ISOLATION AND CHARACTERIZATION OF XYLOGLUCAN AND RHAMNOGALACTURONANS FROM COTTON CELL WALLS OF SUSPENSION CULTURE

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TABLE OF CONTENTS

Chapter Page

LIST OF TABLES

Table Page

Table Page

Table Page

LIST OF FIGURES

Figure Page

Figure Page

Figure Page

Figure Page

Figure Page

Figure Page

LIST OF ABBREVIATIONS

Ara	arabinose
CMC	carboxymethyl cellulose
EG	endoglucanase
EPG	endopolygalacturonase
Fuc	fucose
DOM	degree of methylesterification
Gal	galactose
GalA	galacturonic acid
Glc	glucose
GlcA	glucuronic acid
HG	homogalacturonan
HF	hydrogen fluoride
HRGP(s)	hydroxyproline-rich glycoprotein(s)
Hyp	hydroxyproline
LSIMS	liquid secondary ion mass spectroscopy
Man	mannose
M.W.	molecular weight
PA-XG	2-aminopyridine-derivatized XG
Rha	rhamnose
RGI	rhamnogalacturonan I
RGII	rhamnogalacturonanII
RPC	reverse phase chromatography

TNBS	2,4,6-trinitrobenzene-sulfonic acid
TNP-CMC	2,4,6-trinirophenyl-carboxymethyl-cellulose
Vi	included volume
Vo	void volume
wt%	weight percentage determined by weighing
XG	xyloglucan
Xyl	xylose

CHAPTER I

INTRODUCTION

Virtually every higher plant cell is encased in a cellulosic wall. However, it is difficult to define the plant cell wall precisely from either structural or compositional points of view as it is not a static organelle. During growth, the wall's polymeric constituents vary and hence its chemical properties are in constant flux. In addition, the structure of some of its components has not been well defined. The physical properties of cell walls are also altered as a consequence of cell metabolism (Alberghina *et al.*, 1973, Darvill *et al.*, 1980). Developmentally, the cell walls may be divided into primary wall and secondary wall depending upon their mechanical properties and chemical compositions (Preston, 1974). The primary cell wall is a mechanically dynamic structure encasing the cell and determining the shape and size of the cell during the period of rapid expansion that follows cell division. The secondary cell wall is, relative to the primary cell wall, a mechanically static structure that determines the shape and size of the mature cell.

Basically, cell walls are composed of complex carbohydrates, proteins, lignin, water and minerals. Complex carbohydrates, the main (~80-90%) constituents of cell walls, include cellulose, hemicellulose and pectins. The proteins (~10% of cell wall) are mainly hydroxyproline-rich glycoproteins, and lignin exists mainly in the secondary cell walls.

Configurationally, the cell wall may be viewed as a network of microfibrils embedded in a matrix. The microfibrils are essentially cellulose consisting of chains of glucose molecules, and the matrix is composed of hemicellulose, pectic substances and glycoproteins. The hemicellulose consists primarily of xyloglucans and the pectin of

homogalacturonans and rhamnogalacturonans. In the primary cell wall the cellulose microfibrils are more widely dispersed by the matrix components, whereas cellulose and lignin (a polymer of aromatic alcohols) dominate the secondary cell wall.

Since 1950s, considerable information has been obtained about the structure and the function of plant primary cell walls. The cellulose is the simplest and the best known of all plant cell wall polysaccharides. It consists of long chains of glucose molecules linked through β-1,4-glucosidic bonds. The aggregation of these chains forms the structures called microfibrils which are embedded in a complex polysaccharides and proteins matrix. The xyloglucan (XG), a major component of hemicellulose, is composed of a β-1,4-glucan backbone which is the same as what of the cellulose chain but shorter, frequently linked with some sidechains (1 to 3 residues long) consisting of xylose, galactose, fucose and less frequently arabinose at the 6 position of backbone glucose (Fry, 1989). The structure of pectin is considered to consist of three distinct regions (McNeil *et al.*, 1984). One region is almost a homopolymer of galacturonic acid (HG) linked α-1,4 with some rhamnose residues interspersed within it. A second region has been called rhamnogalacturonan I (RGI), which is composed of a backbone of a repeating disaccharide of galacturonic acid linked α-1,2 to rhamnose with sidechains containing arabinose and/or galactose to some of the rhamnose residues (Lau *et al.*, 1987a). The third region, designated rhamnogalacturonan II (RGII), is a small, complex polymer containing some unusual sugars such as ketoneoxyocturonate, aceric acid, and methylated sugars (Darvill *et al.*, 1978, Melton *et al.*, 1986 and Spellman *et al.*, 1983). The glycoproteins, the major component of proteins in cell wall, contain a high percentage of hydroxyproline and the repeating amino acid sequence of Ser-Hyp_4 in which most of the hydroxyprolyl and some of the seryl residues are glycosylated with oligoarabinans (Lamport, 1977).

The polymers in cell walls could be held together by both covalent and noncovalent bonds, including glycosidic bonds, coupled phenols, hydrogen bonds and ionic bonds. However, little is known of the cross-linking in detail. A better knowledge of the

chemistry of these cross-links will help understanding of the extensibility, digestibility and adherence of cell walls.

There have been many efforts to study the function of cell walls along with the investigation of their structure. The primary cell wall defines not only the rate of growth of plant cells but their size and shape, too. The wall that is laid down during cell growth has a great effect on the size and the shape of the plant cells and thus on the whole plant. Actually the tremendous diversity of different species in the plant kingdom is determined more by their size and shape than any other criteria (McNeil, *et al.*, 1984).

The walls of plant cells are also the major physical barrier to potential pathogens of plant tissues. Most of the cell wall polysaccharides possess extreme structural complexity (McNeil *et al.*, 1984, and Selvendran *et al.*, 1987). As a consequence, it is evident that pathogens encounter a large array of differently-linked glycosyl residues and non-carbohydrate substituents during their attempts to penetrate and degrade plant cell walls. The structural complexity of cell wall polysaccharides and non-carbohydrate substituents present difficult-to-degrade polysaccharide substrates that may control the release of biologically active oligosaccharide fragments from the walls (Hahn, *et al.*, 1981). Moreover, the amount of hydroxyproline-rich glycoproteins (HRGP) in the cell wall has been shown to increase after wounding or upon infection with pathogens, and a correlation has been reported between increased HRGP levels and resistance to infection (Stuart and Varner, 1980; Klis *et al.*, 1983; Condit *et al* 1987; and Esquerre-Tugaye *et al.*, 1979) . It was also reported that some proteins in the cell wall can inhibit specifically the wall-degrading enzymes secreted by pathogens (Albersheim and Anderson, 1971; Hoffman and Turner, 1982; Brown and Adikaram, 1982, 1983; Degra, *et al.*, 1988)) and some enzymes within the cell walls can degrade the walls of pathogens (Cline and Albersheim, 1981). Thus, when plant cell walls are exposed to attack by pathogens, it appears that there is a considerable interaction between molecules of the plant cell walls and the pathogens.

Isolation of the complex polysaccharides from primary cell walls is a prerequisite to characterization of their detailed structures. This step however has proven fairly difficult due to their cross linking and their complexity in structure. Xyloglucans can be solubilized from the cell wall by concentrated alkali. KOH (4.3 M) with 0.1 M $NaBH_4$ solubilized most of the xyloglucan from the cell walls of *Phaseolus aureus* hypocotyls (Kato and Matsuda, 1976), whereas more concentrated (24%) KOH extracted essentially all the xyloglucans from suspension cultured soybean cell walls (Hayashi *et al.*, 1980). In addition, xyloglucans were isolated by sequential treatment with alkali, endopolygalacturonase and a series of chromatographies (York *et al*, 1984, Bauer and Albersheim, 1973, Akiyama and Kato, 1982). Pectic polysaccharies can be extracted from the walls in many different ways. Classical methods include hot water extraction, chelating agent extraction, use of specific enzymes, periodate oxidation, and partial acid hydrolysis. Treatment of cell walls with anhydrous hydrogen fluoride (HF) at different temperatures allows the solubilization of pectins in defined segments which are more easily characterized (Komalavilas and Mort, 1989).

The characterization of cell wall polysaccharides has been advanced greatly with the availability of the powerful spectroscopic methods such as mass spectroscopy and NMR spectroscopy. The general structural analysis of the cell wall polysaccharides includes the application of enzymes, specific chemical cleavage agents, gas chromatography, HPLC, mass and NMR spectrometry.

The progress in investigation of structures of cell wall polymers is still quite preliminary. Much more remains to be determined about the structure of these cell wall polymers and their relations to each other to develop a complete picture of the wall which will help explaining their biological roles in such processes as growth, disease resistance, and maintenance of cell shape.

This work reports the extraction and the characterization of xyloglucan and rhamnogalacturonans from cotton cell walls of suspension culture. The reasons for using

cotton suspension cultured cell walls are as follows. First, there are a number of research groups at Oklahoma State University working with cotton, whose interests include the genetic improvement of cotton, the role of phytoalexins in resistance to bacterial disease (blight), electron microscopy of responses in the plant, and the differences in responses of tissue cultured cotton cell lines to the bacteria. Second, the suspension cultured cells are relatively uniform and plentifully available from Dr. Mitchell's laboratory.

The available information about the structure of cotton primary cell wall polysaccharides is very limited. The xyloglucan in the cell walls of cotton fiber was extracted with strong alkali and characterized by endoglucanase treatment followed by chromatography. Based on sugar analysis by gas chromatography, the structure of xyloglucan was suggested to consist of deca-, nona-, octa-, and hepta-saccharides in the ratio of 2 : 7 : 1 : 3, respectively (Hayashi and Delmer, 1988). Treatment of suspension culture cotton cell walls with anhydrous hydrogen fluoride at -23^{0}C gives rise to a mixture of acetylated and nonacetylated disaccharides of galacturonic acid and rhamnose (Komalavilas and Mort, 1989). This result suggests the presence of rhamnogalacturonan I that would generate these disaccharides in the cotton cell walls.

The results of this research are organized in two major sections. The first section describes the extraction and the characterization of xyloglucan from cotton suspension cultured cell walls. Two methods were used to isolate xyloglucan fragments from the walls. One is to treat the endopolygalacturonase-pretreated residue with strong alkali to solubilize a complex of rhamnogalacturonan I and xyloglucan. Further digestion of this complex with a "purified" cellulase produced both RGI and XG fragments. The other method is to treat the endopolygalacturonase-pretreated residue directly with the "purified" cellulase to solubilize XG fragments. The XG fragments extracted in this way still keep any of their acetyl groups which they may originally contain. The analysis of these XG oligosaccharides is described in detail to provide information about the quantitative distribution of these oligomers and the ratio between the acetylated and the nonacetylated

nonamers, the most abundant oligomers in all cell wall xyloglucan. Based on the extraction patterns and the chromatographic analysis of the xyloglucan, indirect evidence is provided for the covalent cross linking between xyloglucan and rhamnogalacturonan I in cotton cell walls.

The second section reports the isolation and the partial characterization of rhamnogalacturonans, especially the backbone of rhamnogalacturonan I. The isolation of RGI, for the first time, from cotton cell walls in two different ways allowed the further characterization of this complex polysaccharide. Glycosyl composition and partial glycosyl linkage analysis helped in determining the properties of both backbone and sidechains of RGI in cotton cell walls. Degree of methylesterification (DOM) determination and ion exchange chromatography of the RGI isolated by method 2 indicated that there were some highly methylesterified homogalacturonans which were covalently linked with RGI either as long polygalacturonans or as short oligogalacturonans. Treatment of this RGI with HF at -23°C which specifically cleaved rhamnose residues in the backbone and the neutral sugars of the sidechains gave rise to rhamnose-terminated oligogalacturonans (accounting for ~17% by weight of the treated RGI) with sizes ranging from 5 to 15 residues in length. GalA in these oligomers was determined to be ~40% methylesterified. Based on the results of sugar composition and HF treatment analysas, the backbone of RGI in cotton cell walls is proposed to consist of the Rha-GalA repeating units which are interspersed by oligogalacturonans (5-15 residues in length on average) in about every 10 units (20 residues in length). The extraction and a very preliminary characterization of rhamnogalacturonan II are also reported in this section. The structural information obtained about RGI, homogalacturonan and RGII is compared with that from other plant cell walls, especially from sycamore. Indeed, much more work is needed in the future to completely characterize the structure of polysaccharides in cotton cell walls.

CHAPTER II

LITERATURE REVIEW

The cell walls of plants are fundamentally involved in many aspects of plant biology, including the morphology, growth, and development of plant cells and interaction between plant hosts and their pathogens. Due to its multifaceted biological and pathological importance, the structure and function of the plant cell walls has long been the object of intense study. A couple of structural models of plant cell walls have been introduced. In 1973 Albersheim and his coworkers (Bauer *et al,*.1973, Talmadge *et al.* ,1973, Keegstra *et al.*, 1973) proposed a model of primary wall using growing sycamore cells. The structure of the wall was characterized by hydrolysis with purified glycanases and subsequent chemical analysis of the released fragments. Reconstruction of the fragments led to the suggestion that rhamnogalacturonan, arabinogalactan, xyloglucan, and hydroxyproline-rich glycoprotein (extensin) were interconnected by covalent bonds, whereas hydrogen bonds were the interconnection between xyloglucan and cellulose. Three years later, Bailey and his coworkers (Monoro *et al.*, 1976) proposed a model of the primary cell walls of lupin and mung-bean hypocotyls. The analysis of polyglacturonan and xyloglucan extracted by different methods suggested that xyloglucan was associated with cellulose by more than hydrogen bonding, and a covalent linkage was likely involved. It was also suggested in this model that there was no covalent linkage between polygalacturonan and extensin, and the covalent bonds between xyloglucan and polygalacturonan were not involved in binding either into the wall structure. However, some xyloglucan might provide a covalently bonded bridge between extensin and cellulose.

Although much more information about the cell wall structures has been obtained since then, we are still far away from constructing a completely correct model of cell walls due to lack of detailed structural information about the wall polymers, and the exact nature of the chemical bonds between them. With the emerging of advanced analytical techniques such as GC, HPLC, MS, NMR and gene technology, a new and exciting phase of study on cell walls is coming.

Cellulose

Cellulose is the fibrillate component of all higher plant walls, varying in amount from approximately 2-4% in cereal endosperm walls (Fincher and Stone, 1986) to approximately 94% in the secondary walls of cotton seed hair (Meinert and Delmer, 1977). It is particularly abundant in secondary cell walls, and usually accounts for about 20-30% of most primary cell walls. Cellulose is the best known polysaccharide in cell walls. Chemically, it is a linear polymer of (1-4)-linked β-D-glucopyranosyl residues. The glucan chains exist as extended ribbons with a 2-fold screw axis (i. e., 2 residues / turn) stabilized by intramolecular hydrogen bonding. In nature, cellulose chains are packed in an ordered manner to form compact aggregates (microfibrils) which are stabilized by both inter- and intramolecular hydrogen bonding.

The diameter, degree of polymerization, and crystallinity of the cellulose microfibrils are highly variable and depend on the source and age of the tissue. Generally, secondary cell walls, relative to primary cell walls, have a higher degree of polymerization, degree of crystallinity, and the thicker microfibrils (Atalla and Vanderhart, 1984, Preston, 1974).

Xyloglucan

Xyloglucan, a hemicellulose, makes up about 20-25% (dry weight) of the primary cell walls in dicotyledons (McNeil *et al.*,1984) and somewhat less (2-5%) in grasses (Kato

and Matsuda, 1985). It occurs uniformly across the thickness of the primary wall, and also in the middle lamella (Moore, *et al.*, 1986)

The best studied xyloglucans are those from the primary walls of suspension cultured sycamore cells (Bauer *et al.*, 1973; York, *et al.*, 1988), peas (Hayashi,T., and Maclachlan, G., 1984) and of legume seedling stems (Kato and Matsuda, 1980b). The backbone of xyloglucan is chemically identical with cellulose, i.e., a β-(1-4) linked D-glucan. (Kato and Matsuda, 1980a). Reported lengths of the backbone vary from about 200 to 3000 residues, from this it can be calculated that a xyloglucan molecule is about 0.15 to 1.5 μm long (each glucose residue is ~ 0.5nm long) (Hayashi; *et al,* 1980; Nishitani and Masuda, 1982, and 1983). About 60-75% of the glucose residues in dicotyledons and 36-38% of the glucose residues in grasses present in the xyloglucan backbone have an α-D-xylopyranose residue attached at position 6. About 30-50% of these xylose residues have, attached to their position 2, a β-D-galactopyranose residue or, more rarely, an L-arabinofuranose residue, and most of these galactose residues are linked with an α-L-fucose residue (Bauer *et al,* 1973; O'Neill and Selvendran, 1983, 1985). In addition, the attachment of a L-arabinofuranose residue to the backbone glucose at position 2 has also been reported recently (Kiefer, *et al.*, 1990).

The backbone of xyloglucan is proposed to have at least the following sidechains: (a) L-Fuc*p*-α- (1-2)-D-Gal*p*-β- (1-2)-D-Xyl*p*-α- (1--; (b) D-Gal*p*-β- (1-2)-D-Xyl*p*-α- (1--; (c) L-Ara*f*-?- (1-2)-D-Xyl*p*-α- (1--; (d) D-Xyl*p*-α- (1--; and (e) L-Ara*f*-α- (1-- . A heptamer, consisting of 4 glucose residues and 3 (d) sidechains, and a nonamer consisting of 4 glucose residues, 1 (a) sidechain and 2 (d) sidechains appear to be the unit structures common to most xyloglucans. The other oligomers, including a decamer (1 (a), 1 (b), and 1 (d)), octamer (1 (b) and 2 (d)). heptadecamer (1 (a), 1 (e), and 5 (d)) have also been identified in different plant cell wall xyloglucans. Moreover, one or two acetate groups have been found to be linked through ester bonds to some of the galactosyl residues at position 3, or 4 in sycamore cell walls (York *et al.*, 1988).

Xyloglucan probably has both structural and regulatory roles in plant cell walls. The binding of xyloglucan to cellulose fibers through hydrogen or/and covalent bonding would possibly limit the self-association of cellulose fibers and might provide sites for other covalent cross-linking of cellulose fibers. Moreover, since it constitutes about 20-25% of the primary walls (in dicotyledons), it must contribute substantially to the mechanical strength of the wall fabric. Its degradation should, therefore, loosen the wall and render it more susceptible to turgor-driven growth. This may be one of the mechanisms for auxins to promote cell expansion by increasing the concentration of β-1,4 glucanase which degrades xyloglucans (Fry, 1985).

A totally new biological function of xyloglucan was recently introduced by the remarkable observation that a cellulase-generated nonasaccharide from *Acer* xyloglucan is a potent anti-auxin (York *et al.*, 1984). The fucose residue of the nonasaccharide seems to play a crucial role in its biological activity. This was confirmed by the observation that both the trisaccharide (Fuc-Gal-Glc) and the pentasaccharide (Fuc-Gal-Xyl-Glc-Glc) had anti-anxin activity, whereas the octasaccharide without fucose was inactive. It appears possible that these active oligomers act in a feedback control loop ensuring that any excess of auxin does not result in excessive growth. This provided the observations for the hypothesis that certain cell wall polysaccharides from uninfected plant tissue represent the lock-up form of a new class of diffusible, regulatory molecules, the "oligosaccharins ".

Pectic Polysaccharides

The pectic polysaccharides are major components of all primary cell walls of dicots. They are probably the most complex polysaccharides in the walls, and have not been characterized in much detail. The sugar residues comprising the pectic polysaccharides include galacturonic acid (the most abundant one), rhamnose, arabinose, galactose, xylose, perhaps glucose, and a combination of methylated sugars. The structure of pectic polysacharide in the primary cell walls of dicots is now proposed by Albershim's group

(McNeil *et al.*, 1984) to consist of three distinct regions, i.e., homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II.

Homogalacturonans are polymers consisting only or predominantly of α-1-4-linked-D-galacturonic acid residues. The extraction of homogalacturonans from cell walls includes the applications of acid hydrolysis, mild alkali treatment and pectic enzyme digestion (e.g., polygalacturonase cleaves α-1,4 linkage of non-methylesterified galacturonic acid residues, and pectin lyase cleaves α-1,4 linkage of methylesterified galacturonic acid residues). But no pure homogalacturonan has been isolated from the wall without treatments that are likely to break covalent bonds. The degree of polymerization (DP) of homogalacturonan has not been well characterized although the extraction of 25 up to 100 DP from citrus fruit cell walls and suspension cultured *Rosa* cell walls has been reported (Chambat and Joseleau, 1980; Rees, 1982; Dea and Rees, 1973). The distribution of methyl esterification in homogalacturonan probably varies in different plant cell walls. It was reported that a high degree of methyl esterification was found in citrus homogalacturonan with a pattern of $[\ (MeGalA)_4\text{-}GalA]_n$ or $[\ (MeGalA)_5\text{-}GalA)\ ]_n$, (deVries *et al.*, 1986), whereas a much lower degree was found in cotton suspension culture cell walls from Mort's laboratory (Maness and Mort, 1989).

The region with predominant non-methylesterified homogalacturonans can form a so-called "calcium gel" by the cooperative interaction between calcium and carboxyl groups on the unbranched, unesterified segments of the polygalacturosyl chains. This rigid, insoluble gel plays an important role in the structure of cells (Rees, 1982; Davis *et al.*, 1980).

A number of studies have demonstrated that fragments of homogalacturonan elicit phytoalexin accumulation in soybean, pea and castor bean (Hahn *et al.*, 1981; Walker-Simmons *et al.*, 1983; and Jin and West, 1984). The oligosaccharides that result in maximum phytoalexin accumulation range between 10 to 13 galacturonic acid residues in length. Their phytoalexin-inducing activity is eliminated by treatment with

endopolygalacturonase, suggesting that the oligosaccharide itself is the active species. Oligogalacturonides isolated from plant cell walls have also been shown to activate other plant defense responses, such as the eliciting of lignification (Robertson, 1986, 1987; Ryan *et al.*, 1981) and stimulation of the accumulation of protease inhibitors (Robertson, 1986, 1987; Ryan *et al.*, 1981; Bishop, *et al.* 1981, and Walker-Simmons *et al.*, 1983).

Rhamnogalacturonan I, another kind of pectic polysaccharide, was first isolated from the primary walls of suspension cultured sycamore cells, and later from walls of rice endosperm cells and suspension cultured maize cells (McNeil *et al.*, 1980; Shibuya and Nakane 1984, and Thomas *et al.*, 1989). The degree of polymerization of RGI is about 2000, and it consists of D-galacturonic acid, L-rhamnose, D-galactose, L-arabinose and small amounts of L-fucose residues. The backbone is composed of repeating 2-linked rhamnosyl and 4-linked galacturonic acid residues. The galacturonic acid residues of the RGI backbone from cotton suspension culture cell walls are frequently acetylated at position 3 (Komalavilas and Mort, 1989). The sidechains attached to position 4 of some of the backbone rhamnosyl residues are mainly composed of arabinosyl and galactosyl residues, and small amounts of glucosyl residues. These sidechain oligosaccharides appear to be irregular, ranging from 1 to 15 residues in length, and at least 30 different ones have been identified by FAB-MS analysis (Lau *et al.*, 1987a).

Rhamnogalacturonan II is quite different from RGI in both size and glycosyl compositions. It has been isolated from the primary cell walls of dicots, monocots, and gymnosperms upon digestion with endo-1, 4-α-polygalacturonase (Darvill *et al.*, 1978; and Thomas *et al.*, 1989). This small complex polysaccharide (molecular weight ~5, 000), account for about 3-4% of the plant cell wall, contains at least 11 different sugars, including the unusual ones like 2-0-methylfucose, 2-0-methylxylose, apiose, aceric acid, 3-deoxyl-D-manno-2-octulosonic acid (KDO) and 3-deoxyl -D-lyxo-2-heptulosaric acid (DHA) (Darvill *et al.*, 1978; Spellman *et al.*, 1983; York *et al.*, 1985; and Stevenson *et al.*, 1988). There are at least 20 differently-linked glycosyl residues in this polysaccharide that

has ~30 glycosyl residues in *toto*. It also sets the standard for greatest degree of branching with 22% branched residues and 6% doubly branched. RGII is thought to be constructed of a linear α (1-4)-linked octa- or nonagalacturonide backbone with sidechains attached to O-2 and/or O-3 of most of the backbone galacturonic acid residues (Stevenson *et al.*, 1988).

RGII probably plays a role in resistance of plant cell walls to attack by some pathogen-secreted enzymes. This was suggested by the fact that RGII remains largely unmodified in Pectinol-AC, a mixture of cell wall-degrading enzymes secreted by *Aspergillus niger* when this fungus is grown on plant residues that have been pressed to release their liquid. This resistance of RGII to fungal enzyme digestion may be due to its high content of unusual glycosyl residues and linkages, and non-glycosidic substitutes (Spellman *et al.*, 1983).

Some other polysaccharides were also identified in the cell walls of different plant species. (1-4)-β-D-mannans, the main constituents of endosperm walls of some plant seeds, are linear extended ribbon-like molecules consisting of (1-4)-linked β-D-mannopyranosyl residues (Meier, 1958). Galactomannans have the same backbone as the β-D-mannans, but substituted with single galactosyl residues at position 6 of some backbone mannose residues(Stephen, 1983). Calloses are polymers of (1-3)-linked β-D-glucopyranosyl residues, occurring in higher plants as a component of special walls at particular developmental stages (Clarke and Stone, 1988). Xylans, the major noncellulosic polysaccharides of primary walls of grasses and secondary walls of all angiosperms, are composed of a linear (1-4)-linked β-D-xylopyranosyl backbone, substituted by mono- or oligosaccharide sidechains as well as by O-acetyl groups and phenolic acids (Bacic and Stone, 1981). Arabinans ((1-4)-linked α-L-arabinofuranosyl residues), galactans ((1-4)-linked β-D-galactpyranosyl residues), and arabinogalactans (galactans with α-L-arabinofuranosyl sidechains) are also found in many plant primary cell walls (Wood and Siddiqui, 1972; and Clarke *et al.*, 1979).

Glycoproteins

There are at least three types of glycoproteins (structural proteins: hydroxyproline-rich, and glycine-rich glycoproteins; receptors and enzymes) in plant cell walls (Bacic *et al.*, 1988), but among these the family of hydroxyproline-rich glycoproteins (HRGPs, extensin) are the best characterized class. Extensins were originally identified by Lamport, who characterized short hydroxyproline-rich peptides and glycopeptides obtained from plant cell wall hydrolysates (Lamport, 1977). Extensin appears to be an extended rod-like molecule coated on the surface with covalently attached sugar residues. A significant advance in our understanding of the structure of extensin was achieved by Chen and Varner (1985a, and b) who obtained cDNA and genomic clones for a wound-induced extensin from carrot and thus the full sequence of the 306-amino acid protein (M.W. 34,000). The most abundant repeat sequences of the extensin studied are the pentapeptide (Ser-Hyp-Hyp-Hyp-Hyp), some tetrapeptide (Tyr-Lys-Tyr- Lys) and the tripeptide (Thr-Pro-Val), but no Gln, Asn, Asp, Trp, or Cys are present. Carbohydrate sidechains attached to hydroxyproline residues are mostly mono-, di-, tri- or tetra-arabinofuranosides, and these sugars make up to two-thirds of the extensin molecule. Some of the serine residues are also substituted with single α-D-galactopyranosyl units (Lamport *et al.*, 1973).

