectic acid were mixed with NaCl or KCl, the material that formed was considered a gel because the suspensions were translucent and there was little settling of the material for extended periods after formation. Although no detectable

precipitate or gel was formed when apple pectic acid and 1 M NH_4Cl were mixed, a precipitate was observed at higher concentrations of NH_4Cl. In contrast to the results with NaCl and KCl, the precipitate with NH_4Cl increased with time and was more like a true precipitate, that is, the suspensions were more opaque and the material settle soon after formation. Both LiCl and CsCl up to 2 M did not cause any detectable gel formation or precipitate with apple pectic acid at concentrations up to 1 mg of polysaccharide per mL.

The putative pectic polysaccharide gel that formed around beads of DEAE-Trisacryl when NaCl and KCl were used was apparently firmer than that which formed around beads of DEAE-Sephadex since in the experiments with apple pectic acid a gap did not form in the DEAE-Sephadex resin bed as it did in the DEAE-Trisacryl bed even though DEAE-Sephadex contracted more than DEAE-Trisacryl at the same concentration of NaCl or KCl. A firmer gel could also account for the lower recovery of apple pectic acid from DEAE-Trisacryl (6.3 and 32%) compared to DEAE-Sephadex (8.6 and 54%). A firmer gel may form because of the smaller wet bead size of DEAE-Trisacryl. However, Redgwell and Selvendran observed that the recovery of an onion pectic polysaccharide fraction from DEAE-Sephadex was lower (59%) than from DEAE-Trisacryl (88%) when phosphate buffer at pH 6.3 and 1 M NaCl were used (15).

Quantitative recovery of six different pectic polysaccharides from DEAE-columns was achieved when NH_4^+ ion was present in the eluent. In contrast, incomplete recovery, and in one case no recovery, was obtained with five of the polysaccharides when Na^+ or K^+ ion was present in the eluent. With one polysaccharide, citrus pectin, NH_4^+, Na^+ and K^+ ions were equally effective in the elution of the polysaccharide. Li^+ and Cs^+ ions acted similar to NH_4^+

ion. We postulated that a polysaccharide gel formed on the DEAE-column when Na^+ or K^+ ion was present in the eluent and the gel was responsible for the incomplete elution of sample. We also proposed that NH_4^+ ion had the ability to dissolve the gel.

REFERENCES

1. Neukom, H., Deuel, H., Heri, W.J., and Kundig, W. (1960) *Helv. Chim. Acta* **43**, 64-71.
2. Siddiqui, I. R. and Wood, P.J. (1974) *Carbohydr. Res.* **36**, 35-44.
3. Aspinall, G. O. and Jiang, K.-S. (1974) *Carbohydr. Res.* **38**, 247-255.
4. Aspinall, G. O., Fanous, H.K. and Sen, A.K. (1983) in *Unconventional Sources of Dietary Fiber* (ed. I. Furda) *Am. Chem. Soc. Sym. Series* **214**, Am. Chem. Soc., Washington, D. C., pp 33-48.
5. Stevens, B. J .H. and Selvendran, R. R. (1984) *Phytochem.* **23**, 107-115.
6. Kato, Y. and Nevins, D. J. (1989) *Plant Physiol.* **89**, 792-797.
7. Stevens, B. J. H. and Selvendran, R. R. (1984) *Carbohydr. Res.* **128**, 321-333.
8. Stevens, B. J. H. and Selvendran, R. R. (1984) *Carbohydr. Res.* **135**, 155-166.
9. Aspinall, G. O. and Fanous, H. K. (1984) *Carbohydr. Polymers.* **4**, 193-214.
10. Saulnier, L. and Thibault, J.-F. (1987) *Carbohydr. Polymers* **7**, 329-343.
11. Massiot, P., Rouau, X., and Thibault, J.-F. (1988) *Carbohydr. Res.* **172**, 229-242.
12. Marcelin, O., Saulnier, L., and Brillouet, J.-M. (1991) *Carbohydr. Res.* **212**, 159-167.
13. Rombouts, F. M. and Thibault, J.-F. (1986) *Carbohydr. Res.* **154**, 177-187.
14. Renard, C. M. G. C., Voragen, A. G. J., Thibault, J. -F. and Pilnik, W. (1990) *Carbohydr. Polymers* **12**, 9-25.
15. Redgwell, R. J. and Selvendran, R. R. (1986) *Carbohydr. Res.* **157**, 183-199.
16. Ryden, P. and Selvendran, R. R. (1990) *Carbohydr. Res.* **195**, 257-272.
17. Ryden, P. and Selvendran, R. R. (1990) *Biochem. J.* **269**, 393-402.

18. Blumenkrantz, N. and Asboe-Hansen, G. (1973) *Anal. Biochem.* **54**, 484-489.

19. Kindel, P. K. and Watson, R. R. (1973) *Biochem. J.* **133**, 227-241.

20. Kindel, P. K., Cheng, L. and Ade, E. R. (1995) *Phytochemistry*, Vol. **41**, No. **3**, 719-723.

CHAPTER IV

A MICRO-SCALE METHOD FOR DEPOLYMERIZATION OF PECTIC POLYSACCHARIDES

ABSTRACT

A micro-scale method utilizing methanolysis, methylester formation, reduction and hydrolysis was developed for depolymerizing pectic polysaccharides. The neutral monosaccharides that were formed were converted to alditol acetates for analysis by gas chromatography and mass spectrometry. For routine analysis, samples of 0.11-0.14 mg were used and all reactions were performed in a single vial. The depolymerization of the polysaccharides and the reduction of the carboxyl groups of the galacturonosyl residues were virtually complete and sample recovery was good.

INTRODUCTION

When chromatographic methods such as GC and HPLC are used to determine the sugar composition of a pectic polysaccharide the sample must first be depolymerized. Typically this is done by acid hydrolysis or methanolysis. Problems with acid hydrolysis are that the glycosidic linkage of uronic acid residues is quite resistant to acid hydrolysis (1), and the released sugar acids decompose at a faster rate than the corresponding neutral sugars (2). One way investigators have attempted to solve these problems is by reducing the uronic acid residues to neutral sugar residues before hydrolysis. The method of Taylor and Conrad (3), which involves the use of a water-soluble carbodiimide and the reduction of a putative intramolecular ester by $NaBH_4$, is widely used but has some disadvantages. The method requires a relatively large amount of sample (0.1 mmole of uronosyl units), simultaneous treatment of multiple samples is difficult, sample loss occurs during the desalting step and complete reduction is not obtained with a single treatment and consequently the process must be repeated one or more times (3-7). Anderson and Stone (8) modified the method for use with smaller samples by using buffer to control the pH, but our results with several pectic polysaccharides showed the buffer failed to maintain the proper pH and complete reduction of carboxyl groups was not achieved. A method that involved methyl esterification followed by reduction with $LiAlH_4$ (9) or $NaBH_4$ (10) was used successfully to reduce the carboxyl groups of uronic acid residues of oligosaccharides. However, it was not capable of completely reducing the carboxyl groups of three pectic polysaccharides tested (this paper). De Ruiter *et al.* (11) described a

method involving methanolysis, TFA hydrolysis and the use of HPAEC-PAD to separate and measure the amount of mono- and oligosaccharides produced from the polysaccharides. The separation of mono- and oligosaccharides of galacturonic acid was good with this method, but the separation of neutral monosaccharides was not. Since the methylester of uronic acid residues that are formed during methanolysis should be quickly hydrolyzed in the subsequent TFA hydrolysis step and released as free uronic acids, the method could still result in almost as great a loss of uronic acid by decomposition as direct acid hydrolysis of pectic polysaccharides. Consequently, it was not surprising to find that sample recovery was low with the method (this paper). In this paper, we report a micro-scale method that effectively depolymerized three pectic polysaccharides tested. The method involves methanolysis/methyl esterification, reduction with $NaBH_4$ (or $NaBD_4$), a repeat of these two steps, acid hydrolysis and preparation of the alditol acetates. The entire procedure, including preparation of alditol acetates, was performed in a single vessel.

MATERIALS AND METHODS

Materials. Apple pectic acid were obtained from Aldrich and PGUA was obtained from Sigma. A cell wall pectic polysaccharide was isolated from *Lemna minor*; it had a $\overline{M}_w$ of 66,400, consisted of 97 mole% galacturonic acid and small amounts of apiose and xylose, and had a degree of methyl esterification of less than 1.0%. A stock solution of methanolic HCl (3.8 M) was prepared by dissolving the HCl gas generated from the

reaction of H_2SO_4 with NH_4Cl in methanol. Immediately before diluting, the concentration of HCl in the stock solution was redetermined by titration.

Depolymerization of pectic polysaccharides. Cell wall pectic polysaccharide from *L. minor*, 0.4 mg, and polygalacturonic acid and apple pectic acid, each 0.5 mg (samples of 0.11-0.14 mg were used in the routine analysis where only GC-MS analysis was performed), were subjected to the following three depolymerization methods. Method I: The methanolysis and acid hydrolysis procedure of De Ruiter *et al.*(11). Method II: The samples were methylesterified, reduced and acid hydrolyzed as described by Hollingsworth *et al.*(10). Method III (proposed method): Dry samples were treated with 0.5 mL of 2 M HCl in dry methanol and 0.05 mL of trimethylorthoformate at 80°C for 8 h with sonication at 80°C for 5 min after 1 h. *t*-Butanol was added and the samples were dried in ambient air with N_2. The samples were reduced with 0.8 mL of $NaBD_4$ in aq. 75% (v/v) ethanol (2 mg/mL) at 22°C for 10 h. The samples were acidified with glacial acetic acid (usually two drops), mixed and dried with N_2 at 30°C or less. Methanol (1 mL) was added and the samples were dried at 30°C or less. Addition of methanol and drying was repeated four times and the samples were desiccated *in vacuo*. The above procedure of depolymerization and reduction was repeated once and the samples were hydrolyzed with 0.5 mL of 2 M TFA at 110°C for 0.5 h. The hydrolyzed samples were analyzed by GC-MS (Appendix B) after conversion of sugars to alditol acetates (12,13) and by HPAEC-PAD. The total carbohydrate and uronic acid of the samples were measured before and after depolymerization by the method of Dubois *et al.* (14) and Blumenkrantz and Asboe-Hansen (15), respectively, and the results were used to calculate percent recovery of sample and

percent uronic acid remaining in the sample (16, Appendices C and D). Sample recover is the sum of the mole of Api, Gal, and AUA after depolymerization divided by the sum of the mole of Api, Gal, and AUA before depolymerization and times 100 (Appendix D).

Establishing conditions for Method III. For the first methanolysis/methyl esterification, 0.5 mL of 2 M HCl in methanol was added to vials containing 0.11-0.14 mg of cell wall pectic polysaccharide from *L. minor*. The samples were heated at 80°C for the time periods shown in Table 4.2 and were dried with N_2 and reduced with $NaBH_4$ as described above in Method III. After reduction, the samples were hydrolyzed with 2 M TFA for the time periods shown in Table 4.2 and dried with N_2 at 30°C or less. The second methanolysis/methyl esterification was with either 2 M or 0.18 M HCl/methanol and time and temperature were varied as shown in Table 4.2. The samples then were reduced with $NaBH_4$ as in Method III. Additional information on optimum conditions was obtained with apple pectic acid. Samples of 0.13 mg were treated as described in Method III except the time periods of both methanolysis/methyl esterification treatments were varied as shown in Figure 4.1.

HPAEC-PAD. Depolymerized samples were dissolved in 1 mL of water and were analyzed by HPAEC-PAD (Dionex Bio LC system, Dionex Corp.). The HPAEC-PAD system was equipped with a CarboPac PA1 column (4 × 250 mm) and a CarboPac PA1 guard column and was operated at 22°C. The flow rate was 1 mL/min, samples of 20 μL were injected, and the column was developed by using, in order, the following times and eluents: 0-18 min, 20 mM NaOH; 18-45 min, a double gradient consisting of 20 to 100 mM NaOH and 0 to 1 M sodium acetate, both increased linearly; 45-55 min, the

concentration of NaOH and sodium acetate was kept unchanged; 55-60 min, the concentration of NaOH and sodium acetate was decreased linearly to 20 mM and 0 M, respectively. Chromatograms were recorded and integrated with both a HP3390 integrator and a computer. The latter was connected to the HPAEC-PAD system through a SRI interface and operated with PEAK-II software (SRI Instruments, Torrance, California). Standard samples of galactose and galacturonic acid were used to calibrate the HPAEC-PAD system and to quantify these two sugar in samples.

Standard sugar mixture treated by Method III. Standard mixtures containing 60 to 80 μg each of rhamnose, fucose, arabinose, xylose, apiose, mannose, PGUA, and glucose were treated as follows: (i) 60 μg of *myo*-inositol was added (as an internal standard) and the sample was treated by Method III and (ii) the standard sample mixture was treated by Method III and then 60 μg of *myo*-inositol was added. In the preparation of the peracetates (12,13) the following changes were made: (i) the quantity of reagents used was 2/5 that used by Kindel and Cheng (12) and (ii) the dichloromethane solution containing the alditol peracetates was 0.6 mL and it was vigorously back-extracted with 3 mL of water four times. The products were analyzed by GC (13, Appendix B). A response factor (GC peak area/mass of sugar) for each sugar was calculated.

RESULTS

Comparison of Methods I, II and III. Samples were incompletely depolymerized when Methods I and II were used since significant amounts of incompletely hydrolyzed

known oligogalacturonic acids and unknown products were detected (Table 4.1). The unknown products that eluted in oligogalacturonic acids region were considered to be galacturonic acids-containig oligosaccharides different than the oligogalacturonic acid standards. For Methods I and II, the unidentified charged products accounted for 6.0-15% and 7.1-9.4%, respectively, of the total product peak area detected with the three polysaccharides tested. With Method III, depolymerization of the three pectic polysaccharides was virtually complete since no oligogalacturonic acids were detected and the unidentified peaks in oligogalacturonic acid region were negligible (<0.18 of the total peak area, Table 4.1). The recovery of the sample after being depolymerized by Method I was 48-54%, while the recovery of samples in Methods II and III was 77-90% and 80-85%, respectively (Table 4.1). Although Method II gave a high recovery of sample, the conversion of sugar acid to neutral sugar was very incomplete, as shown by the Gal/GalA mole ratios of between 10/90 and 20/80 for the products (Table 4.1). In contrast, carboxyl reduction of the three pectic polysaccharides analyzed by Method III is virtually complete, since the mole ratio, Gal/GalA, in the depolymerized samples was 97-98/2.9-2.1 (Table 4.1). For all three methods and samples tested, peaks of unknown compounds were also detected by HPAEC-PAD in the region where neutral sugars eluted. The peaks in neutral sugar region were not identified but were also observed in the GalA control. These peaks are not listed in Table 4.1.

Optimization of conditions for Method III. When Method III was used but the second methanolysis was conducted with 0.18 M HCl in methanol at 45°C for 2 h and the samples were hydrolyzed with 2 M TFA at 110°C for 0.5, 1.0 and 1.5 h before second

Table 4.1. Products detected after depolymerization of three pectic polysaccharides by Methods I - III.

Sugar detected[a]	Polygalacturonic acid			Apple pectic acid			*L. minor* pectic polysaccharide		
	Method I	Method II	Method III	Method I	Method II	Method III	Method I	Method II	Method III
Gal	NA[b]	26.7	99.3	NA	39.2	99.1	NA	11.5	99.4
GalA	85.3	61.0	0.54	82.7	50.4	0.76	91.9	71.3	0.63
di-galA	0.73	2.70		0.49	1.86		1.62	4.14	
tri-galA	0.30	1.30		1.44	1.02		0.45	2.12	
tetra-galA	0.24	0.38		0.50	0.33			0.60	
penta-galA		0.16			0.14			0.31	
hexa-galA								0.59	
Unidentified peaks in oligo-uronic acid region	13.4	7.73	0.14	14.9	7.07	0.18	6.03	9.41	0
Mole ratio of Gal/GalA in product	NA	10.1/89.9	97.9/2.1	NA	16.7/83.3	97.1/2.9	NA	20.4/79.6	97.6/2.4
Sample recovery after depolymerization (%)[c]	54.2	76.6	80.2	53.4	82.1	84.8	48.4	89.5	81.5

[a] Numbers are the percent of each compound present based on the area under the peaks from HPAEC-PAD analysis. Sugars were identified by retention times from HPAEC-PAD.

[b] NA = not applicable.

[c] Sample recovery was calculated as described in Materials and Methods.

methanolysis, the uronic acid remaining in the sample after reduction was 21%,19% and 16% (Table 4.2). When the same conditions were used but the time of the second methanolysis was increased to 14 h plus 10 h at 22°C, the uronic acid remaining only decreased from 19% to 14% (1 h TFA samples, Table 4.2). When the temperature of the second methanolysis was increased from 45°C to 80°C and methanolysis times of 2 and 4 h were used, 16 and 14%,respectively, of the uronic acid residues still remained in the samples (Table 4.2). However, when the concentration of HCl/methanol in the second methanolysis was 2 M and each methanolysis was for 1 h at 80°C, the uronic acid remaining in the sample was 5.6% (Table 4.2). Further reduction of the remaining uronic acid residues was difficult. When the times of the first and second methanolysis were increased to as much as 15 and 8 h, respectively, the uronic acid residues remaining in the sample still did not decrease below 3.6% (Table 4.2). A time course experiment examining the depolymerization of apple pectic acid showed that there was only a slight decrease in the uronic acid content of the depolymerized samples when the two methanolysis times were increased from 1.5 h : 1.5 h to 10 h : 10 h (Figure 4.1). At the time the experiments with 0.18 M HCl in methanol were done, hydrolysis with TFA was performed after the first methanolysis (Table 4.2). Later experiments with 2 M HCl in methanol showed this hydrolysis was not necessary (Table 4.2) and it was eliminated from Method III.

Standard sugars mixture treated with Method III. The GC response factors for samples taken through Method III were similar to those of the standard sample hydrolyzed only with 2 M TFA at 120°C for 1 h (Figure 4.2), the condition (17) widely used by

Table 4.2. Optimization of conditions for depolymerization by Method III.

Time of first methanolysis/ methyl esterification (h)	Time of TFA hydrolysis (h)	Second methanolysis/methyl esterification			GalA remaining after depolymerization (%)[a]	Sample recovery (%)[a]
		HCl/MeOH (M)	temperature (°C)	time (h)		
16	0.5	0.18	45	2	20.9	89.6
16	1.0	0.18	45	2	18.8	83.5
16	1.5	0.18	45	2	15.8	83.6
16	1.0	0.18	45	14[b]	14.3	85.7
16	1.0	0.18	80	2	15.6	75.2
16	1.0	0.18	80	4	14.0	75.4
1	0.5	2.0	80	1	5.6	78.9
1	omitted	2.0	80	1	5.8	83.8
3	0.5	2.0	80	3	4.8	79.3
3	omitted	2.0	80	3	4.3	84.9
8	omitted	2.0	80	8	3.6	82.1
15	omitted	2.0	80	8	4.1	83.7

[a] Uronic acid remaining and sample recovery were calculated as described in the Materials and Methods.

[b] After methanolysis for 14 h at 45°C, the sample stood for 10 h at 22°C before reduction.

Figure 4.1. Galacturonic acid remaining after depolymerization of apple pectic acid by Method III used with varying methanolysis times. Times of the first methanolysis are indicated in the figure. Times of the second methanolysis were: 0.25 h (—□—), 0.75 h (···▲···), 1.5 h (···○···), 3 h (---●---) and 10 h (-✚-). GalA remaining (%) after depolymerization was calculated as described in the Materials and Methods.

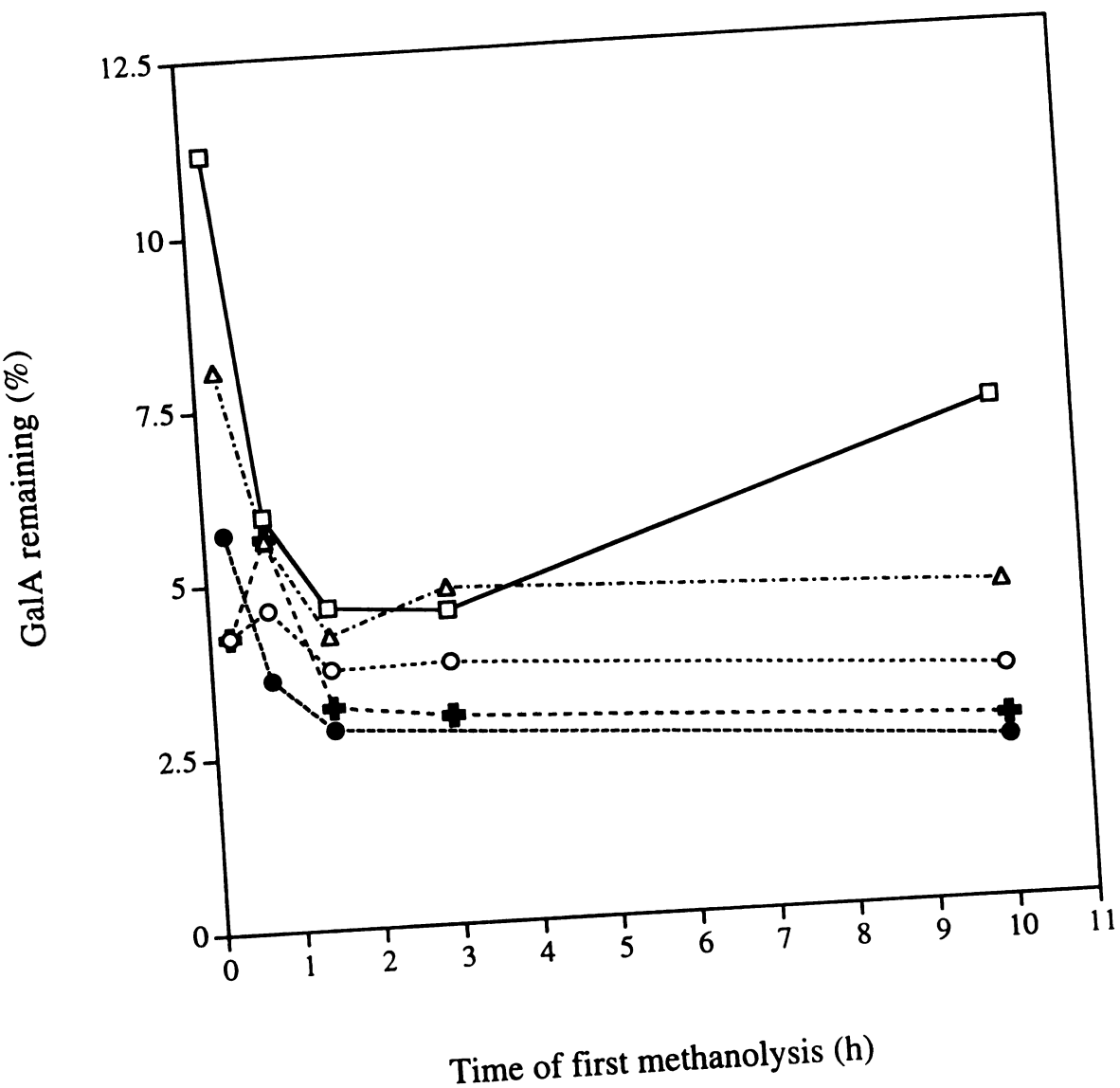
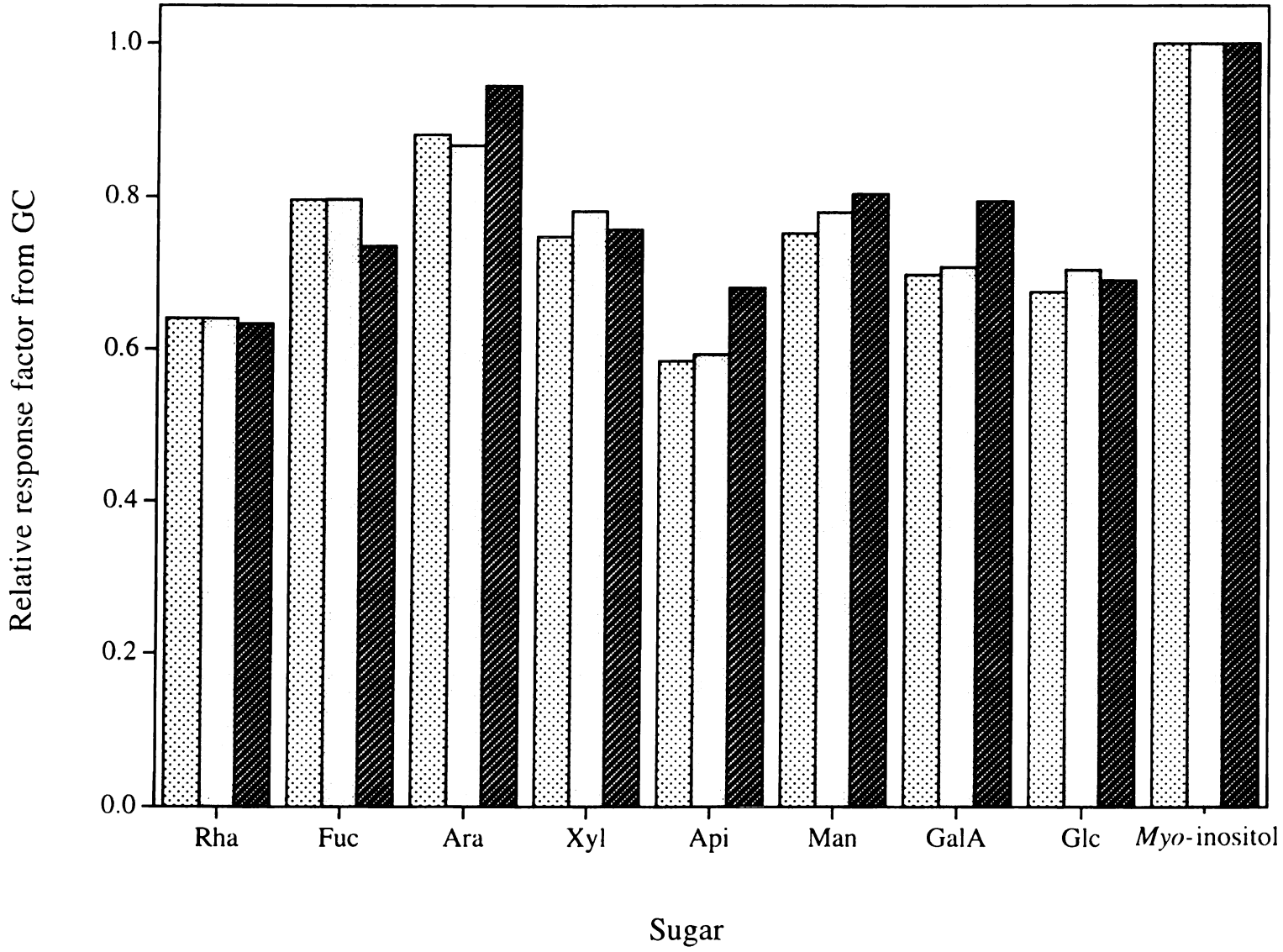

12.5
10
7.5
5
2.5
0
0
1
2
3
4
5
6
7
8
9
10
11
GalA remaining (%)
Time of first methanolysis (h)

Figure 4.2. GC response factors of eight alditol peracetates and *myo*-inositol peracetate (as internal standard) based on area/mg of sample. Response factors of alditol acetates are relative to *myo*-inositol which was set to unity. Response factors of alditol peracetates when *myo*-inositol was added after ▩ and before ▩ the sugars were treated by Method III. Response factors of sugars not treated by Method III but hydrolyzed with 2 M TFA at 120°C for 1 h and converted directly to alditol peracetates ▨.


Relative response factor from GC
1.0
0.8
0.6
0.4
0.2
0.0
Rha
Fuc
Ara
Xyl
Api
Man
GalA
Glc
Myo-inositol
Sugar

researchers to hydrolyze neutral polysaccharides. Addition of internal standard (*myo*-inositol) before and after depolymerization by Method III did not have a significant effect on the response factors.

DISCUSSION

In our proposed method the pectic polysaccharides were partially depolymerized and methylesterified by the first methanolysis and then the esterified carboxyl groups were reduced. The hydrolysis with TFA cleaved the remaining mainly reduced oligosaccharides as well as the methyl glycosides. Although the total time of methanolysis (16 h) in our proposed procedure is the same as in Method I, the second methanolysis was conducted after most of the uronic acid residues were reduced to neutral sugar residues. Since the glycosidic linkages of neutral sugars are much more susceptible to acid hydrolysis than those of uronic acids (1) and since the released neutral sugars are less susceptible to acid degradation than are uronic acids (2), the conversion of uronic acid to neutral sugar in our procedure both enhanced the depolymerization of the pectic polysaccharides as well as reduced the loss of released sugar during both the methanolyses and the TFA hydrolysis. The procedure resulted in complete depolymerization of polysaccharides (no oligosaccharides were observed) and a much higher sample recovery (80-85%) than the procedure of De Ruiter *et al.* (11) (48-54%), at least for the three pectic polysaccharides tested (Table 4.1).