In addition to the structural function, considerable information has been obtained to suggest that extensin plays a role in resistance of cell walls to infection. The application of antibodies to extensin has shown localized deposition of extensin at or near sites of infection (Esquerre-Tugaye *et al*, 1979). The mRNA encoding presumptive extensins has been observed to increase in response to infection with fungus (Corbin *et al.*, 1987). The accumulation of extensin in plant cells and tissues can be induced by fragments of both fungal and plant cell walls (Showalter *et al.*, 1985; Roby *et al.*, 1985; and Corbin *et al.*, 1987). Moreover, the recent discovery that the extensins induced upon infection of *Phaseolus vulgaris* have higher order hexadeca repeats containing Tyr_3 blocks has led to

the suggestion that extensin could play a role in plant defense by facilitating inter- (if isodityrosine exists intermolecularly), and intramolecular isodityrosine cross-linking and/or provide sites for deposition of lignin (Corbin *et al.*, 1987; Whitmore, 1978).

Other than extensins, there are some other proteins present in cell walls, including enzymes such as malate dehydrogenase (Groos and Janse, 1977), peroxidases (Fry, 1980), phosphatases (Crasnier, 1980), protease, glycosyl hydrolases (Fincher and Stone, 1981), transferases, endoglycanases, and some glycoproteins which, unlike extensin, are low in hydroxyproline, and some are rich in glycine instead. (McNeil, *et al.*, 1984; Brown and Kimmins, 1981; and Varner and Cassab, 1986).

Cross-linking of wall polymers

Although plentiful information about the chemistry of primary cell wall polymers has been obtained, little is known of the molecular organization and interaction (cross-linking) of these wall components. Attempts to constitute a complete model of cell wall structure require an understanding of both physical (non-covalent) and chemical (covalent) associations between wall polymers.

Non-covalent associations between wall polymers are mainly hydrogen bonds and ionic bonds, including calcium bridges. Hydrogen bonding is not only the major interaction between cellulose fibers and xyloglucans (Albersheim *et al.*, 1973), but probably between most of the wall polymers, especially in the formation of gel, a three-dimensional network of polysaccharides (Rees, 1969, 1975, 1982). Homogalacturonan molecules consisting predominantly of non-methylesterified galacturonic acid are cross-linked to form calcium gel by a so-called calcium bridge, the ionic bond formed by calcium ion (Ca^{2+}) and carboxyl groups of galacturonic acid (Jarvis, 1982, 1984; and Rees, 1982).

There are numerous observations which indicate that primary cell wall polymers may also interact with each other by covalent cross-linking (Fry, 1986). Isodityrosine, a compound discovered by Lamport many years ago, was identified to form intramolecular

cross links in extensin between the tyrosines within the sequence tyr-lys-tyr- (Fry, 1982); but no intermolecular cross link has yet been found. The fact that extensin not extracted by salt is also insoluble in chaotropic agents, boiling SDS, acidified phenol, reducing agents, and even anhydrous hydrogen fluoride, strongly suggests that extensin is covalently wall-bound (Fry, 1982; Monro *et al.*, 1976; Mort and Lamport, 1977). The observation that this extensin can be solubilized with $NaClO_2$ (sodium chlorite), which concomitantly splits isodityrosine, but not peptide bonds, supports the idea that isotyrosine holds extensin in the wall matrix (Fry, 1982; O'Neill and Selvendran, 1980). In addition, peroxidase, which catalyzes the formation of isodityrosine in extensin *in vitro*, was suggested to cross-link extensins via isodityrosine bridges (Cooper and Varner, 1984; Everdeen *et al.*, 1988).

Covalent cross-linking between wall polysaccharides has not been demonstrated, although some circumstantial evidence has been obtained to propose the existence of this kind of cross-linking. Glycosyl cross-linking between xyloglucan and pectic polysaccharides proposed by Albersheim's group has been questioned, since the source of the minor sugars (glucose, xylose (or methylxylose), and fucose (or methylfucose) in the fractions that were thought to be the linkage point could have been the pectin RGII rather than xyloglucan (Fry, 1986). Ferulic acid and *p*-coumaric acid were found esterified with galactosyl and arabinosyl residues of pectic polysaccharides from primary cell walls of sugar beet (Fry, 1984, Rombouts and Thibault, 1986a, b, c, d). The oxidative dimerization of these substituted molecules was suggested to cause cross-linking of polysaccharides. Other possible covalent cross-linking mechanisms between wall polymers are the tyrosyl-feruloyl cross link formed by oxidative coupling between protein and polysaccharide and cross-linking formed via reduction of the propenyl portion of polysaccharide-bound ferulic acid by a cysteinyl sulfhydryl of the protein (Fry, 1984).

CHAPTER III

MATERIALS AND METHODS

Cell Walls of Cotton Suspension Culture

Source of Cell Walls

Suspension cultured cells are a relatively homogeneous source of primary cell walls. A large quantity of cells can be cultured and harvested under well-defined conditions. Cell walls of suspension cultures of two varieties of cotton (*Gossypium hirsutum* L.), Acala 44 and Im 216, susceptible and resistant respectively, to the bacterial pathogen *Xanthomonas campestris* pv. *malvacearum,* the causal agent of cotton blight, were used for cell wall preparation. These suspension cultured cells were obtained from Dr. Earl D. Mitchell's laboratory.

Preparation of Cell Walls from Suspension Cultured Cells

Acala 44 and Im 216 cotton suspension cells were grown as described by Ruyack *et al.*, 1979. Two to three week old suspension cultured cell in late log phase were used for cell wall preparation. Cell walls were prepared as described by York *et al.* (1986), except that instead of a pressure bomb, a Polytron homogenizer (Brinkmann Instruments Ins., Westbury, NY) was used to break the cells. Cultured cells were collected on a coarse scintered glass funnel and washed with 100 mM potassium phosphate (pH 7) five times, and with 500 mM potassium phosphate buffer (pH 7) four times to remove extracellular debris. The cells were suspended in one volume of 500 mM phosphate buffer and broken using a Polytron for 10 min at high speed, keeping the sample cold (in ice) during the

procedure. Preparations were homogenized until microscopic examination indicated that cell breakage was complete. The suspension of broken cells was then centrifuged at 2000 x g for 10 min .

The supernatant was decanted and the pellet was washed twice by resuspending in five volumes of the 500 mM phosphate buffer and centrifuging at 2000 x g for 10 min. The washing procedure was repeated five times using distilled water. The washed cell wall pellet was suspended by vigorous stirring in 5 volumes of 1:1 chloroform-methanol and placed in a coarse scintered glass funnel. The organic solvent was removed by applying gentle suction to the funnel, and the cell walls were resuspended in five volumes of acetone. Cell walls were repeatedly washed with and resuspended in acetone before air drying.

Cell walls were tested for the presence of starch by an iodine test. Residual starch was removed by treating the walls with α-amylase (Bacillus type II-A, from Sigma Chemical Co.) as described by York *et al.*, 1986. The cell walls (10 mg/ml) were suspended in potassium phosphate buffer (100 mM, pH 7.0) containing α-amylase (50 units/ml). The suspension was stirred for 48 hr at 25^0C. The enzyme treated cell walls were centrifuged at 10, 000 x g for 10 min . The cell walls were then washed extensively with distilled water followed by acetone and air dried.

Purification of Endopolygalacturonase (EPG)

Purification and Storage of EPG

An *Escherichia coli* clone (HB101/ pAKC213::Tn5-2) expressing a *peh* gene from *Erwinia carotovora subsp. carotovora,* kindly provided by Dr. A. K. Chatterjee, was used as a source of EPG enzyme (Willis *et al.,* 1987). Bacterial cultures, subcultured from colonies grown on L-agar and supplemented with the antibiotics tetracycline (10 μg/ml), kanamycin (50 μg/ml), and ampicillin (50 μg/ml), were grown in L-broth at 30^0C for 36 h.

Periplasmic shock fluids, containing greater than 80% of the total enzyme activity, were prepared as described by Witholt *et al.* (Witholt *et al.,* 1976). Typically, 800 ml of periplasmic shock fluids were prepared from 2 liters of bacterial culture.

Purification of the EPG was accomplished in a two-step procedure with crosslinked polypectate as an affinity adsorbant (Maness and Mort, 1989). The crosslinked polypectate was prepared from apple pectin (Sigma Chemical Co.) as described by Romobouts *et al.* (Romobouts *et al* , 1979) and modified by Barash *et al.* (Barash *et al.*, 1984). In the initial purification step up to 1 liter periplasmic shock fluid was added to 50 g (dry wt) crosslinked polypectate, previously equilibrated with 100 mM Tris, pH 8.0, and stirred slowly for 5 min and the supernatant discarded. The crosslinked polypectate was then rinsed three times with 100 mM Tris, pH 8.0 (250 ml). The adsorbed enzyme was eluted with stirring using 100 mM Tris, pH 8.0, plus 1.0 M NaCl (150 ml). The eluent was passed through a 1-µm filter and then concentrated to about 5 ml with a YM10 µltrafilter (10,000 M_r cutoff; Amicon Corp., Cambridge, MA). The final purification step consisted of application of the partially purified enzyme to a column packed with the crosslinked pectate (1.8 X 27 cm) equilibrated with 100 mM Tris, pH 8.0. The column was then rinsed overnight with 100 mM Tris, pH 8.0, at a flow rate of about 0.3 ml / min. The enzyme was eluted with a 300 ml linear gradient from 0 to 1.0 M NaCl in the Tris buffer. Six-milliliter fractions were collected and the active fractions were combined, concentrated as before to 1 or 2 ml, and then stored in 10% (v/v) glycerol at -70°C. This enzyme preparation exhibited a single protein band upon SDS-electrophoresis and had an apparent molecular weight of 45,000.

Assay of EPG

Enzyme sample plus buffer (0.1 ml) were added in 0.4 ml assay stock solution containing Aldrich pectic acid and incubated at room temperature for 15 min. The reaction was stopped by adding 0.5 ml Nelson-Somogyi reagent and heating at 100^{0}C for 10 min.

After cooling, 1 ml arsenomolybdate reagent was added and mixed. If necessary the sample could be centrifuged to remove unreacted pectate and then absorbance read at 500 nm. The enzyme activity was determined by comparing to the standard which was made with 10-100 µl of a 2.5 mM galacturonic acid stock solution. The amount which released 1 µmole reducing suger per min was defined as one unit.

Purification of β-1,4-endoglucanase (EG)

Purification of EG

An *Escherichia coli* clone (C600SF8) expressing a β-1,4-endoglucanase gene from *Bacillus subtilis* DLG kindly provided by Dr. Glenn H. Chambliss, was used as a source of β-1,4-endoglucanase. Bacterial cultures, subcultured from colonies grown on LB medium, were first grown in liquid LB medium in small containers (20-mL tubes) at 37° C with vigorous aeration for 24 hours, and then transfered to bigger containers (1000-mL flasks) to grow for another 24 hours under the same condition. The bacterial culture (typically two liters in 4 1000-mL flasks) was centrifuged at 1465 X g for 10 min to remove the medium. Bacteria were then suspended in 20 mM sodium acetate buffer , pH 5.2, followed by vortexing for 5 min. The suspension was centrifuged at 1465 X g for 10 min, and the β-1,4-glucanase-containing supernatant was concentrated on a YM10 µltrafilter (10,000 M_r cutoff Amicon Corp. Cambridge MA) to about 3 ml. The concentrated solution was applied to a Bio-Gel P-100 gel filtration column (2.5 X 60 cm, Bio-Rad Laboratories, Richmond, CA) with the same buffer to separate from other contaminated proteins. Based on the Bio-Rad protein assay (Bradford, 1976) and β-1,4-glucanase assay described below, the fractions which contained the fairly purified β-1,4-glucanase were pooled and concentrated with the concentrater mentioned above. This purified enzyme showed a major band with a M.W. of ~39,000 on SDS-PAGE.

Assays of EG

Nelson-Somogyi Method for Determination of Reducing Sugars: The assay was carried out as described by Spiro (1966). Between 1 and 100 µl of β-1,4-glucanase-containing solution was pipetted into a 15-mL tube along with 500 µl of 0.1% carboxymethyl cellulose (CMC) (Sigma No. C-8758) as a substrate and 50 mM sodium acetate buffer, pH 5.2 to give a total volume of 1 ml. The mixture was incubated at room temperature for 15 min. After addition of 0.5 ml of Somogyi-Nelson reagent to the mixture, the solution was heated at 100°C in a Multi-Blok heater for 10 min. The solution was then cooled, mixed with 1.0 ml of arsenomolybdate solution on a vortex mixer. The absorbance was read at 500 nm and compared with standard curve which was made of 9% glucose (0 - 50 µmoles). The amount which released 1 µmole of reducing sugar in 1 hour was defined as one unit.

Assay of EG Using Trinitrophenyl Carboxymethyl Cellulose (TNP-CMC) CMC is chemically modified to include trinitrophenyl groups which absorb at 344 nm. The CMC used to prepare the TNP derivative is fibrous and insoluble. β-1,4-glucanase effects solubilization of short-chain fragments which carry the chromophoric TNP groups.

TNP-cellulose was prepared as described by Huang and Tang (Huang and Tang, 1976). Suction dried CM-cellulose (10 g) prewashed with 500 ml of 0.5 N NaOH, 500 ml of 0.5 N HCl and 4000 ml of water to remove the fines was resuspended in 30 ml of 0.2 M sodium-borate buffer, pH 9.0. The suspension was stirred gently, and 0.6 g of TNBS was added. The pH was adjusted to a value between 8.5 to 9.0 with 4 N NaOH. The reaction mixture was mixed by rotating the container (aluminum-foil wrapped) for 3 h at room temperature. The fibrous suspension was washed successively with 500 ml of 0.2 M Na-borate buffer, pH 9.0, 6 liters of distilled water, and 1 liter of acetone. The air-dried TNP-cellulose, which has an orange color, contained about 0.12 mmol of TNP-moiety in 1 g of TNP-cellulose. The product was stable when stored in a brown bottle at 4°C.

The β-1,4-endoglucanase assay was carried out basically as described by Chambliss *et al.* (Chambliss *et al.*, 1984). Two ml of 1% TNP-CMC, suspended in 50 mM sodium phosphate buffer, pH 6.2, was mixed with 100 µl of β-1,4-glucanase solution (in 20 mM NaOAc, pH 5.2). The suspension was gently stirred at room temperature for 30 min. Solids were then removed by filtering the suspension with a Whatman No.4 filter paper, and the absorbance of the supernatant was measured at 344 nm against a blank. One unit of TNP-CMC degrading activity is defined as the amount of enzyme releasing in 1 min soluble color (light-yellow) corresponding to a specific absorbance of 0.01 at 344 nm.

Determination of Sugar Composition

Between 10 and 100 µg of dry sugars were placed in Teflon-lined, screw-cap glass vials containing 50 or 100 nmoles of *myo*-inositol as an internal standard. Methanalysis and derivatization were carried out by a modification of the method of Chaplin (1982). One hundred µl of 1.5 M methanolic HCL and 25 µl of methylacetate were quickly added to the sample and the sealed vials were heated in a heating block at 80^0C for 10 to 20 h. After removing from the heating block and cooling to the room temperature, 3 to 5 drops of *t*-butanol were added to each vial and the sample was evaporated under a stream of nitrogen. Trimethylsilylating reagent was prepared fresh in the exhaust hood by mixing 1 part of Tri-Sil concentrate (Pierce Chemical Co., Rockford, IL) with 3 parts of dry pyridine. Twenty-five µl of this reagent were added quickly to the sample and allowed to react for at least 15 min at room temperature. The derivatized samples were then evaporated gently under a nitrogen stream and redissolved in 10 to 100 µl of isooctane. A 1 µl aliquot was injected into a fused silica capillary column (30 m X 0.25 mm i.d, Durabond-1 liquid phase, J &W scientific, Inc., Rancho Cordova, CA) installed in a varian 3300 gas chromatographer.

Determination of Cellulose

Cellulose content was determined as described by Updegaraff (1969). Dry Acala 44 cell walls of suspension culture (10 mg) was suspended in 1.5 ml acetic nitric reagent (a mixture of 50 ml of 80% acetic acid and 5 ml of conc. nitric acid), and allowed to react in a boiling water bath for 15 min. Supernatant was removed by centrifuging at high speed for 5 min. 67% H_2SO_4 (2 ml) was then added in the residue and allowed to react for 1 h at room temperature. After diluted to 100 ml with distilled water, the solution was centrifuged to remove any precipitate. This dilution (1 ml) was then further diluted with 4 ml water, and mixed well with 10 ml cold anthrone. The mixture was allowed to react in a boiling water bath for 16 min, cooled to room temperature, and then read at 620 nm to compare to a standard curve made with pure cellulose (0 - 150 μg).

Extraction of Xyloglucan

Digestion of Cell Walls with Endopolygalacturonase

Dry cell walls were suspended in ca. 30 ml of 50 mM ammonium acetate buffer, pH 5.2, in 250-mL centrifuging bottles (500 mg cell wall per bottle), and subjected to 30 min in a NAPCO vacuum oven at room temperature to wet the walls completely. Ten units of EPG was added in each bottle and incubated at room temperature for 3 h with gentle stirring. After incubation, the reaction mixture was centrifuged at 13200 X g for 10 min and the supernatant was saved. The above procedure was then repeated twice and the final residue was washed with 100 ml of water and lyophilized. The supernatants were combined and then either dialyzed against distilled water or freeze dried twice to remove the salts.

Isolation of Xyloglucan-Rhamnogalacturonan I (XG-RGI) Complex

The residues of EPG-treated cell walls were suspended in a solution of 24%KOH, containing 0.1%$NaBH_4$ to prevent peeling reaction, and allowed to react at room temperature for 18 h with stirring. After filtering the reaction mixture with a scintered glass funnel and washing three times with distilled water, the supernatant was neutralized with concentrated acetic acid to pH 5.5 and dialyzed against distilled water in a dialysis membrane of Mr 3,500 cutoff (Spectrum Industrial Inc., L.A, CA). The solubilized material was further fractionated by DEAE-Sephadex anion exchange chromatography, and the unbound polysaccharides (accounting for about 85% by weight of the applied material) were then subjected to gel filtration chromatography on Bio-Sil TSK400. The XG-RGI complex eluted predominantly in the void volume and a fraction, arabinose-rich sugars and protein, eluted ater.

Generation of XG Fragments (Method I)

The XG-RGI complex fractionated from TSK400 gel filtration chromatography was digested with a chromatographically purified cellulase (Worthington Biochemical Co., Freedhold, NJ) by dissolving in 50 mM ammonium acetate buffer, pH 5.2, and incubating with the enzyme (50:1 by wight) at room temperature for 24 h. A few drops of toluene were added to the solution to prevent contamination. After digestion, the cellulase and the undigested polymer (RGI) were separated from xyloglucan fragments by gel filtration chromatography on TSK400 as described above.

Generation of XG Fragments (Method II)

Direct digestion of the EPG-treated cell wall residue with the "purified" cellulase in the same way as mentioned above solubilized XG fragments along with about 40% of

RGI. The RGI and the cellulase were separated from XG fragments by gel filtration chromatography on Fracto-gel HW40S HPLC.

Labeling of Oligosaccharides

The xyloglucan fragments were labeled with 2-aminopyridine (2-AP) at their reducing termini by reductive amination as described by Hase *et al.* (Hase *et al.*, 1981). Samples (0.5-5 mg) were weighed on a Cahn 29 electro-balance and placed into 1-mL reaction vials (Pierce Chemical Co.). Samples were then dissolved in the aqueous 2-aminopyridine (50 µl) (Aldrich Chemical Co; prepared by dissolving 1 g of 2-aminopyridine in 0.8 ml 6 N HCl and 1.6 ml H_2O, pH of reagent ca. 7.0), sealed securely with Teflon-lined caps and incubated at 70°C for overnight (15-20 h). The excess 2-AP was removed by one of two alternate methods: 1) the reaction mixture was passed through the HW40S gel filtration column mentioned above or 2) the reaction mixture was passed through a cation exchange cartridge (Extract clean from Alltech) in the ammonium form and eluted with water (Maness *et al.*, 1991).

Separation of Derivatized XG Fragments by Reverse Phase HPLC (RPC)

The 2-aminopyridine derivatized xyloglucan fragment mixture extracted by the two methods mentioned above was separated efficiently by reverse phase HPLC chromatography (see "Analytical Methods").

Identification of XG Fragments by Liquid Secondary Ion Mass Spectrometry (LSIMS)

The pyridylamino derivatives of xyloglucan (PA-XG) oligosaccharides separated by RPC were tentatively identified by liquid secondary ion mass spectrometry. The mixture of underivatized xyloglucan oligosaccharides from Acala 44, Im 216 cotton cell

walls and from tamarind cell walls were also identified by LSIMS. Since only molecular weight information was obtained from the LSIMS, structures of these oligomers were inferred from previously proven structures of xyloglucan fragments obtained from sycamore and tamarind cell walls.

Isolation of Rhamnogalacturonan I

Digestion of Cell Wall with Endopolygalacturonase (EPG)

This was carried out as described in "Extraction of xyloglucan".

Isolation of RGI from EPG-treated Residue (Method 1)

RGI was first co-solubilized with xyloglucan from EPG-treated wall residue by 24%KOH-0.1%$NaBH_4$ as described in "Extraction of Xyloglucan". RGI-XG complex was then digested with the "purified" cellulase followed by gel filtration chromatography on a TSK400 HPLC column to remove xyloglucan fragments and enzyme (refer to "Generation of XG fragments (method I)").

Isolation of RGI from EPG-treated Residue (method 2)

RGI was also isolated by directly digesting the EPG-treated wall residue with cellulase as described in "Generation of XG Fragments (method II)". The extract of this enzyme treatment was subject to chromatography on a HW40S HPLC gel filtration column to remove XG fragments, cellobiose and glucose. Further gel filtration HPLC of TSK 400 was used to separate the RGI from cellulase.

Isolation of Rhamnogalacturonan II

Treatment of Cell Walls with EPG

RGII, non-methylesterified GalA, and a little RGI were solubilized from the walls by EPG as described previously (refer to "Extraction of Xyloglucan").

Application of Chromatography

To separate RGII from HG and RGI, the EPG-solubilized fraction, after dialysis against distilled water in a Mr 3,500 cutoff membrane, was subjected to a series of chromatographies including DEAE-Sephadex anion exchange, Bio-gel A-5m gel filtration and TSK 2000SW HPLC gel filtration (see "Analytical Methods").

Partial Acid Hydrolysis of RGII

Purified RGII (1.0 mg) was partially hydrolyzed with 200 µl of 0.1M TFA for 24 h at 50^0C. The hydrolysate was then neutralized with NaOH to pH 7 to prepare it for further analysis (Thomas *et al.*, 1989).

Determination of Degree of Methylesterification (DOM)

DOM OF The RGI isolated by method 2, RGII, and the oligogalacturonan generated by treatment of the above RGI with HF at -23^0C was determined as follows: The sample (0.2-0.5 mg) was suspended in 1.0 M imidazole-HCL buffer, pH 7.0 (200 µl/mg sample) and cooled on ice. Sodium borohydride (40 mg) was then added and vial was capped loosely and incubated for at least 1 h on ice. Glacial acetic acid (100 µl/40 mg borohydride) was then added slowly to the cooled sample to decompose the excess borohydride. An equal volume of distilled water was then added, and the reduced pectin was precipitated by adding 3 to 4 volume of 95% of ethanol. The sample was further

desalted by resuspending in water and reprecipitating two more times with ethanol, and then dried under a stream of nitrogen.

Glycosyl compositions of both reduced and non-reduced samples were determined by methanolysis-TMS derivatization followed by gas chromatography. Galactose and galacturonic acid values were expressed as residues per residue of rhamnose present in samples. The equation used for calculation of DOM is:

$$DOM = \frac{[\text{galactose(R)} - \text{galactose(N)}]}{[\text{galactose(R)} - \text{galactose(N)} + \text{galacturonic acid(R)}} \text{x} 100$$

where [galactose (R) - galactose (N)] represents methylesterified galacturonic acid in the native sample.

Demethylesterification

RGI isolated by method 2 and the oligomers of galacturonic acid generated by HF solvolysis at -23^{0}C were demethylesterified by dissolving them in 0.05 M NaOH for 15 min at room temperature. The pH was then adjusted to 6-7 with acetic acid. Dialysis against water with 3, 500 MW cutoff dialysis tubing was used to remove the salt.

Methylation Analysis

Methylation

Methylation analysis of polysaccharides (XG fragments, RGII and RGI) was performed mainly as described by Kvernheim (Kvernheim, 1987). Freeze dried samples (200-500 μg) were flushed with argon for 2 min in a normal 10-mL test tube securely sealed by a rubber cap. DMSO (400 ml) was then added using a glass syringe with argon flushing. To dissolve the sample, the tube was either sonicated or heated at 50^{o}C in a heating block for a few hours. After cooling to room temperature, butyllithium (150 μl,

15% in hexane) was added with argon flushing, and shaken in a shaker at 37°C for 2 h. After cooling in an ice bath (until frozen), methyliodide (200 µl) was added with a glass syringe. The samples were allowed to thaw and react at room temperature for 30 min.

Extraction of Methylated Sugars

Sep-Pak C_{18} Cartridges (Waters Associates Inc. Milford MA) were used for the extraction of methylated sugars (Mort *et al.*, 1983). The Sep-Pak cartridges were preconditioned with (i) 5 ml of ethyl acetate, (ii) 5 ml of methanol, and then (iii) 15 ml of water before the sample was applied. The samples were diluted with 5 ml of water to ensure a high enough solvent polarity for the sugars to be adsorbed and then applied slowly to the Sep-Pak with a 10 ml glass syringe. The Sep-Pak was next washed with 15 ml of water, and the methylated sugars were then eluted by the washing of 3 ml of water-methanol (1;1 v/v); 3 ml of methanol and 3 ml of methanol-chloraform (1:1 v/v), respectively, depending on the sizes of sugars. The eluted sugars were dried by evaporating the solvent with a nitrogen stream.

Hydrolysis

The dry methylated sugars were hydrolyzed by adding 100 µl of 88% formic acid and reacting for 1 h at 100°C (small oligosaccharides do not need this step). After evaporating the acid with a nitrogen stream, 100 µl of 2 N trifluoroacetic acid was added, and hydrolysis was continued for 1.5 h at 121°C. After cooling, 250 nmoles of inositol were added and the acid was evaporated with a nitrogen stream.