Methyl esterification with 0.18 M HCl in dry methanol at 45°C is rigorous enough to

make the methylester of small oligosaccharides that contain some uronic acid residues (10) but is not adequate for methylesterifying the pectic polysaccharides and partially depolymerized pectic polysaccharides tested since the mole ratio, Gal/GalA, in the final product in Method II ranged from 20/80 to 10/90 (Table 4.1). After a first methanolysis with 2 M HCl in methanol, increasing the temperature of methyl esterification in the second methanolysis from 45°C to 80°C or extending the time of the treatment to 14 h plus 10 h at 22°C did not substantially improve methyl esterification since the GalA remaining in samples after depolymerization decreased only from 19% to 16% and 14% (Table 4.2). Strengthening the methanolysis/methyl esterification conditions from 0.18 M to 2 M HCl in dry methanol and 80°C resulted in more complete methyl esterification since the ratio of Gal/GalA now ranged from 98/2 (Table 4.1) to 96/4 (Table 4.2) even though the time of both the first and second methanolysis/methyl esterification was as short as 1 h each. This indicated the concentration of HCl/methanol was more important than temperature or time of methanolysis in the conversion of GalA units to Gal. Since the glycosidic linkage to neutral sugars is more susceptible to methanolysis and acid hydrolysis than is the same linkage to uronic acids, it is not surprising that virtually complete reduction of the galacturonic acid residues in Method III resulted in almost complete depolymerization (Table 4.1).

Acetylation of eight monosaccharides with or without first treating the sample by Method III did not result in significant change for the GC response factors for alditol acetates. These results indicated Method III (i) did not interfere with the followed acetylation process and (ii) did not cause significant sample loss of the tested sugars.

Addition of *myo*-inositol before or after Method III did not resulted in significant change the GC responce factor of *myo*-inositol compared to those of sugar alditol acetates; this showed Method III did not cause significant loss of myo-inositol.

Although the separation of oligogalacturonic acids with a degree of polymerization up to 15 with the HPAEC-PAD system is fast and complete (data not presented), the separation of the eight neutral monosaccharides examined by this system is incomplete. Complete separation of the common alditol acetates and the ease of interfacing a gas chromatograph to a mass spectrometer still make GC-MS a more accurate system than HPLC-MS for sugar composition analysis and identification in most cases. The advantages of the proposed depolymerization method over others in use (3,11) are: (i) more complete depolymerization of pectic polysaccharides, (ii) greater conversion of uronic acid residues to neutral sugars, (iii) reactions are performed in a single vial and dialysis is not necessary, (iv) 0.11-0.14 mg or less of sample is needed, (v) multiple samples can be treated simultaneously and (vi) recovery of sample is higher.

REFERENCES

1. BeMiller, J. N. (1967) *Adv. Carbohydr. Chem.*, **22**, 25-108.
2. Biermann, C. J. (1988) *Adv. Carbohydr. Chem. Biochem.*, **46**, 251-271.
3. Taylor, R. L., and Conrad, H. E. (1972) *Biochemistry*, **11**,1383-1388.
4. Schols, H. A., Posthumus, M. A. and Voragen. A. G. (1990) *Carbohydr. Res.* **206**,117-129
5. Kiyohara, H., Cyong J. C. and Yamada, H. (1988) *Carbohydr. Res.*, **182**, 259-275.
6. Saulnier, L., Brillouet, J. M. and Joselean, J. P.(1988) *Carbohydr. Res.*182, 63-78
7. Lan, J. M., Michael, M., Darvill, A. G. and Albersheim, P. (1985) *Carbohydr. Res.*, **137**, 111-125.
8. Anderson, M. A. and Stone, B. A. (1985) *Carbohydr. Polymers*, **5**,115-129.
9. Adams, G. A. and Bishop, C. T. (1956) *J. Am. Chem. Soc.*, **78**, 2842-2844.
10. Hollingsworth, R. I., Abe, M., Sherwood, J. E. and Dazzo, F. B. (1984) *J. Bacteriol.*, **160**, No. **2**, 510-516.
11. De Ruiter, G. A., Schols, H. A., Voragen, A. G. J. and Rombouts, F. M. (1992) *Anal. Biochem.* **207**, 176-185.
12. Kindel, P. K. and Cheng, L. (1990) *Carbohydr. Res.*, **199**, 55-65.
13. Cheng, L. and Kindel, P. K. (1996) *Carbohydr. Res.*, **290**, 67-70.
14. Dubois, M. G., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.*, **28**, 350-356.

15. Blumenkrantz, N. and Asboe-Hansen, G. (1973) *Anal. Biochem.*, **54**, 484-489.

16. Kindel, P. K., Cheng, L. and Ade, B. R. (1996) *Phytochemistry*, **Vol. 41, No. 3**, 719-723.

17. Albersheim, P., Nevins, D. J., English, P. D. and Karr, A. (1967) *Carbohydr. Res.*, **5**, 340-345.

CHAPTER V

HOMOGENEITY AND STRUCTURE OF THE 22°C AMMONIUM OXALATE-SOLUBLE PECTIC POLYSACCHARIDES OF *Lemna minor*

[Adapted from Cheng, L. and Kindel, P. K., *Carbohydr. Res.*, 1996, submitted]

ABSTRACT

The homogeneity of pectic polysaccharides from *Lemna minor* purified through DEAE-Trisacryl Plus M column chromatography was determined by analyzing the polysaccharide material in individual column fractions. Determining the $\overline{M}_w$ of such polysaccharide material by using multi-angle laser light scattering detector in conjunction with high performance size exclusion chromatography showed that four pectic polysaccharides were present in the 22°C ammonium oxalate-soluble fraction prepared from purified cell walls of *L. minor*. The four pectic polysaccharides were designated PS-I, -II, -III and -IV and the peak $\overline{M}_w$ ranges of the purified polysaccharides were: PS-I, 50,200 - 75,400; PS-II, 18,800 - 99,700; PS-III, 24,200 - 150,000; and PS-IV, 6,170 to at least 163,000. PS-IIb and PS-IVb, major components of PS-II and PS-IV, respectively, were basically homogeneous with respect to sugar composition and degree of methyl esterification, each however was different chemically. The average sugar composition of the polysaccharide material analyzed that constituted PS-IIb was: GalA (54.5±3.2%) (std. dev.), Api (39.3 ± 1.4%), Xyl (2.5% ±1.4%), Ara (1.4% ± 0.8%), and Rha (2.3 ± 0.3%) while that of PS-IVb was: GalA (96.3% ± 0.8%), Api (2.1 ± 0.7%), and Xyl (1.6 ± 0.3%). Small amounts (<0.5%) of other common sugars were detected in the two polysaccharides. The degree of methyl esterification of both PS-IIb and PS-IVb was basically zero. The isolation of pectic polysaccharide of high galacturonic acid content (96%) and low degree of methyl esterification (~0%) while using mild conditions is new. PS-IIb and PS-IVb were heterogeneous with respect to size of molecules. The peak $\overline{M}_w$ range of PS-IIb was 28,100 - 99,700 while the range for PS-IVb was 53,300 to at least 163,000. A simple, mild procedure for quantitatively isolating and purifying pectic

polysaccharides from the 22°C ammonium oxalate-soluble fraction was developed.

INTRODUCTION

Pectic polysaccharides constitute one of the three major polysaccharide components of primary cell walls of plants (1). It is well known that a portion of the cell wall pectic polysaccharides is solubilized by chelating agents at room temperature. Recently, the kinetics of the solubilization process were reported (2) (Appendix A). Kinetic experiments with *L. minor* (duckweed) and *Apium graveolens* (celery) showed that solubilization of the 22°C ammonium oxalate-soluble fraction occurs rapidly and is almost complete in 15 min and that further solubilization virtually ceases after 30 min (Appendix A). With purified cell walls of fresh *L. minor* and store-purchased *A. graveolens*, 32% and 22%, respectively, of the total anhydrouronic acid of the cell wall is solubilized in 30 min. These results show that on the basis of solubility, the pectic polysaccharides of the 22°C chelator-soluble fraction are different from the pectic polysaccharides remaining in the cell wall, at least in these two plants. Whether they also differ structurally is not yet known.

Purification and characterization experiments indicate that the 22°C chelator-soluble fraction consists of more than one component (3-11); however, the results do not rule out the possibility that there is a continuum in the structure of the pectic polysaccharide material in the 22°C chelator soluble fraction and discrete polysaccharides are not present. Typically the last step in the purification of pectic polysaccharides from this fraction is DEAE column chromatography. The homogeneity of the pectic polysaccharides eluted from the columns

has not been established. The goal of this study was to determine the degree of homogeneity of the isolated pectic polysaccharides and then determine their structure. Determining the degree of homogeneity first will simplify the interpretation of structural results. Homogeneity was established by determining different properties of the pectic polysaccharide material in the individual DEAE column fractions and then comparing each property across column fractions. If a property of the pectic polysaccharide material in a contiguous set of fractions was the same, the pectic polysaccharide material in these fractions would be homogeneous with respect to that property. The $\overline{M}_w$, sugar composition, and degree of methyl esterification were the properties determined in this study. Appropriate fractions were also combined as individual polysaccharides for further analysis. The pectic polysaccharides were isolated from the 22°C ammonium oxalate-soluble cell wall fraction of *L. minor*. Since the recovery of pectic polysaccharide material from the DEAE column was quantitative, all the pectic polysaccharides in the fraction were examined. A second goal of the work was to simplify the purification procedure of pectic polysaccharides from cell walls while still maintaining non-degradative conditions.

MATERIALS AND METHODS

General method. *L. minor* was grown as described elsewhere (12). Total carbohydrate and uronic acid were determined by the methods of Dubois *et al.* (13) and Blumenkratz and Asboe-Hansen (14), respectively. DEAE-Trisacryl Plus M, polygalacturonic acid and citrus pectin were purchased from Sigma Chemical Co. Apple pectic acid and D-galacturonic

acid·H_2O were purchased from Aldrich Chemical Co. and Pfanstiehl Laboratories, Inc., respectively. Information on the above three polysaccharides has been given previously (15).

Isolation of pectic polysaccharides. Cell walls were isolated from fresh *L. minor* (597 g) as described by Kindel *et al.* (Appendix A and Ref. 2). The cell walls were extracted immediately with 1070 mL of 0.05 M ammonium oxalate (pH 5.5) at 22°C for 30 min, and the suspension was filtered with 15 μm Nylon mesh (3-15/6, Tetko, Inc.). The cell wall residue was resuspended in 850 mL of 0.05 M ammonium oxalate (pH 5.5), swirled several minutes, and the suspension was filtered. The combined filtrates were filtered successively with 5 and 1.2 μm membrane filters (MF-Millipore, Millipore Corp.). A small portion of the final filtrate was removed for determination of uronic acid. The remainder was applied directly to a DEAE-Trisacryl Plus M column, 2.9 cm (i.d.) x 24 cm (height), and the column was washed with 200 mL of 0.05 M ammonium oxalate (pH 5.5) (column buffer) and then developed with 4800 mL of column buffer containing a linear gradient of 0 to 0.5 M NH_4Cl. Fractions of 20 mL were collected at 1.06 mL/min and analyzed for uronic acid. The $\bar{M}_w$, sugar composition, and degree of methyl esterification of the polysaccharide material in selected column fractions were determined.

Rechromatography of pectic polysaccharides with DEAE-Trisacryl Plus M. Portions (0.5 mL) of DEAE-Trisacryl column fractions 195-224 and 287-320 (fraction numbers refer to Figure 5.3 in Results) were combined to give two polysaccharide samples called PS-IIb and PS-IVb, respectively. PS-IIb and PS-IVb were dialyzed in water, ammonium oxalate was added to each to a final concentration of 0.05 M and the pH of the solutions was adjusted to 5.5 with oxalic acid. Each sample was applied to a separate column of DEAE-

Trisacryl Plus M [0.7 cm (i.d.) x 8 cm (height)]. Each column was washed with 0.05 M ammonium oxalate (pH 5.5) (column buffer), and developed with 100 mL of column buffer containing a linear gradient of 0 to 0.5 M NH_4Cl. Fractions of 1 mL were collected at 3.6 mL/h and were tested for uronic acid.

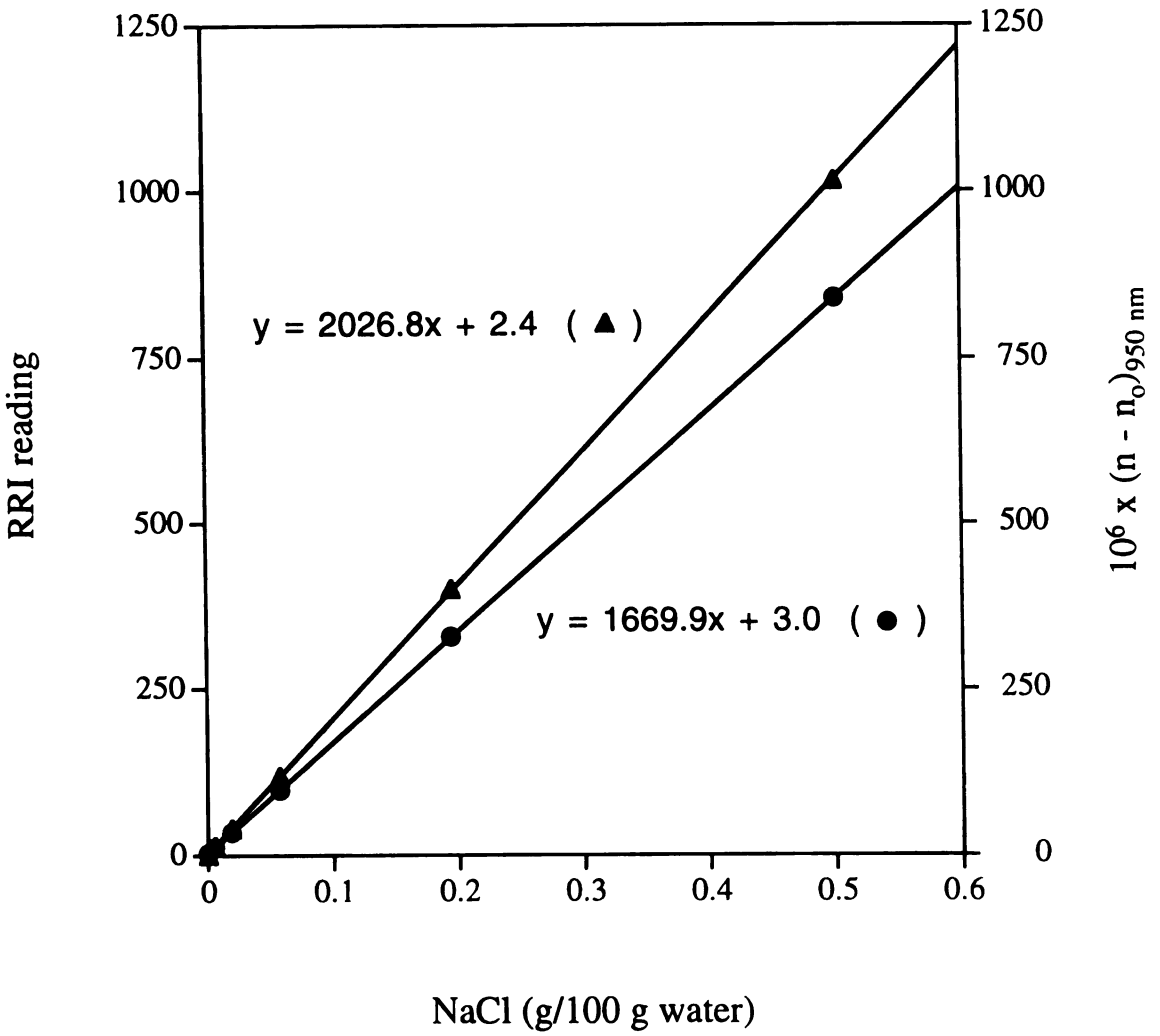
Refractive index increment (dn/dc) of polysaccharide samples. The differential refractometer (Knauer, λ_{max} from a light-emitting diode = 950±30 nm) was calibrated with NaCl. The Cauchy dispersion formula (terms of higher order than B/λ^2 were not used) was used to extrapolate the refractive index data of Kruis (16) at 9 concentrations of NaCl (0.09412 to 6.9092 g/100 g H_2O) to 950 nm. A plot of $(n-n_o)_{950\,nm}$ (from extrapolation) versus concentration of NaCl was prepared, and the following polynomial in concentration was obtained:

$$(n-n_o)_{950\,nm} \times 10^6 = 0.65345C^3 - 21.987C^2 + 1680.6C + 2.6472$$

where $(n-n_o)_{950\,nm}$ is the difference in refractive index between solution (n) and solvent (n_o) at 950 nm and C is the concentration of NaCl in g/100 g H_2O.

The instrument relative refractive index (RRI) reading of 5 concentrations of NaCl (0.0056, 0.0190, 0.0571, 0.1942 and 0.5009 g/100 g H_2O) that were passed through the differential refractometer at 2 mL/min and the $(n-n_o)_{950\,nm}$ of these 5 concentrations calculated from the above polynomial were plotted separately against the concentration of NaCl (Figure 5.1). Because the change in slope of the line was so small over the concentration range of NaCl used, linear regression analysis of the data was performed. The slope of the former plot, $(dRRI/dc)_{NaCl}$ = 2026.8 RRI units (g NaCl/100 g $H_2O)^{-1}$, and the slope of the latter, $(dn/dc)_{NaCl}$ = 1669.9 x 10^{-6} (g NaCl/100 g $H_2O)^{-1}$, were used in the equation below to

Figure 5.1. Relationship between RRI reading (▲) or calculated refractive index at 950 nm $(n-n_o)_{950\ nm}$ (●) and concentration of NaCl. The calculation of $(n-n_o)_{950\ nm}$ and the linear regressions are described in the Materials and Methods.


1250
1000
750
500
250
0
0
0.1
0.2
0.3
0.4
0.5
0.6
y = 2026.8x + 2.4 (▲)
y = 1669.9x + 3.0 (●)
RRI reading
10^6 x (n - n_o)$_{950\ nm}$
NaCl (g/100 g water)

determine the refractive index increment of pectic polysaccharide samples.

A portion (2 mL, 0.53 - 0.83 mg of pectic polysaccharide) of each of fractions 222, 223, 284 and 290 from the DEAE column chromatography (fraction numbers refer to Figure 5.3 in Results and samples were called polysaccharides F222, F223, F284 and F290, respectively; polysaccharide material in other column fractions were named similarly) was dialyzed against 0.35 M NH_4Cl (column buffer) for 28 h with dialysis tubing having a molecular weight cut-off of 6000-8000. The dialyzed samples were passed through the differential refractometer at a rate of 2 mL/min. The relative refractive index readings were obtained with 0.35 M NH_4Cl as the reference and the sample dry weights were calculated from total carbohydrate and uronic acid determinations. The dn/dc values of the polysaccharides were calculated with the equation:

$$(dRRI/dc)_{NaCl}/(dn/dc)_{NaCl} = (dRRI/dc)_{polysaccharide}/(dn/dc)_{polysaccharide}$$

where $(dRRI/dc)_{polysaccharide}$ is the slope from linear regression analysis of the data in the plot of relative refractometer reading versus concentration of the pectic polysaccharide tested, and $(dn/dc)_{polysaccharide}$ is the refractive index increment of the polysaccharide. The dn/dc values of the four pectic polysaccharides and polygalacturonic acid are given in Table 5.1. Since the dn/dc of the polysaccharide material in every column fraction examined by MALLS (multi-angle laser light scattering) was not determined, appropriate dn/dc values of Table 5.1 were averaged and average values were used to calculate the $\bar{M}_w$ of polysaccharides.

Determination of $\bar{M}_w$ and HPSEC. The $\bar{M}$, $\bar{M}_w$ and polydispersity of the polysaccharide(s) in DEAE column fractions 189, 207, 214, 217, 220, 223, 227, 270, 278, 282, 284, 286, 288, 290, 294, 296 and 298 of the DEAE column chromatography (fraction

Table 5.1. Refractive index increment of pectic polysaccharides

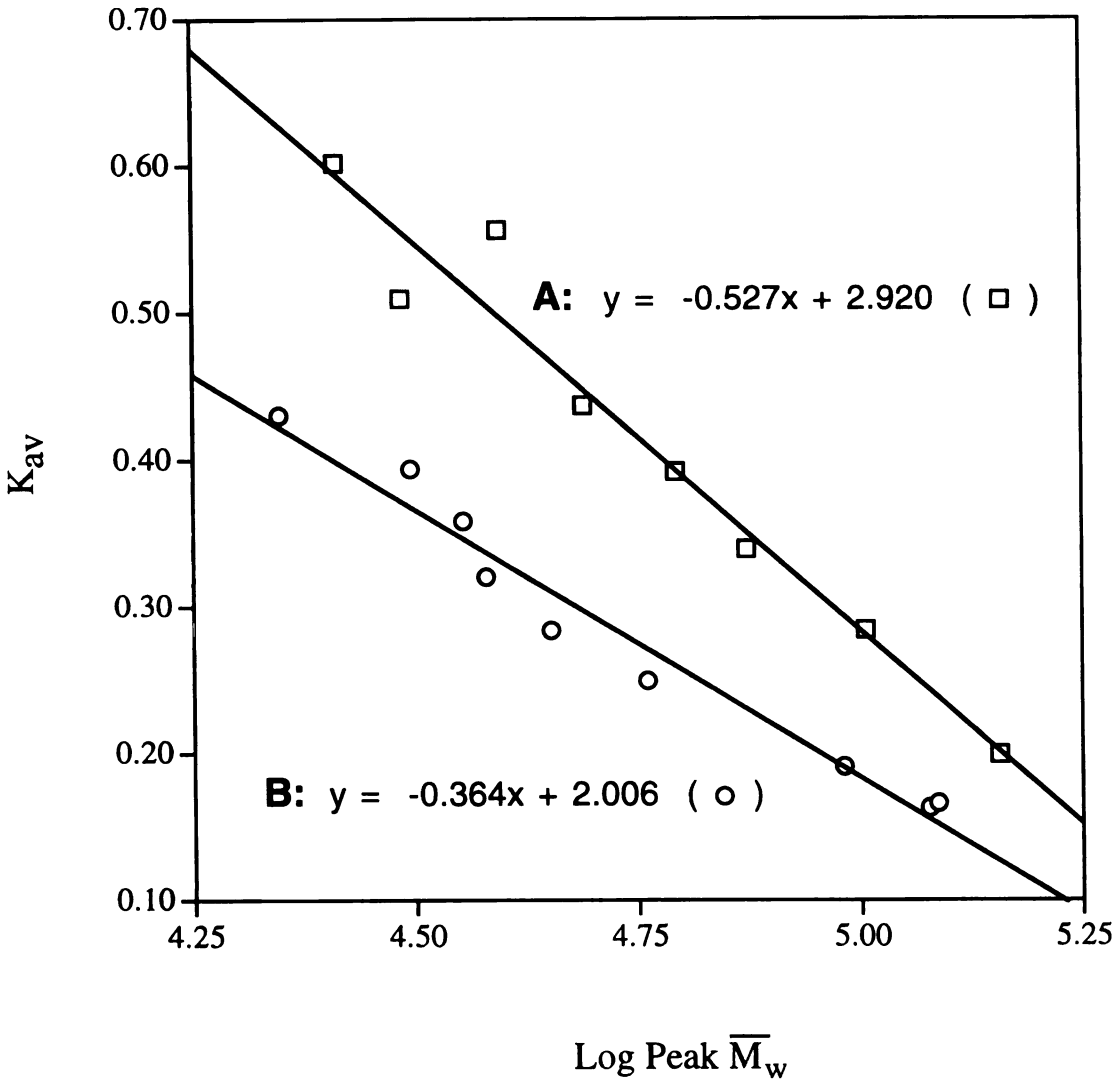
Sample	dn/dc
polygalacturonic acid	0.1757
F222[a]	0.1072
F223[a]	0.1088
F284[a]	0.1526
F290[a]	0.1784

[a]Polysaccharide material in corresponding DEAE column fractions of Figure 5.3.

numbers were referred to Figure 5.3 in Results) were determined with a miniDawn MALLS detector (Wyatt Technology Corp.) used in conjunction with an in-line differential refractometer and an in-line HPSEC column [G4000PW$_{XL}$, 7.8 mm (i.d.) x 30 cm (height), particle size: 10 μm] (TosoHaas). Centrifuged samples of 100 μL from the above DEAE column fractions were chromatographed on the G4000PW$_{XL}$ column shortly after being collected and before being frozen. The column was developed with 0.35 M NH_4Cl at a rate of 0.6 mL/min. HPSEC solvents were filtered through a 0.22 μm filter (Durapore-hydrophilic, Millipore Corp.). The light scattering and refractometry signals for polysaccharide material eluting from the column were collected by a computer (IBM compatible PC). The refractometry signal was also recorded with an integrator (Model 3390A, Hewlett-Packer Co.). The computer data were processed with ASTRA 4.0 and EASI software (Wyatt Technology Corp.) and $\overline{M}_w$, $\overline{M}_n$, and polydispersity and peak $\overline{M}_w$ and peak $\overline{M}_n$ were calculated. Peak $\overline{M}_w$ is defined as the $\overline{M}_w$, determined either directly from light scattering data or indirectly from the two equations of linear regression described below, of a narrow band of polysaccharide material at the top of the polysaccharide peak (band) eluted from the HPSEC column, with polysaccharide being detected by refractometry (see Figure 5.4 of Results). Peak $\overline{M}_w$ values were calculated because retention times from HPSEC were used to calculate $\overline{M}_w$ values of samples not analyzed by MALLS.

Two different linear regression equations of K_{av} [$K_{av} = (V_e - V_o)/(V_t - V_o)$; where V_e, V_o and V_t were elution volumes of pectic polysaccharide, Dextran T-2000, and D-xylose, respectively, determined by HPSEC] versus peak $\overline{M}_w$ (from MALLS) were established and used to calculate the peak $\overline{M}_w$ of column fraction samples (Figure 5.2). Equation A (Figure

Figure 5.2. Relationship between K_{av} and Log $\overline{M}_w$ for those pectic polysaccharide fractions analyzed by HPSEC and MALLS. The polysaccharide fractions used to obtain linear regression equations A and B are given in the Materials and Methods. The calculation of $\overline{M}_w$ is described in the Materials and Methods.


0.70
0.60
0.50
0.40
0.30
0.20
0.10
K_{av}
A: y = -0.527x + 2.920 (□)
B: y = -0.364x + 2.006 (o)
4.25
4.50
4.75
5.00
5.25
Log Peak $\overline{M}_w$

5.2) was derived from the results of HPSEC and MALLS for F189, F207, F214, F217, F220, F223, F227, and F270 and was used to determine the peak $\overline{M}_w$ of the polysaccharide materials in the indicated column fractions of Figure 5.3 that were part of PS-I, PS-II, and PS-III. Equation B (Figure 5.2) was derived from the results of HPSEC and MALLS for F278 (band 2), F282 (band 2), F-284 (band 2), F286, F288, F290, F294, F296, and F298 and was used to determine the peak $\overline{M}_w$ of the polysaccharide material in the indicated column fractions of Figure 5.3 that was part of PS-IV. The selection of data used to establish linear equations A or B in Figure 5.2 was based on that the plotted values in the figure and were most appropriately represented by the two linear equations. PS-I through PS-IV and bands 1 and 2 are defined in the Results.

Depolymerization of pectic polysaccharides and preparation of alditol acetates. Pectic polysaccharide samples (approximately 0.14 mg dry weight) were dialyzed against water, freeze-dried, depolymerized and converted into aditol acetates as described in Chapter IV of this thesis.

Gas chromatography and mass spectrometry. Alditol acetates were separated isothermally at 230°C on a DB-225 fused silica capillary column (30 m x 0.25 mm, i.d.; film thickness, 0.15 μm) (J&W Scientific) attached to a gas chromatograph, Model 5840A (Hewlett-Packard Co.), equipped with a splitter, a flame-ionization detector and a 5840A data terminal. Helium was the carrier gas and the flow rate was 1.45 mL/min. Samples injected into the gas chromatograph ranged from 0.5 to 2.5 μL and the split ratio ranged from 1:10 to 1:20. The FID signal was recorded with a computer (IBM compatible PC) through a interface A/D board (Model 110 BUS, SRI Instrument) and processed by PeakSimple II

software (SRI Instrument).

Electron impact mass spectrometry of alditol acetates was performed with a JEOL JMS-HX110, double-focusing mass spectrometer (JEOL USA) interfaced to a gas chromatograph, Model 5890A (Hewlett-Packard Co.), which was equipped with a splitless injector and a DB-225 fused silica capillary column (30 m x 0.32 mm, i.d.; film thickness, 0.25 μm). Helium was the carrier gas and the flow rate was 4 mL/min. The proportion of galactose units to galacturonic acid units in samples was determined by selective ion monitoring. The ratio, 6,6-dideuteriogalactitol acetate/6,6-dideuteriogalactitol acetate + galactitol acetate, was the mean of the ratios calculated from two ion clusters: (i) ions *m/z* 217, 218 and 219 and (ii) ions *m/z* 289, 290 and 291 (Appendix E). Standard galactitol acetate was prepared from D-galacturonic acid·H_2O and $NaBD_4$ at the same time samples were prepared. The value of the ratio for the standard was set to 100% and the samples were adjusted accordingly.

Determination of degree of methyl esterification. The procedure of Wood and Siddiqui (17) was used to determine the methyl ester content of polysaccharide samples. The degree of methyl esterification is the molar ratio of methyl ester/uronosyl unit for each sample, expressed as a percent.

^1H NMR spectroscopy. Pectic polysaccharide material from column fractions 289, 290 and 292 of Figure 5.3 was dialyzed against water and adjusted to pH 6.0. For F289 and F292 the pH adjustment was by evaporation and addition of D_2O 4 times and then addition of NaOD in D_2O. F290 was adjusted with NaOH before evaporation and then subjected to the same evaporation and D_2O addition as F289 and F292. The pH of F290 remained at 6.0. Polygalacturonic acid and apple pectic acid in water were adjusted to pH 5.0 with NaOH,

lyophilized and approximately 5 mg of each was dissolved in 0.8 - 1.0 mL of D_2O. F223 was treated the same except no pH adjustment was made. The samples were analyzed with a VXR-500 NMR spectrometer (Varian Instruments) at 90°C. The reference frequency was the HDO (in D_2O) peak at 3.937 ppm. In a separate analysis, the HDO reference frequency (3.937 ppm) was obtained by reference to a *p*-dioxane peak which was locked at 3.530 ppm at 90°C.

RESULTS

Isolation of pectic polysaccharides. The pectic polysaccharides solubilized from purified cell walls by 0.05 M ammonium oxalate at 22°C were chromatographed on a DEAE-Trisacryl Plus M column. The column chromatogram, based on determination of uronic acid, is shown in Figure 5.3. Recovery of uronic acid from the column was 99.4%. Although not determined in this experiment, recovery of total carbohydrate in analogous experiments was also quantitative and the total carbohydrate elution profile had the same general shape as the uronic acid profile. Uronic acid and total carbohydrate (in previous experiments) were not detected in column fractions 1 through 110. Two large peaks, a small peak and a small shoulder on the rising side of the second large peak can be seen in the uronic acid profile (Figure 5.3).

$\bar{M}_w$, $\bar{M}_n$ and Polydispersity. The $\bar{M}_w$, $\bar{M}_n$, and polydispersity of the polysaccharide samples that were analyzed directly by MALLS are given in Table 5.2. The $\bar{M}_w$, $\bar{M}_n$ and polydispersity for the peak region of these samples are also given in Table 5.2. These peak $\bar{M}_w$ values are plotted in Figure 5.3 as well so they can be compared to the peak $\bar{M}_w$ values

Figure 5.3. Column chromatogram of the 22°C ammonium oxalate-soluble fraction from purified cell walls of *L. minor*. The column was DEAE-Trisacryl Plus M. Column operation and determination of uronic acid (○) were as described in the Materials and Methods. Salt concentration (·-·-·-) was measured with an in-line conductivity meter. Peak $\overline{M}_w$ values for the four pectic polysaccharides calculated from the plots, K_{av} versus peak $\overline{M}_w$, are designated as follows: PS-I (◆), PS-II (▲), PS-III (■), and PS-IV (●) with peak $\overline{M}_w$ values for the same polysaccharide connected by a dotted line (·····). Peak $\overline{M}_w$ values obtained directly from MALLS are designated by (✚). The vertical dotted lines and double-headed arrows (↔) designate the column fractions constituting PS-IIb and PS-IVb.

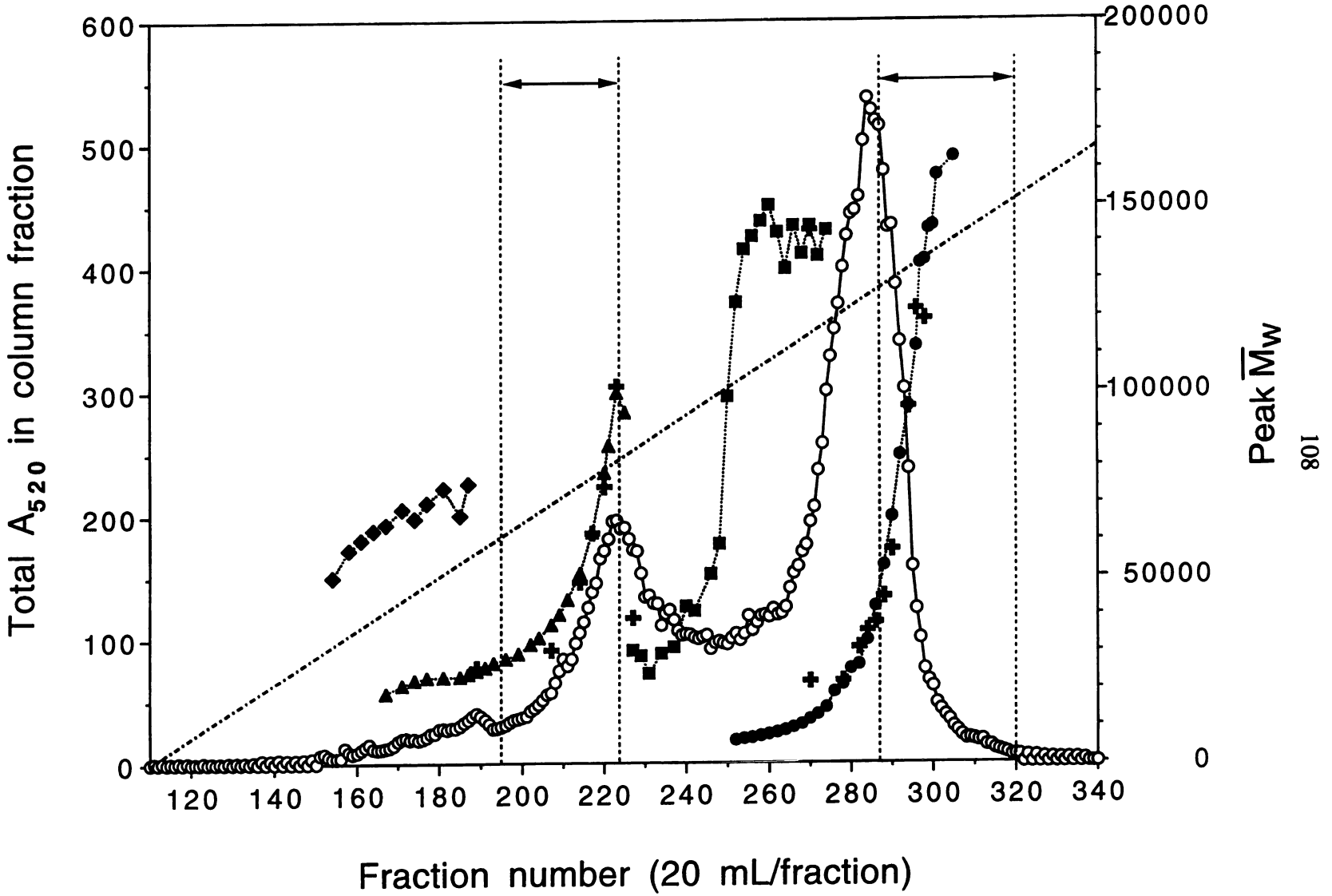

Total A_{520} in column fraction
Peak $\overline{M}_w$
10^8
Fraction number (20 mL/fraction)
600
500
400
300
200
100
0
200000
150000
100000
50000
0
120
140
160
180
200
220
240
260
280
300
320
340

Table 5.2. The $\bar{M}_w$, $\bar{M}_w$ and polydispersity ($\bar{M}_w/\bar{M}_n$) of selected pectic polysaccharide samples from the DEAE column chromatogram of Figure 5.3

sample		*dn/dc* used	Entire sample			Peak (top) region of sample			Mass of aggragation in sample (%)
			$\bar{M}_w$	$\bar{M}_n$	$\bar{M}_w/\bar{M}_n$	$\bar{M}_w$	$\bar{M}_n$	$\bar{M}_w/\bar{M}_n$	
F189		0.108[b]	42240	26000	1.625	25740	23800	1.082	4.50
F207		0.108[b]	41650	31050	1.324	30540	29950	1.020	0.18
F214		0.108[b]	56790	50020	1.135	48890	48430	1.009	1.37
F217		0.108[b]	70100	63670	1.101	62090	62000	1.001	0.27
F220		0.108[b]	80910	72940	1.109	74610	74530	1.001	0.69
F223		0.108[b]	106000	93290	1.136	101500	101400	1.001	0.98
F227		0.108[b]	56020	34370	1.630	39270	37500	1.047	0.52
F270	band (peak) 1	0.130[c]	192200	106300	1.808	143400	142400	1.007	0.43
	band (peak) 2	0.153	27150	23340	1.163	22120	20940	1.056	n.a.[e]
F278	band 2[a]	0.153	26860	25180	1.067	22210	22100	1.005	0.71
F282	band 2[a]	0.153	35700	33610	1.062	31230	31160	1.002	1.92
F284	band 2[a]	0.153	41450	38860	1.067	35770	35700	1.002	0.30
F286		0.166[d]	70410	44300	1.589	38000	37930	1.002	0.24
F288		0.166[d]	72520	47840	1.516	44910	44880	1.001	0.93
F290		0.178	219300	67610	3.111	57630	57460	1.003	0.76
F294		0.178	191500	109700	1.745	95850	90360	1.061	0.89
F296		0.178	230900	132100	1.748	122200	113200	1.080	1.63
F298		0.178	250000	127800	1.916	119500	117200	1.019	0.18

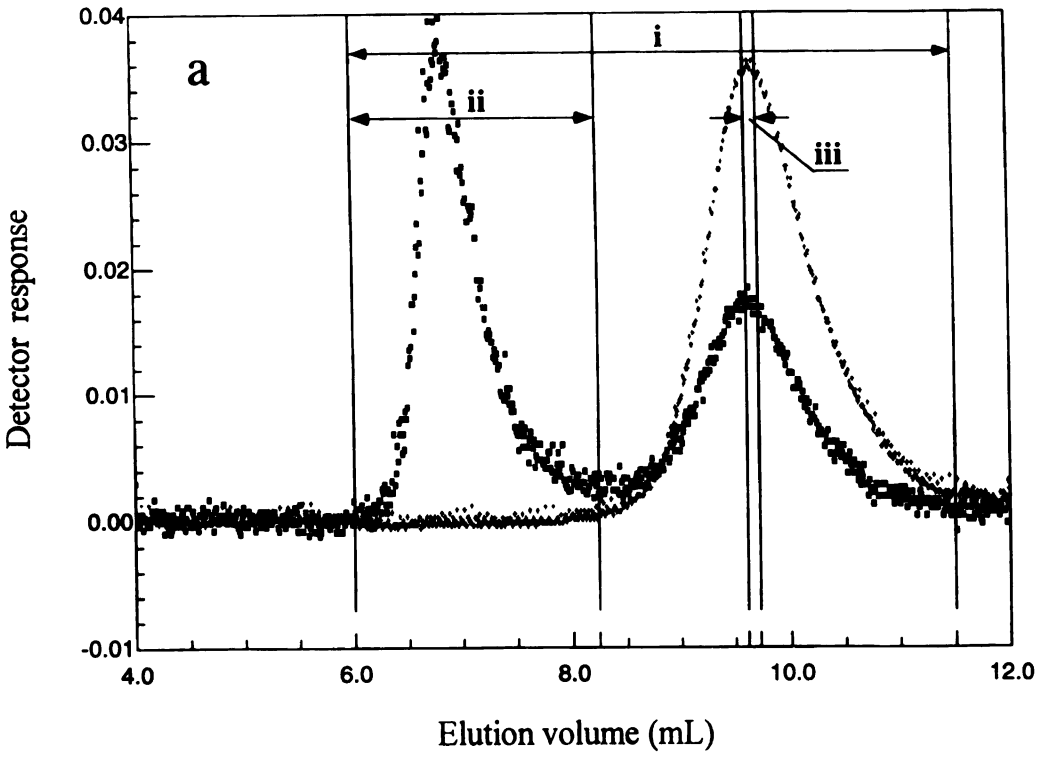
[a]In these samples band 1 was a shoulder
[b]Average dn/dc of F222 and F223 of Table 5.1
[c]Average dn/dc of F222, F223, and F284 of Table 5.1
[d]Average dn/dc of F284 and F290 of Table 5.1
[e]Not applicable

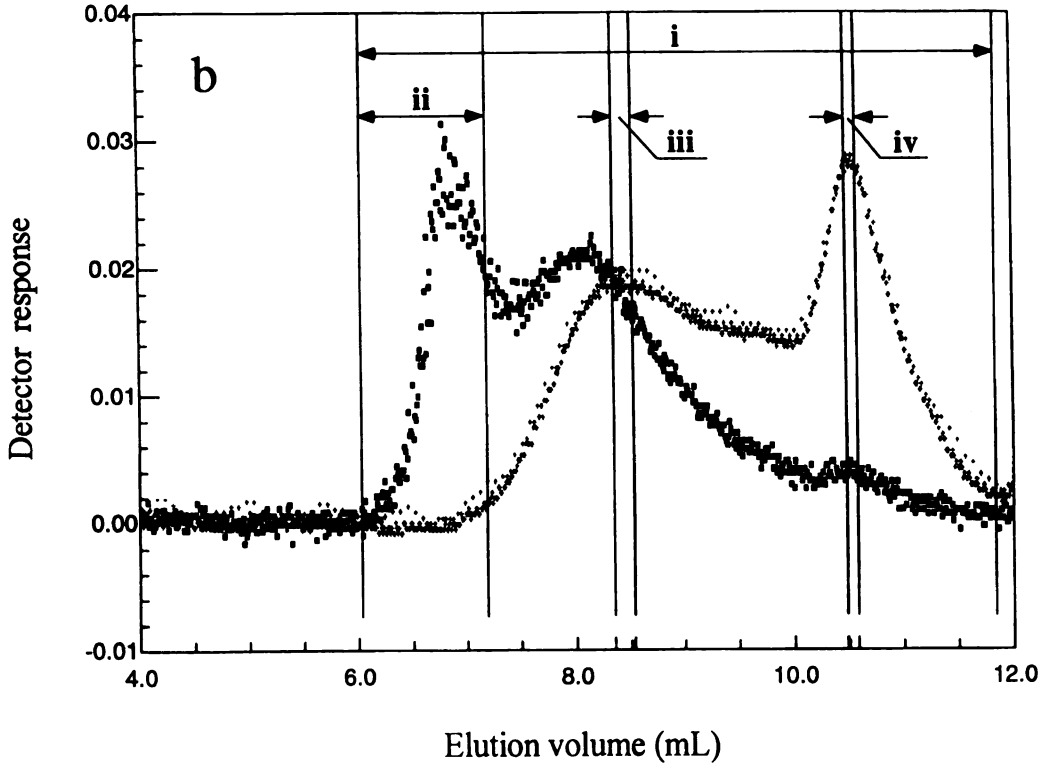
for these samples that were calculated from equations A and B (Figure 5.2). These latter peak $\overline{M}_w$ values are also plotted in Figure 5.3. The peak $\overline{M}_w$ values for all other samples are also reported in Figure 5.3. These, of course, were calculated by using equations A and B (Figure 5.2).

In all of the samples examined by MALLS some aggregated pectic polysaccharide material was observed (Figure 5.4a). The aggregated material was readily detected by a light scattering but the mass was too small to be detected by refractometry. The material present in the aggregated form ranged from 0.18% to 1.9% of the total mass in the sample for all samples except polysaccharide F189, which was 4.50% due to low sample concentration and a high ratio of noise/signal (Table 5.2). Excluding the aggregated material, the elution profiles from light scattering and refractometry of all the samples shown in Table 5.2, except F227, F270, F278, F282, and F284, each had a single peak, indicating the samples were homogeneous (Figure 5.4a). The light scattering and refractometry profiles showed that samples F270 (Figure 5.4b), F278, F282 and F284 consisted of at least two polysaccharides that were not completely separated. F227 migrated as a single band on HPSEC but the increase in broadness of the band compared to F223 and the large change in $\overline{M}_w$ between F227 and F225 — which resulted in F227 being part of PS-III and F225 being part of PS-II (see next section) — indicated that F227 consisted of more than one polysaccharide.

The profile areas used to calculate the $\overline{M}_w$ and $\overline{M}_n$ of the samples in Table 5.2 were based on the refractometry profiles. For a majority of the samples this excluded the aggregated material; however, for samples F288-F298 there was some overlap of aggregated material with material detected by refractometry. Although the overlap increased the $\overline{M}_w$ values of

Figure 5.4. HPSEC column chromatograms of *L. minor* pectic polysaccharides F217 (a) and F270 (b). Column operation is described in the Materials and Methods Polysaccharide was detected by MALLS (▪ ▪ ▪ ▪) and refractometry (+ + + +). For F217, bands between arrows are: (i), the entire sample including aggragation used to calculate $\overline{M}_w$; (ii), region used to calculate the aggragate material in the sample; (iii), region used to calculate peak $\overline{M}_w$ of the sample. For F270 (i) and (ii) are as for F217; (iii) and (iv) are the regions used to calculate peak $\overline{M}_w$ of the two polysaccharides, PS-III and PS-IV, respectively, in the sample.


a
i
ii
iii
0.04
0.03
0.02
0.01
0.00
-0.01
4.0
6.0
8.0
10.0
12.0
Detector response
Elution volume (mL)


b
i
ii
iii
iv
0.04
0.03
0.02
0.01
0.00
-0.01
4.0
6.0
8.0
10.0
12.0
Detector response
Elution volume (mL)

these samples, the basic trend of increasing $\overline{M}_w$ values across these samples was not altered. For samples F270, F278, F282 and F284 the refractometry areas used for calculating $\overline{M}_w$ were selected either on the basis of the point minimum refractometry response between peaks (F270) or where the refractometry response changed sharply but only gave a shoulder (F278, F282, F284).

HPSEC. The polysaccharide material in each of column fractions 154 - 164 (Figure 5.3) migrated as a single band on HPSEC indicating a single pectic polysaccharide was present. The peak $\overline{M}_w$ of the polysaccharide material in these fractions increased from fraction 154 through 164. The polysaccharide material in each of column fractions 167-187 consisted of at least two pectic polysaccharides as indicated by the presence of two peaks on the HPSEC column chromatograms (chromatograms not shown). These results indicated two different pectic polysaccharides had eluted from the DEAE column. The first to elute was called PS-I, the second, PS-II (Figure 5.3). In any DEAE column fraction containing both, the V_e of PS-I on HPSEC was always smaller than the V_e of PS-II. The V_e values of both polysaccharides continually decreased from column fraction 167 through 187 and this is reflected in their generally increasing peak $\overline{M}_w$ values across these fractions (Figure 5.3). In fractions followed DEAE column fraction 187, PS-I was no longer detectable by HPSEC. The polysaccharide material in column fractions 196-223 migrated as a single band on HPSEC. The peak $\overline{M}_w$ values of the polysaccharide material in these fractions linked smoothly with the peak $\overline{M}_w$ values for PS-II material in column fractions 167-189 therefore the polysaccharide material in fractions 196-223 is considered to be part of PS-II (Figure 5.3). PS-IIb, described earlier, contained the majority of the mass of PS-II but did not

contain detectable amounts of PS-I.

The polysaccharide material in column fractions 225 and 227 was considered to be different because of the substantial change in peak $\overline{M}_w$ from 94,000 for the material in column fraction 225 to 30,000 for the material in column fraction 227 and from the fact that the peak $\overline{M}_w$ of the polysaccharide material in the column fractions immediately preceding 225 was about 97,000 while the peak $\overline{M}_w$ of the material in the column fractions immediately following 227 was about 27,000. Two components may have been present in column fractions 225 and 227 but a separation by HPSEC was not observed. For DEAE column fractions 229 - 242, a single pectic polysaccharide appeared to be present as indicated by the single peak on the HPSEC column chromatogram; however, the peak was broad. The peak $\overline{M}_w$ values of the polysaccharide material in the fractions are given in Figure 5.3. As is seen in Figure 5.3, the values linked together smoothly and progressively increased across the fractions. Since the peak $\overline{M}_w$ values of the pectic polysaccharide material in the initial fractions of column fractions 229 - 242 was so much lower than the peak $\overline{M}_w$ values of the PS-II material in column fractions 222 and 224, the polysaccharide material in column fractions 229 - 242 was considered to be part of a third pectic polysaccharide, called PS-III.

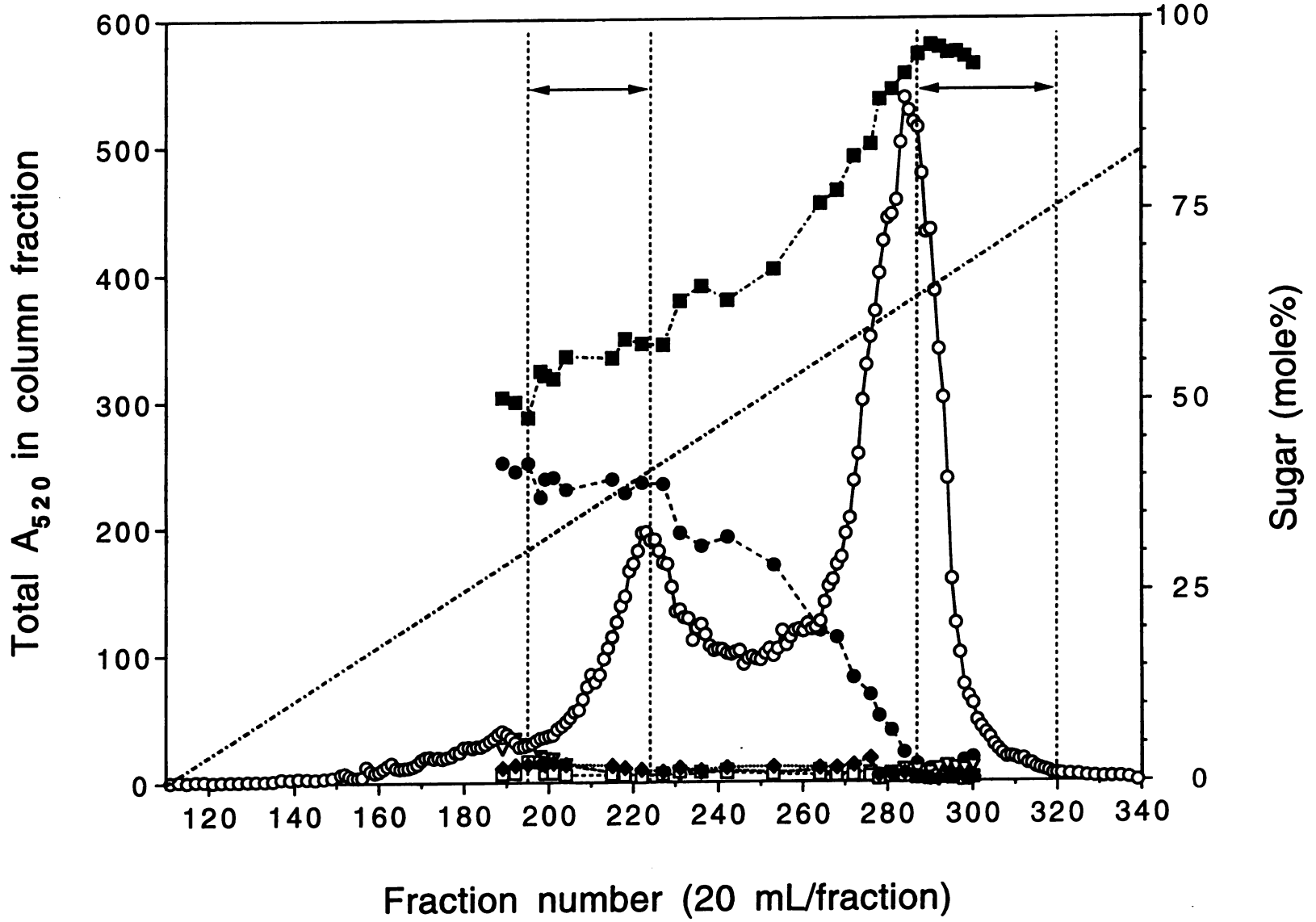
The polysaccharide material in each of column fractions 248-274 also consisted of two pectic polysaccharides as indicated by the presence of two peaks on the HPSEC column chromatograms. The situation was similar to what was found in fractions 167 - 187. The polysaccharide material in column fractions 248 - 274 that eluted first from the HPSEC column had peak $\overline{M}_w$ values that linked smoothly with those of the PS-III material in column fractions 227 - 242 and therefore was considered to be the remainder of PS-III (Figure 5.3).

The polysaccharide material in these fractions that eluted second from the HPSEC column was considered to be a fourth pectic polysaccharide, called PS-IV, and the peak $\overline{M}_w$ values of the PS-IV material in these fractions linked smoothly with the peak $\overline{M}_w$ values for PS-IV material in DEAE column fractions 276 - 305 (Figure 5.3). As stated earlier, aliquots of column fractions 287-320 were combined to give PS-IVb, which did not contain detectable amounts of PS-III. HPSEC chromatograms showed that PS-III material was visible as a shoulder on the PS-IV peak until DEAE column fraction 284.

The results in Figure 5.3 show that four pectic polysaccharides were present in the 22°C ammonium oxalate-soluble cell wall fraction of *L. minor*. Their peak $\overline{M}_w$ ranges were: 50,200-75,400; PS-II, 18,800 - 99,700; PS-III, 24,200 - 150,000; and PS-IV, 6,170 to at least 163,000. The results in Table 5.2 show that peak $\overline{M}_w$ values are only slightly less than the $\overline{M}_w$ values for the entire sample for most of the samples constituting PS-II and PS-III. The differences are greater for those samples that are part of PS-IV. Consequently the $\overline{M}_w$ ranges when the entire band of each sample is used will be somewhat higher than the peak $\overline{M}_w$ ranges stated above. $\overline{M}_w$ values of samples constituting PS-I were not determined because of insufficient quantity of sample for MALLS-refractometry analysis.

Sugar composition of polysaccharides. The sugar composition of the polysaccharide material in selected column fractions of Figure 5.3 was determined and the results are shown in Figure 5.5. Galacturonic acid is the major component of PS-II, PS-III, and PS-IV. Substantial amounts of apiose are present in PS-II and PS-III but very little is present in PS-IV, assuming those fractions analyzed were representative of all fractions constituting PS-IV. Small amounts of xylose, arabinose, and rhamnose were also detected in PS-II and PS-III

Figure 5.5. Sugar composition of the pectic polysaccharide(s) in individual column fractions from the DEAE-Trisacryl Plus M column chromatography (Figure 5.3). The profiles, total A_{520} (○) and salt concentration (·-·-·-) are those of Figure 5.3. Sugars are: galacturonic acid (■), apiose (●), xylose (▽), rhamnose (◆) and arabinose (□). Sugar composition was determined as described in the Materials and Methods. The vertical dotted lines and double-headed arrows have the same meaning as in Figure 5.3.


Total A_{520} in column fraction
Sugar (mole%)
Fraction number (20 mL/fraction)
600
500
400
300
200
100
0
100
75
50
25
0
120
140
160
180
200
220
240
260
280
300
320
340

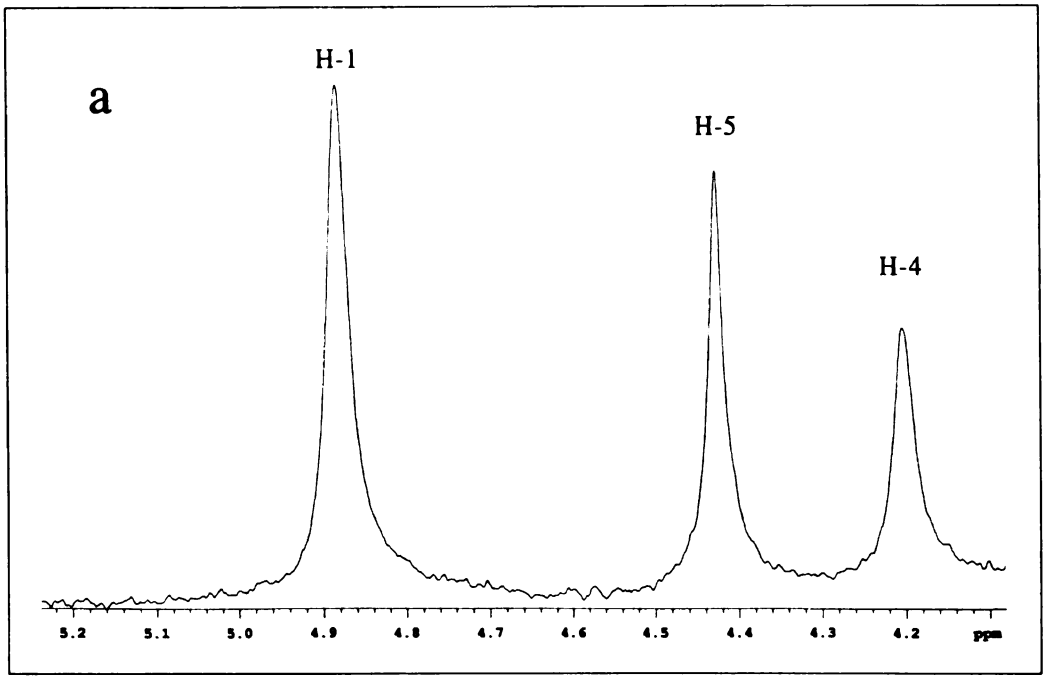
and a small amount of xylose was detected in PS-IV. The presence of these sugars was confirmed by mass spectrometry. Gas chromatographic analysis also detected arabinose and rhamnose in PS-IV and fucose, mannose, and glucose in all three polysaccharides, each at less than 0.5 mole%, but their presence has not been confirmed by mass spectrometry. The sugar composition across the samples constituting PS-IIb and PS-IVb was reasonably constant (Figure 5.5).