Reduction

Ten microliters of 1M ammonium hydroxide was added in the methylated and hydrolyzed samples, followed by addition of 100 µl of 0.3 M potassium borohydride in dimethyl sulfoxide (freshly prepared). The mixture was allowed to react for 1.5 h at 40°C.

After reaction, excess potassium borohydride was decomposed by adding 10 µl of glacial acetic acid (Harris *et al.*, 1984).

Acetylation

The reduced samples from the previous step were acetylated using the procedure of Blakeney *et al.*, (1983). 20 µl of 1-methylimidazole and 200 µl of acetic anhydride were added to each reduced sample. The reaction was allowed to proceed for 10 min at room temperature. Excess acetic anhydride was decomposed by addition of 5 ml of distilled water. The alditol acetates were purified using Sep-Pak C18 cartridges. Alditol acetates were eluted in 2-3 ml of methylene chloride. The water on top of methylene chloride was sucked out with a pasteur pipet ,and the excess water was absorbed by adding a pinch of anhydrous sodium sulfate. After evaporation of methylene chloride with a nitrogen stream, the samples were dissolved in isooctane for gas chromatography analysis.

Selective HF Treatment of RGI

RGI was treated with anhydrous liquid HF as described by Mort (Mort, 1983). Twenty milligrams of dried RGI were put in a Teflon reaction vessel with a stirring bar. The whole apparatus was evacuated and leaks in any part of the system were checked and corrected. The reaction vessel was immersed in a -23°C immersion cooler using a cooling bath of 95% ethanol. Temperature was maintained by the cooler and a temperature regulator (FTS system, Inc. NY). Liquid HF (2 ml) which was already cooled with dry ice and acetone (70°C) was transferred to the reaction vessel together with 50 µl of water. The reaction was allowed to continue for 30 min at -23°C and then stopped by adding 50 ml of cold ether (cooled by adding dry ice to the ether) to the reaction mixture. The quenched reaction mixture was kept for 15 min at room temperature before the HF-ether complex was evaporated under vacuum into a liquid-nitrogen trap. The sugars were dissolved in water and freeze dried.

Analytical Methods

Gas Liquid Chromatography

The trimethyl silyl derivatives of sugars and the methylated sugars were separated on a fused silica capillary column (30 m X 0.25 mm i.d, Durabond-1 liquid phase, J &W scientific, Inc., Rancho Cordova, CA) installed in a Varian 3300 gas chromatograph equipped with an on column injector and helium carrier gas. One µl aliquots of the samples were injected at 105°C, and the temperature was immediately raised to 160°C at the rate of 10°C/ min and held for 4 min, and then raised 1°C/min to 190°C. After that, temperature was immediately raised to 240°C at the rate of 10°C/ min and held for 10 min to clean the column. Peaks were integrated using a Varian 4290 integrator.

Liquid Chromatography Gel Filtration

The RGII fraction eluted at ca.0.45-0.75 M NaCl in 50 mM sodium acetate pH5.2 buffer from DEAE-Sephadex chromatography was applied to a column (2.5 X 60 cm) of Bio-gel A-5m (Bio-Rad Laboratories, Richmond, CA) in 50 mM sodium acetate pH5.2, and eluted with the same buffer. Fractions (2.5 ml) were collected, and the sugars in the fractions were tested by the phenol-sulfuric acid method (Ashwell, 1966) as follows. Fifty microliters of sample was mixed with 450 µl of water and 12.5 µl of phenol reagent (80% by weight in distilled water), followed by 1.25 ml of concentrated sulfuric acid. Sample was vortexed and the color was measured at 485 nm after 30 min. Sodium ions in the pooled fractions from the chromatography were then removed by passage through a small column of AG-50 WX-8 (H^+) cation exchange resin (Bio-Rad Laboratories, CA) prior to lyophilization.

Ion Exchange

The EPG-solubilized fractions were separated on a column (2.5 X 30 cm) of DEAE-Sephadex anion exchanger (A-25-120, 200-400 μm, Sigma Chemical Co. St. Louis, MI). Samples were applied in a weak buffer of 25 mM sodium acetate, pH 5.2, and eluted with a gradient of 0.0 to 1.0 M sodium chloride in the same buffer. The 24%KOH-solublized fractions from EPG-treated residues and the RGI isolated by method 2 were also applied to this DEAE-Sephadex anion exchange column in the same way as mentioned above.

High Performance Liquid Chromatography (HPLC)

HW40S Gel Filtration

The cellulase-solubilized fractions from EPG-treated wall residues and from RGI-XG complex, the fractions of HF-treated RGI at -23°C and the mixture of 2-aminopyridine-labeled xyloglucan fragments were separated on Fracto-gel HW40S gel filtration column (50 X 1cm) from Supply Co Inc, Beflefonte, PA. The column was equilibrated with 50 mM ammonium acetate buffer, pH 5.2, with a flow rate of 0.5 ml/min. The sugar were detected by a refractive index monitor and collected in 0.5 ml per tube. The molecular weight standard used was made with pullulan (Polymer Laboratories Technical Center, Amherst, MA). Pullulan, or polymaltotriase, is a linear macromolecule polysaccharide consisting of links of maltotriose.

TSK2000SW Gel Filtration

The fraction 3 from Bio-gel A-5m gel filtration chromatography of RGII-containing polysaccharides was farther fractionated on a Beckman TSK2000SW gel filtration column (7.5 mm X 30 cm). The elution buffer was 0.3 M sodium acetate, pH 5.2, and the flow

rate was 1 ml/min. Sugars were detected by refractive index detector and the M.W. standard used was made with pullulan.

TSK400 Gel Filtration

The cellulase-solubilized fractions from EPG-treated residues and the 24%KOH-solubilized fractions from EPG-treated residues were fractionated using a Bio-Sil TSK400 gel filtration column (7.5 X 30 cm) from Bio-Rad, Richmond, CA. The samples were loaded and eluted with a buffer of 50 mM ammonium acetate, pH 5.2, with a flow rate of 1 ml/min, and collected in 1 ml/tube. Sugar detection and M.W. standard measurement were the same as with the HW40S system.

Reverse Phase Chromatography (RPC)

The 2-aminopyridine derivatized XG fragments (PA-XG oligosaccharides) were separated using a Bakerbond Wide-pore octadeyl-silica reverse phase column (250 X 4.6 mm from J.T. Baker Inc. Philipsburg, NJ or a Zorbax ODS-Silica Column 250 X 4.6 mm from Du Pont, Wilmington DE). The PA-XG oligosaccharides were eluted with a gradient of consecutive linear segments of increasing acetonitrile concentration: from 0 to 3.2% (v/v) in 15 min, from 3.2 to 6.8%, (v/v) in 10 min, from 6.8 to 13.2% (v/v) in 15 min followed by isocratic elution at 13.2% acetonitrile for 5 min. In all cases, solvents A and B were water and 40% (v/v) acetonitrile in water, respectively, both at 0.01% (v/v) TFA. The PA-XG fragments were detected with a dual beam variable-wavelength UV detector, and the interesting peaks were collected.

Dionex CarboPac PA-1 Ion Exchange Chromatography

The RGI isolated by method 2 was chromatographed on a CarboPac PA-1 HPLC ion exchange column using a Dionex Bio-LC Carbohydrate System. A linear gradient of ammonium acetate, pH 5.2 from 0.03 to 1.00 M over 50 minutes followed by a steady

elution of 1.00 M for 10 more minutes was used to elute the sample. A Dionex UV detector was used to monitor the chromatography by taking advantage of the small UV absoption of the RGI. Flow rate was 1.0 ml per min, and 1.0 ml fraction was collected Sugar was tested by the Phenol-sulfuric acid method mentioned before. The de-methylesterified RGI was also subject to the same chromatography.

The demethylesterified fraction 1 from Fracto-Gel HW40S HPLC of HF-treated methylesterified RGI and "standard" homogalacturonans (pectic acid from Aldrich Chemical Company Inc.) were fractionated on this column by a program developed by Hotchkiss and Hicks (Hotchkiss and Hicks, 1990). A gradient of potassium oxalate was used as follows: inject at 0.025 M, after 1 min a linear increase to 0.1 M over 8 min, a linear increase to 0.2 M over 30 min, a linear increase to 0.25 M over 40 min, and a linear increase to 0.28 M over 15 min. The sugars were detected by a pulse amperometric detector (PAD) with a 20% of the eluant (the eluant was split using a Kel-F tee) plus a constant concentration (1 ml/min) of 1 N NaOH, which was used to make the eluant alkaline for the electrochemical detector.

The fraction 3, 4, 5, and 6 of HF-treated RGI were also fractionated by this column. A linear gradient of sodium acetate, pH 5.2, was applied from 0.00 - 0.00 M over first 10 min, 0.00 - 0.25 M over 10 min, 0.25 - 0.5 M over 30 min, 0.5 - 0.75 M over 20 min, and o.75 - 1.0 M over 10 min (totally 80 min). Sugars were detected by PAD detector as described above.

Liquid Secondary Ion Mass Spectrometry (LSIMS)

The sugar fragments, including xyloglucan fragments generated by method I and method II, 2-AP-derivatized xyloglucan fragments, RGII fragments generated by mild acid hydrolysis, and RGI fragments generated by HF solvolysis, were identified by LSIMS. The sugar samples were dried using a SpeedVac Concentrator (from Savant Instrument Co., Farmingdale, NY). The dried samples were then dissolved in 2 to 5 μl of either water

or methanol, and 1 µl of the solution was mixed with 1 µl of thioglycerol on a stainless steel target before injection. Spectra were obtained on a ZAB 2SE mass spectrometer (from VG, Manchester, UK) using cesium ions at 35 Kv for the ionization, and were collected in the positive ion mode. Both $M+H^+$ and $M+Na^+$ ions were observed in most of the cases.

Gas Chromatography and Mass Spectrometry (GC-MS).

The partially methylated alditol acetate derivatives of xyloglucan, RGI and RGII from cotton suspension culture cell walls were subjected to GC-MS to identify their glycosyl-linkage compositions. One or two microliters of the derivatized sample dissolved in isooctane were injected into a Hewlett Packard 5890 gas chromatograph which was connected to a VG Tritech TS-250 Trisector tandem mass spectrometer (VG Tritech, Manchester, UK) with EBE geometry. The samples were injected at 105^0C and the temperature was immediately raised to 160^0C at the rate of 10^0C/min and held for 4 min, and then raised 1^0/min to 200^0C. After that, temperature was raised 10^0C/min to 240^0C and held for 10 min. The glycosyl-linkages of the different sugar residues were identified based on their mass fragmentation patterns.

NMR Spectroscopy

^{1}H and ^{13}C n.m.r. spectra were recorded with a Varian (Palo Alto, CA) XL-400 n.m.r spectrometer (400 MHz). Some of the ^{1}H spectra were recorded at both 25^0C, or room temperature, and 70^0C. O-linked hydrogen atoms were exchanged for deuterium atoms in the following way. Samples (1-20 mg) were dissolved in 99.9% D_2O (Aldrich Chemical Co.) and freeze dried. The samples were then redissolved in the D_2O followed by the freeze drying for another time before n.m.r. recording. Sodium 2,2,3,3-tetra deuterio-4,4-dimethyl-4 silapentanoate (TSP) was sometimes used as the internal standard (0.00 ppm).

CHAPTER IV

RESULTS AND DISCUSSION

Endopolygalacturonase Treatment of Cell Walls

One gram of Acala 44 cotton suspension cultured cell walls was treated with 50 units of the endopolygalacturonase purified from *E. coli.* (gene cloned from *E. carotovara)* in a volatile buffer of 50 mM ammonium acetate, pH 5.2. The mole percent and the weight percent of the sugars in the extract and in the residue of the enzyme treatment are shown in Table 1. The extract, accounting for about 28% of by weight of the treated walls , contains predominantly galacturonic acid (81% of the weight of the extract), arabinose (~5%), rhamnose (~6%), xylose (~4%), galactose (~4%) and a little non-cellulosic glucose (cellulosic glucose was not detected because methanol-HCl did not hydrolize the insoluble cellulose fibers). The galacturonic acid extracted in this way represents approximately 80% of the total galacturonic acid present in cotton cell walls (Figure 61). The other 20% of GalA, mainly present in rhamnogalacturonan I, could be extracted together with xyloglucan by strong alkali from the walls. Im 216 suspension culture cotton cell walls were also treated with the endopolygalcturonase in the same way as for Acala 44. The mole percent and the weight percent of the sugars in the residue and in the extract which accounded for 25% by weight of the treated walls, were calculated and compared with that of Acala 44 (Table 1). The results indicate that the sugar compositions of the EPG extract from Im 216 cell walls are very similar to that from Acala 44 cell walls.

TABLE 1

MOLE PERCENT OF SUGARS IN COTTON SUSPENSION CULTURE CELL WALLS AND IN THE SOLUBLE AND THE INSOLUBLE PORTIONS OF THE WALLS TREATED WITH EPG

	Acala 44 Cotton Cell Walls			Im 216 Cotton Cell Walls		
Residue	Intact Walls	Soluble	Insoluble	Intact Walls	Soluble	Insoluble
Ara	17.5	4.6 (0.6)[a]	19.9 (1.2)[b]	10.5	4.6 (0.7)[c]	14.0 (0.5)[d]
Rha	10.6	5.9 (0.7)	14.6 (1.4)	5.6	6.4 (0.6)	8.2 (0.3)
Fuc	2.5	0.7 (0.5)	2.6 (0.3)	2.1	1.9 (0.0)	1.7 (0.2)
Xyl	14.1	4.5 (1.0)	22.5 (1.2)	9.3	6.7 (0.5)	26.8 (0.1)
GalA	37.6	78.6 (1.0)	22.5 (1.2)	28.5	74.1 (1.5)	13.7 (1.0)
Gal	8.3	3.4 (0.8)	10.5 (1.5)	9.3	3.5 (0.3)	10.3 (0.3)
Glc (non-cellu)	8.7	2.3 (0.5)	7.5 (1.2)	31.8	2.7 (0.2)	17.2 (2.0)
Cellulose[e]	24 wt%					
wt%[f]	100	28	72	100	25	75

[a,b] Standard deviation of the triplicate samples.

[c,d] Standard deviation of the duplicate samples.

[e] Determined as described by Updegraff, 1969.

[f] Measured by weighing individual fractions.

Analysis of the Endopolygalacturonase Extract

After dialysis against water in a Mr 3,500 cutoff membrane to remove salts, the supernatant of the EPG-treated Acala 44 cell walls was fractionated on a column of DEAE-Sephadex anion exchange (Figure 1). The glycosyl compositions (mole%) of the fractions from the chromatography are shown in Table 2.

The fractions 2 and 3, accounting for about 15% by weight of the EPG-treated walls, contain predominantly galacturonic acid (92 mol% on average). DOM determination of the two fractions with the selective reduction method shows no methylesterification on the GalA residues (data not shown). ^{1}H n.m.r spectra of the fractions indicate that there is no acetylation on the GalA (signals at 2.1-2.2 ppm) in fraction 2, and very low degree of acetylation on the GalA in fraction 3 (Figure 2 and Figure 3). Further analysis of the ^{1}H n.m.r spectra demonstrates that the fraction 2 consists of trigalacturonides, and that the fraction 3 consists of tetragalacturonides, respectively.

The unbound sugar fraction represents only about 3% of the weight of the treated walls. It has a sugar composition similar to that of what has been called rhamnogalacturonan I except for the presence of about 15% of xylose and 6% of glucose which are not expected in RGI. Digestion of this fraction by a "purified" Worthington cellulase mentioned earlier followed by the HPLC gel filtration on a Bio-Sil TSK400 column produced four fractions. The sugar compositions of the fractions in mole percent are given in Table 3. Fraction 1 and 2, eluting early, showed very similar sugar compositions and elution patterns to those of the RGI isolated from the EPG-treated residue with other methods which will be discussed later. This indicates that a little RGI (~10%) was solubilized from the cotton cell walls by endopolygalacturonase. This result contrasts with the fact that ~50% of the RGI of sycamore cell walls was extracted by EPG treatment (McNeil *et al.*, 1980), suggesting that a different cell wall construction between sycamore and cotton may exist. The RGI in cotton may be more firmly bound in cotton cell walls

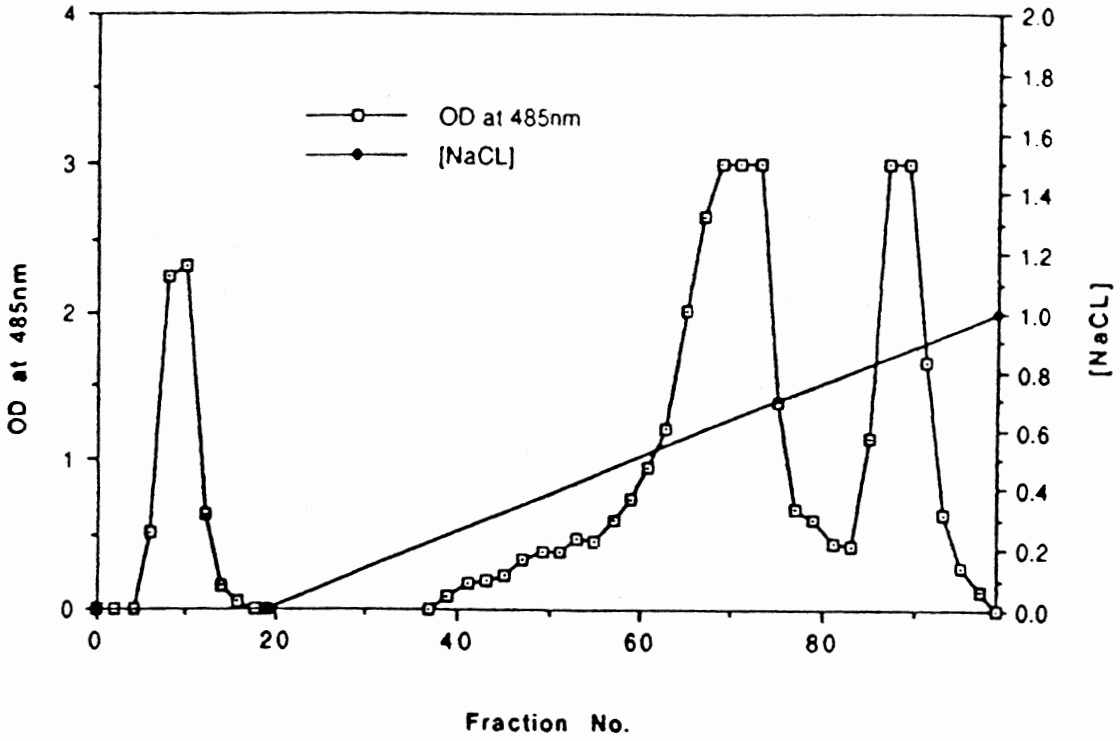


Figure 1. Chromatogram of the Extract of EPG-treated Acala 44 Cotton Cell Walls on DEAE-Sephadex Anion Exchange Column. Tubes 5-18 were pooled into Fn (neutral sugars), 40-60 into fraction 1, 61-76 into fraction 2 (trimers of GalA), 77-84 into fraction 3 (tetramers of GalA), and 85-100 into fraction 4 (RGII-containing polysaccharides).

TABLE 2

GLYCOSYL COMPOSITIONS (MOLE%) OF THE DIFFERENT DEAE-SEPHADEX FRACTIONS OF THE EPG EXTRACT FROM ACALA 44 AND IM 216 COTTON CELL WALLS

	Acala 44					Im 216			
	Unbound	Bound Sugars				Unbound	Bound Sugars		
Residue	Sugars	Fra 1	Fra 2	Fra 3	Fra 4	Sugars	Fra 1	Fra 2	Fra 3
Ara	15.9	1.4	0.6	3.6	8.7	7.6	1.7	5.9	12.4
Rha	11.8	0.8	0.4	3.1	14.4	-	0.9	6.8	22.7
Fuc	trace	-	-	-	1.0	1.9	-	1.0	1.8
Xyl	14.8	3.4	1.9	5.5	6.1	29.2	2.4	5.9	3.9
GalA	36.0	90.7	97.1	85.8	63.4	-	94.0	77.4	44.6
Man	1.9	-	-	-	-	10.6	-	-	-
Gal	13.3	1.7	-	1.9	6.4	11.0	1.0	3.0	9.6
Glc (non-cellu)	6.3	2.0	-	-	-	39.6	-	-	-
Unusual Sugars	-	-	-	-	yes	-	-	-	yes
wt%	11	2	45	11	31	7	49	25	19

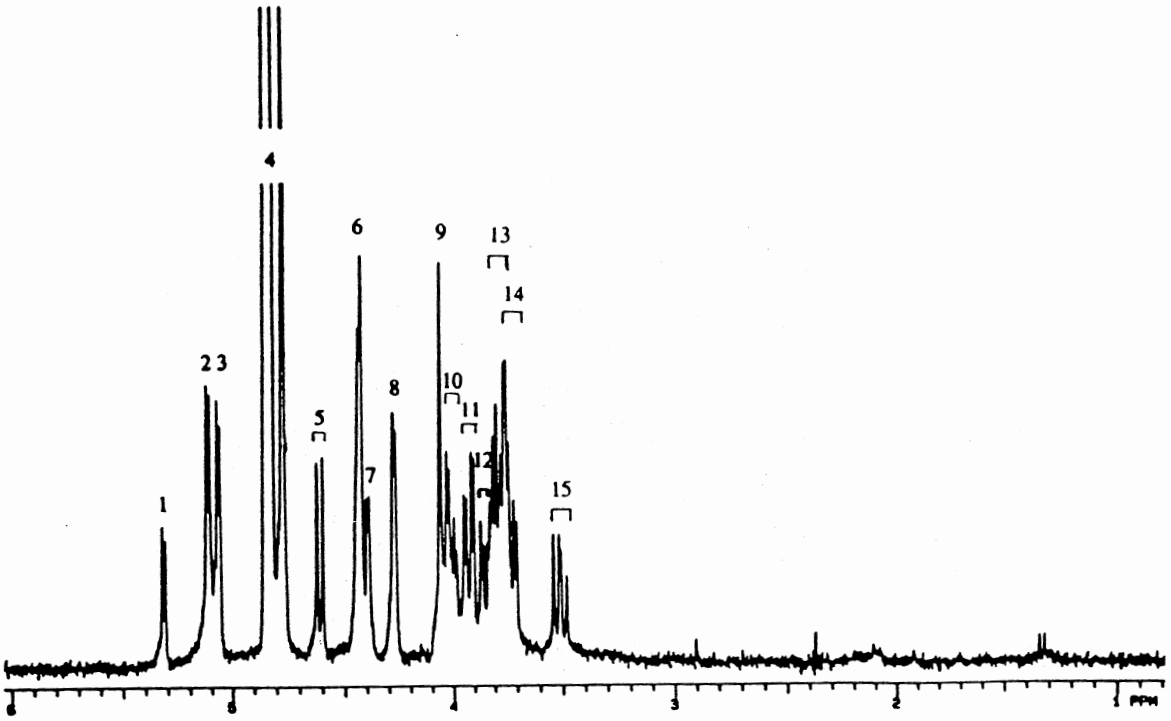


Figure 2. ^{1}H-n.m.r.Spectrum of the Fraction 2 from DEAE Sephadex Anion Exchange Chromatography of the EPG-extracted Materials from Cotton Suspension Culture Cell Walls. The spectrum was recorded on a 300 MHz n.m.r spectrometer at 25^{0}C in D_2O using TSP as a internal standard at 0.00 p.p.m. 1) H-1 of α-R; 2) H-1 of IR; 3) H-1 of NR; 4) H-5 of IR, H-5 of NR, and H_2O; 5) H-1 of β-R; 6) H-4 of IR, H-4 of α-R and H-5 of α-R; 7) H-4 of β-R; 8) H-4 of NR; 9) H-5 of β-R; 10) H-3 of α-R; 11) H-3 of NR; 12) H-2 of α-R; 13) H-2 of IR; 14) H-2 of NR, and H-3 of β-R; and 15) H-2 of β-R. R = Reducing end GalA; IR = Internal GalA; and NR = Non-reducing end GalA.

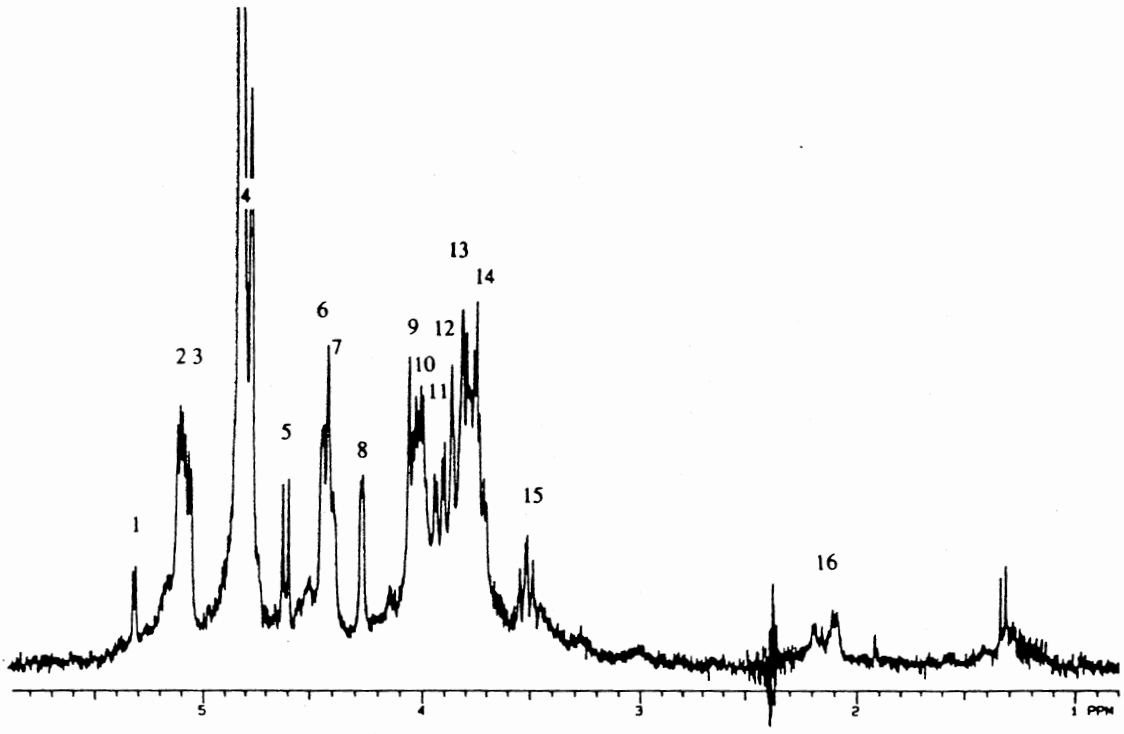


Figure 3. ^{1}H-n.m.r. Spectrum of the Fraction 3 from DEAE Sephadex Anion Exchange Chromatography of the EPG-extracted Materials from Cotton Suspension Culture Cell Walls. The spectrum was recorded on a 300 MHz n.m.r spectrometer at 25^{0}C in D_2O using TSP as a internal standard at 0.00 p.p.m. 1) H-1 of α-R; 2) H-1 of IR; 3) H-1 of NR; 4) H-5 of IR, H-5 of NR, and H_2O; 5) H-1 of β-R; 6) H-4 of IR, H-4 of α-R and H-5 of α-R; 7) H-4 of β-R; 8) H-4 of NR; 9) H-5 of β-R; 10) H-3 of α-R; 11) H-3 of NR; 12) H-2 of α-R; 13) H-2 of IR; 14) H-2 of NR, and H-3 of β-R; and 15) H-2 of β-R; 16) CH_3 of acetate. R = Reducing end GalA; IR = Internal GalA; and NR = Non-reducing end GalA.