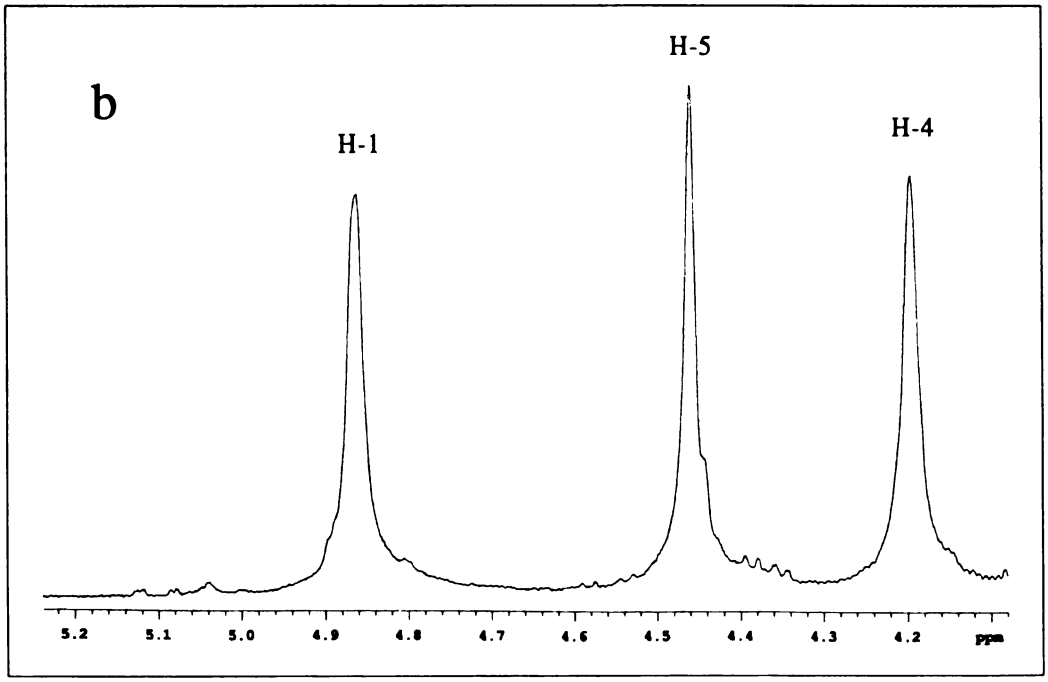
The polysaccharide material in 23 column fractions between fractions 207 and 301 of Figure 5.3 was tested for galactose and none was detected. Approximately every third fraction was analyzed except between fractions 231 and 284 where approximately every fifth fraction was tested. Selective ion monitoring analysis showed that 100.4 ± 0.8% (std. dev.) of the galactitol peracetate, on a mole basis, was derived from the galacturonic acid residues of the polysaccharide material and none from galactose residues. For column fractions 189 and 199, 91.3% and 96.7%, respectively, was derived from galacturonic acid residues and 8.7% and 3.3% from galactose residues.

Degree of methyl esterification. The degree of methyl esterification of the polysaccharide material in column fractions 208, 215, 218, 222, 287, 289, 290, 292, 294 and 297 of Figure 5.3 ranged from 0 to 1.6% and averaged 0.25 ± 0.56% (std. dev.).

^{1}H NMR spectroscopy. The low-field portion of the 500 MHz ^{1}H NMR spectra for F289, F290, F292, polygalacturonic acid and apple pectic acid were similar. The signals for H-1, H-4 and H-5 were each a single symmetrical peak with no sign of splitting in the H-5 and H-1 signals. The ^{1}H NMR spectra of F289 and polygalacturonic acid are shown in Figure 5.6. The spectrum of F223, a pectic polysaccharide containing a high amount of

Figure 5.6. The low-field region of the 500 MHz ^{1}H NMR spectra of *L. minor* pectic polysaccharide F289 (a) and polygalacturonic acid (b). The signals assigned to the protons on C-1, C-4 and C-5 of galacturonosyl residues are labeled H-1, H-4 and H-5, respectively.


a
H-1
H-5
H-4
5.2
5.1
5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
ppm


b
H-5
H-1
H-4
5.2
5.1
5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
ppm

apiose and presumably small amounts of other neutral sugars, was too complex to determine whether there was any splitting of the H-5 and H-1 signals (data not shown).

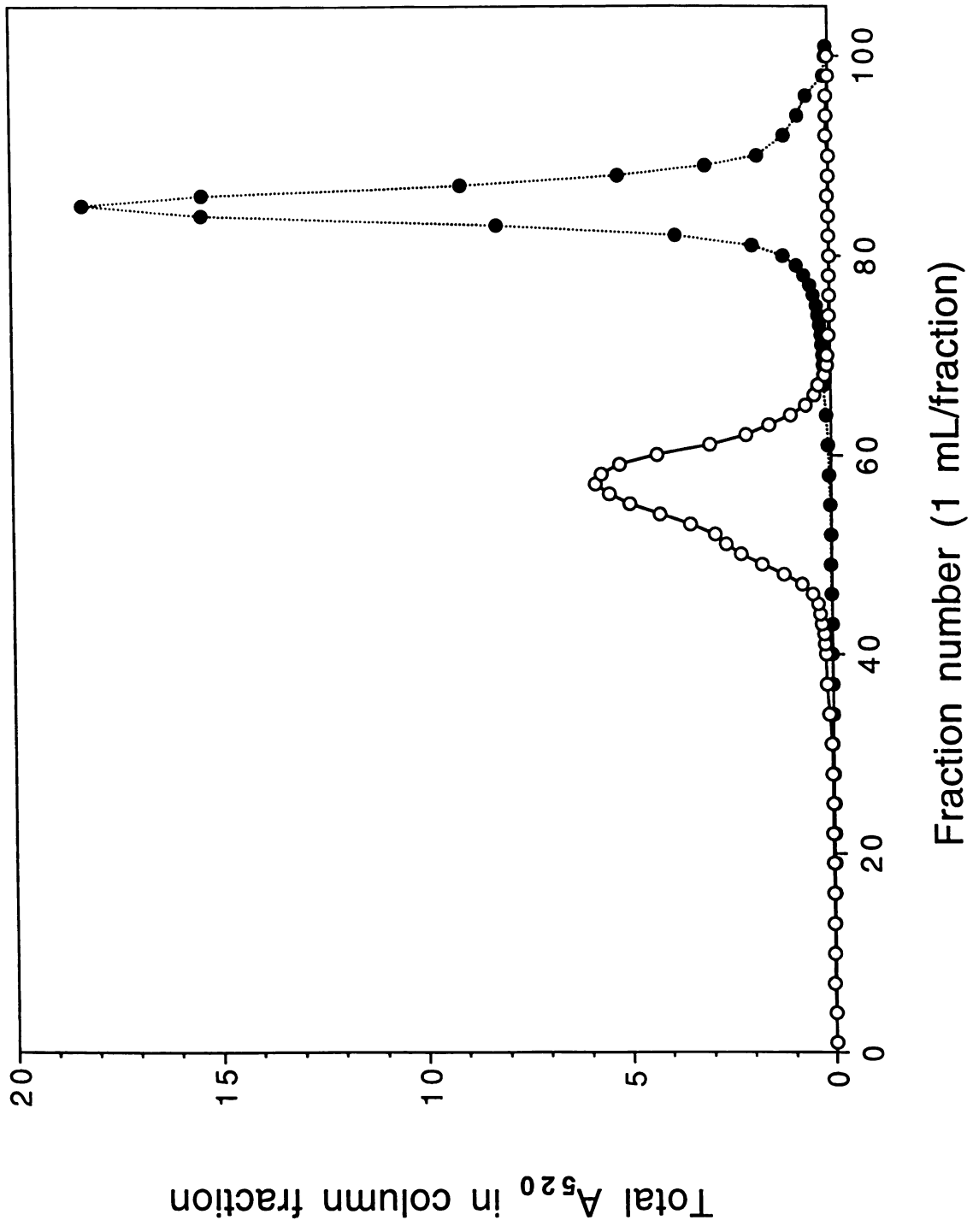
Rechromatography of pectic polysaccharides. Rechromatography of PS-IIb and PS-IVb on DEAE-Trisacryl Plus M showed that each migrated as a single band that for PS-IIb was almost symmetrical and for PS-IVb was symmetrical (Figure 5.7). Recovery of PS-IIb and PS-IVb from the columns, based on determination of uronic acid, was 96% and 92%, respectively.

DISCUSSION

Procedures were developed for determining the homogeneity of plant cell wall pectic polysaccharide material eluted from DEAE columns. The homogeneity of these polysaccharides has not been determined previously. The use of MALLS together with HPSEC for testing the homogeneity of the eluted polysaccharide material led to the unexpected finding that four pectic polysaccharides are present in the 22°C ammonium oxalate-soluble fraction from cell walls of *L. minor*. These results show there is not a continuum in structure for pectic polysaccharides in this fraction.

Two major bands of pectic polysaccharide material, PS-IIb and PS-IVb, were eluted from the DEAE column that were considered to be homogeneous with respect to sugar composition and degree of methyl esterification; each, however, was different chemically. The average sugar composition of the polysaccharide material in the column fractions analyzed that constituted PS-IIb (Figure 5.3) was: GalA (54.5 ± 3.2%) (std. dev.), Api (39.3

Figure 5.7. Column chromatograms of PS-IIb (○) and PS-IVb (●). The column was DEAE-Trisacryl Plus M. The two samples were run on separate but identical columns and the columns were developed identically. Sample preparation, column operation, and analyses were performed as described in the Materials and Methods.


Total A_{520} in column fraction
0
5
10
15
20
0
20
40
60
80
100
Fraction number (1 mL/fraction)

± 1.4%), Xyl (2.5 ± 1.4%), Ara (1.4 ± 0.8%), and Rha (2.3 ± 0.3%). The average sugar composition of the polysaccharide material in the column fractions analyzed that constituted PS-IVb (Figure 5.3) was: GalA (96.3 ± 0.8%), Api (2.1 ± 0.7%), and Xyl (1.6 ± 0.3%). The inclusion of xylose, arabinose, and rhamnose as constituents of PS-IIb and xylose as a constituent of PS-IVb is tentative since data establishing them as structural components of the polysaccharides have not yet been obtained. In addition, small amounts of fucose, mannose, and glucose and arabinose, rhamnose, fucose, mannose, and glucose were tentatively detected in PS-IIb and PS-IVb, respectively. Whether these minor sugars are constituents of the polysaccharides is also not known. The degree of methyl esterification of the pectic polysaccharide material in the column fractions constituting both PS-IIb and PS-IVb was basically zero. The ^{1}H NMR results are in agreement with the above results. The lack of detectable splitting in the H-5 and H-1 signals of F289, F290, and F292, the similarity of their spectra to those of polygalacturonic acid and apple pectic acid and the lack of signals for the other sugars also indicate the degree of methyl esterification and the neutral sugar content of PS-IVb are low. The lower limit of detection of methyl esterification with a 400 MHz instrument is approximately 8% (18). The spectrum of F223 indicates that one or more sugars, in addition to galacturonic acid, are present in PS-IIb.

PS-IIb and PS-IVb are heterogeneous with respect to size of molecules. The peak $\overline{M}_w$ of the polysaccharide molecules constituting PS-IIb ranged from 28,100 - 97,000 while those constituting PS-IVb ranged from 53,300 to at least 163,000. The $\overline{M}_w$ range of cell wall pectic polysaccharides from other plants determined by an absolute method such as MALLS has not been reported. Light scattering was used to determine that the $\overline{M}_w$ of a commercial sugar

beet pectin is 266,000 (19). The other light scattering studies on pectic polysaccharides were done with material from fruit tissue (19-23).

Pectic polysaccharide samples in individual column fractions, before being combined as part of PS-IIb and PS-IVb, each migrated as a single band on HPSEC. The combined samples, PS-IIb and PS-IVb, also each migrated as a single symmetrical or almost symmetrical peak when chromatographed on ion-exchange (Figure 5.7) and HPSEC (data not shown) columns. These results showed that PS-IIb and PS-IVb were free of contaminants that migrated differently on these columns. This indicated further that they were highly purified.

A pectic polysaccharide like PS-IVb, that is, one of high galacturonic acid content (≥ 96 mole %) and low degree of methyl esterification (~0%) and isolated under mild conditions, has not been reported previously. The isolation of homogalacturonans has been reported but the strong isolation conditions suggest these were derived from parent polysaccharides by degradation during the isolation process (24-26). The degree of methyl esterification of these isolated homogalacturonans was not reported. A pectic polysaccharide of high galacturonic acid content (94 mole %) was isolated from *Zea mays* L. shoots under relatively mild conditions but the degree of methyl esterification was also not reported (27). Pectic polysaccharides of high galacturonic acid content (≥ 90 mole%) have been isolated from various fruit tissues but all have either a high degree of methyl esterification (≥ 44 %) or were isolated from cell fractions with a high degree of methyl esterification (≥ 54 %) (8,28-30). PS-IVb from *L. minor* was discovered only after conditions were found that gave quantitative recovery of pectic polysaccharides from the DEAE-Trisacryl Plus M column

(15). This type of pectic polysaccharide is probably present in other plants but has escaped detection because of its tight binding to anion-exchange columns.

In the procedure developed for isolating pectic polysaccharides, all steps that involved concentration, dialysis, or freeze-drying of polysaccharide solutions were eliminated as were those that involved precipitation of polysaccharides from solution. The cell walls were isolated from fresh plants at 0-4 °C and pH 5.5, the extraction time with ammonium oxalate was short, 30 min (2), and the extraction was performed at 22 °C. The extract was applied directly to the DEAE column and the column buffer was the same as the extracting agent (0.05 M ammonium oxalate). The recovery of pectic polysaccharides from the DEAE column was quantitative and the column was operated at pH 5.5. The procedures and conditions used for isolation of the pectic polysaccharides eliminated losses and minimized degradation reactions of the polysaccharides.

Three general types of pectic polysaccharides have been reported to be present in plant cell walls. They are commonly designated RG-I, RG-II, and homogalacturonan. Our results show four pectic polysaccharides are present in the 22 °C ammonium oxalate-soluble fraction from cell walls of *L. minor*. Based on our structural results, PS-IV would be classified as a homogalacturonan. PS-II contains rhamnose but the rhamnose residues are linked terminally and are not within the backbone (Chapter VI), which means PS-II can not be classified as a RG-I or RG-II. Pectic polysaccharides containing rhamnosyl residues only as terminal groups have not been reported. There is not sufficient homogeneity and structural information to classify PS-I and PS-III. As stated earlier, the light scattering results show there is not a continuum in structure between these four pectic polysaccharides.

A simple, mild procedure for quantitatively isolating pectic polysaccharides from the 22°C ammonium oxalate-soluble fraction prepared from purified cell walls of *L. minor* was developed. Four different pectic polysaccharides were isolated. Procedures for determining the homogeneity of the isolated pectic polysaccharides were developed. Two of the pectic polysaccharide fractions isolated, PS-IIb and PS-IVb, were chemically homogeneous with respect to sugar composition and degree of methyl esterification but were heterogeneous with respect to the size of their molecules.

REFERENCES

(1) Carpita, N. C. and Gibeaut, D. M. (1993) *Plant J.*, **3**, 1-30.

(2) Kindel, P. K., Cheng, L. and Ade, B. R. (1996) *Phytochemistry*, **41**, 719-723.

(3) Souty, M. , Thibault, J. -F., Navarro-Garcia, G., Lopez-Roca, J. -M. and Breuils, L. (1981) *Sciences des Aliments*, **1**, 67-80.

(4) Redgwell, R. J. and Selvendran, R. R. (1986) *Carbohydr. Res.*, **157**, 183-199.

(5) Saulnier, L. and Thibault, J. -F. (1987) *Carbohydr. Polymers*, **7**, 329-343.

(6) Renard, C. M. G. C.,Voragen, A. G. J.,Thibault, J. -F. and Pilnik, W. (1990) *Carbohydr. Polymers*, **12**, 9-25.

(7) Ryden, P. and Selvendran, R. R. (1990) *Carbohydr. Res.*, **195**, 257-272.

(8) Seymour, G. B., Colquhoun, I. J., DuPont, M. S., Parsley, K.R. and Selvendran, R. R. (1990) *Photochemistry*, **29**, 725-731.

(9) Ryden, P. and Selvendran, R. R. (1990) *Biochem. J.*, **269**, 393-402.

(10) Coimbra, M. A., Waldron, K. W. and Selvendran, R. R. (1994) *Carbohydr. Res.*, **252**, 245-262.

(11) Gooneratne, J., Needs, P. W., Ryden, P. and Selvendran, R. R. (1994) *Carbohydr. Res.*, **265** 61-77.

(12) Kindel, P. K. and Watson, R. R. (1973) *Biochem. J.*, **133**, 227-241.

(13) DuBois, M., Gilles, K. A., Hamilton, J. K., Roberts, P. A. and Smith, F. (1956) *Anal. Chem.*, **28,** 350-356.

(14) Blumenkratz, N. and Asboe-Hansen, G. (1973) *Anal. Biochem.*, **54**, 484-489.

(15) Cheng, L. and Kindel, P. K. (1995) *Anal. Biochem.*, **228**, 109-114.

(16) Kruis, A. (1936) *Z. physik. Chem.* (B), **34**, 13-50.

(17) P. J. Wood and I. R. Siddiqui, (1971) *Anal. Biochem.*, **39**, 418-428.

(18) Grasdalen, H., Bakøy, O. E. and Larsen, B. (1988) *Carbohydr. Res.*, **184**, 183-191.

(19) Thibault, J. -F., Renard, C. M. G. C., Axelos, M. A. V., Roger, P. and Crépau, M. -J. (1993) *Carbohydr. Res.*, **238**, 271-286.

(20) Chapman, H. D., Morris, V. J., Selvendran, R. R. and O'Neill, M. A. (1987) *Carbohydr. Res.*, **165**, 53-68.

(21) Kontominas, M. G. and Kokini, J. L. (1990) *Lebensm.-Wiss. u. Technol.*, **23**, 174-177.

(22) Smith, J. E. and Stainsby, G. (1977) *British Polymer J.*, **9**, 284-289.

(23) Doco, T. and Brillouet, J. -M. (1993) *Carbohydr. Res.*, **243**, 333-343.

(24) Bhattacharjee, S. S. and Timell, T. E. (1965) *Can. J. Chem.*, **43**, 758-765.

(25) Zitko, V. and Bishop, C. T. (1965) *Can. J. Chem.*, **43**, 3206-3214.

(26) Chambat, G. and Joseleau, J. -P. (1980) *Carbohydr. Res.*, **85**, C10-C12.

(27) Kato, Y. and Nevins, D. J., (1989) *Plant Physiol.*, **89**, 792-797.

(28) Vries, J. A. De, Voragen, A. G. J., Rombouts, F. M. and Pilnik, W. (1981) *Carbohydr. Polymers*, **1**, 117-127.

(29) Redgwell, R. J., Melton, L. D. and Brasch, D.J. (1988) *Carbohydr. Res.*, **182**, 241-258.

(30) Westerlund, E., Åman, P., Andersson, R. and Andersson, R. E. (1991) *Carbohydr. Polymers*, **15**, 67-78.

CHAPTER VI

STRUCTURAL ANALYSIS OF PECTIC POLYSACCHARIDES

PS-IIb AND PS-IVb

ABSTRACT

Methylation analysis and partial hydrolysis followed by poly-[1,4-α-D-galacturonide] glycanohydrolase (EC 3.2.1.15) digestion were performed on PS-IIb and PS-IVb, two pectic polysaccharides purified from cell walls of *L. minor*. Glycosyl-linkage composition of the two was deduced from the electron impact mass spectrometry of partially *O*-methylated alditol acetates derivatives. With 97% galacturonosyl residues (4-linked:terminal:2,4-linked :3,4-linked residues = 92.1:1.9:1.7:1.3) and only 0.5%, 1.0% and 1.6%, respectively, of terminal rhamnosyl, terminal apiosyl and internal apiosyl residues, PS-IVb is virtually a homogalacuronan. In contrast, PS-IIb is highly branched with side chains mainly of 1,3'-linked apiobiose (the mole% of teminal apiose and internal apiose is 21.3% and 27.7%, respectively) attached almost solely at the 2-*O*-position of every other galacturonosyl residues of the backbone. Direct digestion of these two polysaccharides with poly-[1,4-α-D-galacturonide] glycanohydrolase released 98.5% of the galacturonosyl residues of PS-IVb while only 12% of the galacturonosyl residues of PS-IIb were released. However, after the side chains were removed from PS-IIb by partial acid hydrolysis, the enzyme digestion released all the galacturonosyl residues. The results indicate both PS-IIb and PS-IVb have a backbone of α-1,4-linked-D-galacturonosyl residues. PS-IIb but not PS-IVb has side chains of apiose and apiobiose. The side chains of PS-IIb cover most of the backbone and occur on approximately every the other galacturonosyl residue.

INTRODUCTION

Pectic polysaccharides are usually the most abundant component of plant primary cell wall. The major types of pectic polysaccharide fractions isolated from plant cell walls are homogalacturonan (1-5), RG-I (6-11) and RG-II (12-17). However, no pure homogalacturonan with zero degree of methyl esterification has been isolated from primary cell walls without using treatments that are likely to cleave covalent bonds. The rhamnosyl residues in RG-I are 1,2-linked and approximately half of them are branched, containing glycosyl substitutes at *O*-4 (3). A large portion of the rhamnosyl residues of RG-II are 3-, 3,4-, 2,3,4- and terminally linked.

Pectic polysaccharides solubilized from the cell wall of *L. minor* with 0.5% ammonium oxalate have been found to be different from the above three types; they contain mainly galacturonosyl and apiosyl residues and were partially characterized as apiogalacturonans (18-20). Beck (18) detected that apiose (25.2-27.9% of the apiogalacturonans), xylose (8.3%), galactose (2.8%) and apiose-free oligo- and mono galacturonic acid by paper chromatography after mild acidic hydrolysis, autohydrolysis and pectinase (a mixture of exo- and endo-polygalacturonase) digestion of the apiogalacturonans. Based on these results Beck suggested that the apiogalacturonan he isolated was "an unesterified α-1,4 linked polygalacturonic acid to which monomeric side-groups (75% apiose and 25% xylose residues) are attached" (18). The apiogalacturonan fraction isolated and purified from cell walls of *L. minor* by Hart and Kindel (19,20) consisted mainly of D-galacturonosyl and apiosyl residues (apiose accounted for 7.9-38.1% of the apiogalacturonans). Mild acid

hydrolysis of the apiogalacturonans gave three products: apiose, apiobiose and galacturonan (20). The galacturonan was hydrolyzed with a crude pectinase, providing some evidence that the galacturonosyl residues were α-1,4 linked. Periodate oxidation together with proton NMR analysis suggested that apiobiose had the structure, β-D-Api*f*-(1→3')-D-Api. However, complete methylation analysis was not performed by either Beck's or Kindel's group. It was not established whether the apiosyl and apiobiosyl residues were linked to C-2 or C-3 of the residues of the galacturonan backbone. In addition, the homogeneity of the apiogalacturonans had not been determined when the polysaccharides were partially characterized previously. With current techniques such as GC-MS and HPSEC-MALLS, we [Cheng and Kindel (21), Chapter V] re-examined the isolation and purification procedures and have purified two chemically different pectic polysaccharides, PS-IIb and PS-IVb, from cell walls of *L. minor*. With a peak $\overline{M}_w$ range of 28,100 -99,700, PS-IIb consisted mainly of galacturonosyl (54.5±3.2%) and apiosyl (39.3±1.4%) residues although small amounts of rhamnosyl (2.3±0.3%), xylosyl (2.5±1.4%) and arabinosyl (1.4±0.8%) residues have also been detected with GC-MS. PS-IVb is virtually a homogalacturonan since 96.3% (±0.8) of the polysaccharide is galacturonosyl residues. Api, 2.1±0.7%, Xyl, 1.6±0.3% and other neutral sugars, < 0.5% were also found. PS-IVb has a peak $\overline{M}_w$ range of 53,300 to at least 163,000. In this study, methylation analysis, partial hydrolysis and polygalacturonase digestion followed by HPAEC-PAD were performed on these two pectic polysaccharides.

MATERIALS AND METHODS

General method. Total carbohydrate and uronic acids were measured with the methods of Dubois *et al.* (22) and Blumenkrantz and Asboe-Hansen (23), respectively.

Materials. Sodium borohydride, sodium borodeuteride (98% D), anhydrous dimethyl sulfoxide, methyl iodide were obtained from Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide, pectinase (purified, poly-[1,4-α-D-galacturonide]glycanohydrolase, EC 3.2.1.15) and bovine serum albumin was obtained from Sigma. DEAE-Trisacryl Plus M column fractions 195-222 and 287-327 (Chapter V, Figure 5.3), 4 mL each fraction, were combined as PS-IIb and PS-IVb, respectively. The samples were dialyzed against distilled water for 45 h with 10 water changes and tested for uronic acid and total sugar.

Carboxyl reduction. The dialyzed samples PS-IIb and PS-IVb were evaporated to ~10 mL under 40°C and the carboxyl groups reduced basically as described by Taylor and Conrad (24) except that after 173 mg of EDAC was added, 0.01 M and 0.05 M HCl was added dropwise by hand to maintain the pH at 4.75 for the 2 h, and 0.5 M and 5 M HCl was added dropwise to maintain the pH at 7.0 while about 25 mL of 2 M $NaBH_4$ was added over a 2 h period. The samples were dialyzed in tubing with a 6000-8000 molecular weight cut-off against distilled water for 36 h and evaporated under 40°C to about 10 mL. The carboxyl-reduction procedure was repeated once. Uronic acid and total sugar were determined for the dialyzed samples and the above procedure of carboxyl reduction was repeated once for PS-IVb. The final volume of PS-IVb after evaporation was 15.9 mL.

Methylation. Samples of carboxyl-reduced PS-IIb, 2 mL (0.826 mg/mL by total sugar

and uronic acid tests), and PS-IVb, 3.7 mL (0.439 mg/mL by total suagr and uronic acid tests), were lyophilized to dryness in 1 DRAM vials and dried *in vacuo* over P_2O_5 for 2 days. The polysacharides were methylated by a modification of the procedure of Ciucanu and Kerek (25) as described by Needs and Selvendran (26) except the following changes were made: Each samples was dissolved in 1 mL of anhydrous DMSO under N_2 , sonicated for 5 min and stirred slowly overnight. After 100 mg of fine powdered anhydrous NaOH was added (under N_2), the sample was briefly vortexed, sonicated for 20 min, stirred for 1 h and the sonication and stirring were repeated once with the last stirring being 2 h. Methyl iodide, 0.8 mL, was added dropwise over 2 min to the sample which was held by a pre-cooled (in freezer for 15 min) metal block while being stirred under N_2. The mixture was briefly vortexed, sonicated for 15 min and stirred for 30 min. Methyl iodide, 0.25 mL, was added and the sample was vortexed, sonicated and stirred as above. The mixture was transfered to 2 DRAM vials, 3 mL of water was added and the solution was extracted with chloroform three times each time with 1.5 mL. The chloroform phase was washed with water (3 × 3 mL) and reduced to 1 mL with N_2. The water phase was diluted to 15 mL and slowly applied to a Sep-Pak C_{18} Cartridge (Waters Corp., Milford), which was pre-treated as described (27). The cartridge was washed with 20 mL water and methylated polysaccharides were eluted with acetonitrile and ethanol as described (27). The effluent was reduced to 1 mL with N_2, combined with the chloroform phase and the solution taken to dryness.

Hydrolysis, reduction and acetylation. Per-O-methylated PS-IVb was treated with 2 M HCl in dry methanol at 80°C for 4 h and the sample was dried with N_2 at 22°C. Methylated PS-IIb and methanolysis-treated, methylated PS-IVb were hydrolyzed with 0.5

mL of 2 M TFA at 120°C for 90 min and the samples were dried with N_2 at 22°C. $NaBD_4$, 2.5 mg in 0.25 mL of 5% (v/v) 14 M ammonium hydroxide in ethanol, was added and the samples were vortexed and stood at 22°C for 3 h. Acetic acid, 25 μL, was added and the samples were brought just to dryness with N_2 at 22°C . Acetic acid-methanol (1:9, v/v), 0.5 mL, was added to the samples and they were dried with N_2 at 22°C. The addition and evaporation of 1:9 (v/v) acetic acid-methanol was repeated three times and followed by two cycles of evaporations to dryness with 1 mL of methanol. Pyridine-acetic anhydride (2:1, v/v), 0.25 mL, was added and the samples were heated at 80°C for 45 min. After the vials were cooled to 22°C, 2 mL of water was added and the mixture was extracted with chloroform (3 × 1 mL). The chloroform extract was reduced to 1.5 mL with N_2 and washed with water (3 × 3 mL). The PMAAs in the chloroform phase were transferred to a 0.5 DRAM vial, the solution was reduced to ~0.15 mL with N_2 and analyzed.

GC-MS determination of the partial methylated alditol acetates. Separation and identification of PMAAs were by GC (FID) and GC-MS basically as was done with alditol acetates (28), except that a 30 m DB-225 capillary column (0.25 mm, i. d., for GC and 0.32 mm, i. d., for GC-MS) was used. For GC the temperature was started at 180°C and increased at a rate of 2.5°C/min to 230°C. For GC-MS the initial temperature was 100°C, and was increased at a rate of 45°C/min to 170°C then increased at a rate of 2.5°C/min to 225°C. The molar ratio of the PMAAs was determined from the individual GC peaks by using the molar response factors calculated on the basis of effective carbon response (e.c.r) as described (29-31).