TABLE 3

GLYCOSYL COMPOSITIONS OF THE FRACTIONS OF THE CELLULASE-TREATED UNBOUND FRACTION FROM DEAE-SEPHADEX OF THE EPG EXTRACT FROM ACALA 44 CELL WALLS

	Fra 1		Fra 2		Fra 3		Fra 4	
Residue	Mole%	Rel Mole	Mole%	Rel Mole	Mole%	Rel Mole	Mole%	Rel Mole
Ara	16.7	0.6	19.8	0.9	9.4	1.1	13.8	0.8
Rha	27.1	1.0	21.2	1.0	8.7	1.0	3.7	0.2
Fuc	1.1	0.1	-	-	1.2	0.1	1.8	0.1
Xyl	6.2	0.2	4.0	0.2	11.5	1.3	16.7	1.0
GalA	37.8	1.4	32.1	1.5	39.6	4.6	29.1	1.7
Man	-	-	1.0	-	10.1	1.2	4.9	0.3
Gal	10.0	0.4	20.8	1.0	16.7	1.9	10.7	0.6
Glc	1.1	0.1	1.1	0.1	2.7	0.3	15.8	1.0
wt%	37		10		22		31	

than that in sycamore cell walls. Fraction 3, containing ~40 mol% of mannose, is a combination of the cellulase and the sugars which are either from the enzyme, a glycoprotein, or from the contaminants of the enzyme preparation. A small amount of xyloglucan fragments generated by the cellulase was eluted in fraction 4. These xyloglucan fragments may have been firmly associated with RGI before cellulase digestion.

Fraction 4, eluting last from the DEAE-Sephadex column with a high concentration of sodium chloride , contains some unusual sugars (see "Partial Analysis of RGII") in addition to the arabinose, rhamnose, fucose, xylose, galacturonic acid and galactose (Figure 4). Further analysis (see "Isolation of RGII") demonstrated that almost all of the rhamnogalacturonan II in cotton cell walls was present in this fraction.

The sugars solubilized from Im 216 cotton suspension culture cell walls by EPG were also subjected to the DEAE-Sephadex anion exchange chromatography (Figure 5). The sugar compositions of the fractions from the chromatography are shown in Table 2. Similar results for these fractions were obtained. Fractions 2, 3, and 4 consist of trigalacturonides, tetragalacturonides, and the RGII-containing polysaccharides, respectively. However, the unbound sugar fraction which is relatively rich in xylose, glucose and fucose may be richer in xyloglucan than rhamnogalacturonan I when compared to the same fraction from Acala 44 cell walls described earlier.

Co-solubilization of XG with RGI by Strong Alkali Treatment

Strong alkali has been used in the extraction of xyloglucan from different plant cell walls by several research groups (Fry, 1986). KOH (24%), containing 0.1%$NaBH_4$ to prevent the peeling reaction, has been reported to extract almost all the xyloglucan from pea and cotton fiber cell walls (Hayashi and Maclachlan, 1984, Hayashi and Delmer, 1988). In order to isolate xyloglucan from cotton cell walls, the residue of the above EPG-treated Acala 44 cotton cell walls were treated with 24%KOH-0.1%$NaBH_4$ at room temperature

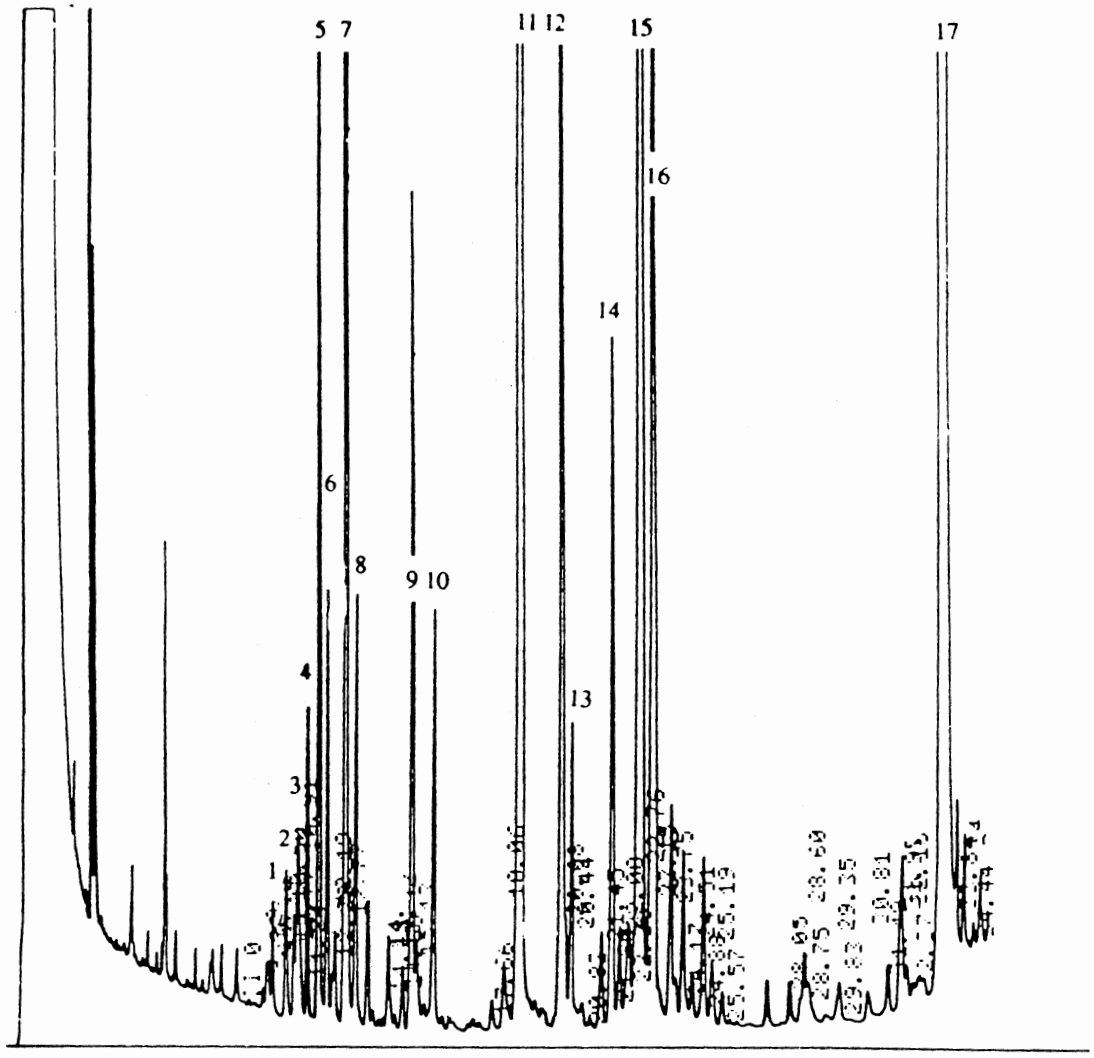


Figure 4. Gas Chromatogram of the Trimethyl Silyl Derivatives of the Methyl Glycosides of the Fraction 4 from DEAE-Sephadex Chromatography of the EPG-extracted Materials from Cotton Suspension Culture Cell Walls. Peak 1, 2, 3, and 4 were from the putative unusual sugars; peak 5 and 6 were identified as Ara; 7 and 8 as Rha; 9 and 10 as Xyl; 11, 12, and 15 as GalA; 13, 14 as Gal; 16 as GalA+Gal, and 17 as inositol.

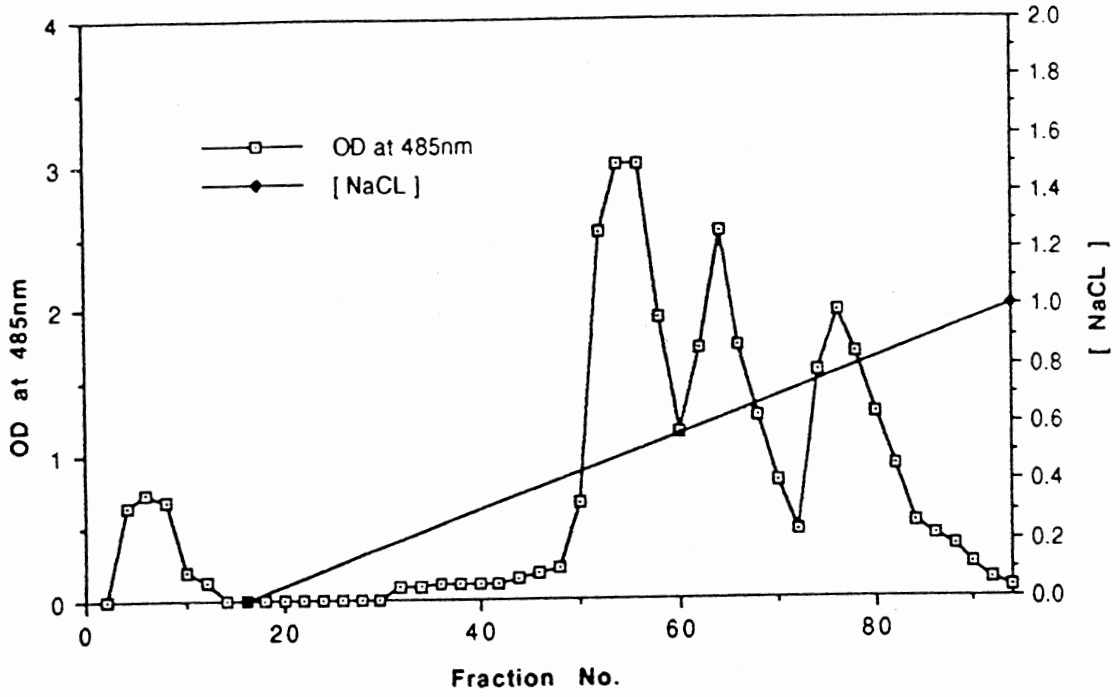


Figure 5. Chromatography on a DEAE-Sephadex Anion Exchange Column of the EPG Extract from Im 216 Cotton Cell Walls. Tubes 5-15 were pooled into Fn (neutral sugars), 50-60 into fraction 1 (trimers of GalA), 61-72 into fraction 2 (tetramers of GalA), and 73-90 into fraction 3 (RGII-containing polysaccharides).

for 24 h. The mole percent of sugars in the soluble and the insoluble fractions of the treatment are presented in Table 4. The insoluble fraction, representing about 38% in Acala 44 (based on three experiments) of the dry weight of the original walls, was mainly composed of glucose in addition to a very small amount of xylose, galacturonic acid and rhamnose, suggesting that almost all the sugars in this fraction were from cellulose. The soluble fraction, accounting for about 34% of the dry weight of the walls, was not only rich in xylose and glucose but also in rhamnose, arabinose, galactose and galacturonic acid. Based on the sugar compositions, it appeared that the strong alkali had extracted almost all of the xyloglucan and pectic polysaccharides from the EPG-treated cotton cell walls. The residue of EPG-treated Im 216 cotton cell walls was also treated with the strong alkali, and similar results were obtained in terms of sugar composition and weight percentage. The mole percent and weight percent of sugars in the soluble and the insoluble fractions of this treatment are presented in Table 4.

Chromatography of the strong alkali solublized materials from the residue of EPG-treated Acala 44 cotton cell walls on a Bio-Sil TSK400 HPLC gel filtration column gave rise to two major peaks with the ratio of peak areas of 3:1 (Figure 6). The sugar compositions of the two peaks (fraction 1 and 2) from the chromatography are shown in Table 5. Fraction 1, eluted in the void volume, contains the sugars Ara, Rha, GalA, Gal, Xyl, Glc, and Fuc in the mole percentage of 8.6%, 12.7%, 19.7%, 13.3%, 27.3%, 15.0%, and 3.4%, respectively, suggesting a combination of xyloglucan and rhamnogalacturonan I (XG-RGI complex). The absence of the unusual sugars in this fraction indicated there was no RGII-like polysaccharides present in the fraction. Fraction 2, eluted close to the included volume, contains 42 mol% of Ara and shows a high UV absorbance (280 nm). Hydroxyproline (Hyp.) estimation indicated that the Hyp in this fraction represented about 0.15% by weight of the total treated cell walls, suggesting that more than 50% of the Hyp was solubilized in this fraction (Hyp makes up ~0.3% of the

TABLE 4

GLYCOSYL COMPOSITIONS (MOLE%) OF THE SOLUBLE AND THE INSOLUBLE FRACTIONS AFTER TREATMENT OF THE EPG-TREATED CELL WALL RESIDUE BY 24% KOH-0.1% $NaBH_4$

	Acala 44		Im 216	
Residue	Soluble	Insoluble	Soluble	Insoluble
Ara	18.9	-	12.4	2.6
Rha	13.2	2.6	9.0	-
Fuc	3.0	-	3.3	-
Xyl	24.8	9.1	24.1	6.7
GlcA	trace	trace	trace	trace
GalA	18.1	2.5	11.5	-
Gal	11.1	trace	11.6	5.8
Glc	10.9	85.7	28.0	85.0
wt% of the treated wall	33.6	38.0	34	32

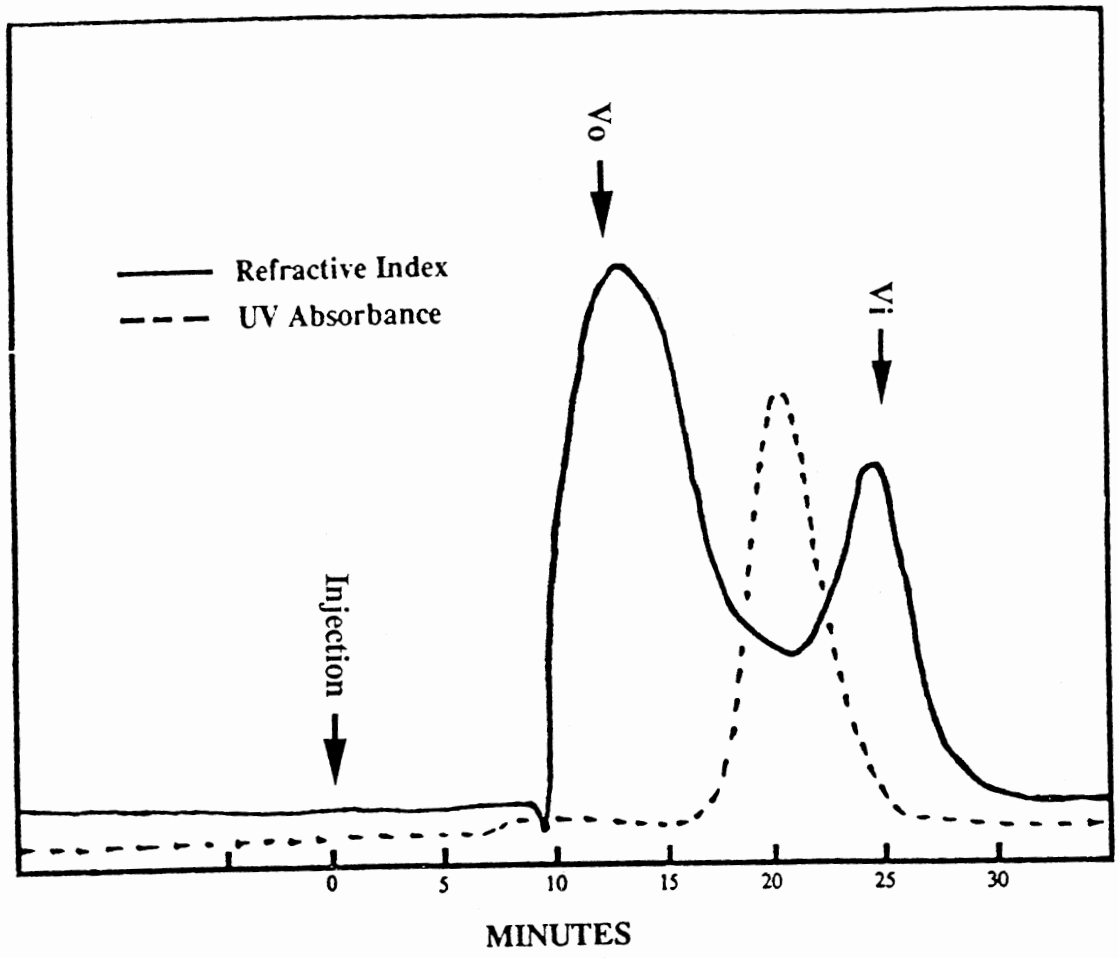


Figure 6. Chromatography on a Bio-Sil TSK400 HPLC Gel Filtration Column of the Fraction Solubilized from the EPG-treated Cell Wall Residue by 24%KOH-0.1%$NaBH_4$. Tubes 10-21 were pooled into fraction 1 (XG-RGI complex), and 22-30 into fraction 2 (protein-polysaccharide complex).

TABLE 5

GLYCOSYL COMPOSITIONS (MOLE%) OF BIO-SIL TSK400 GEL FILTRATION FRACTION OF THE MATERALS SOLUBILIZED FROM THE EPG-TREATED RESIDUE BY 24% KOH-0.1% $NaBH_4$

Residue	Fra 1	Fra 2
Ara	8.6	42.2
Rha	12.7	8.3
Fuc	3.4	-
Xyl	27.3	18.4
GalA	19.7	12.7
Man	-	2.3
Gal	13.3	6.5
Glc	15.0	9.6
wt%	75	25

weight of the cotton cell walls). the UV absorbance and the high Hyp content demonstrated that the strong alkali had solubilized a considerable amount of glycoproteins.

Generation of Xyloglucan Fragments from XG-RGI Complex (Method I)

Treatment of XG-RGI Complex with *E.coli* -cloned Endoglucanase

To obtain xyloglucan fragments from the xyloglucan-containing fraction (XG-RGI complex) generated by the TSK400 chromatography of the alkali solubilized materials from the EPG-treated walls, the complex was treated with an endoglucanase purified from *E.coli* into which an β-1,4-endoglucanase gene of *B. subtilis* DLG had been cloned, and then fractionated on a Bio-Sil TSK400 HPLC gel filtration column (Figure 7). Sugar composition analysis of the fractions from the chromatography (Table 6) indicated that the xyloglucan associated with RGI was only partially degraded by this enzyme. The lower activity of this endoglucanase is probably caused by a need for more than one consecutive unbranched glucose residue for activity.

Treatment of XG-RGI Complex with Worthington Cellulase

Chromatographically purified Worthington cellulase was found to be more powerful than the *B. subtilis* enzyme in digesting xyloglucans. Treatment of XG-RGI complex with this enzyme cleaved xyloglucan molecules into small fragments which were further fractionated by Fracto-gel HW40S HPLC gel filtration chromatography (Figure 8). The mole percent of the sugars in the different fractions from this separation are presented in Table 7. Fraction 1, eluting in the void volume, contained RGI and the cellulase. Fraction 2 through 5 contained the cellulase-generated xyloglucan fragments with different sizes. Fraction 6, eluting in the included volume, contains monomers of Ara, Fuc, Xyl, Gal and Glc, accounting for only ~0.2% of the weight of the cellulase-treated

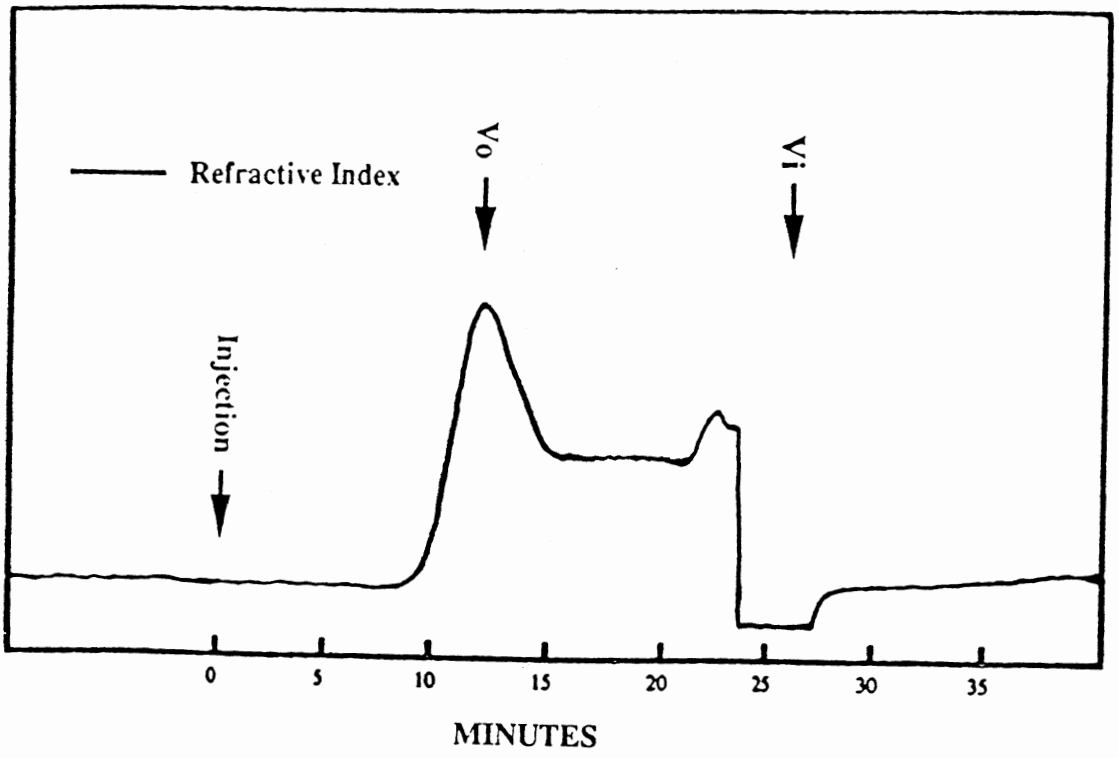


Figure 7. Chromatography on a Bio-Sil TSK400 HPLC Gel Filtration Column of the XG-RGI Complex Treated with an Endoglucanase Purified from *E. coli.* into Which an Endoglucanase Gene of *B. subtilis* DLG had been Cloned. Tubes 10-15 were pooled into fraction 1, 16-20 into fraction 2, 21-23 into fraction 3, and 24-28 into fraction 4.

TABLE 6

GLYCOSYL COMPOSITIONS OF FRACTIONS FROM BIO-SIL TSK400 HPLC GEL FILTRATION OF THE XG-RGI COMPLEX TREATED BY AN ENDOGLUCANASE PURIFIED FROM *E. coli* INTO WHICH AN β-1,4 ENDOGLUCANASE GENE OF *B. subtilis* DLG HAD BEEN CLONED

Residue	Fra 1	Fra 2	Fra 3	Fra 4
Ara	11.3	4.9	2.7	2.5
Rha	16.7	7.5	1.2	1.0
Fuc	1.4	3.6	4.1	2.8
Xyl	11.4	26.1	41.8	37.5
GalA	37.6	13.8	5.1	4.0
Man	-	1.6	1.0	-
Gal	13.3	11.9	10.2	12.8
Glc	8.3	30.6	33.9	38.9

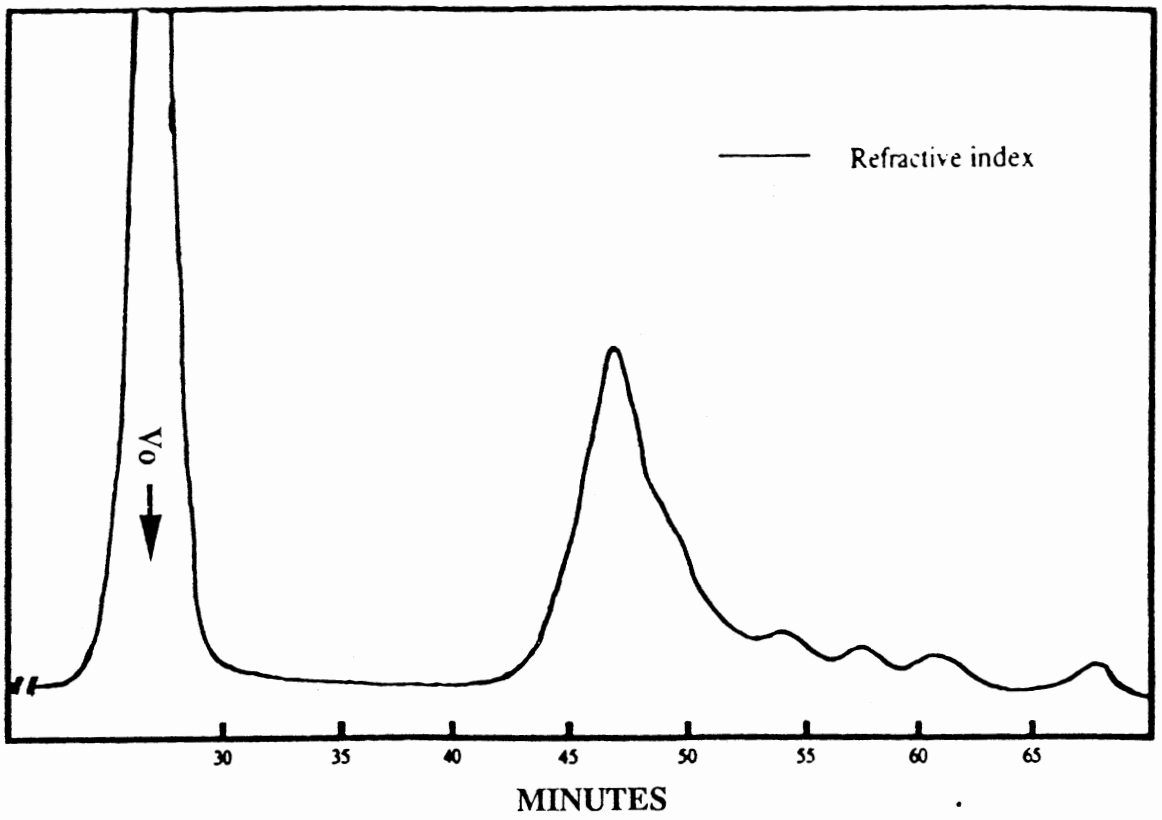


Figure 8. Chromatography on a Fracto-gel HW40S HPLC Gel Filtration Column of the Cellulase-digested XG-RGI Complex Solubilized by Strong Alkali from the EPG-treated Wall Residue. Tubes 26-40 were pooled into fraction 1, 44-52 into fraction 2, 53-56 into fraction 3, 57-59 into fraction 4.

TABLE 7

GLYCOSYL COMPOSITIONS (MOLE%) OF FRACTO-GEL HW40S GEL FILTRATION FRACTIONS OF THE CELLULASE-DIGESTED XG-RGI COMPLEX FROM BOTH ACALA 44 AND IM 216 COTTON WALLS

	Acala 44						Im 216		
Residue	Fra 1	Fra 2	Fra 3	Fra 4	Fra 5	Fra 6	Fra 1	Fra 2	Frac 3
Ara	8.0	-	-	-	-	20.8	11.3	1.5	2.2
Rha	21.5	-	-	-	-	-	18.7	-	-
Fuc	-	4.2	2.9	-	-	5.5	-	6.2	2.7
Xyl	5.8	27.9	34.8	38.7	21.1	16.6	8.9	34.2	42.1
GalA	43.2	-	-	-	-	-	36.3	1.4	-
Man	3.5	-	-	-	-	-	6.1	-	-
Gal	14.1	12.8	12.5	3.9	-	31.4	14.8	9.9	5.4
Glc	1.6	55.1	59.7	57.4	57.4	25.8	2.3	46.7	47.1
wt%	58	3	32	4	2	2	-	-	-

polysaccharides. This indicates that this cellulase is relatively free of glycosidase activities other than perhaps β-glucosidase.

The XG-RGI complex solubilized by 24%KOH-0.1%$NaBH_4$ from cotton Im 216 suspension culture cell walls was also treated with the cellulase in the same way . The same xyloglucan fragments were obtained using the same fractionation procedures (data not shown).

Generation of Xyloglucan Fragments from the EPG-treated Wall Residues (Method II)

Xyloglucan fragments were also isolated from the EPG-treated wall residues by directly digesting them with the Worthington cellulase. In this case about 54% of the weight of the cell wall material was solubilized by the enzyme, including pectic polysaccharides, cellulose in the form of cellobiose and glucose as well as xyloglucan oligosaccharides. The separation of the xyloglucan fragments from the other sugars was achieved in the same way as in method I by using Fracto-gel HW40S HPLC gel filtration chromatography. The advantage of this method is that the xyloglucan fragments produced, unlike those generated by strong alkali which would hydrolyze acetate esters, do not lose their original acetyl groups because only the specific enzymes were employed.

Analysis of Xyloglucan Fragments

Identification of Xyloglucan Fragments by LSIMS

The xyloglucan fragments generated by method I were identified by Liquid Secondary Ion Mass Spectroscopy (LSIMS). Figure 9 shows the mass spectrum of the xyloglucan fragments of fraction 2, the major xyloglucan oligosaccharide-containing fraction fractionated in the HW40S HPLC gel filtration chromatography. Six significant peaks with the masses of 1063, 1085, 1225, 1247, 1371 and 1393 were identified in this

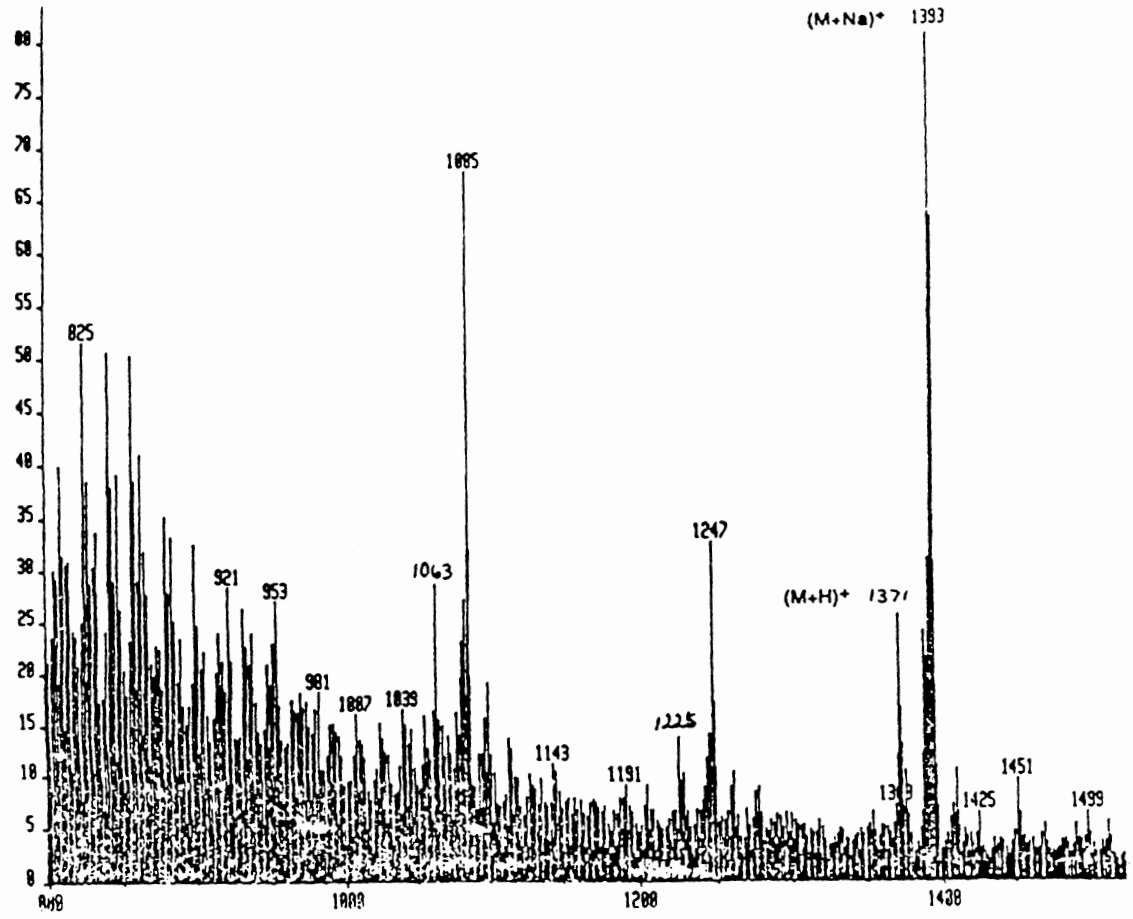
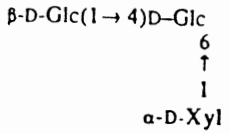


Figure 9. Mass Spectrum of the Xyloglucan Fragments (Fraction 2 of the HW40S) Generated by Method I. Peak with mass of 1063 corresponds to the $[M+H^+]$ of heptamer, 1085: $[M+Na^+]$ of heptamer, 1225: $[M+H^+]$ of octamer, 1247: $[M+Na^+]$ of octamer, 1371: $[M+H^+]$ of nonamer, 1393: $[M+Na^+]$ of nonamer.

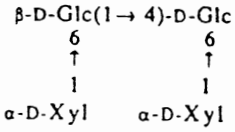
spectrum. The masses of 1063, 1225 and 1371 are consistent with the [M+H$^+$] of hepta-, octa- and nonasaccharides of xyloglucan (Figure 10), respectively, and whereas the masses of 1085, 1247 and 1393 were found to be consistent with the [M+ Na$^{+)}$ of hepta-, octa-, and nonamer of xyloglucan, respectively (Figure 11). Meanwhile, the peaks with the masses corresponded to the molecular weights of the hexamer (901), pentamer (769), isopentamer (783) and tetramer (607) were also identified in the LSIMS spectra of the xyloglucan fragment-containing fractions eluted later than fraction 2 from the HW40S HPLC chromatography (Figure 12). The fact that small amounts of tetra-, and hexamers were generated by the cellulase indicated that either the cellulase has both activities in cleaving unbranched and branched β-1,4 linkage of glucose residues, or there is another enzyme present in the cellulase preparation which has the latter activity.

The xyloglucan fragments generated by method II were also subjected to the analysis of LSIMS. Since the xyloglucan oligosaccharides generated in this way have never been exposed to alkaline conditions, any esters, e.g. acetate esters, which were originally present on the native polysaccharides should have been retained on the xyloglucan fragments. Acetylated xyloglucan oligosaccharides have been found and characterized from the xyloglucans obtained from the medium around sycamore suspension cultures (York *et al.*, 1988) and by cellulase digestion of cell walls from sycamore suspension cultures (Kiefer, *et al.*, 1989). Only the galactose residue has been found to be esterified, predominantly at position 6 but also position 3 and 4 to a lesser extent. The LSIMS spectrum of the major XG fragment-containing fractions generated by method II from the HW40S HPLC chromatography shows not only the 6 peaks with the masses identical to those of the XG fragments generated by method I, i.e., 1063, 1085, 1225, 1247, 1371 and 1393, but also four more significant peaks with the masses of 1267, 1289, 1413 and 1435 (Figure 13). The first 6 peaks no doubt represent the hepta-, octa- and nonasaccharides of xyloglucan, and the last four peaks, 1267, 1289, 1413, and 1435 were identified as the acetylated octamer ([M+H$^+$], [M+Na$^+$]) and the acetylated nonamer



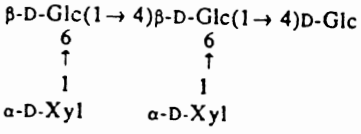
1.

M.W. 474



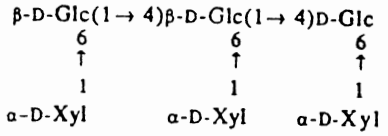
2.

M.W. 606



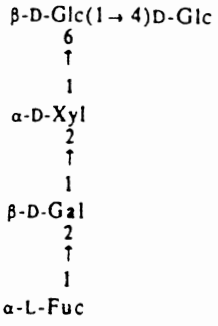
3.

M.W. 768



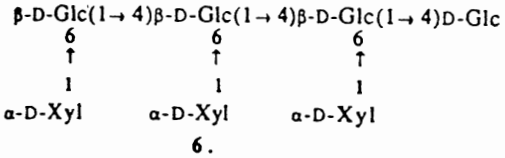
4.

M.W. 900



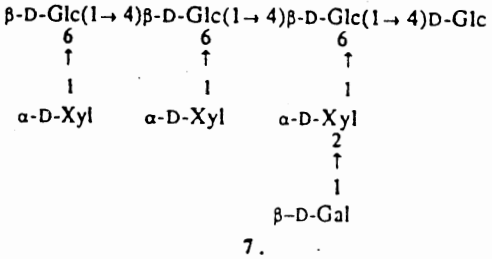
5.

M.W. 782



6.

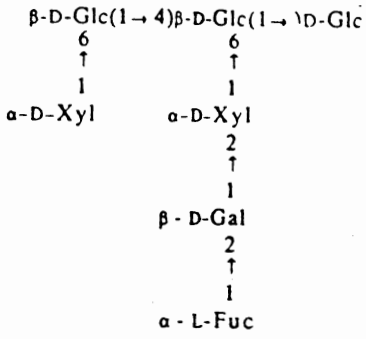
M.W. 1062



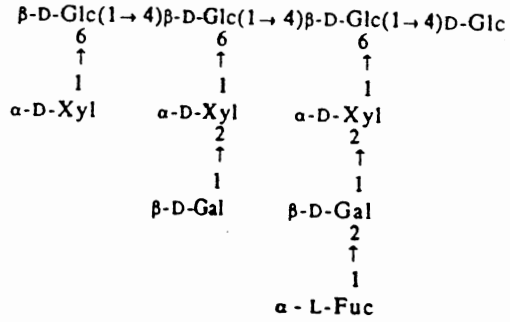
7.

M.W. 1224

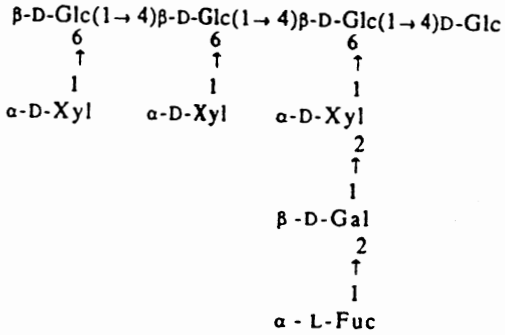
Figure 10. Structures of Oligosaccharides Generated by the "Purified" Cellulase from Cotton and Tamarind Seed Cell Wall Xyloglucan (York *et al.*, 1990)(1).



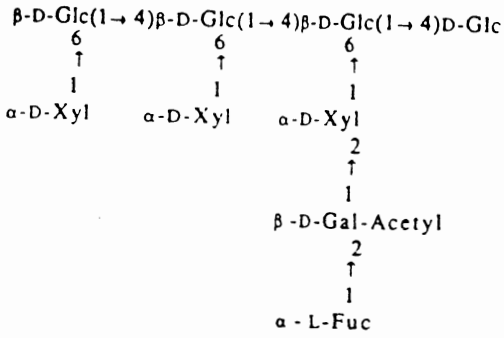
8. M.W. 1076



M.W. 1532 9.

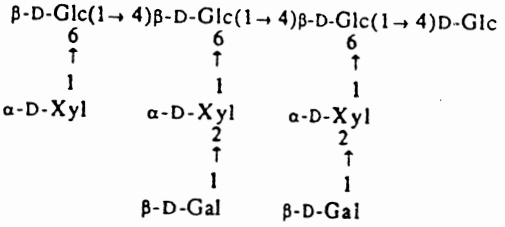


M.W. 1370 10.



10a.

M.W. 1412



11.

M.W.1386

Figure 11. Structures of Oligosaccharides Generated by the "Purified" Cellulase from Cotton and Tamarind Seed Cell Wall Xyloglucan (2).

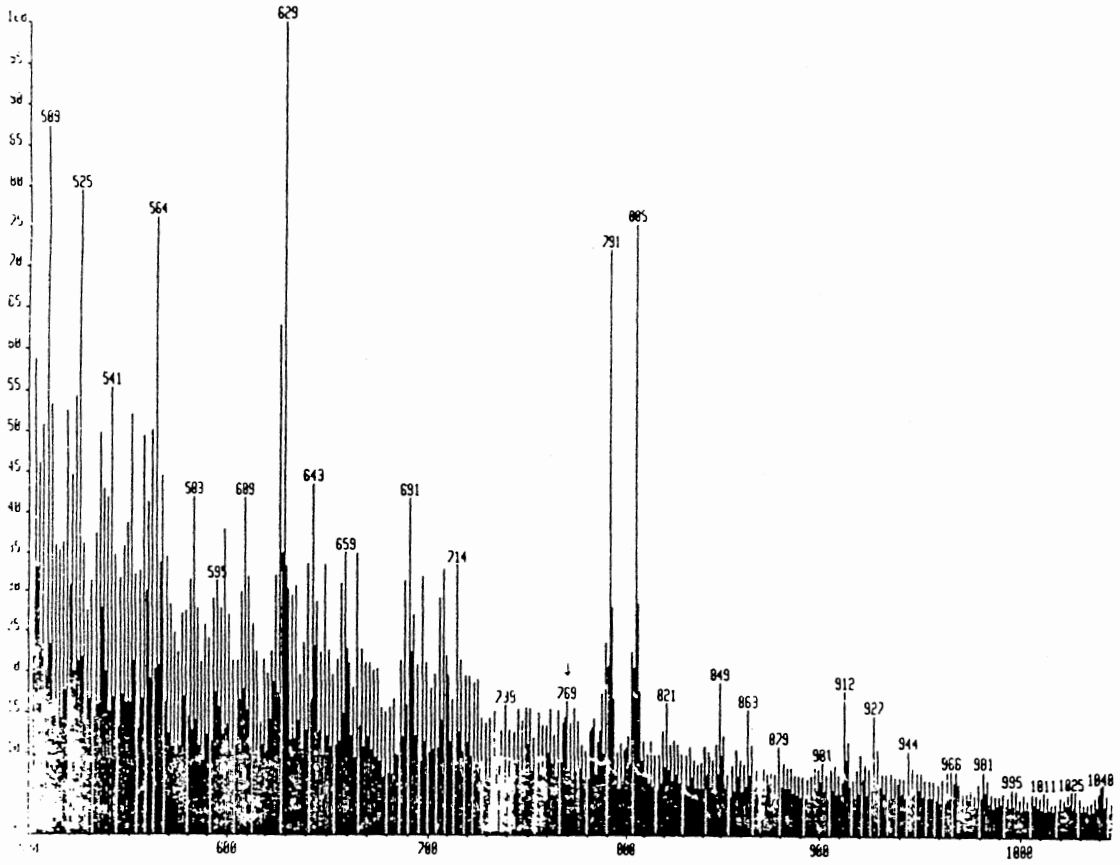


Figure 12. Mass Spectrum of the Xyloglucan Fragments (Fraction 3 and 4 of the HW40S) Generated by Method I. Peak with mass of 629 corresponds to $[M+Na^+]$ of tetramer, 791: $[M+Na^+]$ of pentamer, 805: $[M+Na^+]$ of isopentamer.

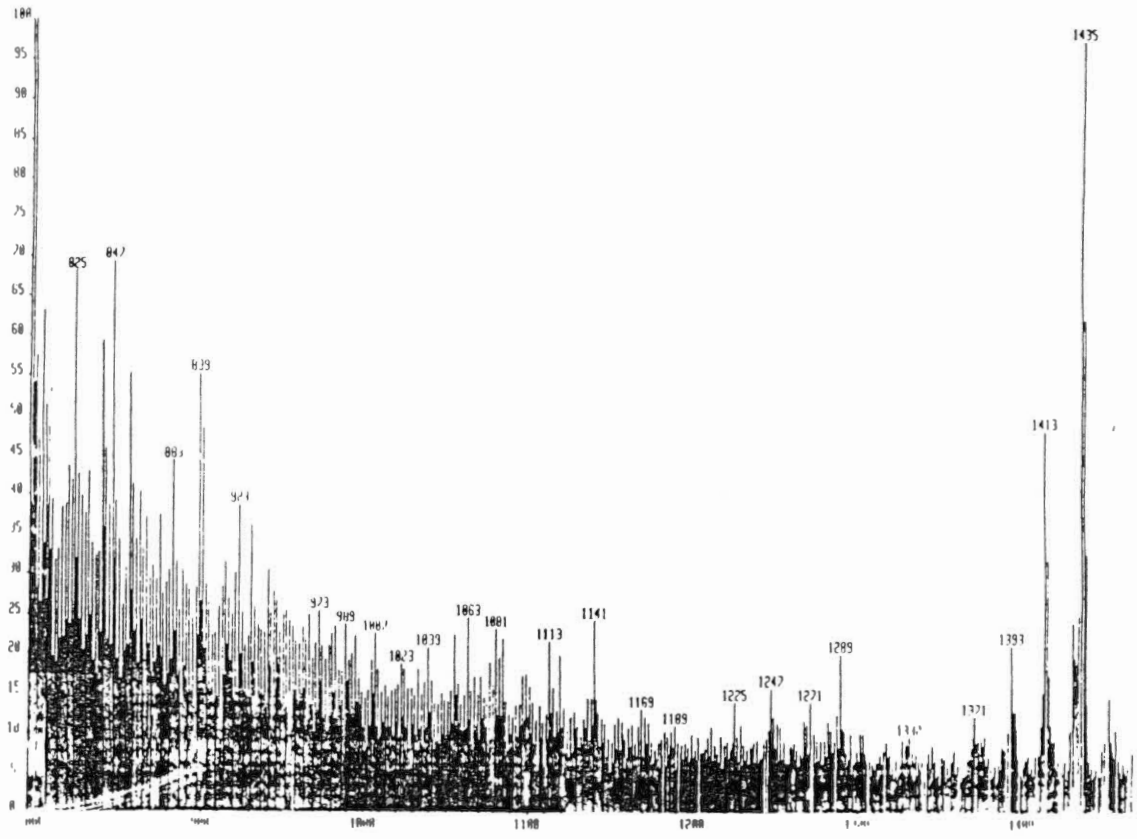


Figure 13. Mass Spectrum of the Xyloglucan Fragments (Fraction 2 of the HW40S) Generated by Method II. The peaks with masses of 1063, 1085; 1225, 1247; and 1371, 1393 correspond to the [M+H$^+$] and [M+Na$^+$] of the hepta-, octa- and nonamers, respectively. The peaks with masses of 1267, 1289; 1413, 1435 correspond to the [M+H$^+$], [M+Na$^+$] of the acetylated octa- and acetylated nonamer, respectively.

([M+H$^+$], [M+Na$^+$]) , respectively. This result demonstrated that the acetylation on cotton cell wall xyloglucan is also most likely on the galactosyl residue because only the octa- and nonamer, which have galactose residues, were found to be acetylated.

The LSIMS spectrum of a mixture of the xyloglucan fragments generated by method I from Im 216 suspension culture cotton cell walls is shown in Figure 14. Compared to Figure 9, the same peaks with the masses consistent with the molecular weights of the hepta-, octa- and nonasaccharides were identified, suggesting that the similar xyloglucan structures are present in Im 216 strain.

Determination of the Glycosyl-linkage Compositions of the XG Fragments by GC-MS

The glycosyl-linkage compositions of the xyloglucan oligosaccharides in fraction 2 (Figure 8) were determined by GC-MS of the partially methylated alditol acetate derivatives. The gas chromatogram of the sugar derivatives is shown in Figure 15. All of the expected glycosyl-linkages of xyloglucan were observed and their adentites confirmed by mass spectral fragmentation patterns (Lindberg, 1972). Peaks with scan numbers of 177, 220, 313, 422, 577, 583, 620, and 816 were identified as terminal xylose, terminal fucose, 2-linked xylose, terminal galactose, 6-linked glucose, 2-linked galactose, 4-linked glucose, and 4,6-linked glucose, respectively.

The mole percent of these different glycosyl-linkage residues of xyloglucan fragments were calculated according to their relative areas from GC chromatogram and their molar flame response factors (Sweet *et al.*, 1975) (Table 8). The molar ratios of these residues were calculated as normalized to one 4-linked glucosyl residue. Assuming that the xyloglucan fragments in the preparation are mainly composed of the nona- and heptasaccharides, the ratios of these different glycosyl-linkage residues are not well consistent with what was expected (Table 8). This probably resulted from the experimental error of the methylation, such as over evaporation of the derivatized sample, and incomplete methylation.

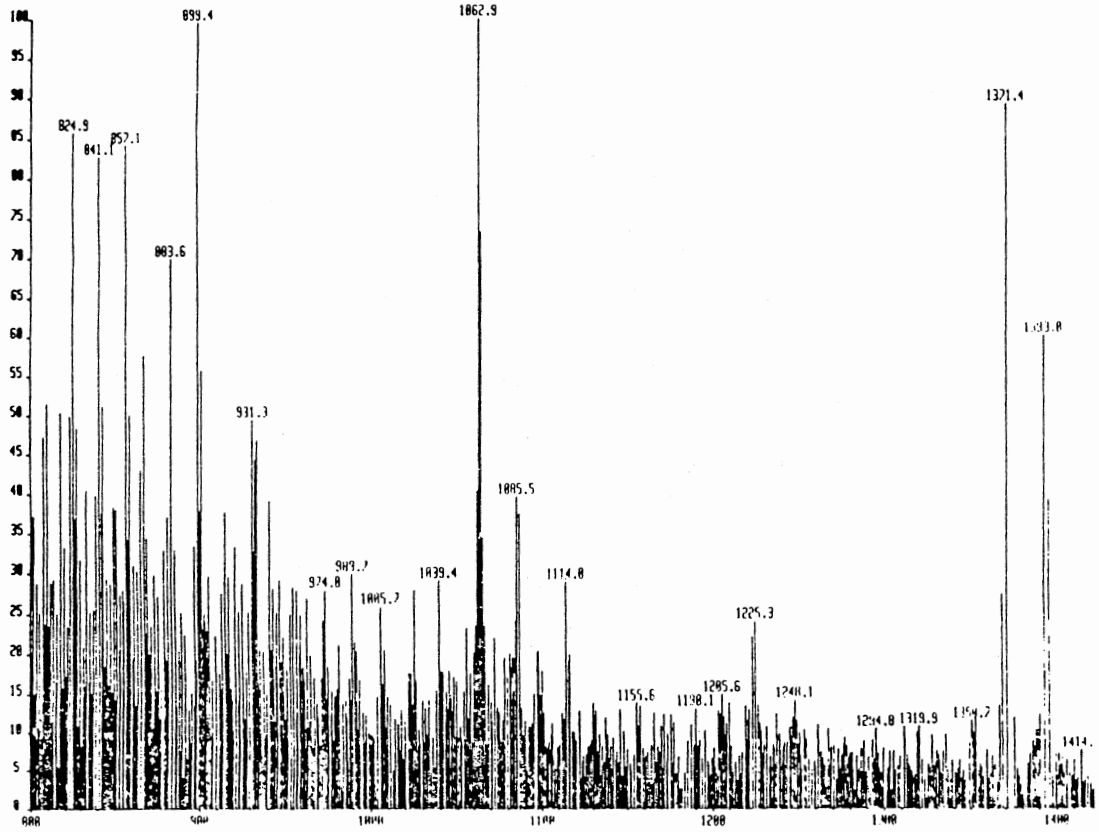


Figure 14. Mass Spectrum of the Xyloglucan Fragments (Fraction 2 of HW40S) Solubilized by the "Purified" Cellulase (Method II) from Im 216 Cotton Suspension Culture Cell Walls. The spectrum was recorded by a ZAB 2SE mass spectrometer. Peaks with masses of 1062.9, 1085.5, 1205.6, 1225.3, 1371.4, and 1393 correspond to the $[M+H^+]$ and $[M+Na^+]$ of hepta-, octa-, and nonamer, respectively.