Pectinase degradation of polysaccharides PS-IIb and PS-IVb. Pectinase (1.1 units/

mg) was dissolved in aq. 0.5% (w/v) BSA containing 50 mM sodium acetate buffer, pH 5.0, at 10 mg/mL. One tenth (0.1) mL of a solution containing either polysaccharide PS-IIb or PS-IVb (1.2 mg/mL, uronic acid and total sugar tests) was diluted with 0.1 mL of 0.1 M sodium acetate buffer, pH 5.0. Pectinase solution or BSA [aq. 0.5% (w/v) solution containing 50 mM sodium acetate, pH 5.0], 10 μL, was added and the mixture was incubated at 30°C for 4 h. Samples of polysaccharides PS-IIb and PS-IVb were also partially hydrolyzed at pH 4 and 100°C for 5 h before treatment with pectinase. The partially hydrolyzed PS-IIb and PS-IVb were digested with pectinase as described above.

High performance anion exchange chromatography with pulse amperometric detection

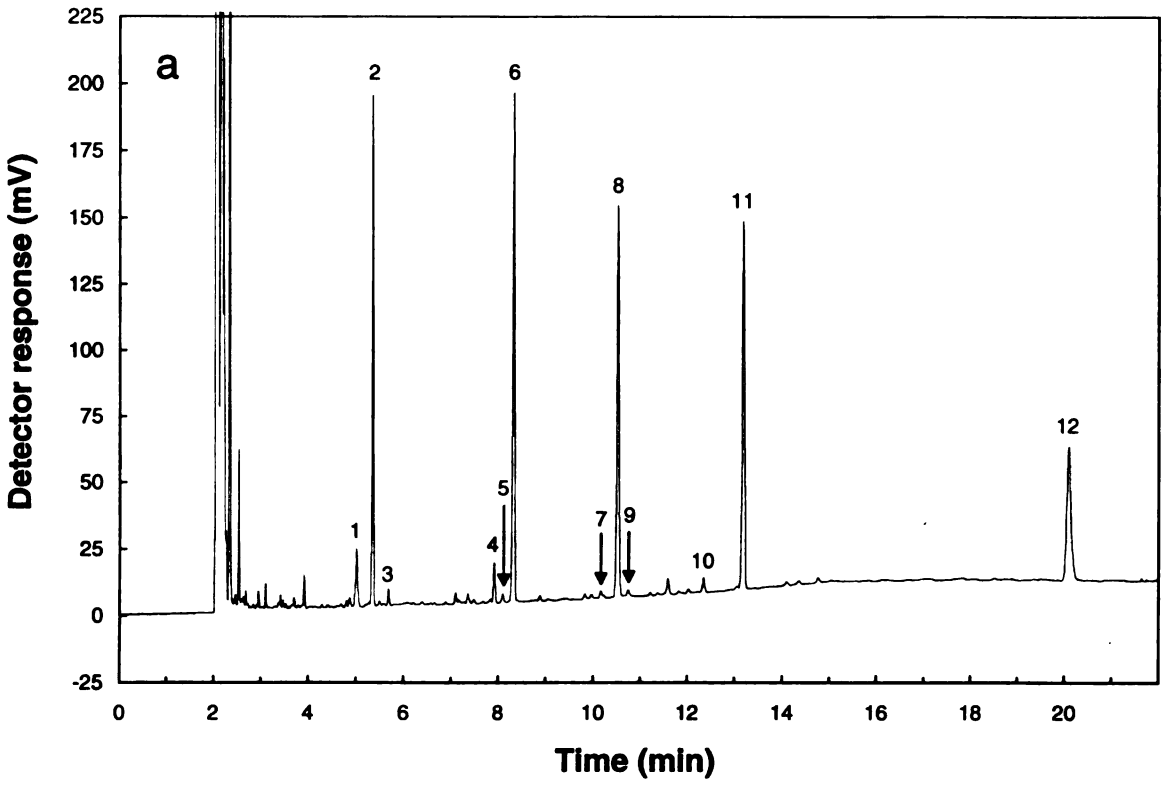
PS-IIb and PS-IVb samples treated with pectinase, partial acid hydrolysis or both, plus controls were diluted 1:3 with water and analyzed by HPAEC-PAD (Dionex system). The Dionex system was equipped with a CarboPac PA1 column (4 mm × 250 mm) and a CarboPac PA1 guard column and was operated at 22°C. Samples of 20 μL were injected and the column flow rate was 1 mL/min. The following eluents were used: 0-18 min, 20 mM NaOH; 18-45 min, concentration of NaOH was increased to 100 mM and sodium acetate was increased from 0 to 1 M, each linearly; 45-55 min, the concentration of NaOH and sodium acetate was kept unchanged; 55-60 min, concentration of NaOH was decreased from 100 mM to 20 mM and that of sodium acetate was decreased to 0 M, each linearly. Chromatograms were recorded and integrated with a HP3390 integrator and also with a computer that was connected to the Dionex system through a SRI interface and operated with PEAK-II software (SRI Instruments, Torrance, California). Standard samples of galactose, apiobiose, and galacturonic acid were used to calibrate and quantify the

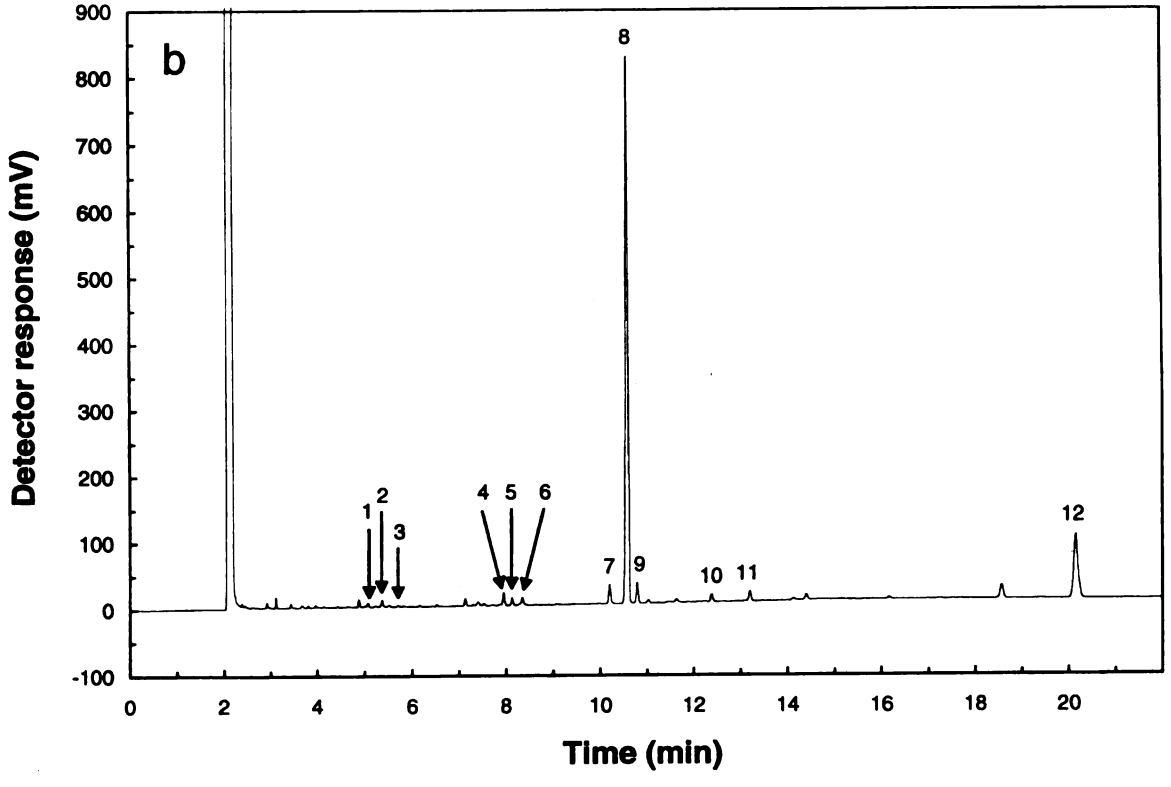
HPAEC-PAD system. The percentage of free GalA released in samples was calculated from GalA detected by HPAEC-PAD and from results of total carbohydrate (22) and uronic acid (23) tests of the original polysaccharides (Appendices C and D).

RESULTS

Methylation analysis. Gas chromatograms of PMAAs from methylation analysis of PS-IIb and PS-IVb are shown in Figure 6.1. The 11 PMAAs were identified by GC-MS analysis. The GC-MS spectra of PMAAs 1, 3-5, and 7-11 are similar to those given by Carpita and Shea (32). PMAAs of apiose were not described in ref. 32 or elsewhere in the literature. Therefore spectra for 1,4-di-*O*-acetyl-(1-deuterio-)-2,3,3'-tri-*O*-methyl apiitol (t-Api*f*, the glycosidic linkage at C-1 is assumed for all PMAA in this study) and 1,3',4-tri-*O*-acetyl-(1-deuterio-)-2,3-di-*O*-methyl apiitol (3'-Api*f*) are not available. Peak 1 (Figure 6.1a), 1,5-di-*O*-acetyl-(1-deuterio-)-2,3,4-tri-*O*-methyl-6-deoxy-hexitol was assigned to t-Rha*p* since sugar composition analysis (Chapter V) showed that no significant amount of any other 6-deoxysugar (such as fucose) was present. Peak 1 of Figure 6.1b had a similar GC retention time and mass spectra (data not shown) as peak 1 of Figure 6.1a and therefore was considered to be t-Rha*p* although sugar composition analysis did not conclusively detect Rha in PS-IVb possibly due to the small quantity present. The following information was used to identify peaks 4, 8, 10 and 11 of Figures 6.1a and b. First, the mass spectrum of each peak was similar to corresponding hexose PMAA described by Carpita and Shea (32). Second, enzyme degradation indicated that

Figure 6.1. Gas-liquid chromatograms of partial methylated alditol acetates derived from pectic polysaccharides PS-IIb (a) and PS-IVb (b). Experimental procedures are described in the Materials and Methods. For both panels, peaks 1 through 12 are: [1], 1,5-di-*O*-acetyl-(1-deuterio-)-2,3,4-tri-*O*-methyl-6-deoxy-hexitol (**t-Rha*p***); [2], 1,4-di-*O*-acetyl-(1-deuterio-)-2,3,3'-tri-*O*-methyl apiitol (**t-Api*f***); [3], 1,5-di-*O*-acetyl-(1-deuterio-)-2,3,4-tri-*O*-methyl pentitol (**t-pentitol*p***); [4], 1,5-di-*O*-acetyl-(1-deuterio-)-2,3,4,6-tetra-*O*-methyl hexitol (**t-Gal*p*A**); [5], 1,4,5-tri-*O*-acetyl-(1-deuterio-)-2,3-di-*O*-methyl pentitol (**4-pentitol*p***, or **5-pentitol*f***); [6], 1,3',4-tri-*O*-acetyl-(1-deuterio-)-2,3-di-*O*-methyl apiitol (**3'-Api*f***); [7], 1,4,5-tri-*O*-acetyl-(1-deuterio-)-2,3,6-tri-*O*-methyl hexitol (**4-hexitol*p***, or **5-hexitol*f***); [8], 1,4,5-tri-*O*-acetyl-(1-deuterio-)-2,3,6-tri-*O*-methyl hexitol (**4-Gal*p*A**); [9], 1,4,5-tri-*O*-acetyl-(1-deuterio-)-2,3,6-tri-*O*-methyl hexitol (**4-hexitol*p***, or **5-hexitol*f***); [10], 1,3,4,5-tetra-*O*-acetyl-(1-deuterio-)-2,6-di-*O*-methyl hexitol (**3,4-Gal*p*A**); [11], 1,2,4,5-tetra-*O*-acetyl-(1-deuterio-)-3,6-di-*O*-methyl hexitol (**2,4-Gal*p*A**); [12], myo-inositol hexaacetate (internal standard).


a
Detector response (mV)
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12


b
Detector response (mV)
Time (min)
1
2
3
4
5
6
7
8
9
10
11
12

galacturonosyl residues were in the pyranose form. Third, hexoses other than Gal*p*A were not conclusively identified by sugar composition analysis (Chapter V). Although peaks from GC with retention times the same as those of mannose and glucose were detected, they were present in a trace amount (< 0.5%) compared to the large amount of GalA in PS-IVb (96.3%) and PS-IIb (54.5%). Since the area ratio of peaks 7, 8, and 9 (PMAAs of hexoses) in Figure 6.1a (PS-IIb) is 1:30:1 and in Figure 6.1b (PS-IVb) it is 1:50:1 and assuming the major PMAA peak of hexoses was derived from GalA, then peak 8 can only be assigned to 4-Gal*p*A. The same reasoning was used to identify peak 11 of Figure 6.1a as 2,4-Gal*p*A. The other two hexose peaks, peaks 7 and 9 have to be the PMAAs derived from Man and Glc residues although the data available do not differentiate between the two. Since the PMAAs of Man and Glc were assigned to PMAA peaks 7 and 9 (Figure 6.1), the terminal and 3,4- linked hexoses (peaks 4 and 10, Figure 6.1) were tentatively identified as t-Gal*p*A and 3,4- linked Gal*p*A, respectively, although standard samples are necessary for positive identification.

The identification of t-Api*f* and 3'-Api*f* in PS-IIb was based on: (i) the mass spectra of the PMAAs and the fragmentations proposed in Figures 6.2 and 6.3, respectively, (ii) the high amount of Api in PS-IIb as determined by sugar composition analysis (Chapter V) and (iii) the earlier isolation of apiobiose from pectic polysaccharides of *L. minor* (20). Ions at *m/z* 101, 146, 161, 205 and 206 (Figure 6.2) are characteristic of t-Api*f* and ions at *m/z* 129 and 189 (Figure 6.3) are characteristic of 3'-Api*f*. Sugar composition results (Chapter V) showed that the Api content of PS-IVb is low (2.1±0.7%). However, the similarity of retention times and mass spectra of two PMAAs from PS-IVb with those of t-Api*f* and 3'-

Figure 6.2. E.i. mass spectrum and proposed fragmentation of 1,4-di-*O*-acetyl-(1-deuterio-)-2,3,3'-tri-*O*-methyl apiitol (t-Api, peak 2 of Figure 6.1a).

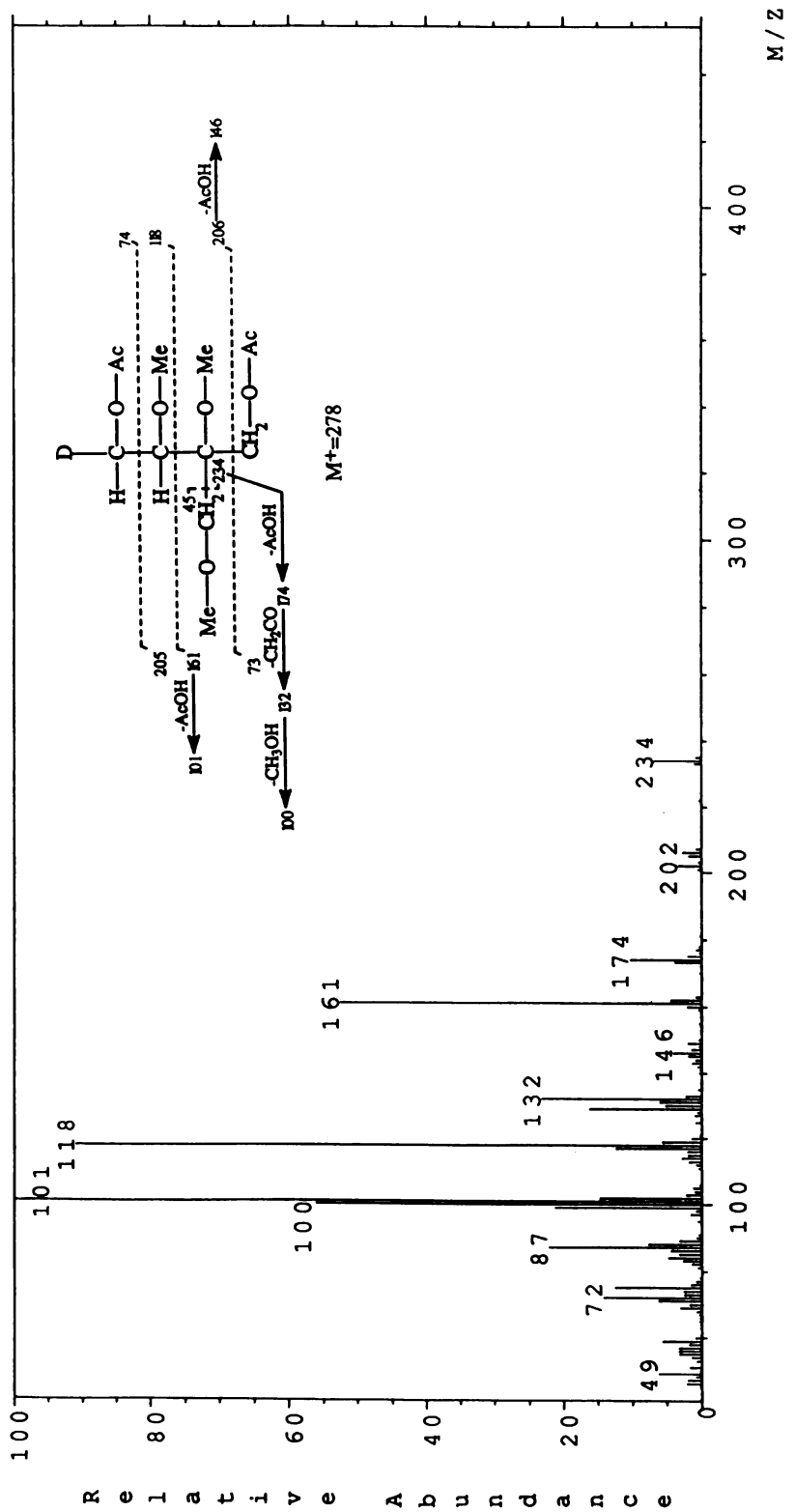
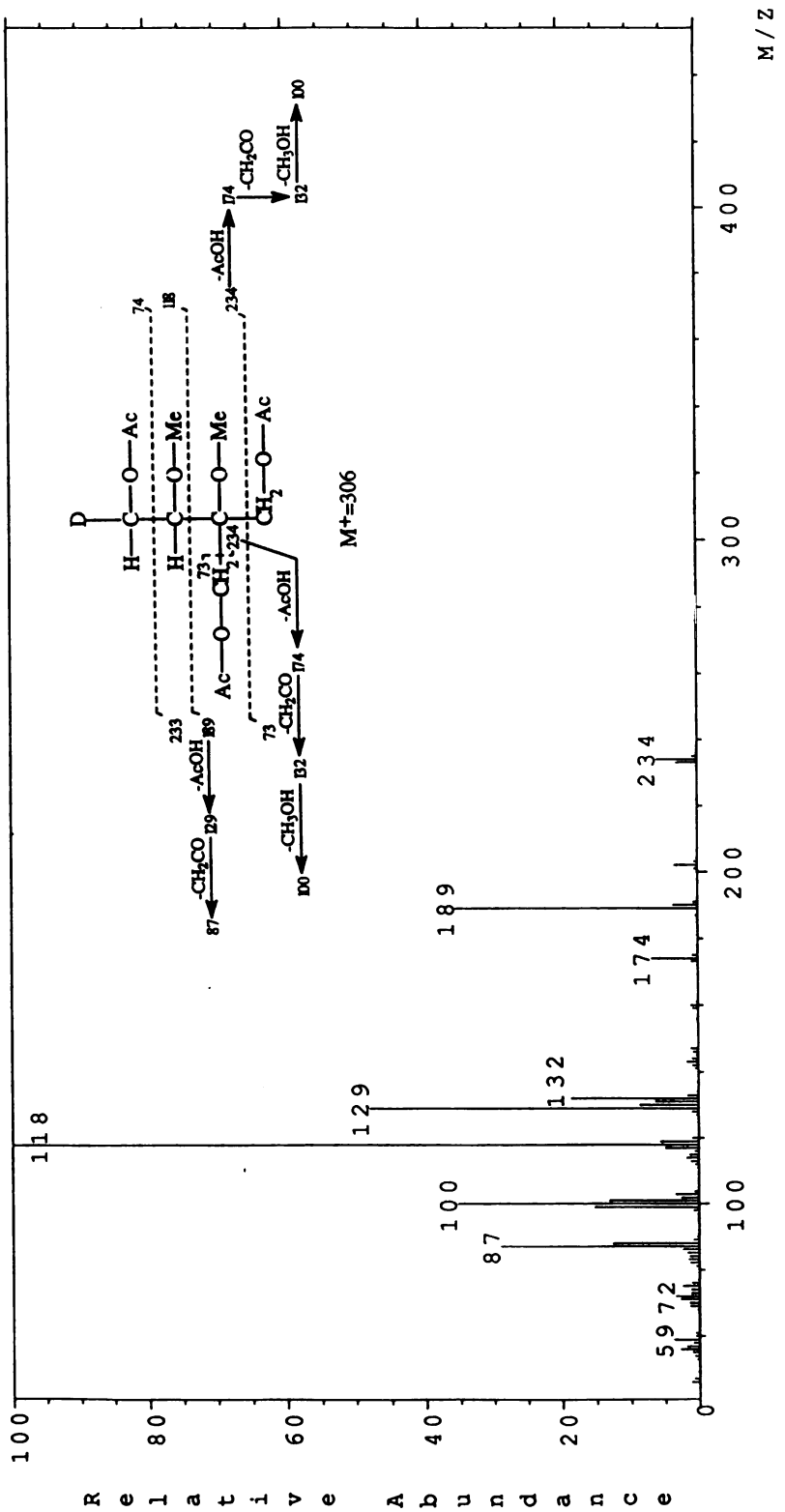

Relative Abundance
100
80
60
40
20
0
100
200
300
400
M / Z
M+=278
-AcOH
-CH3OH
-CH2CO
-AcOH
-AcOH
101
118
132
146
161
174
202
234
49
72
87

Figure 6.3. E.i. mass spectrum and proposed fragmentation of 1,3',4-tri-*O*-acetyl-(1-deuterio-)-2,3-di-*O*-methyl apiitol (3'-Api, peak 6 of Figure 6.1a).


Relative Abundance
M / Z
M+=306
118
129
132
100
87
189
174
234
597
-AcOH
-CH2CO
-CH3OH
-AcOH
-CH2CO
-CH3OH
-AcOH
-CH2CO
-CH3OH

Api*f* from PS-IIb plus criterium (iii) above, identify t-Api*f* and 3'-Api*f* as components of PS-IVb. The glycosyl-linkage compositions of PS-IIb and PS-IVb, calculated by using effective carbon response, are shown in Table 6.1.

Enzyme degradation of polysaccharides PS-IIb and PS-IVb. HPAEC-PAD chromatograms of PS-IIb, pectinase-digested PS-IIb, partially acid hydrolyzed (pH 4.0, 100°C, 5 h) PS-IIb, and PS-IIb partially acid hydrolyzed and then digested with pectinase are shown in Figures 6.4a-d. HPAEC-PAD chromatograms of PS-IVb subjected to the same four treatments are shown in Figures 6.5a-d. Free galacturonic acid was not released in the controls (Figures 6.4a and 6.5a). The amount of GalA released by treatment of PS-IIb and PS-IVb with pectinase alone was 12% (peak 4, Figure 6.4b) and 99% (peak 4, Figure 6.5b), respectively, of the GalA found in the starting polysaccharides. Partial acid hydrolysis of PS-IIb at pH 4.0 and 100°C for 5 h appeared to have released most of the apiose and apiobiose residues (compare peaks 2 and 3, respectively, of Figure 6.4c to those of Figure 6.4d) and only 2.2% of the GalA (peak 4, Figure 6.4c) from the polysaccharide; however, good quantification of apiose and apiobiose was not established due to irregular elution of these compounds with this system. PS-IVb, a virtual homogalacuronan, was quite resistant to acid hydrolysis at pH 4.0 and 100°C for 5 h since only 1.1% of the GalA (peak 4, Figure 6.5c) was released form the polysaccharide. The GalA released from PS-IIb and PS-IVb by partial acid hydrolysis followed by digestion with pectinase was 106% (peak 4, Figure 6.4d and 6.5d) of the GalA found in the starting polysachharides.

Table 6.1. Glycosyl-linkage composition (mol%) of PS-IIb and PS-IVb from methylation analysis.

Peak number in Figure 6.1	Glycosyl linkage	Polysaccharide	
		PS-IIb	PS-IVb
1	t-Rha*p*	3.30	0.48
2	t-Api*f*	21.30	1.02
6	3'-Api*f*	27.65	1.55
4	t-Gal*p*A	2.11	1.87
8	4-Gal*p*A	23.34	92.05
10	3,4-Gal*p*A	0.90	1.30
11	2,4-Gal*p*A	21.40	1.73

Figure 6.4. Column chromatograms from HPAEC-PAD for polysaccharide PS-IIb after various treatments. The chromatograms shown are : (a), PS-IIb (as control); (b), PS-IIb digested with poly-[1,4-α-D-galacturonide] glycanohydrolase; (c), PS-IIb hydrolyzed at pH 4.0 and 100°C for 5 h; (d), PS-IIb digested with poly-[1,4-α-D-galacturonide] glycanohydrolase after hydrolysis at pH 4.0 and 100°C for 5 h. Peaks 1 to 4 are Gal (internal standard), Api, apiobiose and GalA, respectively. Oligouronic acids with a degree of polymerization greater than 15 can not be detected with this system.