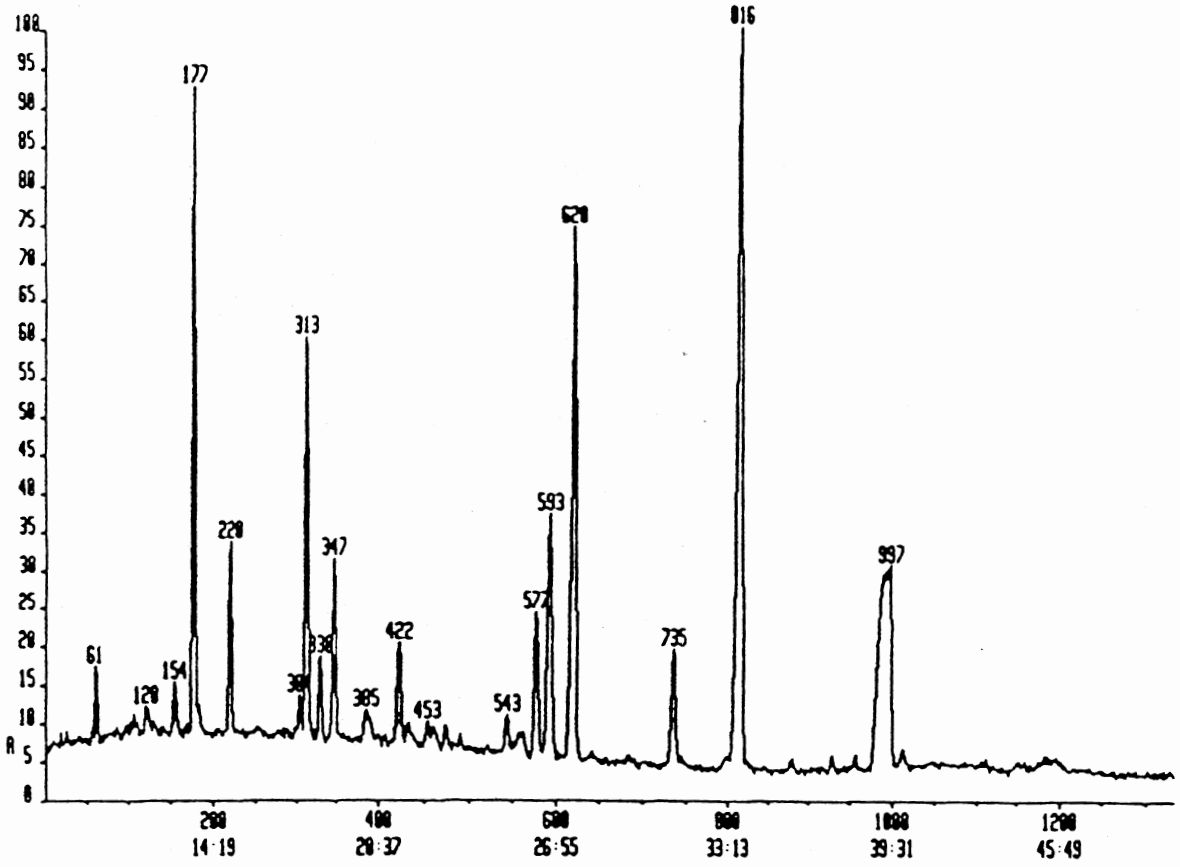


Figure 15. Gas Chromatogram of the Partially Methylated Alditol Acetate of the Xyloglucan Fragments (Fraction 2 of HW40S) Generated by Method II. Peaks were identified from the mass spectra of these signals. 177: t-Xyl, 220: t-Fuc, 313: 2-Xyl, 422: t-Gal, 577: 6-Glc, 583: 2-Gal, 620: 4-Glc, 816: 4,6-Glc.

TABLE 8

GLYCOSYL-LINKAGE COMPOSITIONS OF XYLOGLUCAN FRAGMENTS GENERATED BY METHOD II FROM THE EPG-TREATED WALLS

Glycosyl-linkage	Mole Percent	Molar Ratio*	Expected Molar Ratio
Terminal Xyl	16.3	1.0	2.5
2-linked Xyl	13.2	0.7	0.5
Terminal Fuc	4.0	0.2	0.5
Terminal Gal	2.0	0.1	trace
2-linked Gal	8.6	0.4	0.5
4-linked Glc	18.5	1.0	1.0
6-linked Glc	3.4	0.2	1.0
4,6-linked Glc	33.9	1.8	2.0

* Normalized to that of 4-linked glucose residue.

NMR Analysis of the XG Fragments

The ^1H n.m.r. spectra of the xyloglucan fragments isolated by both method I and method II were recorded on a 400 MHz n.m.r. spectrometer at room temperature in D_2O (figure 16 and 17). The signals were identified mainly by comparing the previously assigned ^1H n.m.r. spectra of xyloglucan fragments (Kiefer *et al.*, 1990 and York *et al.*, 1990). All of the anomeric signals of sugars present in both xyloglucan samples were identified, including H-1 of α-fucosyl residues (1), H-1 of α-glucosyl residues (2), H-1 of α-xylosyl residues with an α-Fuc-(1-2)-β-Gal substituent at C-2 (3), H-1 of terminal, non-reducing α-xylosyl residues (4), H-1 of terminal β-galactosyl residues (5) ?, H-1 of 2-O-linked β-galctosyl residues (6), H-1 of 4, 6-O-linked β-glucosyl residues; H-1 of 4-O-linked β-glucosyl residues; H-1 of 6-O-linked β-glucosyl residues, and H-5 of α-fucosyl residues (7). The small signals between signals 2 and 3 were putatively identified as H-1 of α-xylosyl residues with a β-Gal substituent at C-2. The signal at 1.22 p.p.m. was identified as the methyl group of fucosyl residues (11). As expected, the signals for the acetyl groups present on the galactosyl residues were identified in the spectrum of the sample prepared by method II (8, 9, 10), but not in that of the one from method I. Signals 8, 9, and 10 were tentatively identified as CH_3 of acetate linked to Gal at C-4, C-3, and C-6, respectively. The bulk of signals of the ring protons which have upfield p.p.m compared with the anomeric proton signals have not been interpreted due to their complexity.

Separation of the Xyloglucan Fragments by RPC

The above analysis of the xyloglucan fragments by HPLC chromatography, LSIMS spectroscopy, GC-MS, and n.m.r. spectroscopy revealed that the xyloglucan in cotton cell walls of suspension cultures basically consists of several types of cellulase-generated oligosaccharides, including nona-, octa-, hepta-, penta-, and isopentamers. The

predominant oligosaccharide units comprising the xyloglucan appear to be nona- hepta-, and octamers. However, since the resolution of the gel filtration employed was not high enough to separate these oligomers, and the LSIMS could only provide the information of molecular weight quantitatively, the actual proportions and distributions of these oligosaccharides were still not clear at this point. Separation of xyloglucan oligomers carried out by other researchers has relied mostly on gel filtration on Bio-Gel P-2 or on paper chromatography (Fry, 1989 and Bauer *et al.*, 1973).

Reverse phase chromatography (RPC) HPLC, having a high resolution, was used to separate the xyloglucan fragments from cotton and tamarind seed (El Rassi *et al.*, 1991). This was done completely by El Rassi and his graduate students in his laboratory. The RPC was performed using a shallow gradient with consecutive linear segments of increasing acetonitrile concentration in the eluent. The xyloglucan oligosaccharides applied to this RPC were pre-derivatized with 2-aminopyridine in order to be detected by UV absorption. The RPC chromatogram of the derivatized xyloglucan fragments (PA-XG) generated by method I (non-acetylated) is shown in Figure 18. The peak 2, 3, 4, 5, 6, 7, 8, 9, and 10 were collected and identified by LSIMS as tetra-, penta-, hexa-, isopenta-, hepta-, octa-, isohepta-, deca- and nonasaccharides of xyloglucan, respectively (Figures 10 and 11). Since only molecular weight information was obtained from LSIMS, these structure identifications were actually also based on the analysis of sugar composition, glycosyl-linkage composition, and inferred from previously reported structures of xyloglucan fragments isolated from sycamore and tamarind, using *Trichoderma* endoglucanases (York *et al.*, 1990).

Figure 19 illustrates the oligosaccharides map of the PA-XG fragments produced by method II. The same peaks and eluting patterns were obtained as that of figure 18 (non-acetylated PA-XG) in this chromatogram except a big additional peak (10a) eluted after peak 10. This peak was identified as the acetylated nonasaccharide (refer to Figure 11, structure 10a). However, the acetylated octasaccharide which had been observed by

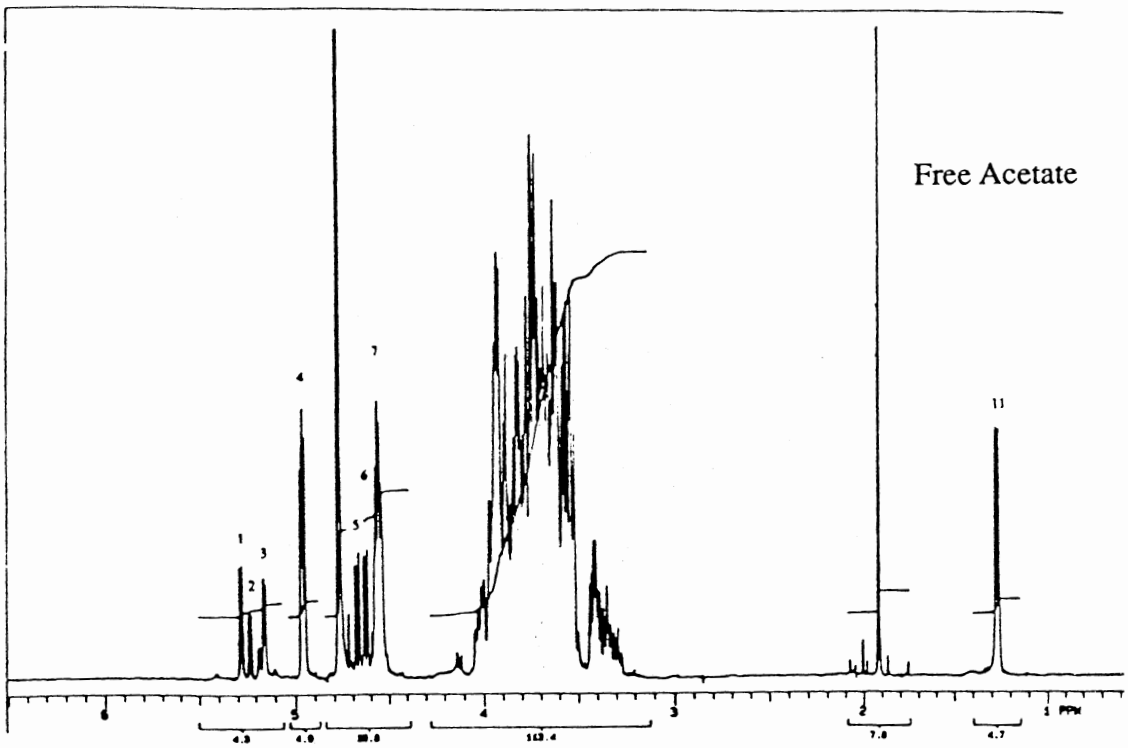


Figure 16. ^{1}H-n.m.r.Spectrum of the Xyloglucan Fragments Generated by Method I. The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25^{0}C in D_2O. Peaks are assigned as follows: 1) H-1 of α-Fuc; 2) H-1 of α-Glc; 3) H-1 of α-Xyl with an α-Fuc-(1-2)-β-Gal at C-2; 4) H-1 of t-α-Xyl; 5) H-1 of t-β-Gal; 6) H-1 of 2-α-Gal; 7) H-1 of 4,6-β-Glc, H-1 of 4-β-Glc, H-1 of 6-β-Glc, H-5 of α-Fuc; 11) CH_3 of Fuc.

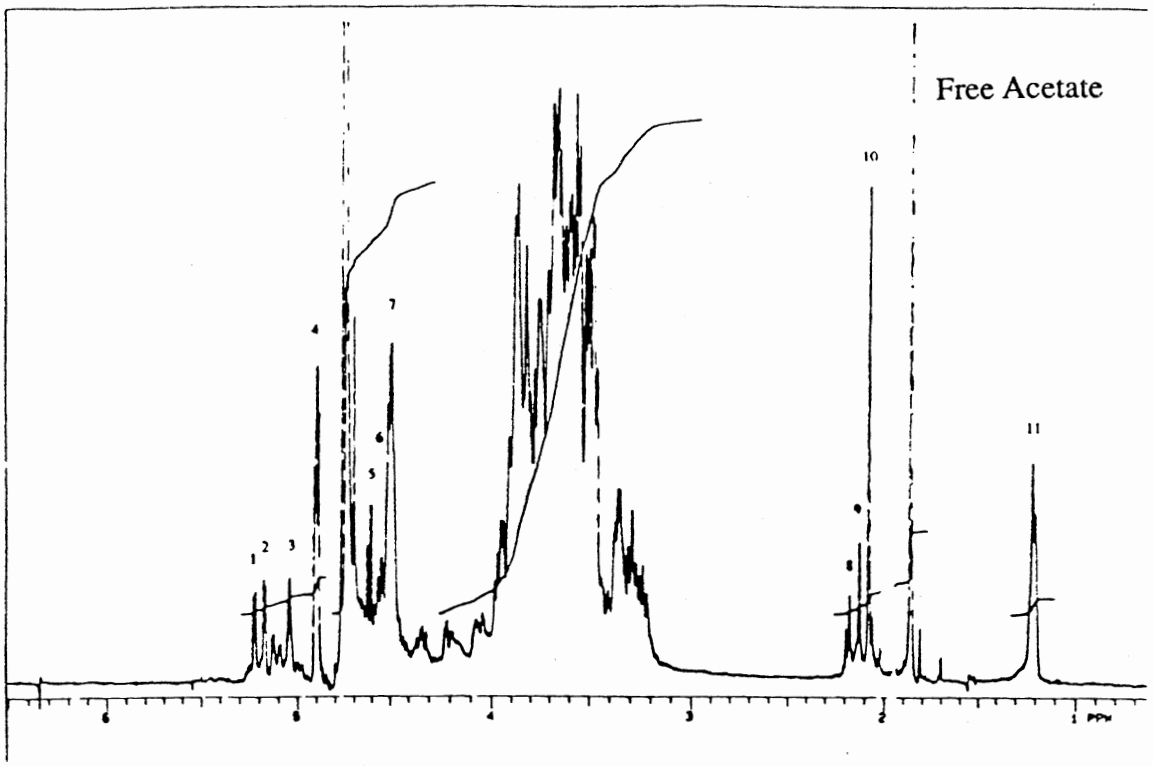


Figure 17. ^{1}H-n.m.r.Spectrum of the Xyloglucan Fragments Generated by Method II. The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25^{0}C in D_2O. Peaks are assigned as follows: 1) H-1 of α-Fuc; 2) H-1 of α-Glc; 3) H-1 of α-Xyl with an α-Fuc-(1-2)-β-Gal at C-2; 4) H-1 of t-α-Xyl; 5) H-1 of t-β-Gal; 6) H-1 of 2-α-Gal; 7) H-1 of 4,6-β-Glc, H-1 of 4-β-Glc, H-1 of 6-β-Glc, H-5 of α-Fuc ; 8, 9, and 10) CH_3 of acetate linked to Gal at different positions; 11) CH_3 of Fuc.

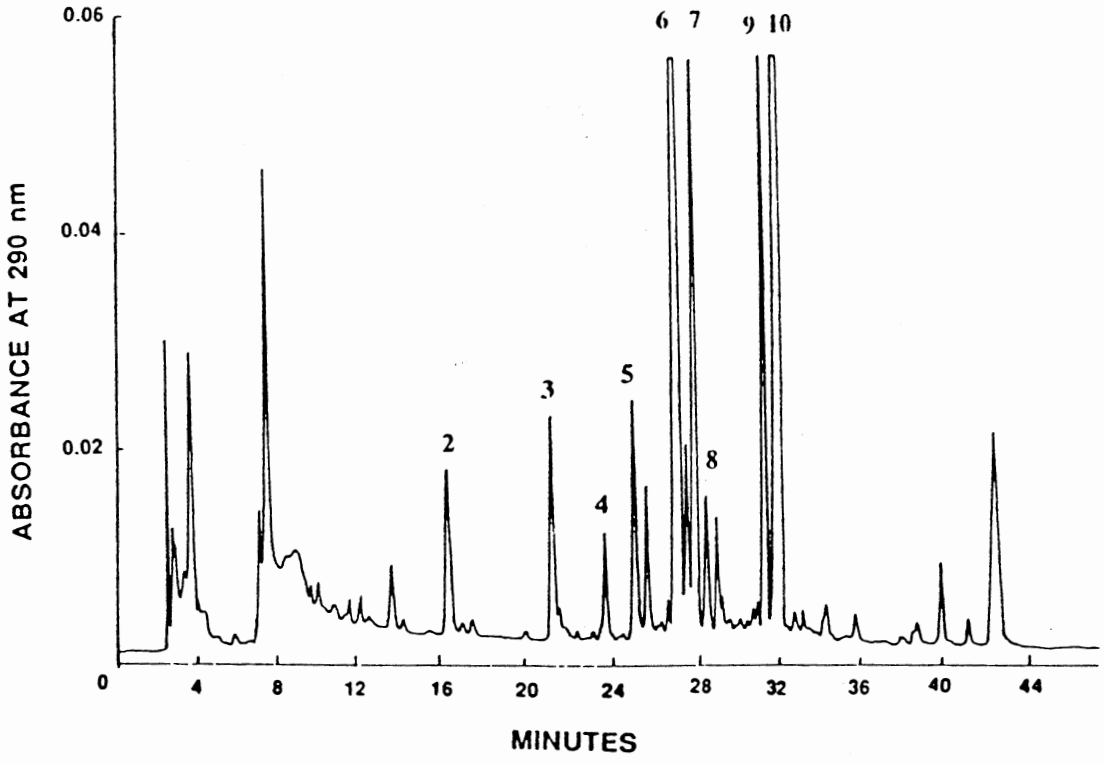


Figure 18. RPC Map of Pyridylamino Derivatives of the Xyloglucan Fragments Generated by Method I. Peaks 2, 3, 4, 5, 6, 7, 8, 9, and 10 are identified as tetra-, penta-, hexa-, isopenta-, hepta-, octa-, isohepta-, deca-, and nonamers of xyloglucan, respectively.

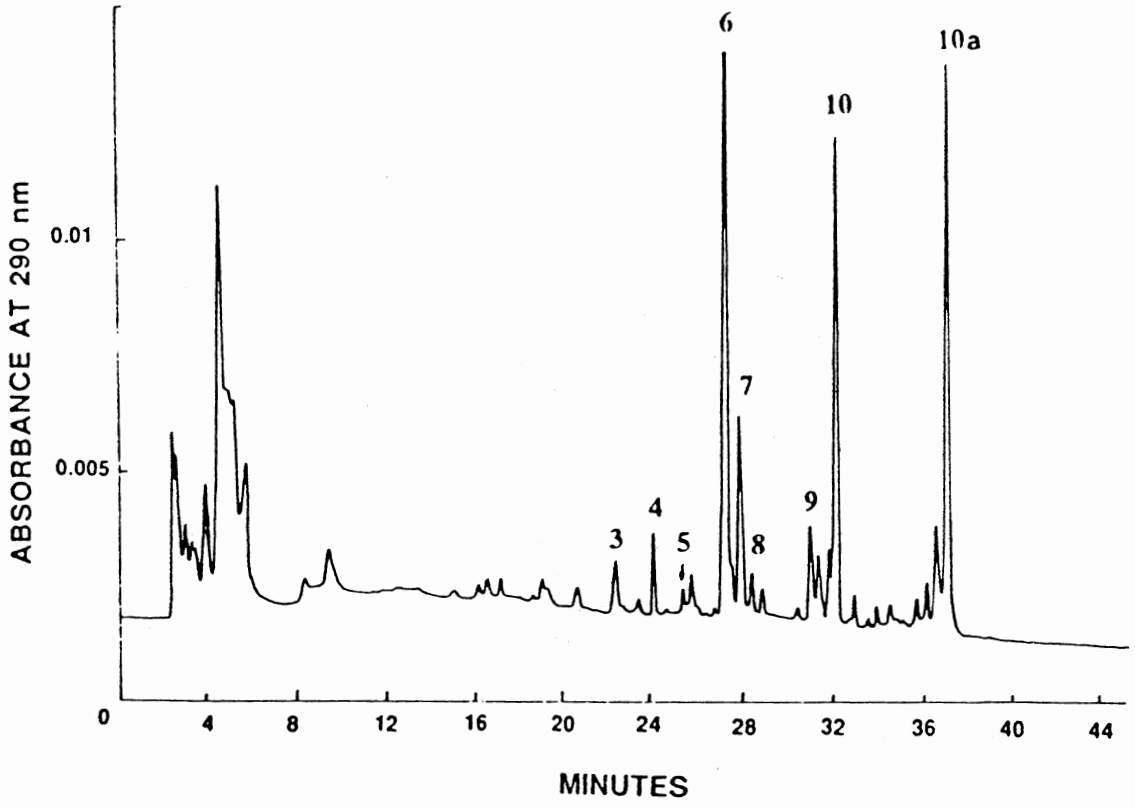


Figure 19. RPC Map of Pyridylamino Derivatives of the Xyloglucan Fragments Generated by Method II. Peaks 3, 4, 5, 6, 7, 8, 9,10 and 10a are identified as ,penta-, hexa-, isopenta-, hepta-, octa-, isohepta-, deca-, nona- and acetylated nonamers of xyloglucan, respectively.

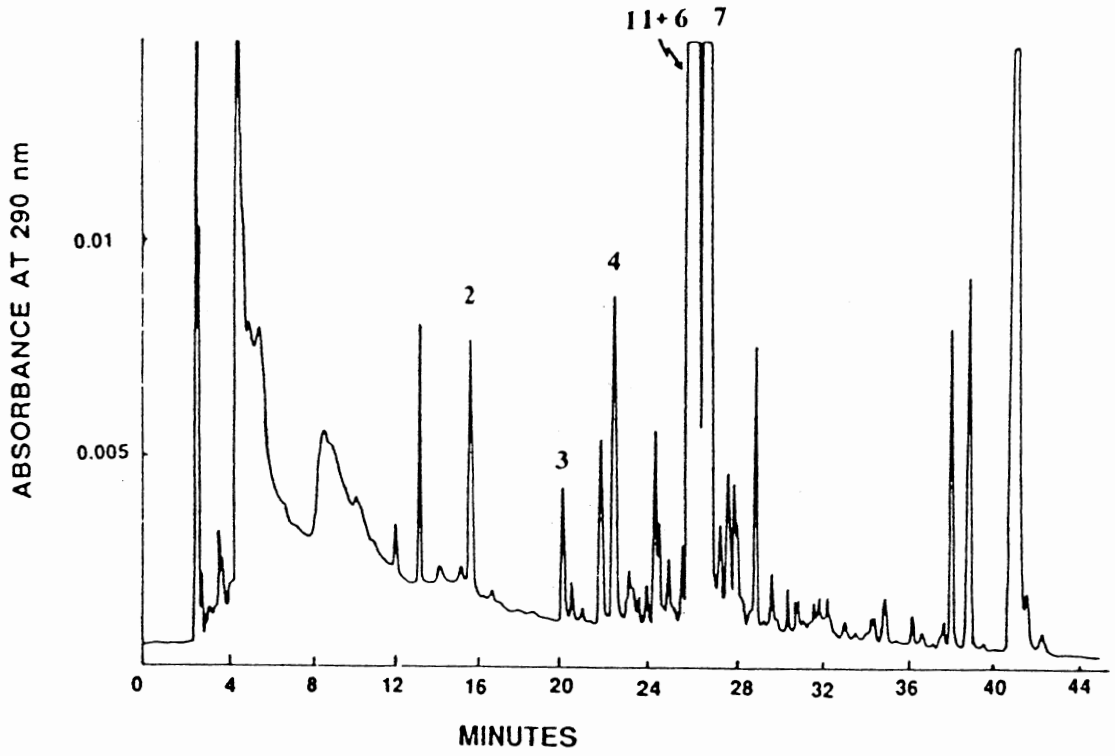


Figure 20. RPC Map of Pyridylamino Derivatives of the Xyloglucan Fragments Generated by Method I from Tamarind Seed Xyloglucan. Peaks 2, 3, 4, 6, 7, and 11 are identified as tetra-, penta-, hexa-, hepta-, octa-, and nonamer with two galactosyl residues attached of xyloglucan, respectively.

LSIMS in the sample mixture was not identified in this chromatogram most likely due to the low abundance of this fragment. Indeed, there are a number of minor peaks in the RPC map which were not identified.

Taking advantage of known structure for tamarind seed xyloglucan, the PA-XG fragments solubilized by the "purified" cellulase from tamarind seed xyloglucan was also subjected to the RPC separation (Figure 20) to confirm the identification of xyloglucan fragments. Due to the lack of fucose in the oligosaccharides of this sample, the PA-XG map is less populated with peaks compared to the map of PA-XG from cotton cell walls. There are no isopentose, isoheptose and the fucosylated nonaose in this chromatogram. However the big peak (11) eluting just before peak 7, was identified to be another nonasaccharide with two galactose residues (Figure 11, structure 11).

It is noted that the chromatographic behavior of the PA-XG fragments is strongly affected by the structures of the xyloglucan oligomers. As expected, due to the non-polar character of the substituent, the presence of an acetyl group on the galactosyl residue in the side chain of the nonasaccharide resulted in a substantial increase in the retention of the oligomer when compared to that of the non-acetylated oligomer. The fucosylated isopentose (structure 5 in Figure 10) is more retarded than pentaose and hexaose. Also isoheptaose (structure 8 in Figure 11), which is fucosylated, is more retarded than heptaose and octaose. This is the expected behavior since the fucosyl residue has a methyl group, and therefore is less polar than any other sugar moieties in the PA-XG oligosaccharides. This imparts to the fucosylated oligosaccharides stronger interaction with the RPC column than that exhibited by the unfucosylated xyloglucan fragments of similar or slightly larger size. It is also apparent from the comparison of the retention behavior of the different xyloglucan fragments that a xylosyl residue imparts to the oligomer a smaller retention increment than that a glucosyl residue. On the other hand, a galactosyl residue produces a retention increment smaller than that produced by xylose and much smaller than that of glucose. An irregular behavior, however, is exhibited by the decasaccharide, which is less

retained than the nonasaccharide, despite the fact that the former had an additional galactosyl residue with respect to the latter. This may be explained by organic solvent induced conformational changes. A fucosyl residue seemed to impart a retention increment similar to or greater than the sum of retention increments of a glucosyl and a xylosyl residue (El Rassi *et al.*, 1991).

Proportions of the XG Oligosaccharides

The ratios of the xyloglucan oligosaccharides from cotton cell walls of suspension culture were deduced based on the relative areas of these oligomer peaks in the RPC chromatogram. This is because each oligomer, no matter what size (molecular weight) it has, was linked with only one 2-aminopyridine molecule at its reducing end, and this is the only source of UV absorbance at 290 nm. The molar ratios of these oligosaccharides, therefore, could be expressed by their peak area ratios which were determined exclusively by the UV absorbance of the attached 2-aminopyridines.

The relative peak areas of the different oligosaccharides generated by method I in the RPC map were normalized to that of the most abundant peak, that of the non-acetylated nonasaccharide, and the molar percent of these oligomers were thereby calculated basing on the peak area ratios (Table 9). It is apparent that the most abundant oligosaccharides in the xyloglucan are nonamer (34.2%), and heptamer (29.1%), followed by the octa-, deca-, penta-, isopenta-, tetra-, isohepta-, and hexamers.

The mole percent of the xyloglucan oligosaccharides generated by method II was also determined according to their relative peak areas which were normalized to the sum of the areas of the non-acetylated and the acetylated nonamers in the RPC map of Figure 19 (Table 9). Here again, the nonasaccharides, including the acetylated and the non-acetylated ones, and the heptasaccharides are the most abundant fragments in this xyloglucan preparation, followed by octa-, deca-, penta-, hexa-, isohepta-, and isopentamers. The lower mole percent of the smaller oligosaccharides, such as tetra-, penta-, and hexamers in

the acetylated xyloglucan sample than in the non-acetylated one could have resulted from a difference in the sample preparations. From HW40S HPLC gel filtration chromatography, only the major xyloglucan oligosaccharide-containing fractions were collected for the former sample compared to the collection of the whole xyloglucan fragments for the latter sample. Therefore it is not surprising that some of the small oligomers were not seen in the acetylated xyloglucan sample.

If the molar ratios of the oligomers were normalized to the heptasaccharide in both preparations (Table 10), the molar ratios between the four major oligomers, i.e. nona-, hepta-, octa-, and decamers in both acetylated and the non-acetylated samples were very similar except a slightly lower content of decamer in acetylated sample. This may have been caused by the fact that some of the acetylated decamers were not identified in the RPC chromatography.

These results also agree fairly well with the reported proportions of xyloglucan oligosaccharides generated by Streptomyces endoglucanase from cotton fiber cell walls (Hayashi *et al.*, 1988) and are similar to those obtained from most dicot cell wall xyloglucans (Fry, 1989).

Preliminary Evidence for Covalent Crosslinking between XG and RGI

Co-solubilization of RGI with XG

Covalent crosslinking between xyloglucan and rhamnogalacturonan I in sycamore suspension culture cell walls was proposed in 1973 based on evidence that some xyloglucan preparations contained galacturonic acid, arabinose and galactose could be solubilized by hydrogen bond breaking agents or endoglucanase digestion (Talmadge, *et al.*, 1973). Since then, no more evidence has been reported to support the existence of this kind of crosslinking between the two polysaccharides. During my attempts to isolate

TABLE 9

MOLE PERCENT OF THE XYLOGLUCAN OLIGOSACCHARIDES GENERATED BY CELLULASE FROM COTTON SUSPENSION CULTURE CELL WALLS

	No. of Peck	Generated by Method I		Generated by Method II	
Oligomer	(structure)	Rel Area*	Mole%	Rel Area**	Mole%
Tetra-	2	0.11	3.8	-	-
Penta-	3	0.12	4.1	0.07	3.1
Hexa-	4	0.05	1.7	0.07	3.1
Isopenta-	5	0.11	3.8	0.03	1.4
Hepta-	6	0.85	29.1	0.66	29.6
Octa-	7	0.30	10.3	0.18	8.1
Isohepta-	8	0.08	2.7	0.04	1.8
Deca-	9	0.30	10.3	0.09	4.0
Nona-	10	1.00	34.2	0.45	20.1
Ac-nona-	10a	-	-	0.55	24.6

* Normalized to that of the nonamer.

** Normalized to that of the sum areas of the nona- and acetylated nonamer.

TABLE 10

MOLAR RATIOS* OF THE XYLOGLUCAN OLIGOSACCHARIDES GENERATED BY CELLULASE FROM COTTON SUSPENSION CULTURE AND COTTON FIBER CELL WALLS

	From Cotton Suspension Culture walls		From Cotton Fiber Walls**
Oligosaccharide	Generated by Method I	Generated by Method II	Generated by *Streptomyces* EG
Tetra-	0.1	-	-
Penta-	0.1	0.1	-
Hexa-	0.1	0.1	-
Isopenta-	0.1	0.1	-
Hepta-	1.0	1.0	1.0
Octa-	0.4	0.3	0.4
Isohepta-	0.1	0.1	-
Deca-	0.4	0.2	0.6
Nona-	1.2	0.7	2.3
Ac-nona-	-	0.8	-

* Normalized to that of the heptamers.

** Hayashi *et al.*, 1988.

rhamnogalacturonan I from the EPG-treated cotton cell wall residue by the two methods described earlier, it was interesting to note that rhamnogalacturonan I, which was not extracted by EPG treatment, was solubilized together with xyloglucan by both methods.

Behavior of XG-RGI Complex on Ion Exchange Chromatography

Chromatography of the alkali-solubilized polysaccharides on a DEAE-Sephadex anion exchange column (Figure 21) shows that more than 80% by weight of the applied materials did not bind to the column and eluted with the loading buffer (Table 11). This unbound fraction was determined to contain both xyloglucan and RGI (see "Isolation of RGI"), suggesting that covalent interaction exits between xyloglucan and RGI. The RGI, which contains 41 mol% of galacturonic acid, would be expected to bind to the column, and the neutral xyloglucan should have eluted by the loading buffer. In fact the RGI isolated from the EPG-treated wall residue by cellulase treatment (method 2) did bind to this column firmly, even though the RGI is partially methylesterified (data not shown).

Decrease in Size of the XG-associated RGI after Cellulase Treatment

After Worthington cellulase digestion of the XG-RGI complex which was solubilized by strong alkali (24%KOH) from the EPG-treated wall residue, the xyloglucan fragments were separated from the RGI by Bio-Sil TSK400 HPLC gel filtration chromatography (Figure 23). Comparing the chromatogram of Figure 23 with that of Figure 22 which shows the elution pattern of XG-RGI complex itself on Bio-Sil TSK400 HPLC column, it was noted that the RGI in Figure 23 eluted later than XG-RGI complex in Figure 22. The RGI in this chromatography, on average, shows a molecular weight of ~200, 000 by comparison to pullulan standards, whereas XG-RGI complex shows a molecular weight of >380, 000. The obvious decrease in size of this complex after cellulase digestion suggests that some kind of strong interaction is present between the xyloglucan and RGI molecules. The fact that no rhamnose and little galacturonic acid were

found in fraction 4 (Vi) from Figure 23 eliminates the possibility of digestion of RGI by cellulase even though Qi (personal communication) has found that the cellulase does contain som EPG activity.

Xylose and Glucose in RGI

In comparison with the sugar compositions of RGI from sycamore and Douglas fir suspension culture cell walls (data not shown), the relative amounts of xylose and glucose in the RGI fractions extracted by the both methods from cotton suspension culture cell walls were much more than what were expected, suggesting that xylosyl and glucosyl residues may have been shared by both RGI and XG molecules before cellulase treatment. In other words, these xylose and glucose residues may be the crosslinking points between the two types of wall polysacchrides.

Much more work needs to be done to obtain an understanding of crosslinking between xyloglucan and rhamnogalacturonan I. A better knowledge of the detailed structures of the two polysaccharides will help the progress in the characterization of the crosslinking.

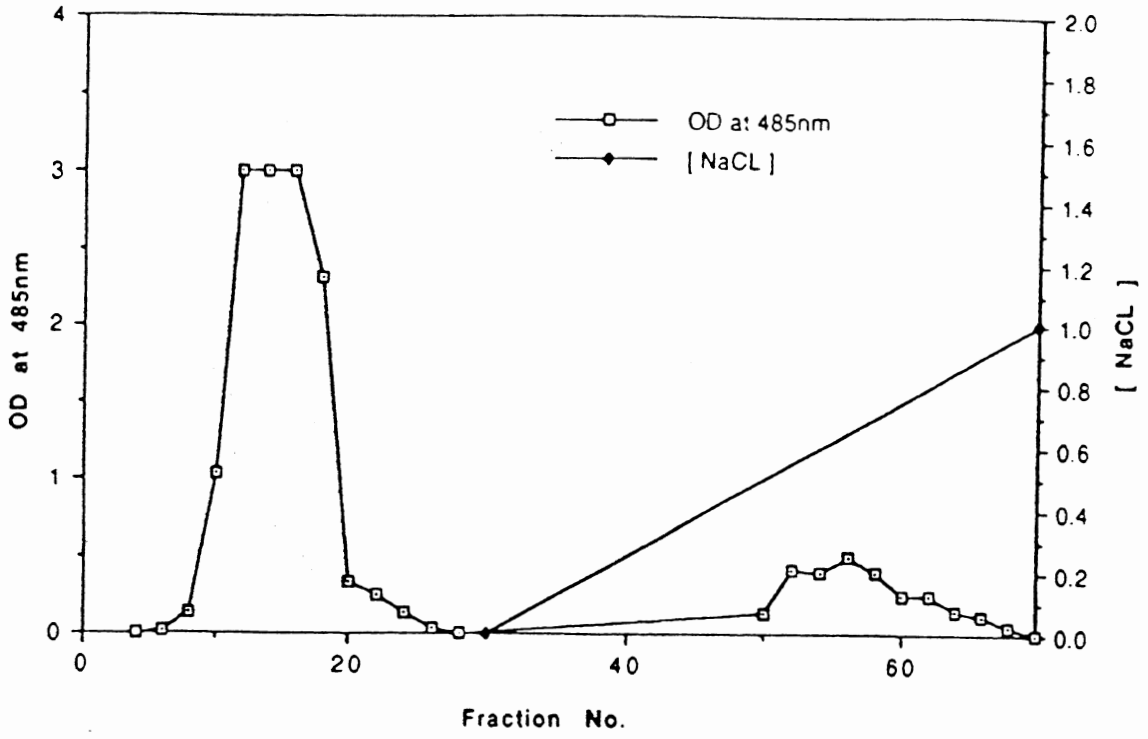


Figure 21. Chromatography on a DEAE-Sephadex Anion Exchange Column of the Material Solubilized by 24%KOH-0.1%$NaBH_4$ from the EPG-treated Cell Wall Residues. Tubes 8-21 were pooled into FN (neutral sugars), and 50-70 into Fc (acidic sugars).

TABLE 11

GLYCOSYL COMPOSITIONS (MOLE%) OF DEAE-SEPHADEX FRACTIONS FROM THE MATERIAL SOLUBILIZED BY 24% KOH-0.1% $NaBH_4$

	Im 216		Acala 44	
Residue	the unbound	the bound	the unbound	the bound
Ara	12.0	10.9	16.6	10.6
Rha	5.9	14.3	11.7	17.8
Fuc	3.9	1.2	3.4	trace
Xyl	27.2	20.9	26.6	16.4
GlcA	3.5	4.4	trace	trace
GalA	8.8	28.5	19.1	38.0
Gal	11.5	12.2	11.4	11.2
Glc	27.6	7.7	11.3	6.0

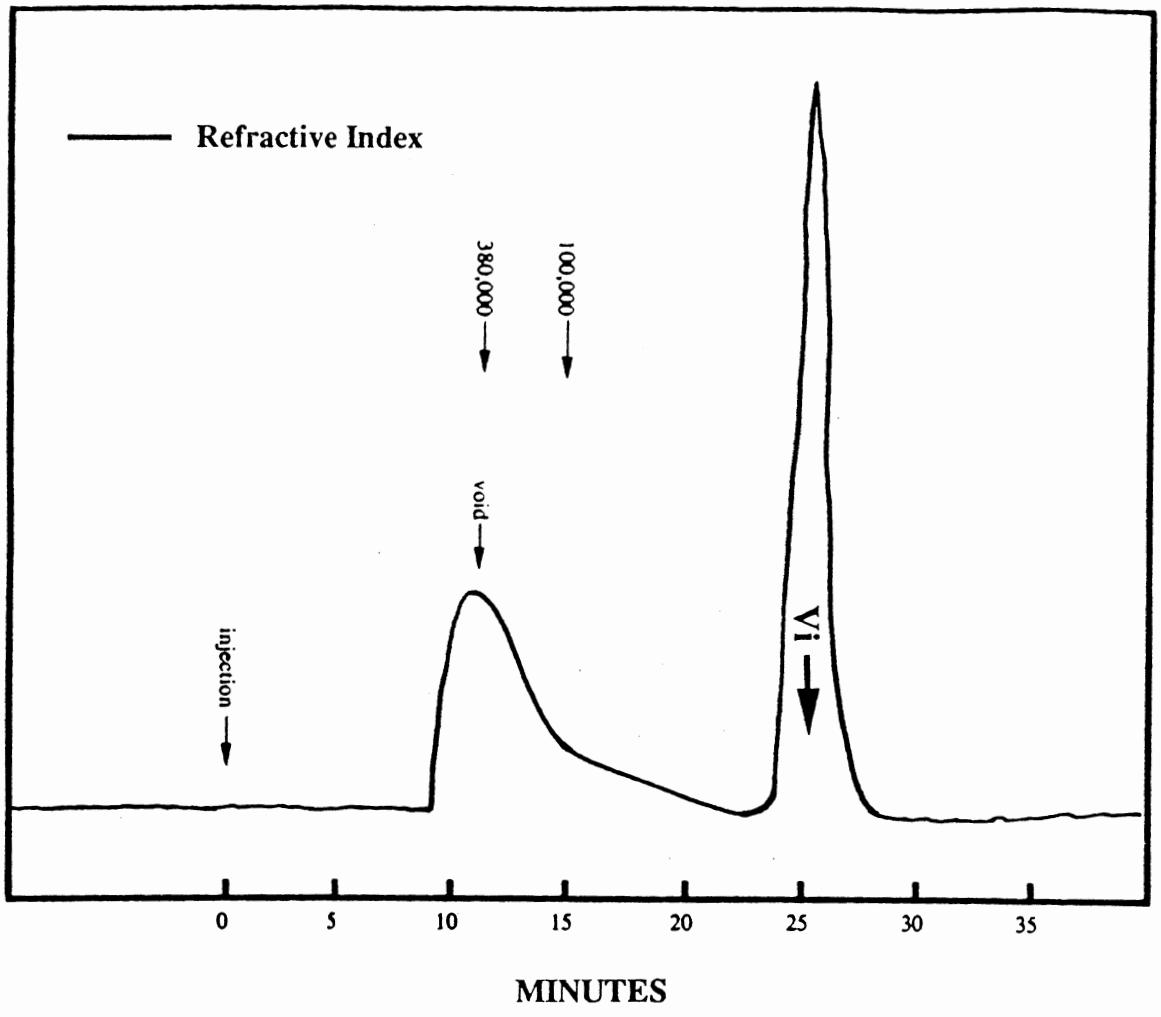


Figure 22. Chromatography on a Bio-Sil TSK400 HPLC Gel Filtration Column of the XG-RGI Complex Solubilized by Strong Alkali from the EPG-treated Wall Residue. Tubes 9- 20 were pooled into fraction 1 (XG-RGI complex), tubes 23-28 into fraction 2 (no sugar found in this fraction).

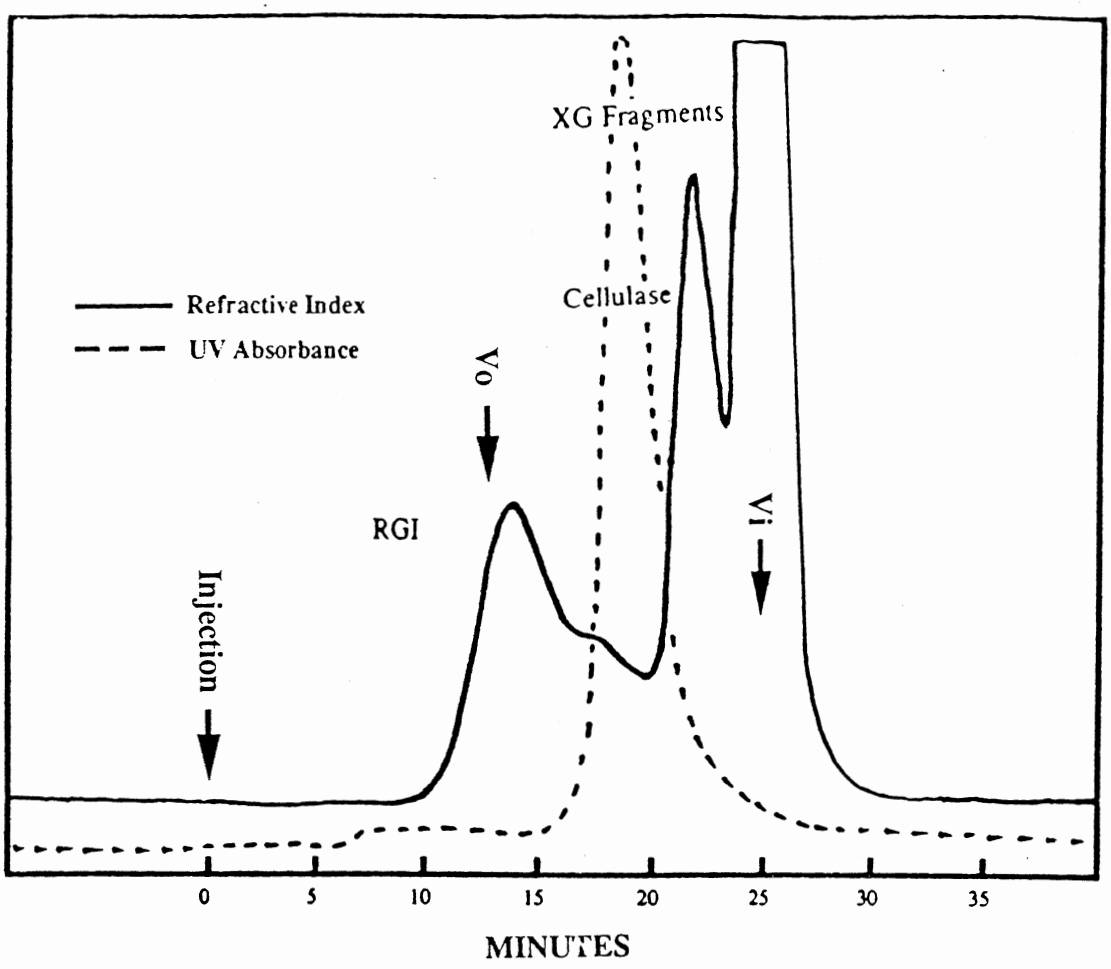


Figure 23. Chromatography on a Bio-Sil TSK400 HPLC Gel Filtration Column of the Cellulase Treated XG-RGI Complex Solubilized by Strong Alkali from the EPG-treated Wall Residue. Tubes 11-16 were pooled into fraction 1 (RGI), tubes 17-20 into fraction 2, 21-24 into fraction 3, and 25-29 into fraction 4.

Isolation of Rhamnogalacturonan I from Cotton Suspension culture Cell Walls

Isolation of RGI from Alkali-solubilized XG-RGI Complex (Method 1)

XG-RGI complex was generated by treating cotton suspension culture cell walls with the endopolygalacturonase, followed by strong alkaline (24%KOH-0.1%$NaBH_4$) solubilization as described as before. Further treatment of the complex with Worthington cellulase, followed by gel filtration chromatography on a Bio-Sil TSK400 HPLC column, separated the RGI from xyloglucan fragments as well as from the enzyme (Figure 23). The mole percent of sugars in fractions 1 to 4 from the chromatography is presented in Table 12. Fractions 1 and 2, accounting for about 50% by weight of the cellulase-treated XG-RGI complex, are rich in rhamnose, galacturonic acid, arabinose and galactose, suggesting that they are RGI-like polysaccharides. The molecular weights of these RGI polysaccharides ranged from 100,000 to 200,000 according to the pullulan standard. But it should be remembered that the pectic polysaccharides may elute earlier than pullulan which is made up of neutral sugars. Fraction 3, having a significant UV absorbance and being very rich in mannose, was presumed to originate from cellulase (a glycoprotein) by comparison with the enzyme control. The sugars in this fraction may be either from the glycosyl moiety of the enzyme or from the impurities of the enzyme preparation, such as the medium used to grow the fungi that produced the cellulase. Fraction 4 contained all the cellulase-solubilized xyloglucan fragments which have been described previously .

Isolation of RGI from the EPG-treated Wall Residues (Method 2)

RGI was also isolated from cotton suspension culture cell walls by directly treating the EPG-treated wall residues with the "purified" cellulase, followed by fractionation with Bio-Sil TSK400 HPLC gel filtration chromatography (Figure 24). The RGI, eluted close to the void volume, exhibited a molecular weight of about 200,000 according to the

TABLE 12

GLYCOSYL COMPOSITIONS (MOLE%) OF BIO-SIL TSK400 GEL FILTRATION FRACTIONS OF THE CELLULASE-DIGESTED XG-RGI COMPLEX

Residue	Fra 1	Fra 2	Fra 3	Fra 4
Ara	9.8	9.7	2.2	5.5
Rha	28.2	29.1	2.8	-
Fuc	-	-	-	3.8
Xyl	4.8	4.9	3.8	32.9
GalA	40.6	38.2	22.7	4.2
Man	-	3.3	44.9	2.2
Gal	14.4	14.8	15.4	11.6
Glc	2.3	-	8.3	39.8

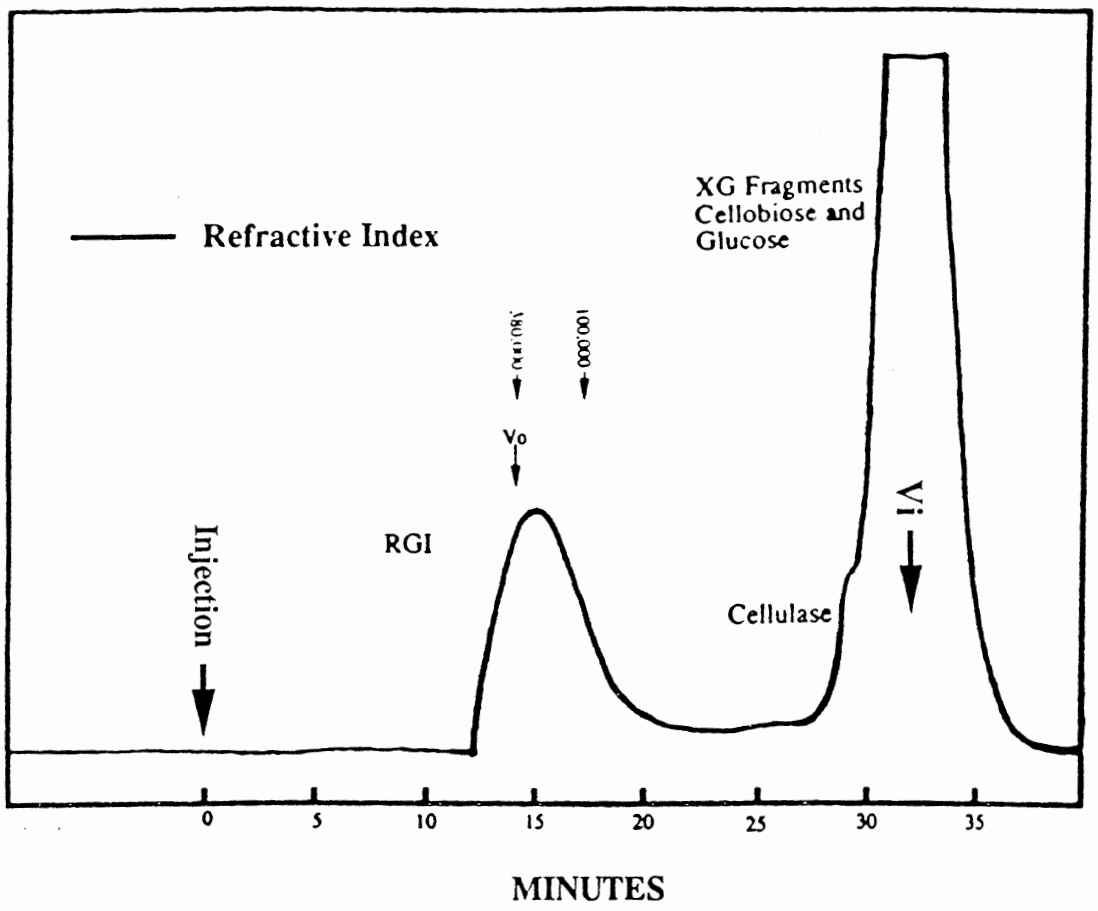


Figure 24. Chromatography on a Bio-Sil TSK400 HPLC Gel Filtration Column of the Fraction Extracted from the EPG-treated Wall Residue by the Cellulase. Tubes 13-21 were pooled into fraction 1 (RGI), and 30-36 into fraction 2 (XG fragments, cellobiose and glucose).

pullulan standard, showing a similar size to that of the RGI isolated by method 1. The sugar compositions of the RGI were also very similar to that of the RGI isolated by method 1 except the former contained a little more galacturonic acid than the latter.

RGI purified in this way represented approximately 40% of the total RGI in the walls. The rest, not solubilized by cellulase, may have been associated with other wall polymers such as extensin. Treating this insoluble material with 24%KOH containing 0.1% $NaBH_4$ solubilized 95% by weight of the material. Fractionation of the soluble material by Bio-Sil TSK400 HPLC gel filtration chromatography gave rise to two fractions with significant UV absorbance (Figure 25). Sugar compositions and hydroxyproline contents of the two fractions are illustrated in Table 13. It is apparent that the sugar compositions of fraction 1 were very similar to these of RGI although the amount of arabinose and galactose residues is slightly less than was expected. On the other hand, fraction 2 was predominantly composed of arabinose. By taking into account the sugar compositions, the hydroxyproline contents and the size estimations, it appears that RGI-like polysaccharides associated with extensin or/and other polymers may have comprised fraction 1, whereas the oligoarabinans associated with extensin segments may have comprised fraction 2. The hydroxyproline content in the two fractions (2.1mg) accounts for about 70% by weight of the total hydroxyproline in the treated walls (1g), assuming that hydroxyproline makes up approximately 0.3-0.4% of the weight of the cotton suspension cultured cell walls.