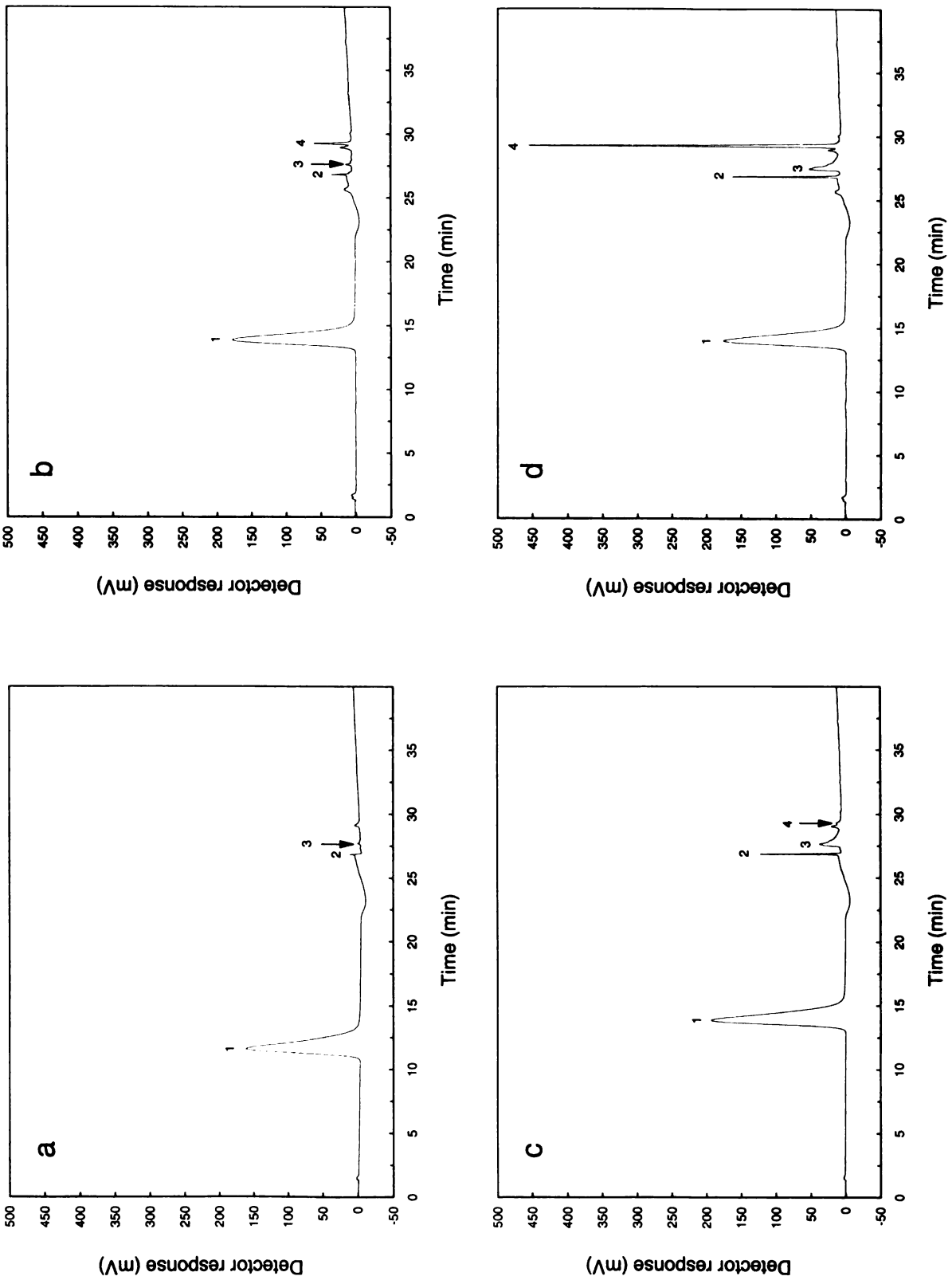
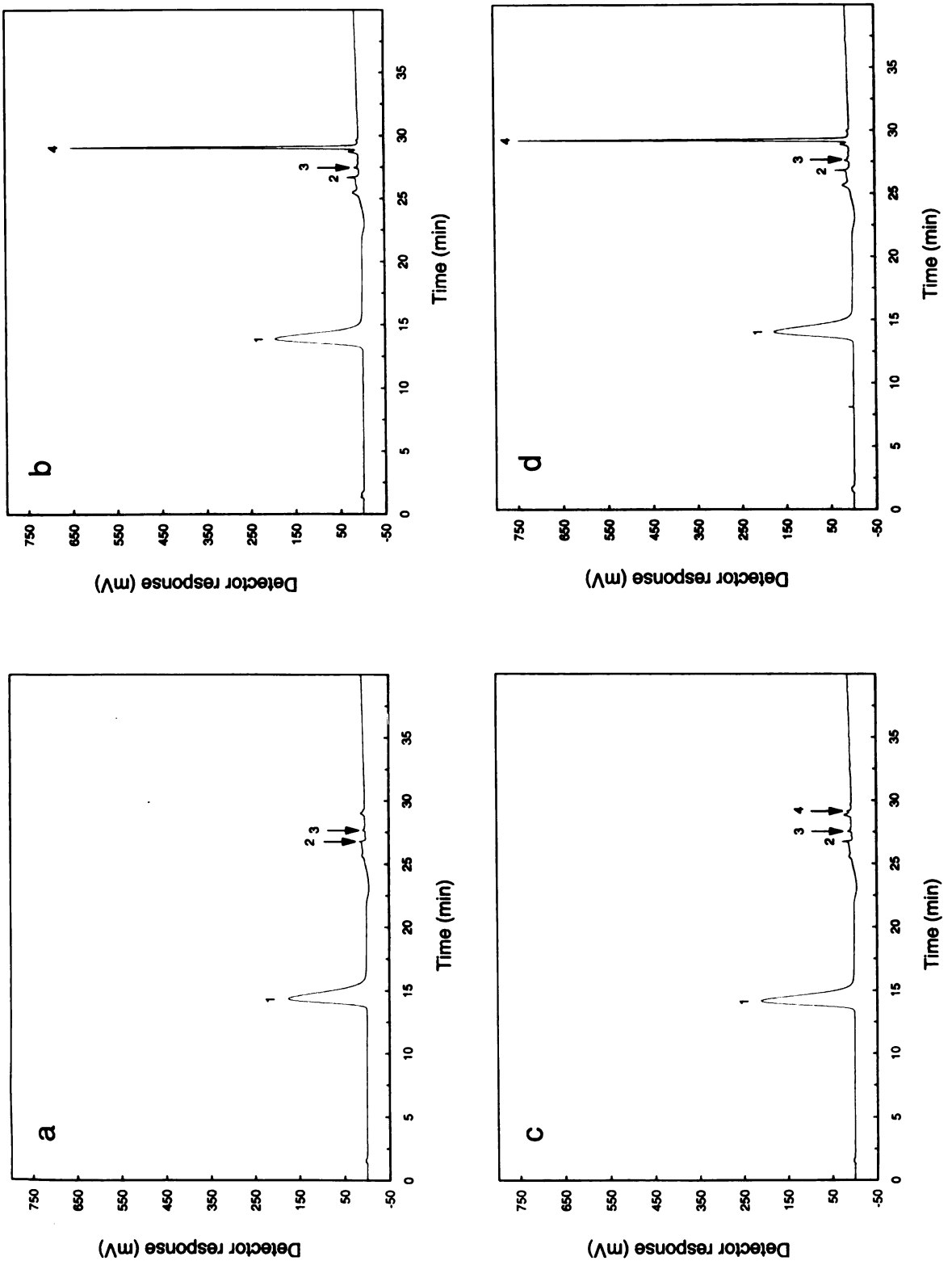

a
b
c
d
Detector response (mV)
Time (min)
1
2
3
4

Figure 6.5. Column chromatograms from HPAEC-PAD for polysaccharide PS-IVb after various treatments. The chromatograms shown are : (a), PS-IVb (as control); (b), PS-IVb digested with poly-[1,4-α-D-galacturonide] glycanohydrolase; (c), PS-IVb hydrolyzed at pH 4.0 and 100°C for 5 h; (d), PS-IVb digested with poly-[1,4-α-D-galacturonide] glycanohydrolase after hydrolysis at pH 4.0 and 100°C for 5 h. Peaks 1 to 4 are Gal (internal standard), Api, apiobiose and GalA, respectively. Oligouronic acids with a degree of polymerization greater than 15 can not be detected with this system.


a
b
c
d
Detector response (mV)
Time (min)
1
2
3
4

DISCUSSION

For PS-IIb, the ratio, PMAAs of apiosyl residues:PMAAs of galacturonosyl residues (49%:48%), does not exactly match but is reasonably close to the ratio, Api:GalA (40%:55%) obtained from sugar composition analysis by GC of alditol acetates. PS-IIb clearly is a highly branched pectic polysaccharide, since the glycosyl-linkage composition (Table 6.1) shows that nearly half (22% out of 48%) of its galacturonosyl residues are branched. Almost all (96%) of the branching is at C-2 of the galacturonosyl residues. Consequently it is not surprising to find that PS-IIb is quite resistant to poly-[1,4-α-D-galacturonide] glycanohydrolase digestion; only 12% of the galacturonosyl residues were released by the enzyme treatment.

The following results indicate that most of the apiose in PS-IIb is present as apiobiosyl side chains that are attached to galacturonosyl residues: (i) the amount of terminal apiose is close to that of internal apiose (t-Api*f*: 3'-Api*f* =21.3%:27.7%); (ii) partial acid hydrolysis of PS-IIb at 100°C appeared to have released most of the apiobiose (compare peaks 2 and 3 of Figure 6.4c to those of Figure 6.4d), but no significant amount of GalA was released; (iii) after partial acid hydrolysis, all galacturonosyl residues were released as free GalA from PS-IIb by treatment with poly-[1,4-α-D-galacturonide] glycanohydrolase. These findings are in agreement with earlier findings (19,20).

The complete release of galacturonosy residues as free GalA from PS-IIb by poly-[1,4-α-D-galacturonide]glycanohydrolase after mild acid hydrolysis indicates that the polysaccharide has a backbone of α-1,4-linked D-galacturonic acid. Since: (i) the ratio of branched

galacturonosyl residues (2,4-Gal*p*A plus 3,4-Gal*p*A) to unbranched residues (4-Gal*p*A) was approximately 1:1 (22%:23%), (ii) the ratio of PMAA of galacturonosyl residues (t-Gal*p*A, 4-Gal*p*A, 3,4-Gal*p*A and 2,4-Gal*p*A) to those of apiosyl residues (t-Api*f* and 3'-Api*f*) was also approximately 1:1 (48%:49%), and (iii) untreated PS-IIb was quite resistant to the enzyme digestion, the apiobiose side chains must be relatively evenly distributed along the backbone on approximately every other galacturonosyl residue.

Since: (i) internal apiose (3'-Api*f*, 27.7%) was somewhat higher than the terminal apiose (21.3%), (ii) rhamnosyl residues (3.3%) were solely terminal-linked, and (iii) the release of free GalA from the backbone was complete after side chains were removed by partial hydrolysis, it is possible that rhamnosyl residues may be attached to the backbone of the polysaccharide through apiose. However, evidence for a disaccharide of Rha and Api has not been obtained.

Although small amounts of 1,5-di-*O*-acetyl-(1-deuterio-)-2,3,4-tri-*O*-methyl pentitol, 1,4,5-tri-*O*-acetyl-(1-deuterio-)-2,3-di-*O*-methyl pentitol and 1,4,5-tri-*O*-acetyl-(1-deuterio-)-2,3,6-tri-*O*-methyl hexitol were found by methylation analysis and Xly ($2.5 \pm 1.4\%$), Ara ($1.4 \pm 0.8\%$) and trace amounts ($< 0.5\%$) of other hexose, such as Man and Glc, were found by sugar composition analysis, the assignment of the corresponding sugar to these PMAAs has not been possible due to lack of standards. However the finding that complete degradation of PS-IIb by pectinase occurred after partial acid hydrolysis seemed to indicate that these minor components (assuming they are not contaminants) are in side chains that are released by the acid hydrolysis.

All PMAAs obtained from PS-IIb (Figure 6.1a) were also obtained from PS-IVb (Figure

6.1b). The major difference between PS-IVb and PS-IIb is that in PS-IVb the PMAAs obtained in large amounts were all derived from galacturonosyl residues while in PS-IIb they were derived from both galacturonosyl and apiosyl residues. Although small amounts of PMAAs of neutral sugars were detected from methylation analysis of PS-IVb, and Api (2.1%), Xyl (1.6%) and trace amounts (<0.5%) of other neutral sugars were detected by sugar composition analysis (Chapter V), these sugars may actually be present in PS-III and PS-III is contaminating PS-IVb. The overlapping elution of PS-III and PS-IV from the DEAE column (Chapter V) makes this a possiblity. The molar percentage of PMAAs of PS-IVb detected by methylation analysis (97%, t-Gal*p*A, 4-Gal*p*A, 3,4-Gal*p*A and 2,4-Gal*p*A) is similar to the molar percentage of GalA detected in sugar composition analysis (Chapter V). The high content of GalA and the fact that PS-IVb was nearly completely digested by pectinase indicate PS-IVb is a homogalacturonan.

REFERENCES

1. Chambat, G. and Joseleau, J. (1980) *Carbohydr. Res.*, **85**, C10-C12.
2. Kato, Y. and Nevins, D. J. (1989) *Plant Physiol.* **89**, 792-797.
3. McNeil, M., Darvill, A. G., Fry S. C., and Albersheim, P. (1984) ***Ann. Rev. Biochem.*** **53,** 625-663
4. Shibuya, N. and Iwasaki, T. (1978) *Agric. Biol. Chem.*, **42**, 2259-2266
5. Thibault, J.-F, Renard, C. M.G.C., Axelos, M. A. V., Roger, P. and Crépeau, M-J (1993) *Carbohydr. Res.*, **238**, 271-286
6. McNeil, M., Darvill, A. G. and Albersheim, P. (1980). *PLant Physiol.* **66**, 1128-1134
7. McNeil, M., Darvill, A. G. and Albersheim, P. (1982). *Plant Physiol.* **70**, 1586-1591
8. Lau, J. M., McNeil, M., Darvill, A. G. and Albersheim, P. (1985). *Carbohydr. Res.* **137**, 111-125.
9. Lau, J. M., McNeil, M., Darvill, A. G. and Albersheim, P. (1987) *Carbohydr. Res.* **168**, 245-247.
10. Aspinall, G. O. (1980) in *The Biochemistry of Plants, A comprehensive Treatise,* (Preiss, J. Ed.), Vol. **3**, Academic Press, New York, pp. 473-500.
11. Dey, P. M. and Brinson, K. (1984) *Adv. Carbohydr. Chem. Biochem.*, **42**, 265-382
12. Darvill, A. G., McNeil, M. and Albersheim, P. (1978). *Plant Physiol.* **62**, 418-422
13. Spellman, M. W., McNeil, M., Darvill, A. G., Albersheim, P. and Henrick, K. (1983) *Carbohydr. Res.* **122**, 115-129.
14. Spellman, M. W., McNeil, M., Darvill, A. G., Albersheim, P. and Dell A. (1983)

Carbohydr. Res. **122**, 131-153.

15. Ros, J. M., Schols, H. A. and Voragen, A. G. J. (1996) *Carbohydr. Res.* **282**, 271-284.
16. Ishii, S. (1981). *Phytochemistry* **20**, 2329-2333.
17. Ishii, S. (1982). *Phytochemistry* **21**, 778-780.
18. Beck, E. (1967). *Z. Pflanzenphysiol. Bd.* **57**, S. 444-461
19. Hart, D. A. and Kindel, P. K. (1970) *Biochem. J.*, **116**, 569-579
20. Hart, D. A. and Kindel, P. K. (1970) *Biochemistry* **9**, 2190-2196
21. Cheng, L. and Kindel, P. K. (1996) *Homogeneity and Structure of the 22 °C Ammonium Oxalate-soluble Pectic Polysaccharides of Lemna minor*, submitted to *Carbohydr. Res.*
22. M. G. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith (1956) *Anal. Chem.*, **28**, 350-356.
23. N. Blumenkrantz, and G. Asboe-Hansen (1973) *Anal. Biochem.*, **54**, 484-489
24. R. L. Taylor and H. E. Conrad (1972) *Biochemistry*, **11**, 1383-1388.
25. I. O. Ciucanu and F. Kerek (1984) *Carbohydr. Res.*, **131**, 209-217.
26. P. W. Needs and R. R. Selvendran (1993) *Carbohydr. Res.*, **245**, 1-10.
27. T. J. Waeghe, A. G. Darvill, M. McNeil and P. Albersheim (1983) *Carbohydr. Res.*, **123**, 281-304.
28. P. K. Kindel, and L. Cheng (1990) *Carbohydr. Res.*,**199**, 55-65.
29. D. P. Sweet, R. H. Shapiro, and P. Albersheim (1975) *Carbohydr. Res.*, **40**, 217-225.
30. R. F. Addison and R. G. Ackman (1968) *J. Gas Chromatogr.*, **6**, 135-138.
31. R. G. Ackman (1964) *J. Gas Chromatogr.*, **2**, 173-197.

32. Carpita, N. C. and Shea, E. M. *Analysis of Carbohydrates by GLC and MS* (Biermann, C. J. and McGinnis, G. D., Eds). CRC Press, 1990, Chapter 9, 157-216.

CHAPTER VII

CONCLUSIONS AND FUTURE PERSPECTIVE

CONCLUSIONS

Procedures for preparing cell walls from *L. minor* and for extracting the 22°C chelator-soluble pectic polysaccharides of the cell walls were improved. Thirty-two percent (32%) of the total pectic polysaccharides (measured as uronic acid) in purified cell walls of *L. minor* was solubilized with 0.05 M ammonium oxalate (pH 5.5) at 22°C in 30 min.

A fast, simple yet effective procedure of pectic polysaccharide purification was developed. Pectic polysaccharides solubilized by 0.05 M ammonium oxalate were directly applied to a DEAE column whose column buffer was the same as the extraction buffer. All steps that involved dialysis, concentration, and freeze-drying of polysaccharide solutions were eliminated so that the sample loss and degradation reactions of polysaccharides were minimized .

Conditions were established for quantitatively eluting pectic polysaccharides from DEAE-columns. Cations were found to greatly affect the elution of pectic polysaccharides from anion exchange columns. Six plant pectic polysaccharides from four different plant sources (citrus, apple, duckweed, and celery) were quantitatively (or nearly so) eluted from DEAE-Trisacryl columns by 0.5 M NH_4Cl in 0.05 M ammonium acetate buffer (pH 5.5). In contrast, the elution of five of these six pectic polysaccharides was incomplete when up to 1 M NaCl or KCl in acetate buffer were used to develop the DEAE columns. Na^+ and K^+ were found responsible for precipitation and gel formation with the tested pectic polysaccharide in test tubes and apparently also in the DEAE columns. The discovery that NH_4^+, Li^+ and Cs^+ in the eluent resulted in the essentially quantitative elution of pectic

polysaccharides from anion exchange resins solves the long-standing problem of incomplete recovery of pectic polysaccharides from DEAE column chromatography.

Homogeneity with respect to sugar composition, molecular size and degree of methylesterification was established for the pectic polysaccharide material in column fractions after the chelator-soluble pectic polysaccharides of *L. minor* were fractionated by DEAE chromatography. Analysis of individual DEAE-column fractions by HPSEC-MALLS resulted in detection of four pectic polysaccharides, PS-I, PS-II, PS-III and PS-IV, with peak $\overline{M}_w$ ranges of 50,200 - 75,400, 18,800 - 99,700, 24,200 - 150,000 and 6,170 to ≥ 163,000, respectively. PS-IIb and PS-IVb, the major portion of pectic polysaccharides PS-II and PS-IV, respectively, are homogeneous in sugar composition and methylester content, and they both show a single, virtually symmetrical peak when they were rechromatographed on DEAE-Trisacryl Plus M columns.

Primary structures were further established for the two purified *L. minor* pectic polysaccharides, PS-IIb and PS-IVb. PS-IIb is a apiogalacturonan with a peak $\overline{M}_w$ range of 28,100 - 99,700, and it consists of a α-1,4-linked D-GalA*p* backbone with 1,3'-linked apiobiose as the predominant side chains connected to the *O*-2 position of nearly every other galacturonosyl residues of the backbone. The carboxyl groups of GalA residues are free of methylester. Besides GalA (54.5%±3.2%) and Api (39.3%±1.4%), small amounts of Rha (2.3%±0.3%), Xyl (2.5%±1.4), Ara (1.4%±0.8%) and other common neutral sugars (<0.5%) were detected by sugar composition analysis of PS-IIb but the sugars present in small amounts could not be conclusively established as components of PS-IIb. Further degradation of PS-IIb by poly-[1,4-α-D-galacturonide]glycanohydrolase (EC 3.2.1.15) after side chains

containing apiose were removed by mild acid hydrolysis indicated that the sugar detected in small amount, if not contaminants, are present in the side chains. PS-IIb is somewhat different than the three types of plant pectic polysaccharides isolated so far —homogalacturonan, Rhamnogalacturonan-I and Rhamnogalacturonan-II — since apiose is the predominate neutral sugar residue and the small amount of rhamnose (assuming it is a component of PS-IIb) was found to be solely terminal-linked.

PS-IVb is basically an α-1,4-linked homogalacturonan and contains 96.3% GalA and has a peak $\overline{M}_w$ range of 53,300 to at least 163,000. The carboxyl group of the GalA residues are not methylesterified. Although Api (2.1%), Xyl (1.6%) and trace amounts (<0.5%) of other neutral sugars such as t-Rha were detected in sugar composition analysis or in methylation analysis, they appeared to be contaminants rather than components of PS-IVb, since most (~99%) of PS-IVb was degraded by poly-[1,4-α-D-galacturonide] glycanohydrolase to GalA without first a mild acid hydrolysis. The isolation of a pectic polysaccharide of high GalA content basically free of methylester while using mild conditions has not been reported before. In the presence of Na^+ and K^+, the polysaccharide formed what appeared to be a gel and was held on DEAE columns when these ions were in the eluent. Consequently this type of polysaccharide is probably present in other plants but has escaped detection because it was not eluted from ion-exchange columns.

A micro-scale method was developed to depolymerize and reduce pectic polysaccharides before they were converted to alditol acetates for GC analysis. All reactions, including methanolysis/methyl esterification, carboxyl-reduction, hydrolysis and reduction-acetylation, were conducted in an single vial. Compared with other methods tested (1,2), the proposed

method gave better depolymerization of the pectic polysaccharides, increased conversion of uronic acid to neutral sugars, and less sample loss. In addition multiple samples can be processed simultaneously on a micro-scale (0.11-0.14 mg).

FUTURE PERSPECTIVE

Data collected in this research described the basic structure of PS-IIb (an apiogalacturonan) and PS-IVb (a homogalacturonan). However, small amounts of neutral sugars such as Rha, Xyl, and Ara were detected in the polysaccharides by sugar composition analyses. Also, t-Rha*p*, t-pentose and 1,4-linked hexose were found in both PS-IIb and PS-IVb. Are these residues components or contaminants? Most commonly, Rha is found to be 2-linked and 2,4-linked in the backbone of pectic polysaccharides of plants. If t-Rha*p* is a component of PS-IIb and PS-IVb, these two polysaccharides are exceptions to this generality. Is t-Rha linked to Api as a disaccharide side chain? These questions can be answered by separating and analyzing oligosaccharides fragments containing those sugars from controlled acid hydrolysis of PS-IIb and PS-IVb or by selectively degrading the GalA residues of these two polysaccharides for example by lithium in ethylenediamine (3,4).

Although both PS-IIb and PS-IVb were solubilized from cell walls of *L. minor* by a chelator, they are quite different. PS-IVb is a smooth long-chain polymer that lacks branches and methylester groups while PS-IIb is a highly ramified polymer with mainly apiobiose side chains evenly distributed along the PGUA backbone. Why do cell walls of *L. minor* need both? What is their function? The pectic polysaccharide RG-I has been isolated from many

plants and found to have "smooth" and "hairy regions" (5,6). Junction zones are formed in smooth regions through Ca^{2+} bridges (7,8) but are spaced by esterification of the carboxyl groups of GalA residues and by neutral sugar side chains attached to GalA residues so pores are formed in the cell wall (9). Is the function of PS-IVb similar to that of smooth regions and the function of PS-IIb similar to that of hairy and esterified regions? Results with some plants suggests that more junction zones are formed after cell wall elongation (10). Examining the ratio of PS-IIb and PS-IVb in cell walls of *L. minor* at different stages of growth may provide information about the above question. Polyclonal antibodies (anti- RG-I and anti- xyloglucan) have been used with the electron microscope to locate polysaccharides in cell walls of plant (11). The future research should include trying to raise monoclonal antibodies against PS-IIb and PS-IVb and determine their location in cell walls of *L. minor* at different growth stages. Such studies could help us learn when and where these polysaccharides are synthesized and how they are finally positioned.

Fifty-seven percent (57%) of the total pectic polysaccharides of the cell wall were not solubilized by the extractant (0.05 M ammonium oxalate) at 22°C. Efforts should be made to extract and analyze these polysaccharides in order to have a comprehensive knowledge of the pectic polysaccharides of the cell wall of *L. minor*.

The procedures successfully developed in this research with *L. minor* enable us to purify cell walls, extract and purify pectic polysaccharides with a simplified process, and establish homogeneity of polysaccharide samples in column fraction of ion-exchange chromatography. But they all can be used with cell walls from other plants. With the conditions established in this research, complete elution of the pectic polysaccharides that consist of high GalA

units from anion-exchange columns becomes possible. It is desirable to test these procedures and conditions with cell walls from other plants in order to find if chelator-soluble homogalacturonans exist widely in higher plants.

REFERENCES

1. Taylor, R. L., and Conrad, H. E. (1972) *Biochemistry*, **11**,1383-1388.
2. De Ruiter, G. A., Schols, H. A., Voragen, A. G. J. and Rombouts, F. M. (1992) *Anal. Biochem.* **207**, 176-185.
3. Mort, A. J. and Bauer, W. D. (1981) *J. Biol. Chem.*, Vol. **257**, No. **4**, 1870-1875.
4. Lau, J. M., McNeil, M., Darvill, A., and Albersheim, P. (1987) *Carbohydr. Res.*, **168**, 219-243.
5. Schols, H. A. and Voragen A. G. J. (1994) *Carbohydr. Res.*, **256**, 83-59.
6. Thibaul, J.-F., Renard, C. M. G. C., Axelos, A. V., Roger, P. and Crépeau, M.-J. (1993) *Carbohydr. Res.*, **238**, 271-286.
7. Morris, E. R., Powell, D. A., Gidley, M. J. and Rees, D. A. (1982) *J. Mol. Biol.*, **155**, 507-516.
8. Rees, D. A. (1975) *MTP Int. Rev. Sci. Biochem. Ser. One,* Vol. **5**, 1-42.
9. Baron-Eple, O., Gharyl, P. K. and Schindler, M. (1988) *Planta*, **175**, 389-395.
10. Carpita, N. C. and Gibeaut, D. M. (1993) *The Plant Journal*, **3**, 1-30.
11. Moor, P. J., Darvill, A. G., Albersheim, P. and Staehelin, L. A. (1986) *Plant Physiol.*, **82**, 787-794.

APPENDICES

APPENDIX A

SOLUBILIZATION OF PECTIC POLYSACCHARIDES FROM THE CELL WALLS OF *Lemna minor* AND *Apium Graveolens*

(Reprinted from *Phytochemistry*)

Pergamon

0031-9422(95)00675-3

Phytochemistry, Vol. 41, No. 3, pp. 719–723, 1996

0031-9422/96 $15.00 + 0.00

SOLUBILIZATION OF PECTIC POLYSACCHARIDES FROM THE CELL WALLS OF *LEMNA MINOR* AND *APIUM GRAVEOLENS*

PAUL K. KINDEL, LIANG CHENG and BRANDI R. ADE

Department of Biochemistry, Michigan State University, East Lansing, MI 48824-1319, U.S.A.

(*Received in revised form* 15 *July* 1995)

Key Word Index—*Lemna minor*; Lemnaceae; duckweed; *Apium graveolens*; Umbelliferae; celery; cell wall; pectic polysaccharides.

Abstract—The kinetics of solubilization of pectic polysaccharides from purified cell walls of *Lemna minor* (duckweed) and *Apium graveolens* (celery) by ammonium oxalate at 22° was determined. With cell walls of *L. minor*, 30 ± 1 (average of three experiments, average deviation from the mean) and 34 ± 1% of the total anhydrouronic acid of the cell walls was solubilized in 15 min and 5 hr, respectively. Water at 22° solubilized 1.3 ± 0.2% and 1.3 ± 0.4% of the total anhydrouronic acid in 15 min and 5 hr, respectively. With cell walls of *A. graveolens*, 19 ± 1% (average of two experiments, average deviation from the mean) and 23 ± 1% of the total anhydrouronic acid of the cell wall was solubilized in 15 min and 3 hr, respectively. Water at 22° solubilized 0.9 ± 0.6 and 0.9 ± 0.1% of the total anhydrouronic acid in 15 min and 3 hr, respectively. When *trans*-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid was the extractant, recovery values based on weight for the soluble pectic polysaccharide fractions were incorrect (high). Color formation in the uronic acid test was inhibited by sodium dodecyl sulfate. A rapid, two-step procedure for isolating purified cell walls was developed.

INTRODUCTION

The primary cell wall of plants maintains the structure of growing plant cells and is involved in a variety of important biological processes [1]. Pectic polysaccharides* are one of the three polysaccharide constituents of primary cell walls. Little is known about the structural role of pectic polysaccharides in cell walls or about their biosynthesis. Understanding these requires that the structure of the pectic polysaccharides be known. The complete structure of any pectic polysaccharide has not been determined and even less is known about the degree of structural variation in the total complement of pectic polysaccharides present in any one plant. In order to obtain accurate structural information a suitable isolation procedure must be used.

The amount of pectic polysaccharides solubilized from cell walls is dependent both on the origin of the cell walls and on the method of extraction [2–15]. A significant portion of the cell wall pectic polysaccharides is readily solubilized by chelating agents and the extent of their solubilization is easily followed colorimetrically. Surprisingly, the time course of solubilization of pectic polysaccharides from purified cell walls at 22° has not been determined. Experiments of this type will show how distinct the difference in solubility is between cell wall pectic polysaccharides readily solubilized and those that tend to remain in the cell wall. They will also permit rational selection of the shortest possible extraction time needed for complete solubilization of a fraction. Our main goal was to determine the kinetics of solubilization of pectic polysaccharides from purified cell walls of *Lemna minor* (duckweed) and *Apium graveolens* (celery).

Pure cell walls are needed before the kinetics of solubilization of pectic polysaccharides can be determined. Both organic [16, 17] and aqueous solvents have been used in the isolation of cell walls, and both have their disadvantages. When aqueous solvents are used cell wall constituents may be solubilized or enzymatically degraded or both. The solubilization of cell wall pectic polysaccharides during the preparation of cell walls has been reported; however, in no case was the solubilized material firmly identified [2–8]. Ionic detergents such as sodium dodecyl sulfate (SDS) have been used in the isolation of cell walls [18], but we found that SDS binds to isolated cell walls and subsequently interferes with color development in the uronic acid assay (this paper). Our second goal was to develop an aqueous cell wall isolation procedure that was rapid, mild, simple and yielded pure cell walls in good yield.

*Pectic polysaccharides are defined as those containing mainly galacturonic acid units in the main chain (backbone) of the polysaccharide.

RESULTS

Characterization of cell walls

Cell walls prepared from *L. minor* were dark green after treatment with a Waring blender but pale green to white after treatment with both a Waring blender and a French press. This indicated numerous intact cells with intact chloroplasts were present after the homogenization step but few were present after both steps. Examination of both preparations by both regular and confocal light microscopy confirmed that this was the case. Examination of the final cell wall preparation by electron microscopy showed only cell walls and an occasional thick-walled cell were present. Membranes were not observed adhering to cell walls. The final celery cell wall preparations were cream-colored. Some small multicellular pieces of tissue were observed in the celery preparations and based on examination by light microscopy we estimated 80–85% of the cells were broken. The protein content of purified cell walls of *L. minor* and *A. graveolens* was 5.5% and 1.0%, by weight, respectively. Purified cell walls from other plants have similar protein contents [19]. The uronic acid content of the cell walls of *L. minor* and *A. graveolens* was 189 ± 10 mg of anhydrouronic acid per gram of dry cell walls (mean of three experiments) and 251 ± 10 mg per gram (two experiments), respectively.

Solubilization of cell wall pectic polysaccharides

The average results of three experiments measuring the time course of solubilization of pectic polysaccharides from purified cell walls of *L. minor* at 22° with 0.05 M ammonium oxalate (pH 5.5) are shown in Fig. 1. On average 30 ± 1% (average deviation from the mean) of the total anhydrouronic acid of the cell walls was solubilized in 15 min and 33 ± 1% (two experiments for this value), 30 ± 3%, and 34 ± 1% was solubilized in 1, 3 and 5 hr, respectively. Water at 22° solubilized 1.3 ± 0.2% of the total anhydrouronic acid of the cell walls in 15 min and 1.3 ± 0.4% in 5 hr. The total amount of anhydrouronic acid recovered in the 15 min and 5 hr ammonium oxalate soluble fractions and the corresponding insoluble residue fractions was 99 ± 5% and 101 ± 4%, respectively. The total cell wall material solubilized by ammonium oxalate in 15 min and 5 hr was 7.9 ± 1.6% and 6.2 ± 1.2%, respectively. These values are based on the dry weights of the starting cell walls and recovered ammonium oxalate residues.

The average results from two experiments measuring the time course of solubilization of pectic polysaccharides from purified cell walls of *A. graveolens* by 0.05 M ammonium oxalate (pH 5.5) at 22° showed that 19 ± 1%, 22 ± 1%, 22 ± 1%, and 23 ± 1% of the total anhydrouronic acid of the cell walls was solubilized in 15 min, 1 hr, 2 hr and 3 hr, respectively (Fig. 1). Less than 1% of the total anhydrouronic acid of the cell walls was solubilized by 15 min and 3 hr extractions with water at 22°. In the above experiments, anhydrouronic acid values were normalized on the basis of 1 g dry weight of cell walls extracted and then percentage values were calculated. Percent values are relative to the anhydrouronic acid content of dry cell walls, which was set equal to 100%.

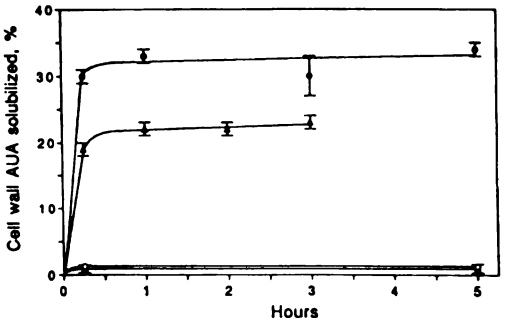


Fig. 1. Time course of solubilization of pectic polysaccharides from purified cell walls *L. minor* (average of three experiments) and *A. graveolens* (two experiments). Purified cell walls of *L. minor* and *A. graveolens* were extracted with ammonium oxalate (●, *L. minor*; ▲, *A. graveolens*) and water (○, *L. minor*; △, *A. graveolens*) as described in the Experimental section. The anhydrouronic acid contents of the dry cell walls and the method of calculating percent values are given in the Results. Dry weights of cell walls were calculated from wet weights and the wet weight/dry weight ratio of the cell walls. For *L. minor* the average wet-weight/dry-weight ratio of the cell walls was 15.3 ± 2.4; for *A. graveolens* it was 12.7 ± 0.4. Error bars show the average deviation from the mean. AUA, anhydrouronic acid.

Analysis of filtrates from the preparation of cell walls for uronic acid

The amount of anhydrouronic acid in the combined, dialysed filtrates obtained during the preparation of cell walls of *L. minor* and *A. graveolens* was 13% (three experiments) and 5.0% (two experiments), respectively, of the total anhydrouronic acid present in the combined filtrates plus cell walls. Percent values were calculated from anhydrouronic acid values normalized on the basis of 1 g dry weight of cell walls extracted.

Effect of various chemicals on the uronic acid test

HCl (1 M), NaCl (1 M) and ammonium oxalate (0.05 M) present individually in samples containing known amounts of galacturonic acid had no effect on color formation in the uronic acid test; control samples were in water. In contrast, 0.0031% and 0.031% (w/v) SDS present in a galacturonic acid solution inhibited color formation 39 and 94%, respectively.

When SDS was used in the isolation of *L. minor* cell walls, measurements of uronic acid in the ammonium oxalate extract were low. Whole plants were homogenized and to one half of the homogenate SDS was added to a concentration of 0.52% (w/v) and to the other half water was added. After stirring for 15 min and centrifug-

ing, the cell wall fraction was washed once with 0.1 M NaCl and once with water and then extracted twice with ammonium oxalate, each time for 4 hr. The amount of uronic acid detected in the ammonium oxalate soluble extracts from cell walls prepared with SDS present, per gram dry weight of cell walls, was 44% (average of two experiments) of that in the water control.

Extraction of cell walls with trans-1,2-*diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA)*

Individual samples of cell walls of *L. minor* were extracted with CDTA and ammonium oxalate for 15 min and 5 hr at 22°. The chelator-soluble fractions were dialyzed for 21 hr in water with six changes and dried to constant weight. The dry weight of the 15 min and 5 hr fractions solubilized by CDTA was 4.7 times and 4.8 times greater, respectively, than the corresponding ammonium oxalate fractions. The values were normalized on a per gram dry weight of cell walls extracted. The anhydrouronic acid and total sugar content of the two extracts obtained at each extraction time were basically the same.

DISCUSSION

The results in Fig. 1 show there is a rapid solubilization of pectic polysaccharides from purified cell walls of *L. minor* and *A. graveolens* by ammonium oxalate at 22° and then the rate of solubilization decreases sharply until solubilization stops. The time needed for complete solubilization of the 22° ammonium oxalate-soluble fraction was approximately 30 min; there was, however, only a slight increase in the percent solubilized between 15 min and 1 hr (Fig. 1). The almost complete cessation of solubilization showed that the difference in solubility between the soluble and insoluble pectic polysaccharides is distinct. When impure preparations of cell walls of *L. minor* were used (preparations obtained following the Waring blender step), the rate of solubilization was not as rapid as that shown in Fig. 1 and pectic polysaccharides continued to be released for the entire 5 hr (data not presented). This result shows the importance of using purified cell walls. We conclude that the chelator-soluble pectic polysaccharides are held in the cell wall by ionic interactions alone.

Extraction times of 1.25–6.5 hr have been used to solubilize the 22° chelator-soluble cell wall fraction [2, 8, 9, 12, 14]. Our results show that such long extraction times are unnecessary and are probably undesirable because of the possibility of polysaccharide degradation. The results in Fig. 1 show that a 30 min extraction with ammonium oxalate at 22° is sufficient to solubilize the chelator-soluble fraction, at least with the two cell walls tested. Since a rapidly-solubilized pectic polysaccharide fraction is present in both a monocot and a dicot, this type of fraction may be present in the cell walls of many higher plants.

The extent to which cell wall pectic polysaccharides are solubilized during the preparation of cell walls is not known. Our results with *L. minor* and *A. graveolens* show that 13% and 5.0%, respectively, of the total uronic acid units present in the cell walls plus filtrates were present in the filtrates. It was not established if the uronic acid material in the filtrates originated from the cell walls. However, no significant amounts of pectic polysaccharides were solubilized by water once the cell walls were isolated (Fig. 1).

HCl and NaCl at 1 M and ammonium oxalate at 0.05 M in the sample solution do not interfere, with color formation in the uronic acid test, but SDS does. CDTA, when used as the extractant, was not completely removed from the 22° chelator-soluble fraction by dialysis in water and therefore incorrect (high) dry weights of the fraction were obtained (data not presented). Similar results have been reported by others [20].

The findings that the 22° chelator-soluble pectic polysaccharide fraction of plant cell walls was solubilized in a short time – slightly more than 15 min – and that solubilization of cell wall material virtually ceased after this time period have not been reported previously. The time needed for solubilization of the 22° chelator-soluble fraction was the same for the two plants. In each plant a specific percentage of the total cell wall pectic polysaccharides was solubilized, however, the percent solubilized was different for the two plants. These findings were obtained only when purified cell walls were used.

EXPERIMENTAL

Materials and general methods. Lemna minor was grown as described elsewhere [21]. *Apium graveolens* was purchased from a local grocery. Dialysis tubing with a 6000–8000 M_r cutoff was from Spectrum Medical Industries, Inc. Nylon mesh (3–15/6) was from Tetko, Inc. Uronic acid and total sugar were determined with 3-hydroxydiphenyl [22] and phenol-sulfuric acid [23], respectively. The determinations of uronic acid and neutral sugar in the combined filtrates were corrected for mutual interference. Equations based on Beer's law were derived for calculating the concn of each sugar type measured in the presence of the interfering sugar type. For the calculations, D-galacturonic acid monohydrate and sucrose were used as the representative uronic acid and neutral sugar types, respectively. The extinction coefficients of these two in both sugar tests were determined and were used in the equations. Bovine serum albumin was used as the protein standard. Nitrogen by the Kjeldahl method was determined by Galbraith Laboratories and the protein content was calculated by multiplying by 6.25. Samples were dried to constant weight under vacuum and over P_2O_5 at 22°. Cell wall prepns were examined by transmitted brightfield microscopy with a standard light microscope and those from *L. minor* were further examined by laser scanning transmitted brightfield microscopy with a Zeiss 10 laser scanning microscope. Cell wall prepns of *L. minor* were also examined by transmission electron microscopy at 10 000 to 59 000 × with a Phillips CM 10 electron microscope operated at 100 kV. Samples were fixed with glutaraldehyde and O_sO_4 and infiltrated

with Quetol 651-vinylcyclohexane dioxide [24]. The sample sections were 90 nm thick and were stained with uranium acetate and lead citrate.

Preparation of cell walls and rate of solubilization of pectic polysaccharides. An experiment with *L. minor* is described; however, the same procedure was used with *A. graveolens* except one extraction time was different. Whole plants of *L. minor* were suspended in water at 22° and the water was decanted. Washing was repeated four times. Plants were freed of excess water with absorbent paper and weighted wet (52 g). Petioles of *A. graveolens* were diced at 4° to cubes 0.5–1.0 cm on a side.

The following was performed at 4°. Plants (47.7 g, wet wt) were suspended in 120 ml of 1.0 M NaCl and homogenized for four, 40-s periods in a Waring blender with 10 sec intervals between periods. The suspension was filtered with 15 μm Nylon mesh, the particulate material was washed with 75 ml of water and the combined filtrates were saved. The cell walls were resuspended in 120 ml of water and passed through a French pressure cell at an average cell pressure of 16 000 pounds/in^2. The suspension was filtered through 15 μm Nylon mesh, the cell walls were washed with 75 ml of water and the combined filtrates were saved. The cell walls were weighed (9.3805 g, wet wt) and examined microscopically.

Two weighed portions of cell walls, each 0.4 ± 0.04 g, wet wt, were dried to constant weight and two weighed portions, each 0.3 ± 0.04 g, wet wt, were hydrolysed as described below. The remainder of the cell walls was divided approximately equally between six, 50 ml beakers (each sample was about 0.85 ± 0.05 g, wet wt) and the wet weights recorded. Four of the cell wall portions were suspended in 0.05 M ammonium oxalate (pH 5.5) (8.5 ml per sample) and stirred for 15 min, 1, 3, and 5 hr at 22°. Two portions were suspended in water (8.5 ml per sample) and stirred for 15 min or 5 hr at 22°. The suspensions were centrifuged at 4° and the supernatant solutions were filtered. The residues were suspended in water and the suspensions were centrifuged and the supernatant solutions filtered. The washing was repeated once and the three filtrates were combined and analysed for uronic acid.

One weighed portion of the washed residue from both the 15 min and 5 hr ammonium oxalate extractions, each approximately 0.3 g, wet wt, was hydrolysed and a second weighed portion (about 0.4 g, wet wt) of each was dried to constant weight. Hydrolysis of samples was by refluxing with 20 ml of 0.25 N HCl for 3 hr at 100° [25]. The hydrolysed samples at 22° were brought to pH 7 with 2.0 N KOH/0.5 M ammonium oxalate and stirred for 10 min. The samples were centrifuged at 4° and the supernatant solutions were filtered. The precipitates were resuspended in water, the suspensions filtered, and the appropriate filtrates combined and analyzed for uronic acid. The results were normalized on a per gram dry wt of cell walls basis and then converted to percent (Fig. 1).

Analysis of the combined filtrates from the preparation of cell walls for sugars. The volumes of the two combined filtrates obtained in the above procedure for the prepn of cell walls were measured. A portion of each was centrifuged and the supernatant solutions were filtered through separate 5.0 μm MF-Millipore filters. The two samples were dialysed in water for 26 hr, their volumes were measured and they were assayed for uronic acid and total sugar.

Acknowledgements—We thank Drs Joanne Whallon and John W. Heckman of the MSU Laser Scanning Microscope Laboratory and the Center for Electron Optics, respectively, for microscope work.

REFERENCES

1. Carpita, N. C., and Gibeaut, D. M. (1993) *Plant* **3**, 1.
2. Jarvis, M. C. (1982) *Planta* **154**, 344.
3. Stevens, B. J. H. and Selvendran, R. R. (1980) *J. Sci. Food Agric.* **31**, 1257.
4. Ring, S. G. and Selvendran, R. R. (1981) *Phytochem.* **20**, 2511.
5. Stevens, B. J. H. and Selvendran, R. R. (1984) *Carbohydr. Res.* **128**, 321.
6. Stevens, B. J. H. and Selvendran, R. R. (1984) *Carbohydr. Res.* **135**, 155.
7. Redgwell, R. J. and Selvendran, R. R. (1986) *Carbohydr. Res.* **157**, 183.
8. Ryden, P. and Selvendran, R. R. (1990) *Biochem. J.* **269**, 393.
9. Souty, M., Thibault, J.-F., Navarro-Garcia, G., Lopez-Roca, J.-M. and Breuils, L. (1981) *Sci. des Alim.* **1**, 67.
10. De Vries, J. A., Voragen, A. G. J., Rambouts, F. M. and Pilnik, W. (1981) *Carbohydr. Polymers* **1**, 117.
11. Aspinall, G. O. and Fanous, H. K. (1984) *Carbohydr. Polymers* **4**, 193.
12. Sauliner, L. and Thibault, J.-F. (1987) *Carbohydr. Polymers* **7**, 329.
13. Marcelin, O., Sauliner, L. and Brillouet, J.-M. (1991) *Carbohydr. Res.* **212**, 159.
14. Ryden, P. and Selvendran, R. R. (1990) *Carbohydr. Res.* **195**, 257.
15. Jarvis, M. C., Hall, M. A., Threlfall, D. R. and Friend, J. (1981) *Planta* **152**, 93.
16. Selvendran, R. R. (1975) *Phytochem.* **14**, 1011.
17. Selvendran, R. R. and DuPont, M. S. (1980) *J. Sci. Food Agric.* **31**, 1173.
18. Selvendran, R. R. and O'Neill, M. A. (1987) in *Methods of Biochemical Analysis* (Glick, D., ed.), Vol. 32, p. 25, John Wiley and Sons, New York.
19. Harris, P. J. (1983) in *Isolation of Membranes and Organelles from Plant Cells* (Hall, J. L. and Moore, A. L., eds.), p. 25, *Academic Press*, New York.
20. Mort, A. J., Moerschbacher, B. M., Pierce, M. L. and Maness, N. O. (1991) *Carbohydr. Res.* **215**, 217.
21. Kindel, P. K. and Watson, R. R. (1973) *Biochem. J.* **133**, 227.

22. Blumenkrantz, N. and Asboe-Hansen, G. (1973) *Anal. Biochem.* **54**, 484.
23. DuBois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. (1956) *Anal. Chem.* **28**, 350.
24. Kushida, H. (1980) *J. Electron Microsc.* **29**, 193.
25. Jarvis, M. C., Forsyth, W. and Duncan, H. J. (1988) *Plant Physiol.* **88**, 309.

APPENDIX B

REEXAMINATION OF THE ACETYLATION OF APIITOL IN THE DETERMINATION OF APIOSE

(Reprinted from *Carbohydrate Research*)

ELSEVIER

Carbohydrate Research 290 (1996) 67–70

CARBOHYDRATE RESEARCH

Note

Reexamination of the acetylation of apiitol in the determination of apiose

Liang Cheng, Paul K. Kindel *

Department of Biochemistry, Michigan State University, East Lansing, MI 48824-1319, USA

Received 22 January 1996; accepted in revised form 27 April 1996

Keywords: Apiose; Apiitol; Xylose; GLC

Apiitol, the sugar alcohol of apiose [3-*C*-(hydroxymethyl)-D-*glycero*-aldotetrose], is considerably more difficult to acetylate completely than sugar alcohols with only primary and secondary hydroxyl groups. We attempted to acetylate apiitol completely by modifying the conditions used by Blakeney et al. [1] for other sugars. When acetylation was performed at 35 °C for 1–20 h, a side-product, 1,2,4-tri-*O*-acetyl-3-*C*-(acetoxymethyl)-3-*O*-(methylthiomethyl)-D-*glycero*-tetritol [herein called 3-*O*-(methylthiomethyl)apiitol tetraacetate], was formed in substantial amounts [2]. Replacing dimethylsulfoxide (Me_2SO) with dimethylformamide avoided the formation of the side-product [2]. Harris et al. [3] reported that the methylthiomethyl ether side-product was not observed when the procedure of Blakeney et al. [1] was used with Me_2SO and the acetylation of apiitol was conducted at 40 and 80 °C for 90 min. In an attempt to reconcile the difference in results we used the conditions of Harris et al. [3] to acetylate apiitol. The methylthiomethyl ether side-product was again observed. At 40 and 80 °C the ether side-product was 30 and 38%, respectively, of the total peak area of the three compounds derived from apiose. The results at 40 °C were similar to those obtained previously at 35 °C [2]. Small amounts of the methylthiomethyl ether (0.2–1.8%) were detected even when acetylation was performed at 22 °C for 10 min. The amounts were dependent on how quickly the sample was cooled back to 22 °C after acetic anhydride was added. These results were obtained with the apiose used in the previous investigation [2] and with apiose purchased as the diisopropylidene derivative. Subjecting apiose to hydrolysis conditions [2 M trifluoroacetic acid (TFA), 120 °C, 1 h] before acetylation

* Corresponding author.

PII S0008-6215(96)00128-0

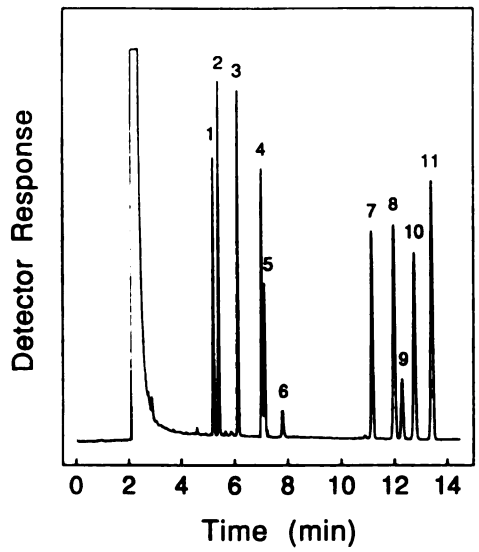


Fig. 1. Gas–liquid chromatogram of alditol peracetates and related compounds. The sample was prepared by the procedure of Blakeney et al. [1] except that the acetylation was performed at 40 °C for 90 min, and the extracted sample in dichloromethane was washed with 5 mL of water three times [11,12]. The sample was analyzed with a DB-225 capillary column operated isothermally at 230 °C. Peaks represent the peracetates of the following alditols, except as noted: 1, rhamnitol; 2, fucitol; 3, arabinitol; 4, xylitol; 5, apiitol; 6, apiitol tetraacetate; 7, mannitol; 8, galactitol; 9, 3-*O*-(methylthiomethyl)apiitol tetraacetate; 10, glucitol; 11, *myo*-inositol hexaacetate.

had no influence on the formation of the three products. The above results are consistent with our earlier results [2] but are contradictory to those of Harris et al. [3].

Harris et al. [3] stated that "the acetylation of sulphoxides in the Pummerer reaction is well documented, but side products are not formed when the reaction is conducted below 80°." Results in the literature do not support this as a general statement. The Pummerer reaction involving Me_2SO and acetic anhydride was shown to occur at 25 °C with formation of the expected sulfide [4]. The formation of the methylthiomethyl ether of a variety of alcohols, including sugars, from Me_2SO and acetic anhydride was also shown to occur at room temperature [5–9]. The rate of reaction was sufficient to detect the methylthiomethyl ether by GLC after 10 min. These results support our observation that the methylthiomethyl ether side-product was formed when more rigorous acetylation conditions were used in the procedure of Blakeney et al. [1].

Apiose can be determined quantitatively by measuring the peak area of apiitol pentaacetate after GLC [2]. Separation of apiitol and xylitol pentaacetates by GLC is difficult and a long sample separation time was required [2,3]. The method has been improved by reducing the separation time to less than 15 min while maintaining almost complete separation of apiitol and xylitol pentaacetates as well as complete separation of the other acetylation products (Fig. 1). For most samples, a DB-225 capillary column operated isothermally at 230 °C will give satisfactory results (Fig. 1). When a new DB-225 capillary column was used at 230 °C, separation of apiitol and xylitol pentaacetates was virtually complete (less than 1% of the total area of the two peaks

overlapped). The DB-225 column lost efficiency for separating the two pentaacetates very slowly. After hundreds of hours of use over 3.5 years, approximately 4% of the total area of the apiitol and xylitol pentaacetate peaks overlapped under the above chromatography conditions; however, there was substantial tailing of apiitol tetraacetate. The high column temperature was needed to separate the apiitol and xylitol pentaacetates. For samples where complete separation of apiitol and xylitol pentaacetates is necessary, both the DB-225 column operated at a lower temperature and a SP-2380 column gave baseline separation of these two pentaacetates as well as the other acetylation products stated in the legend of Fig. 1. The DB-225 column was operated isothermally at 170 °C for 55 min and then the temperature was increased at 1.5 °C/min to 220 °C and held. Under these conditions the order of elution of apiitol and xylitol pentaacetates was the reverse of that obtained when the column was operated isothermally at 230 °C (Fig. 1). The order of elution of the other products remained as shown in Fig. 1. The column chromatography time needed for a single sample was approximately 110 min. Baseline separation of the eight alditol peracetates plus *myo*-inositol hexaacetate was also achieved with a SP-2380 capillary column at 260 °C in 12 min. However, the SP-2380 column lost efficiency relatively rapidly (detectable after approximately 100 h of use) when operated at this temperature.

Harris et al. [3] proposed a method for quantitatively determining apiose based on the peak area of apiitol tetraacetate after GLC. Their method is more involved than the one based on apiitol pentaacetate and small amounts of the ether side-product may be formed.

1. Experimental

Sample preparation.—Apiose was isolated from parsley apiin [10]. 1,2:3,5-Di-*O*-isopropylidene-α-D-apiose was purchased from Pfanstiehl. Me_2SO, acetic anhydride, 1-methylimidazole, TFA and sodium borohydride from Sigma or Aldrich, and acetic acid and ammonium hydroxide were purchased new. Apiose (0.76 mg) and 1,2:3,5-di-*O*-isopropylidene-α-D-apiose (0.81 mg) were hydrolyzed with 2 M TFA at 120 °C for 1 h and the acid was removed with N_2 at 22 °C. Apiose (0.76 mg), hydrolyzed apiose, and hydrolyzed 1,2:3,5-di-*O*-isopropylidene-α-D-apiose were reduced separately and the alditols were acetylated by the method of Blakeney et al. [1], except that the acetylation was conducted at 22, 40, and 80 °C for 10 and 90 min. For samples acetylated at 22 °C, the vials were quickly cooled back to 22 °C by an air stream after acetic anhydride was added. A standard sample containing 0.11–0.12 mg of each of rhamnose, fucose, arabinose, xylose, mannose, galactose, glucose, and *myo*-inositol and 0.46 mg of 1,2:3,5-di-*O*-isopropylidene-α-D-apiose was hydrolyzed with 2 M TFA at 120 °C for 1 h and the acid was removed as described above. The sugars and *myo*-inositol in the hydrolyzed standard were converted to products with the method of Blakeney et al. [1] except the acetylation was conducted at 40 °C for 90 min, and the extracted sample in dichloromethane was washed with 5 mL of water three times [11,12].

Gas chromatography.—Compounds were chromatographed on a DB-225 column [30 m × 0.25 mm (i.d.), 0.15 μm film thickness; J.&W.] attached to a Hewlett–Packard gas

chromatograph, Model 5840A, equipped with a splitter, a flame-ionization detector, and a 5840A data terminal. Helium was used as the carrier gas. When chromatography with the DB-225 column was performed isothermally at 230 °C, the sample size ranged from 1.2 to 4 μL, the carrier gas flow rate was 1.4 mL/min, and the split ratio was 1:5 (for apiose samples acetylated for 10 min at 22 °C) or 1:15 (other samples). For chromatography starting at 170 °C, the DB-225 column was kept at 170 °C for 55 min and then the temperature was increased at 1.5 °C/min to 220 °C and held. The sample size was 0.4 μL, the carrier gas flow rate was 1.7 mL/min, and the split ratio was 1:15. Compounds were chromatographed on a SP-2380 column [30 m × 0.25 mm (i.d.), 0.2 μm film thickness; Supelco] attached to the same system. Chromatography was performed isothermally at 260 °C. Helium was used as the carrier gas at a flow rate of 1.5 mL/min. Apiitol tetraacetate, apiitol pentaacetate and 3-*O*-(methylthiomethyl)apiitol tetraacetate were identified by their retention times from GLC and by their mass spectra [2]. Mass spectrometry was performed as described previously [2].

References

[1] A.B. Blakeney, P.J. Harris, R.I. Henry, and B.A. Stone, *Carbohydr. Res.*, 113 (1983) 291–299.
[2] P.K. Kindel and L. Cheng, *Carbohydr. Res.*, 199 (1990) 55–65.
[3] P.J. Harris, M. Sadek, R.T.C. Brownlee, A.B. Blakeney, J. Webster, and B.A. Stone, *Carbohydr. Res.*, 227 (1992) 365–370.
[4] S. Oae, T. Kitao, S. Kawamura, and Y. Kitaoka, *Tetrahedron*, 19 (1963) 817–820.
[5] K. James, A.R. Tatchell, and (in part) P.K. Ray, *J. Chem. Soc.*, C, (1967) 2681–2686.
[6] P.M. Pojer and S.J. Angyal, *Aust. J. Chem.*, 31 (1978) 1031–1040.
[7] J.D. Albright and L. Goldman, *J. Am. Chem. Soc.*, 87 (1965) 4214–4216; 89 (1967) 2416–2423.
[8] K. Antonakis and F. Leclercq, *Bull. Soc. Chim. Fr.*, (1971) 4309–4310.
[9] K. Yamada, K. Kato, H. Nagase, and Y. Hirata, *Tetrahedron Lett.*, (1976) 65–66.
[10] D.L. Neal and P.K. Kindel, *J. Bacteriol.*, 101 (1970) 910–915.
[11] C. Hoebler, J.L. Barry, A. David, and J. Delort-Laval, *J. Agric. Food Chem.*, 37 (1989) 360–367.
[12] L. Cheng, M.S. thesis, Department of Biochemistry, Michigan State University, East Lansing, Michigan, 1991.

APPENDIX C

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

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

I. BASIC EQUATION

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

$A = b + m \cdot c$ or $c = (A-b)/m$ (1)

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

II. CONCENTRATION OF NEUTRAL SUGAR IN A SAMPLE MIXTURE WITH NEUTRAL SUGAR AND GALACTURONIC ACID

For a sample mainly conatining GalA, Gal and Api, the concentration of Api can be calculated from absorbance in total sugar test:

$$c_{Api} = (c_{GalA} + c_n) \cdot x = \frac{A_{490Api} - b_1}{m_1} \cdot D_1 \quad (2)$$

where c_{Api} = the concentration of Api (mg/mL)
c_{GalA} = the concentration of GalA (mg/mL)
c_n = the concentration of neutral sugars (Api and Gal)
x = percentage (w/w) of Api in the sample
A_{490Api} = absorbance of Api in sample in the total sugar test
m_1 and b_1 = slope and intercept of the standard curve of Api concentration vs. absorbance for the total sugar test
D_1 = fold of dilution of the sample for the total sugar test

Rearrange (2), the absorbance of Api in the total sugar test is:

$$A_{490Api} = \frac{(c_{GalA} + c_n) \cdot x \cdot m_1}{D_1} + b_1 \quad (3)$$

The concentration of Gal can be calculated by:

$$c_{Gal}=c_n-c_{Api}=c_n-(c_{GalA}+c_n)\cdot x=\frac{A_{490Gal}-b_2}{m_2}\cdot D_1 \tag{4}$$

where c_{Gal} = the concentration of Gal (mg/mL)
A_{490Gal} = absorbance of Gal in sample in the total sugar test
m_2 and b_2 = slope and intercept of the standard curve of Gal concentration vs. absorbance for the total sugar test

Rearrange (4), the absorbance of Gal in the total sugar test is:

$$A_{490Gal}=\frac{[c_n-(c_{GalA}+c_n)\cdot x]\cdot m_2}{D_1}+b_2 \tag{5}$$

The concentration of GalA can be calculated by:

$$c_{GalA}=\frac{A_{490GalA}-b_3}{m_3}\cdot D_1 \tag{6}$$

where $A_{490GalA}$ = absorbance of GalA in sample in the total sugar test
m_3 and b_3 = slope and intercept of the standard curve of GalA concentration vs. absorbance for the total sugar test

Rearrange (6), the absorbance of GalA in the total sugar test is:

$$A_{490GalA}=b_3+m_3\cdot\frac{c_{GalA}}{D_1} \tag{7}$$

The total absorbance of the sample in total sugar test, A_{490t}, can be expressed by combination of equation (3), (5) and (7):

$$A_{490t}=A_{490Api}+A_{490Gal}+A_{490GalA}$$

$$=\frac{(c_{GalA}+c_n)\cdot x\cdot m_1}{D_1}+b_1+\frac{[c_n-(c_{GalA}+c_n)\cdot x]\cdot m_2}{D_1}+b_2+b_3+m_3\cdot\frac{c_{GalA}}{D_1} \tag{8}$$

Rearrange (8), the concentration of neutral sugar in the sample is:

$$c_n = \frac{(A_{490t} - b_1 - b_2 - b_3)\cdot D_1 - (m_3 + x\cdot m_1 - x\cdot m_2)\cdot c_{GalA}}{(m_2 + x\cdot m_1 - x\cdot m_2)} \tag{9}$$

III. CONCENTRATION OF GALACTURONIOC ACID IN A SAMPLE MIXTURE WITH NEUTRAL SUGAR AND GALACTURONIC ACID

Similar to equation (2), in a sample mainly conatining GalA, Gal and Api, the concentration of Api can also be calculated from absorbance in uronic acid test:

$$c_{Api} = (c_{GalA} + c_n)\cdot x = \frac{A_{520Api} - b_4}{m_4}\cdot D_2 \tag{10}$$

where A_{520Api} = absorbance of Api in sample in the uronic acid test
m_4 and b_4 = slope and intercept of the standard curve of Api concentration vs. absorbance for the uronic acid test
D_2 = fold of dilution of the sample for the uronic acid test

Rearrange (10), the absorbance of Api in the uronic acid test is:

$$A_{520Api} = \frac{(c_{GalA} + c_n)\cdot x\cdot m_4}{D_2} + b_4 \tag{11}$$

The concentration of Gal can be calculated by:

$$c_{Gal} = c_n - c_{Api} = c_n - (c_{GalA} + c_n)\cdot x = \frac{A_{520Gal} - b_5}{m_5}\cdot D_2 \tag{12}$$

where A_{520Gal} = absorbance of Gal in sample in the uronic acid test
m_5 and b_5 = slope and intercept of the standard curve of Gal concentration vs. absorbance for the uronic acid test

Rearrange (12), the absorbance of Gal in the uronic acid test is:

$$A_{520Gal}=\frac{[c_n-(c_{GalA}+c_n)\cdot x]\cdot m_5}{D_2}+b_5 \tag{13}$$

The concentration of GalA can be calculated by:

$$c_{GalA}=\frac{A_{520GalA-b_6}}{m_6}\cdot D_2 \tag{14}$$

where $A_{520GalA}$ = absorbance of GalA in sample in the uronic acid test
m_6 and b_6 = slope and intercept of the standard curve of GalA concentration vs. absorbance for the uronic acid test

Rearrange (14), the absorbance of GalA in the uronic acid test is:

$$A_{520GalA}=b_6+m_6\cdot\frac{c_{GalA}}{D_2} \tag{15}$$

The total absorbance of the sample in uronic acid test, A_{520t} , can be expressed by combination of equation (11), (13) and (15):

$$A_{520t}=A_{520Api}+A_{520Gal}+A_{520GalA}$$

$$=\frac{(c_{GalA}+c_n)\cdot x\cdot m_4}{D_2}+b_4+\frac{[c_n-(c_{GalA}+c_n)\cdot x]\cdot m_5}{D_2}+b_5+b_6+m_6\cdot\frac{c_{GalA}}{D_2} \tag{16}$$

Rearrange (16), the concentration of neutral sugar in the sample is:

$$c_n=\frac{(A_{520t}-b_4-b_5-b_6)\cdot D_2-(m_6+x\cdot m_4-x\cdot m_5)\cdot c_{GalA}}{(m_5+x\cdot m_4-x\cdot m_5)} \tag{17}$$

Compare eqution (9) and (17), the left side of the equations are equal:

$$\frac{(A_{490t}-b_1-b_2-b_3)\cdot D_1-(m_3+x\cdot m_1-x\cdot m_2)\cdot c_{GalA}}{(m_2+x\cdot m_1-x\cdot m_2)}=\frac{(A_{520t}-b_4-b_5-b_6)\cdot D_2-(m_6+x\cdot m_4-x\cdot m_5)\cdot c_{GalA}}{(m_5+x\cdot m_4-x\cdot m_5)}$$

(18)

Rearrange equation (18), the concentration of GalA is:

$$c_{GalA}=\frac{(m_5+x\cdot m_4-x\cdot m_5)\cdot(A_{490t}-b_1-b_2-b_3)\cdot D_1-(m_2+x\cdot m_1-x\cdot m_2)\cdot(A_{520t}-b_4-b_5-b_6)\cdot D_2}{(m_5+x\cdot m_4-x\cdot m_5)\cdot(m_3+x\cdot m_1-x\cdot m_2)-(m_2+x\cdot m_1-x\cdot m_2)\cdot(m_6+x\cdot m_4-x\cdot m_5)}$$

(19)

Let $A = (m_5 + x \cdot m_4 - x \cdot m_5)$
$B = (m_2 + x \cdot m_1 - x \cdot m_2)$
$C = (m_3 + x \cdot m_1 - x \cdot m_2)$
$D = (m_6 + x \cdot m_4 - x \cdot m_5)$

then equation (19) is:

$$c_{GalA}=\frac{A\cdot(A_{490t}-b_1-b_2-b_3)\cdot D_1-B\cdot(A_{520t}-b_4-b_5-b_6)\cdot D_2}{A\cdot C-B\cdot D} \quad (20)$$

and equation (9) is:

$$c_n=\frac{(A_{490t}-b_1-b_2-b_3)\cdot D_1-C\cdot c_{GalA}}{B} \quad (21)$$

Assume all intercepts = 0, equations (20) is:

$$c_{GalA}=\frac{A\cdot A_{490t}\cdot D_1-B\cdot A_{520t}\cdot D_2}{A\cdot C-B\cdot D} \quad (22)$$

and equation (21) is:

$$c_n=\frac{A_{490t}\cdot D_1-C\cdot c_{GalA}}{B} \quad (23)$$

Equation (22) and (23) were used to calculate GalA and neutral sugars in the sample.

APPENDIX D

A COMPUTER PROGRAM FOR CALCULATION OF THE AMOUNT OF NEUTRAL SUGAR AND URONIC ACID IN SAMPLES

(By Linag Cheng, 1993)

This program was written with Q-BASIC computer language based on the equations of APPENDIX C. A IBM compatible PC with a Q-BASIC software is required to run this program.

A COMPUTER PROGRAM FOR CALCULATION OF THE AMOUNT OF NEUTRAL SUGAR AND URONIC ACID IN SAMPLES

(Liang Cheng)

```
  OPEN "c:\BAS.DATA" FOR OUTPUT AS #1
  PRINT "**********RATIO OF REDUCTION OF URONIC ACID AND SAMPLE RECOVERY**********"
  PRINT
  CO = 1
  PY = 1.027161985#
  TT = .94391393#
  AC = 1.069033889#
  PE = 1.056089744#
30 INPUT "Apiose % ="; p
  X = (p / 100) * 132.11 / ((1 - p / 100) * 176.12 + (p / 100) * 132.11)
  PRINT
  B3 = 8.967299999999999D-03
  M3 = 6.3007503#
  B6 = .00008333#
  M6 = 9.80217172#
  B1 = -.00038863#
  M1 = 3.06564281#
  B2 = .013801#
  M2 = 12.5923371#
  B4 = 0
  M4 = .02922502#
  B5 = 0
  M5 = .25899977#
  INPUT "Please indicate what type of cuvette you used: (COLEMAN = co, PE = pe, Test tube = tt, A.COLEMAN = ac, Pyrex = py)"; Z$
  PRINT
  IF Z$ = "co" THEN GOTO 180
  IF Z$ = "pe" THEN GOTO 150
  IF Z$ = "tt" THEN GOTO 175
  IF Z$ = "ac" THEN GOTO 160
  IF Z$ = "py" THEN GOTO 170
  GOTO 175
150 B6 = B6 / PE
   M6 = M6 / PE
```

```
    B4 = B4 / PE
    M4 = M4 / PE
    B5 = B5 / PE
    M5 = M5 / PE
    GOTO 180
160 B6 = B6 / AC
    M6 = M6 / AC
    B4 = B4 / AC
    M4 = M4 / AC
    B5 = B5 / AC
    M5 = M5 / AC
    GOTO 180
170 B6 = B6 / PY
    M6 = M6 / PY
    B4 = B4 / PY
    M4 = M4 / PY
    B5 = B5 / PY
    M5 = M5 / PY
    GOTO 180
175 B6 = B6 / TT
    M6 = M6 / TT
    B4 = B4 / TT
    M4 = M4 / TT
    B5 = B5 / TT
    M5 = M5 / TT
180 PRINT "Input data for original solution (before reduction)"
    PRINT
    INPUT "A 490 nm ="; A490
    INPUT "Dilution ratio for the total sugar test (1 = no dilution)"; D1
    INPUT "A 520 nm ="; A520
    INPUT "Dilution ratio for uronic acid test (1 = no dilution)"; D2
240 INPUT "Total volume of the sample before reduction (mL)"; V1
    INPUT "Volume of the sample used for colorimetric tests (mL)"; V2
    INPUT "Do you want to force intercepts to be zero"; A$
    IF A$ = "y" THEN GOTO 290
    GOTO 370
290 B3 = 0
    B6 = 0
    B1 = 0
    B2 = 0
```

```
   B4 = 0
   B5 = 0
370 A = (A520 - B6 - B4 - B5) * D2
   B = (M6 + X * M4 - X * M5)
   C = (M5 + X * M4 - X * M5)
   D = (A490 - B3 - B1 - B2) * D1
   E = (M3 + X * M1 - X * M2)
   F = (M2 + X * M1 - X * M2)
   CU = (C * D - A * F) / (C * E - B * F)
   CN = (A - CU * B) / C
   WU = CU * V1
   WN = CN * V1
   PU = (WU / 176.12) / (WU / 176.12 + (WN + WU) * X / 132.11 + (WN - (WN + WU) * X) / 162.14) * 100
   PN = 100 - PU
   WUL = CU * (V1 - V2) * 194.14 / 176.12
   WNA = (CN + CU) * (V1 - V2) * X * 150.13 / 132.11
   WNG = (CN - (CN + CU) * X) * (V1 - V2) * 180.16 / 162.14
   WNL = WNA + WNG
   MONO = WUL + WNL
   PRINT
   PRINT "********** For original sample before reduction **********"
   PRINT
   PRINT
   PRINT TAB(30); "A 490 nm"; TAB(50); "A 520 nm"
   PRINT "Absorbance"; TAB(30); A490; TAB(50); A520
   PRINT "Dilution Ratio"; TAB(30); D1; TAB(50); D2
   PRINT
   PRINT "Total volume of original sample:", V1; "mL"
   PRINT "Sample used for colorimetric tests:", V2; "mL"
   PRINT "Apiose % ="; p
   PRINT
   PRINT TAB(30); "Neutral Sugar"; TAB(50); "Uronic Acid"
   PRINT "Concentration (mg/mL)"; TAB(30); CN; TAB(50); CU
   PRINT "Anhydro sugar (mg)"; TAB(30); WN; TAB(50); WU
   PRINT "Percentage"; TAB(30); PN; TAB(50); PU
   PRINT "Sample left for reduct.(mg)"; TAB(30); WNL; TAB(50); WUL; "Total ="; MONO
   PRINT
   INPUT "Have you tested the reduced material colorimetrically"; B$
   IF B$ = "y" THEN GOTO 750
   PRINT
```

```
   INPUT "Do you want another calculation"; C$
   IF C$ = "n" THEN GOTO 1480
   IF C$ = "y" THEN GOTO 30
750 PRINT "Input colorimetric test data for reduced material"
   PRINT
   INPUT "A 490 nm ="; RA490
   INPUT "Dilution ratio for the total sugar test (1 = no dilution)"; RD1
   INPUT "A 520 nm ="; RA520
   INPUT "Dilution ratio for uronic acid test (1 = no dilution)"; RD2
   INPUT "Total volume of the sample after reduction (mL)"; RV1
   RA = (RA520 - B6 - B4 - B5) * RD2
   RD = (RA490 - B3 - B1 - B2) * RD1
   RCU = (C * RD - RA * F) / (C * E - B * F)
   RCN = (RA - RCU * B) / C
   RWU = RCU * RV1
   RWN = RCN * RV1
   RPU = (RWU / 176.12) / (RWU / 176.12 + (RWN + RWU) * X / 132.11 + (RWN - (RWN + RWU) * X) / 162.14) * 100
   RPN = 100 - RPU
   RCV = (RWU / 176.12 + (RWN - (RWU + RWN) * X) / 162.14 + (RWU + RWN) * X / 132.11) / (WU / 176.12 + (WN - (WU + WN) * X) / 162.14 + (WU + WN) * X / 132.11) * 100
   TW = RWU * 194.14 / 176.12 + (RWN + RWU) * X * 150.13 / 132.11 + (RWN - (RWN + RWU) * X) * 180.16 / 162.14
   PRINT
   PRINT "********** Test results for sample after reduction **********"
   PRINT
   PRINT
   PRINT TAB(30); "A 490 nm"; TAB(50); "A 520 nm"
   PRINT
   PRINT "Absorbance"; TAB(30); RA490; TAB(50); RA520
   PRINT "Dilution Ratio"; TAB(30); RD1; TAB(50); RD2
   PRINT
    PRINT TAB(30); "Neutral Sugar"; TAB(50); "Uronic Acid"
    PRINT
    PRINT "Concentration (mg/mL)"; TAB(30); RCN; TAB(50); RCU
    PRINT "Anhydro sugar (mg)"; TAB(30); RWN; TAB(50); RWU
    PRINT "Percentage"; TAB(30); RPN; TAB(50); RPU
    PRINT
    PRINT "Total recovery of sample", RCV; "%"
    PRINT "Total weight of sample after reduction", TW; "mg"
    INPUT "Do you want to print the results of the calculation"; D$
    IF D$ = "y" THEN 1090
    GOTO 1480
1090 PRINT #1,
```

```
  PRINT #1, "QUANTITY OF URONIC ACID AND TOTAL SUGAR CALCULATED FROM LIANG CHENG'S EQUITION"
  PRINT #1,
  PRINT #1, TAB(22); "Parameters of Standard Curve Used"
  PRINT #1,
  PRINT #1, TAB(5); "Intercept"; TAB(21); "Slope(a.u./1 mg/mL)"; TAB(42); "Sugar"; TAB(50); "Type of test"; TAB(66); "Date"
  PRINT #1,
  PRINT #1, USING "& & & ##.########"; ""; ""; "B3 ="; B3; TAB(21); ""; ""; "M3 ="; M3; TAB(42); "GalA"; TAB(50); "total sugar"; TAB(64); "5/17+19/95"
  PRINT #1, USING "& & & ##.########"; ""; ""; "B6 ="; B6; TAB(21); ""; ""; "M6 ="; M6; TAB(42); "GalA"; TAB(50); "m-OHdiphenyl"; TAB(64); "6/1/95"
  PRINT #1, USING "& & & ##.########"; ""; ""; "B1 ="; B1; TAB(21); ""; ""; "M1 ="; M1; TAB(42); "Apiose"; TAB(50); "total sugar"; TAB(64); "8/20/93"
  PRINT #1, USING "& & & ##.########"; ""; ""; "B2 ="; B2; TAB(21); ""; ""; "M2 ="; M2; TAB(42); "Gal"; TAB(50); "total sugar"; TAB(64); "5/17+19/95"
  PRINT #1, USING "& & & ##.########"; ""; ""; "B4 ="; B4; TAB(21); ""; ""; "M4 ="; M4; TAB(42); "Apiose"; TAB(50); "m-OHdiphenyl"; TAB(64); "8/19/93"
  PRINT #1, USING "& & & ##.########"; ""; ""; "B5 ="; B5; TAB(21); ""; ""; "M5 ="; M5; TAB(42); "Gal"; TAB(50); "m-OHdiphenyl"; TAB(64); "5/22/95"
  PRINT #1,
  PRINT #1, TAB(10); "B6, M6, B4, M4, B5, and M5 were intercetps and slopes for"
  PRINT #1,
 IF Z$ = "co" THEN GOTO 1300
 IF Z$ = "pe" THEN GOTO 1310
 IF Z$ = "tt" THEN GOTO 1335
 IF Z$ = "ac" THEN GOTO 1320
 IF Z$ = "py" THEN GOTO 1330
  GOTO 1335
1300 PRINT #1, TAB(10); "COLEMAN cuvettes"
  GOTO 1340
1310 PRINT #1, USING "& ##.########"; TAB(10); "P.E. cuvettes, according to ratio: CO/PE ="; PE
  GOTO 1340
1320 PRINT #1, USING "& ##.########"; TAB(10); "A. COLEMAN cuvettes, according to ratio: CO/AC ="; AC
  GOTO 1340
1330 PRINT #1, USING "& ##.########"; TAB(10); "Pyrex test tube, according to ratio: CO/PY ="; PY
  GOTO 1340
1335 PRINT #1, USING "& ##.########"; TAB(10); "Non-pryrex test tube, according to ratio: CO/TT ="; TT
1340 PRINT #1,
  PRINT #1,
  PRINT #1, "*************** For original sample before reduction ***************"
  PRINT #1,
  PRINT #1, TAB(30); "A 490 nm"; TAB(50); "A 520 nm"
  PRINT
  PRINT #1, USING "& #.###### #.######"; "Absorbance"; TAB(30); A490; TAB(50); A520
  PRINT #1, "Dilution Ratio"; TAB(30); D1; TAB(50); D2
  PRINT #1,
  PRINT #1, "Total volume of original sample:", V1; "mL"
```

```
   PRINT #1, "Sample used for colorimetric tests:", V2; "mL"
   PRINT #1, "Apiose % ="; p
   PRINT #1,
   PRINT #1, TAB(30); "Neutral Sugar"; TAB(50); "Uronic Acid"
   PRINT #1,
   PRINT #1, USING "& ##.######### ##.#########"; "Concentration (mg/mL)"; TAB(30); CN; TAB(50); CU
   PRINT #1, USING "& ##.######### ##.#########"; "Anhydro sugar (mg)"; TAB(30); WN; TAB(50); WU
   PRINT #1, USING "& ####.### ####.###"; "Percentage"; TAB(30); PN; TAB(50); PU
   PRINT #1,
   PRINT #1, USING "& ##.######### ##.#########"; "Sample left for reduct. (mg)"; TAB(30); WNL; TAB(50); WUL
   PRINT #1,
   PRINT #1,
   PRINT #1, "*************** Results for sample after reduction ***************"
   PRINT #1,
   PRINT #1, TAB(30); "A 490 nm"; TAB(50); "A 520 nm"
   PRINT #1,
   PRINT #1, USING "& #.###### #.######"; "Absorbance"; TAB(30); RA490; TAB(50); RA520
   PRINT #1, "Dilution Ratio"; TAB(30); RD1; TAB(50); RD2
   PRINT #1,
   PRINT #1, "Volume of the sample after reduction:", RV1; "mL"
   PRINT #1,
   PRINT #1, TAB(30); "Neutral Sugar"; TAB(50); "Uronic Acid"
   PRINT #1,
   PRINT #1, USING "& ##.######### ##.#########"; "Concentration (mg/mL)"; TAB(30); RCN; TAB(50); RCU
   PRINT #1, USING "& ##.######### ##.#########"; "Anhydro sugar (mg)"; TAB(30); RWN; TAB(50); RWU
   PRINT #1, USING "& ####.### ####.###"; "Percentage"; TAB(30); RPN; TAB(50); RPU
   PRINT #1,
   PRINT #1, USING "& ####.### &"; "Total recovery of sample"; RCV; "%"
   PRINT #1,
   PRINT #1, USING "& ##.######## &"; "Total sample weight after reduction"; TW; "mg"
   PRINT #1,
   PRINT #1,
   PRINT #1, CHR$(12)
   INPUT "Another calculation ?"; X$
   IF X$ = "n" THEN GOTO 1480
   GOTO 240
1480 END
```

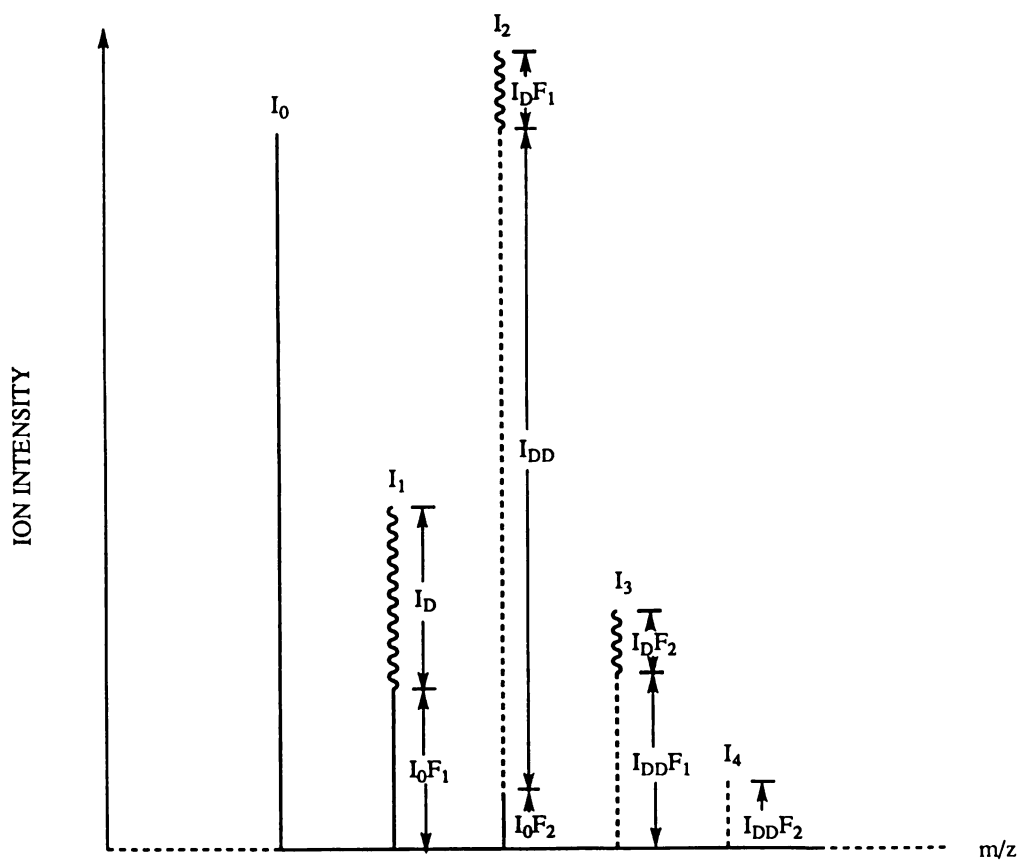
APPENDIX E

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

(By Liang Cheng, 1993)

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

The ratio of GalA/Gal in a pectic polysaccharide is determined from mass spectra of galactitol acetate-D_2/galactitol acetate after the carboxyl groups of GalA residues were reduce with $NaBD_4$ and the sample was depolymerized and acetylated. Ions with 5 different mass should be found in the typical isotope cluster of a nominal fragment ion in a mass spectrum of galactitol acetate-D_2/galactitol acetate derived as above . The ions in the cluster are shown in the diagram:



where I_0 = the intensity of the nominal mass fragment ion.

I_1 = the intensity of the fragment ion is one mass unit larger than I_0 (the species with only ^1H, ^{12}C, or ^{18}O) which includes the following isotopic species:

I_0F_1 = the additional one mass unit due to the presence of one atom of ^2H, ^{13}C, or ^{17}O in the fragment ion.

I_D = the additional one mass unit due to one deuterium atom introduced in to the molecule by $NaBD_4$.

I_2 = the intensity of the fragment ion is two mass unit larger than I_0 which includes following isotopic species:

I_0F_2 = the additional two mass units due to the presence of two atoms of ^{13}C or one atom of ^{18}O in fragment ion.

I_{DD} = the additional two mass units due to two deuterium atoms introduced in to the molecule by $NaBD_4$.

I_DF_1 = the additional two mass units due to the presence of one atom of ^2H, ^{13}C, or ^{17}O in the fragment ion plus one deuterium atom introduced by $NaBD_4$.

I_3 = the intensity of the fragment ion is three mass units larger than I_0 which includes following isotopic species :

$I_{DD}F_1$ = the additional three mass units due to the presence of one atom of ^2H, ^{13}C, or ^{17}O in the fragment ion plus two deuterium atom introduced by $NaBD_4$.

I_DF_2 = the additional three mass units due to the presence of two atoms of ^{13}C or one atom of ^{18}O in the fragment ion plus one deuterium atom introduced by $NaBD_4$.

I_4 = the intensity of the fragment ion is four mass units larger than I_0 which includes following isotopic species:

$I_{DD}F_2$ = the additional four mass units due to the presence of two atoms of ^{13}C or one atom of ^{18}O in fragment ion plus two deuterium atoms introduced by $NaBD_4$.

Since galactitol acetate-D_2 has a symmetrical structure (not considering the deuterium atoms), then for any type of fragment ion with two introduced deuterium atoms there must

be a corresponding fragment 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: $2 \times (I_{DD} + I_{DD}F_1 + I_{DD}F_2)$. The ratio (R) of galactitol acetate-D_2/(galactitol acetate-D_2 + galactitol acetate) can be calculated as follows:

$$R=\frac{2\cdot(I_{DD}+I_{DD}F_1+I_{DD}F_2)}{(I_0+I_1+I_2+I_3+I_4)}=\frac{2\cdot(I_{DD}+I_{DD}\cdot F_1+I_{DD}\cdot F_2)}{(I_0+I_1+I_2+I_3+I_4)}=\frac{2\cdot I_{DD}\cdot(1+F_1+F_2)}{(I_0+I_1+I_2+I_3+I_4)} \quad (1)$$

where F_1 is the expected abundance ratio of a fragment ion that is one mass unit lager due to the natural occurrence of a ^2H, ^{13}C, or ^{17}O compared to the same ion without the natural isotope.

F_2 is the expected abundance ratio of a fragment ion that is two mass unit lager due to the natural occurrence of two ^{13}C or one ^{18}O compared to the same ion without the natural isotope.

Since $I_1 = I_0\cdot F_1 + I_D$, $I_2 = I_0\cdot F_2 + I_{DD} + I_D\cdot F_1$, $I_3 = I_{DD}\cdot F_2 + I_D\cdot F_2$ and $I_4 = I_{DD}\cdot F_2$, replace I_1, I_2, I_3 and I_4 in equation (1) by their constituent ion intensities:

$$R=\frac{2\cdot I_{DD}\cdot(1+F_1+F_2)}{[I_0+(I_0\cdot F_1+I_D)+(I_0\cdot F_2+I_{DD}+I_D\cdot F_1)+(I_{DD}\cdot F_1+I_D\cdot F_2)+I_{DD}\cdot F_2]} \quad (2)$$

Rearrange (2):

$$R=\frac{2\cdot(1+F_1+F_2)}{\frac{I_0\cdot(1-F_1\cdot F_2)+I_1\cdot(1+F_2)+I_2}{I_{DD}}+F_1+F_2} \quad (3)$$

where I_{DD} can not be measured directly but it can be calculated as follows:

$$I_{DD} = I_2 - I_0\cdot F_2 - I_D\cdot F_1 \quad (4)$$

where I_D can not be measured directly but it can be calculated as follows:

$$I_D = I_1 - I_0\cdot F_1 \quad (5)$$

Express I_D in equation (4) with (5):

$$I_{DD} = I_2 - I_0 \cdot F_2 - (I_1 - I_0 \cdot F_1) \cdot F_1 \quad (6)$$

Express I_{DD} in equation (3) with (6):

$$R = \frac{2 \cdot (1 + F_1 + F_2)}{\frac{I_0 \cdot (1 - F_1 \cdot F_2) + I_1 \cdot (1 + F_2) + I_2}{I_2 - I_0 \cdot F_2 - (I_1 - I_0 \cdot F_1) \cdot F_1} + F_1 + F_2} \quad (7)$$

When $NaBD_4$ (98% D) is used, equation (7) should be adjusted as:

$$R = \frac{2 \cdot (1 + F_1 + F_2)}{\frac{I_0 \cdot (1 - F_1 \cdot F_2) + I_1 \cdot (1 + F_2) + I_2}{I_2 - I_0 \cdot F_2 - (I_1 - I_0 \cdot F_1) \cdot F_1} + F_1 + F_2} \cdot \frac{100}{98} \quad (8)$$

When ion pair 217/219 m/z was chosen to represent galactitol acetate/galactitol acetate-D_2, I_0 = intensity of ion 217. Since Ion 217 contains 13 H, 9 C and 6 O, the abundance ratios, F_1 and F_2 can be calculated as:

Ion	I_0	I_1	I_2
Natural D in 13 H		$0.01 \times 13 = 0.13$	
^{13}C in 9 C		$1.1 \times 9 = 9.9$	$0.006 \times (9)^2 = 0.486$
^{17}O in 6 O		$0.04 \times 6 = 0.24$	
^{18}O in 6 O			$0.2 \times 6 = 1.2$
Relative abundance	100	$0.13 + 9.9 + 0.24 = 10.27$	$0.486 + 1.2 = 1.686$
Abundance ratio (F)	1	$F_1 = 0.1027$	$F_2 = 0.01686$

Similarly, when ion pair 289/291 m/z was chosen to represent galactitol acetate/galactitol acetate-D_2, I_0 = intensity of ion 289. Since Ion 289 contains 17 H, 12 C and 8 O, the

abundance ratios, F_1 and F_2 can be calculated as:

Ion	I_0	I_1	I_2
Natural D in 17 H		$0.01 \times 17 = 0.17$	
^{13}C in 12 C		$1.1 \times 12 = 13.2$	$0.006 \times (12)^2 = 0.864$
^{17}O in 8 O		$0.04 \times 8 = 0.32$	
^{18}O in 8 O			$0.2 \times 8 = 1.6$
Relative abundance	100	$0.17 + 13.2 + 0.32 = 13.69$	$0.864 + 1.6 = 2.464$
Abundance ratio (F)	1	$F_1 = 0.1369$	$F_2 = 0.02464$