Analysis of Rhamnogalacturonan I

Glycosyl Compositions of RGI

Glycosyl compositions of the RGIs isolated by the two methods are presented in Table 14. It was noted that the sugar compositions of RGIs from cotton cell walls are similar to those of the RGIs from sycamore cell walls (McNeil *et .al.*,1980, and Ishii *et al.*,

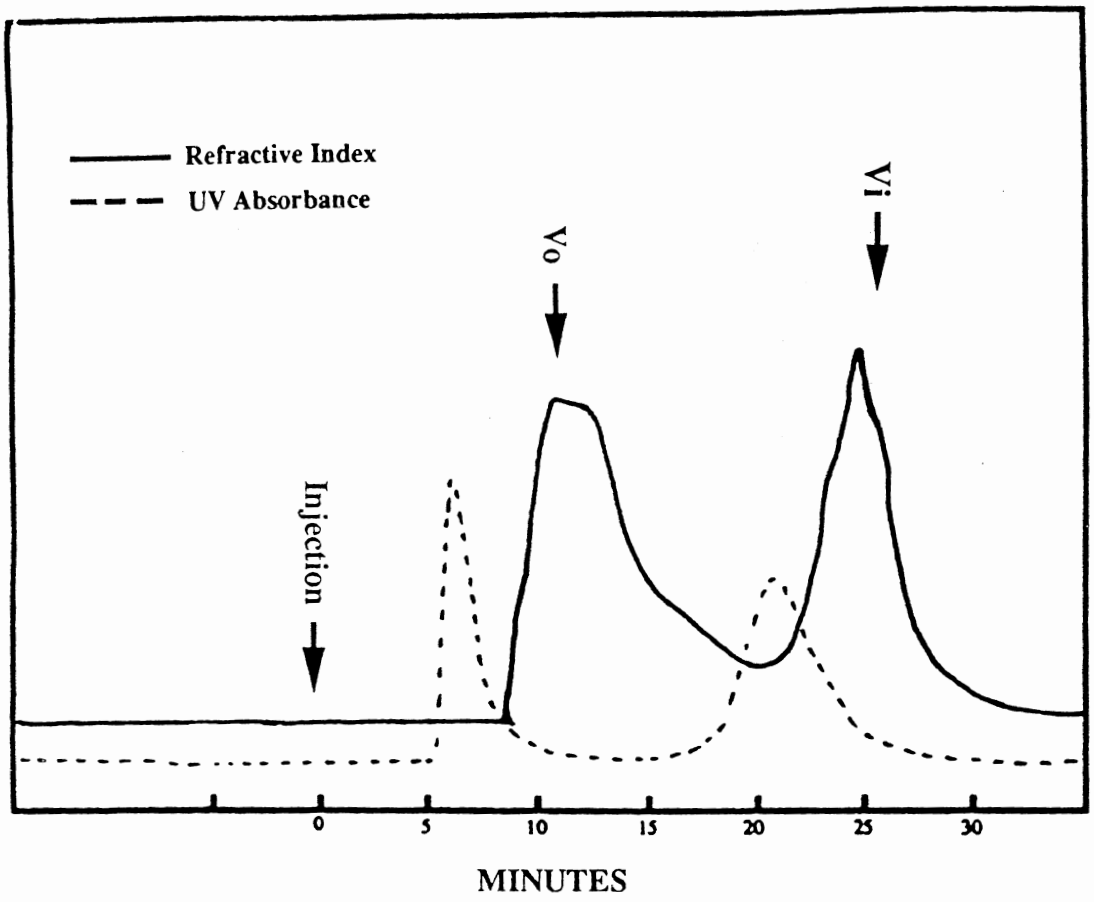


Figure 25. Chromatography on a Bio-Sil TSK400 HPLC Gel Filtration Column of the Strong Alkali-solublized Materials from the Insoluble Portion of the Cellulase-treated Wall Residue. Tubes 10-20 were pooled into fraction 1, and 22-29 into fraction 2.

TABLE 13

GLYCOSYL COMPOSITIONS OF THE FRACTIONS FROM BIO-SIL TSK400 HPLC OF THE 24%KOH-0.1%$NaBH_4$-SOLUBILIZED MATERIAL FROM THE CELLULASE-TREATED RESIDUE OF THE EPG-TREATED RESIDUE

	Fra 1		Fra 2	
Residue	Mole%	Rel Mole to Rha	Mole%	Rel Mole to Rha
Ara	8.3	0.2	69.0	10.3
Rha	38.9	1.0	6.7	1.0
Xyl	4.7	0.1	3.8	0.6
GalA	36.3	1.0	10.1	1.5
Man	-	-	2.5	0.4
Gal	11.8	0.3	2.5	0.4
Glc	-	-	5.4	0.8
wt% of the treated walls	10.7		7.2	
Hyp in the fractions generated from 1 g of cell walls	0.52mg		1.58mg	

TABLE 14

COMPARISON OF GLYCOSYL COMPOSITIONS BETWEEN COTTON AND SYCAMORE SUSPENSION CULTURE CELL WALL RHAMNOGALACTURONAN I

	Cotton Suspension Culture Cell Wall RGI				Sycamore Suspension Culture Cell Wall RGI[d]			
	Isolated by Method 1		Isolated by Method 2		Isolated by EPG		Isolated by EPG+Na_2CO_3	
Residue	Mole%	Rel Mole	Mole%	Rel Mole[c]	Mole%	Rel Mole	Mole%	Rel Mole
Ara	13.4 (4.5)[a]	0.5	13.6 (3.4)[b]	0.7	24	1.6	25	1.9
Rha	26.0 (2.9)	1.0	18.9 (2.1)	1.0	15	1.0	13	1.0
Fuc	0.5 (0.5)	trace	0.6 (0.8)	trace	2.	0.1	1	0.1
Xyl	6.5 (3.8)	0.3	7.7 (0.5)	0.4	1	0.1	6	0.5
GalA	38.5 (4.1)	1.5	38.9 (2.0)	2.1	36	2.4	20	1.5
Gal	12.2 (3.1)	0.5	13.7 (1.5)	0.7	20	1.3	29	2.3
Glc	2.6 (0.4)	0.1	5.0 (2.3)	0.3	1	0.1	6	0.5

[a] Standard deviation of the duplicate samples.

[b] Standard deviation of the triplicate samples.

[c] The molar ratio for all the RGIs in this table is normalized to one of Rha residue.

[d] McNeil *et al.*,1980 and Ishii *et. al.*, 1989.

1989) except that the former contain significantly less arabinosyl and galactosyl residues, which are the major sugar residues for RGI sidechains. This suggests that the RGIs in cotton cell walls may have either fewer or shorter sidechains than the RGIs in sycamore cell walls. In other words, either the degrees of backbone branching or the sidechain structures of the cotton cell wall and the sycamore cell wall RGIs are different from each other to some extent. In addition, the ratios of GalA to Rha residue in the RGIs isolated from cotton cell walls by the two methods, instead of the compositions of 1 : 1 proposed by Albersheim's group for sycamore cell wall RGI, are about 1.5 : 1 and 2 : 1, respectively. However, the published sycamore RGI data also have the ratio greater than 1 : 1 shown in Table 14.

Size Estimation of the RGI

The sizes of the RGIs isolated by the two methods were estimated by the HPLC gel filtration chromatography on a Bio-Sil TSK400 column by comparison with the pullulan standard. The RGI isolated by method 1 shows a molecular weight of about 100,000 - 200,000 (Figure 23), and the RGI isolated by method 2 shows a molecular weight slightly higher than that of the RGI isolated by method 1, closer to 200,000 (Figure 24). Since RGI is a branched and charged polysaccharide, the molecular weight determined here compared to pullulan standard, a neutral polysaccharide, may be lower than the real one. Therefore, an average molecular weight of 200,000 is a reasonable estimate for the RGI in cotton cell walls, and this is very similar to the molecular weight estimated for RGI isolated from sycamore suspension cultured cell walls.

Glycosyl-linkage Compositions of the RGI

Glycosyl-linkage compositions of the RGIs isolated by both methods were determined by GC-MS of the partially methylated alditol acetates as described earlier. The gas chromatograms of the derivatized RGIs purified by method 1 and method 2 are

presented in Figure 26 and Figure 27, respectively. The different linkage components of the RGI were identified according to their fragmentation patterns by mass spectrometry. Mole percent of the glycosyl-linkage compositions of the RGIs isolated with two methods are shown in Table 15. The RGI isolated by the two different methods have almost identical glycosyl-linkage compositions except a slight difference in the proportions. The relative amounts of 2-linked and 2,4-linked rhamnosyl residues in the RGI isolated by method 1 and method 2 are about 1 : 1.1, and 1 : 1.3, suggesting that about half of the rhamnosyl residues in cotton RGI are branched, and that the attachment sites of these branched rhamnoses are at position 4. The absence or failure to identify 2, 3, 4-linked rhamnosyl residue in the both RGIs illustrates that there are very few or no doubly branched rhamnosyl residues in the backbone of cotton RGIs, in contrast to the sycamore RGI isolated with mild alkali, which was reported to contain some doubly branched rhamnosyl residues (Ishii *et. al.*, 1989). The relatively higher content of 5-linked arabinosyl and 4-linked galactosyl residues in the RGI isolated by method 2 than in the RGI isolated by method 1 suggests that the RGI isolated by method 2 has longer sidechains on average than the RGI isolated by method 1. This is also consistent with the fact that the former contains more arabinosyl and galactosyl residues than the latter.

The average length of the sidechains of cotton RGI, however, is apparently much shorter than that of the RGI isolated from sycamore cell walls. This conclusion results from the molar ratio of sidechain glycosyl residues (mainly arabinose and galactose) to that of the branched 2,4-linked rhamnosyl residues in the backbone, which was only about 2 to 1 or 3 to 1. This indicates that the sidechains of cotton cell wall RGI are only about 2 to residues long on average, contrasting to sycamore RGI which has an average sidechain length of 6 residues (McNeil, *et al.*, 1980). Taking into account the branched galactosyl and arabinosyl residues, such as 2,5-linked (~1 mol%) and 3,5-linked (0.5-1.7 mol%) arabinoses, 3,6-linked (1.9 mol%) and 4,6-linked (1.4-4.7 mol%) galactoses, which account for some of the branchpoints other than branched rhamnoses, it appears that there

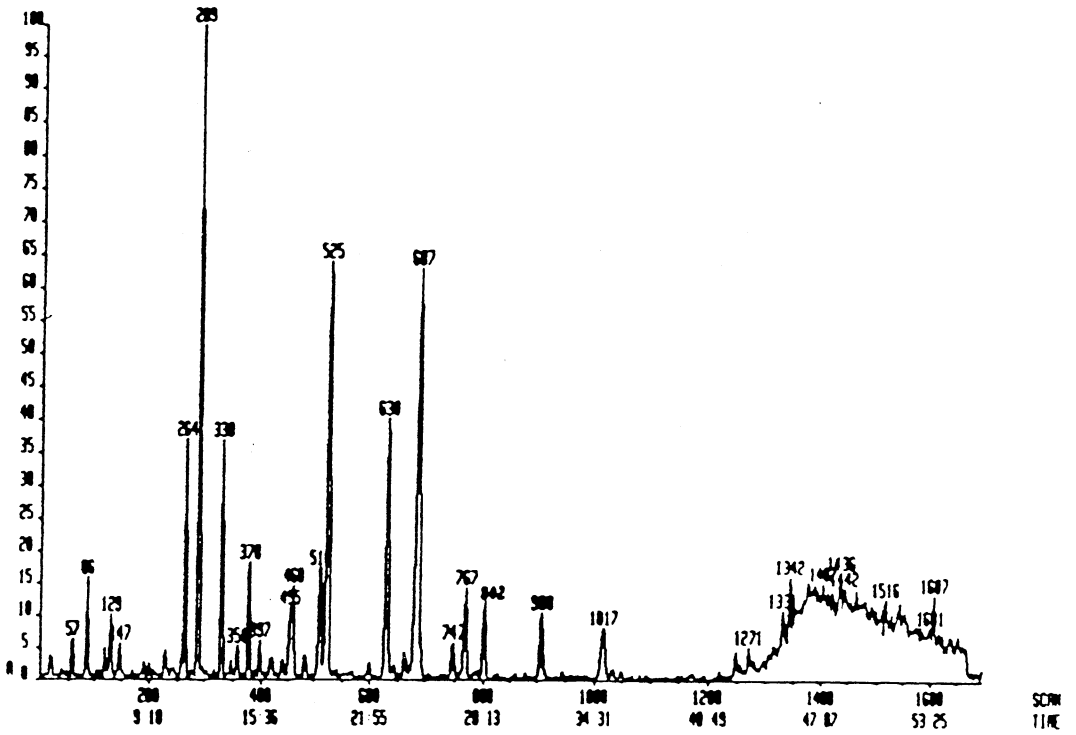


Figure 26. Gas Chromatogram of the Partially Methylated Alditol Acetate Derivatives of the RGI Isolated by Method 1 from XG-RGI Complex by the "Purified" Cellulase Followed by Gel Filtration Chromatography. Pecks were identified from the mass spectra of these signals. 330: t-Ara, 378: t-Xyl, 422: t-Fuc, 460: 3-Ara, 510: 5-Ara, 525: 2-Rha, 630: t-Gal, 644: 3,5-Ara, 661: 2,5-Ara, 687: 2,4-Rha, 767: 4-Gal, 802: 6-Glc, 908: 3,6-Gal, 1017: 4,6-Gal.

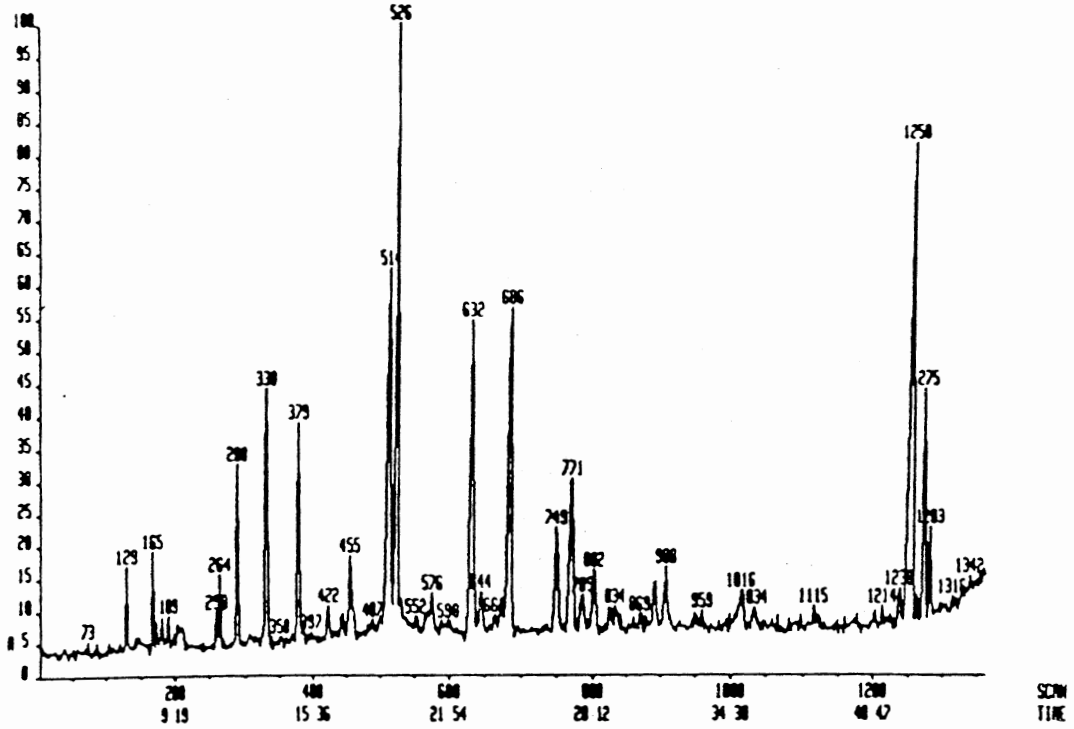


Figure 27. Gas Chromatogram of the Partially Methylated Alditol Acetate Derivatives of the RGI Isolated by Method 2 from the EPG-treated Wall Residues by the Cellulase Followed by Gel Filtration Chromatography. Pecks were identified from the mass spectra of these signals. 330: t-Ara, 379: t-Xyl, 422: t-Fuc, 455: 3-Ara, 510: 5-Ara, 526: 2-Rha, 576: t-Glc, 632: t-Gal, 644: 3,5-Ara, 661: 2,5-Ara, 686: 2,4-Rha, 771: 4-Gal, 785: 4-Glc,802: 6-Glc, 890: 6-Gal ,908: 3,6-Gal, 1016: 4,6-Gal.

TABLE 15

COMPARISON OF THE GLYCOSYL-LINKAGE COMPOSITIONS (MOLE%) OF THE RGIs ISOLATED FROM COTTON AND SYCAMORE SUSPENSION CULTURE CELL WALLS

Residue	Deduced Linkage	Cotton RGI (Method 1)	Cotton RGI (Method 2)	Sycamore RGI (EPG)
Rha	Terminal	-	-	2.2
	2-linked	22.7	17.7	9.5
	2,4-linked	23.8	13.4	9.8
	2,3,4-linked	-	-	0.7
Fuc	Terminal	0.4	1.0	1.7
Xyl	Terminal	4.4	7.4	2.4
Ara	Terminal	9.8	7.7	11.6
	2-linked	-	-	2.5
	3-linked	4.8	4.0	2.5
	5-linked	6.4	14.8	14.1
	2,5-linked	1.0	1.0	0.8
	3,5-linked	0.5	1.7	4.3
Gal	Terminal	13.9	12.3	7.7
	2-linked	-	-	0.7
	4-linked	trace	6.6	10.3
	3-linked	-	-	3.3
	6-linked	3.8	2.5	9.2
	3,6-linked	-	1.9	2.9
	4,6-linked	4.7	1.5	2.6
	2,6-linked	-	-	0.9
Glc	Terminal	-	1.5	-
	4-linked	-	3.2	-
	4-linked	3.8	2.2	-

must be some structurally different sidechains attached to the backbone of the RGI although they may not be as many as in sycamore RGI. This further suggests that a considerable fraction of the RGI sidechains must consist of only one residue since more residues must be in the branched sidechains.

NMR Analysis of the RGI

The ^{1}H n.m.r. spectra of the RGIs isolated by method 1 and method 2 were recorded with a Varian XL-400 n.m.r. spectrometer at $70^{0}C$, and are illustrated in Figure 28 and Figure 29, respectively. The spectra of the RGIs isolated by both methods are similar except the RGI isolated by method 2 showed a signal for acetate ester in its spectrum. Due to the complexity of the RGI n.m.r. spectra, in which many signals having the same or close chemical shifts together, only the major signals were putatively identified on the basis of their specific chemical shifts, of their behavior in 2D-n.m.r spectra (not shown). Signal 1 was identified as H-1 of arabinose, signal 2 was identified as H-1 of α-rhamnose, signal 3 as H-1 of galacturonic acid, signal 4 as H-5 of galacturonic acid, and H-1 of β-galactose (or glucose), signal 5 as H-4 of galacturonic acid, signal 6 as H-2 of α-rhamnose, signal 7 as H-3 of galacturonic acid, signal 8 as H-3 of α-rhamnose, signal 9 as H-4 of α-rhamnose, signal 10 as acetate ester, signal 12 as the methyl group from the 2,4-linked rhamnose and signal 13 as the methyl group from the 2-linked rhamnose. Signal 13 has about duble intensity of signal 12, indicating that the ratio of 2-linked Rha to 2,4-linked Rha is about 0.5 : 1. This is not consistent with the result (less reliable) of the glycosyl-linkage analysis by GC-MS. The identification of the signals for acetate ester in the spectrum of RGI isolated by method 2 indicates the presence of acetylation in the RGI molecules. The signals with different chemical shifts for acetate ester suggest that more than one type of acetylation, in other words, acetylation at different positions, or different sugar residues, may exist in the RGI molecules.

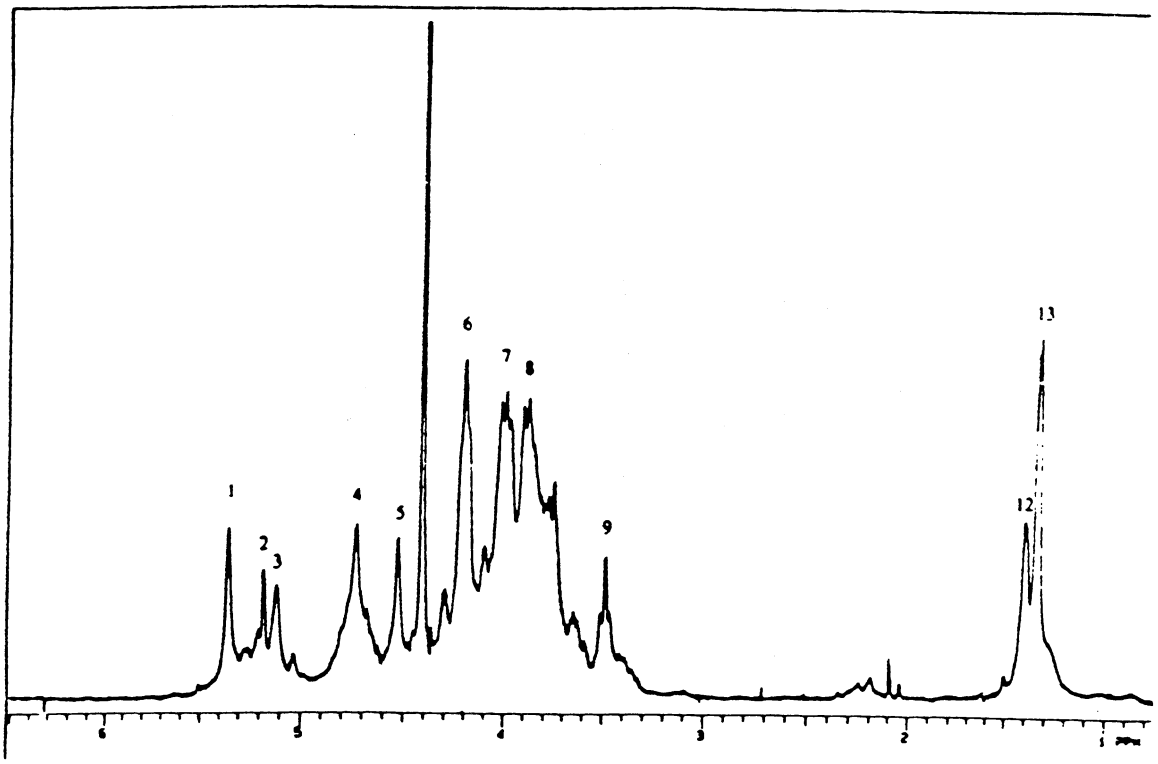


Figure 28. ^{1}H-n.m.r. Spectrum of the RGI Isolated by Method 1. The spectrum was recorded on a Varian XL-400 n.m.r. spectrometer at 70^{0}C in D_2O. Peaks are assigned as follows: 1) H-1 of Ara, 2) H-1 of α-Rha, 3) H-1 of GalA, 4) H-5 of GalA, and H-1 of β-Gal (or Glc), 5) H-4 of GalA, 6) H-2 of α-Rha, 7) H-3 of GalA, 8) H-3 of α-Rha, 9) H-4 of α-Rha, 12) CH_3 of the 2,4-α-Rha and 13) CH_3 of the 2-α-Rha.

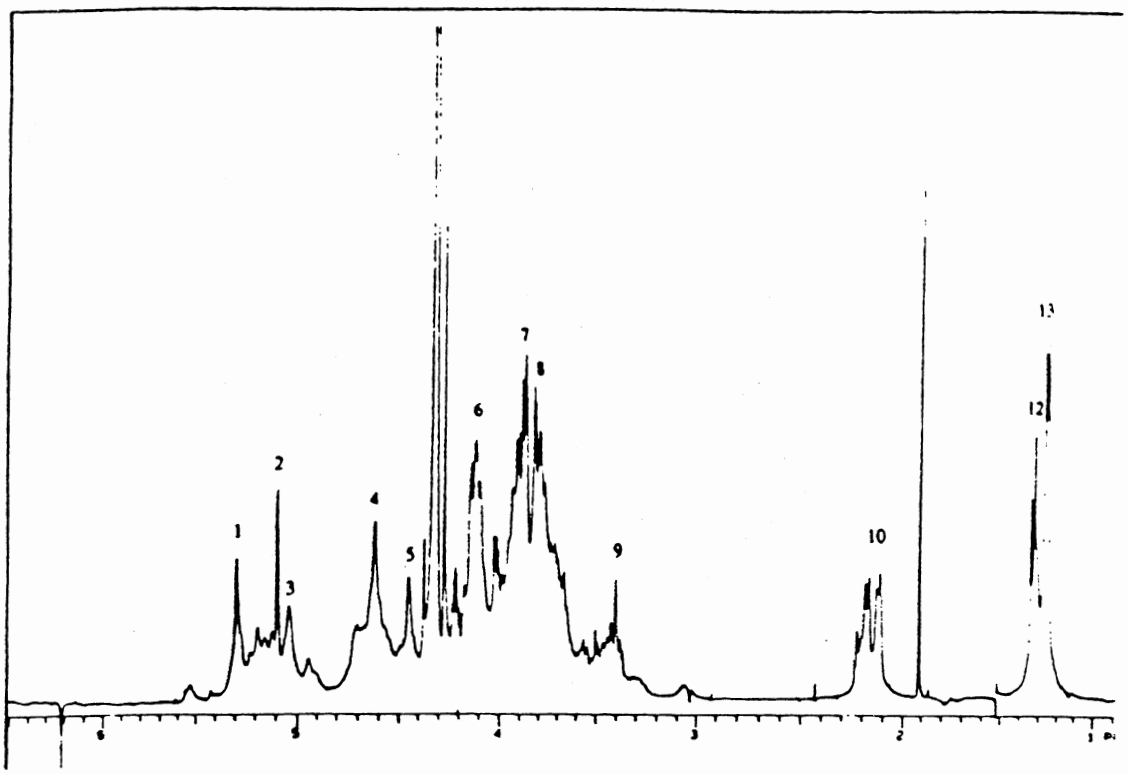


Figure 29. ^{1}H-n.m.r. Spectrum of the RGI Isolated by Method 2. The spectrum was recorded on a Varian XL-400 n.m.r. spectrometer at 70^{0}C in D_2O. Peaks are assigned as follows: 1) H-1 of Ara, 2) H-1 of α-Rha, 3) H-1 of GalA, 4) H-5 of GalA, and H-1 of β-Gal (or Glc), 5) H-4 of GalA, 6) H-2 of α-Rha, 7) H-3 of GalA, 8) H-3 of α-Rha, 9) H-4 of α-Rha, 10) CH_3 of acetate ester, 12) CH_3 of the 2,4-α–Rha and 13) CH_3 of the 2-α-Rha.

DOM Determination of the RGI Isolated with Method 2

The degree of methylesterification of the RGI isolated with method 2 was determined by the selective reduction of methyl esterified galacturonic acid residues to galactose (Maness and Mort, 1989). About 13% of the galacturonic acid residues in the RGI was found to be methylesterified.

Analysis of the RGI with Ion Exchange Chromatography

DEAE-Sephadex Chromatography

From sugar composition analysis of the RGIs isolated by both methods it was found that the molar ratio of galacturonic acid and rhamnose residues was about 2 : 1 and about 1.5 : 1, respectively (Table 14). Assuming that the backbone of RGI is composed of the repeating units of GalA-Rha as reported by Albersheim's group (McNeil *et. al.*, 1980, 1982, 1884), the extra galacturonic acid which accounts for about 10-20% of the total sugars in the RGI, has to be explained. In order to determine if there were homogalacturonans which co-isolated with RGI together, the RGI isolated by method 2 was subjected to DEAE-Sephadex ion exchange chromatography (Figure 30). Sugar compositions of the fractions from the column are shown in Table 16. Fraction 1, which was eluted at concentrations of about 0.3 to 0.5M ammonium acetate, contained a higher ratio of galacturonic acid to rhamnose (2.5 : 1) than fraction 2 which was eluted with 0.6 to 0.8M ammonium acetate. The latter had the ratio of 1.5 : 1.

DOM analysis indicates that the galacturonic acid in fraction 1 has a DOM of 13%, and the galacturonic acid in fraction 2 has a DOM of 11%. If the RGI were co-isolated with homogalacturonans, the deduced homogalacturonans in fraction 1 would have a DOM of 26%, and that in fraction 2 would have a DOM of 22% (GalA in the RGI backbone has no methylesterification) (Komalavilas and Mort, 1989). The elution patterns for the two fractions, therefore, may be explained by their difference in DOM, in other words, the RGI

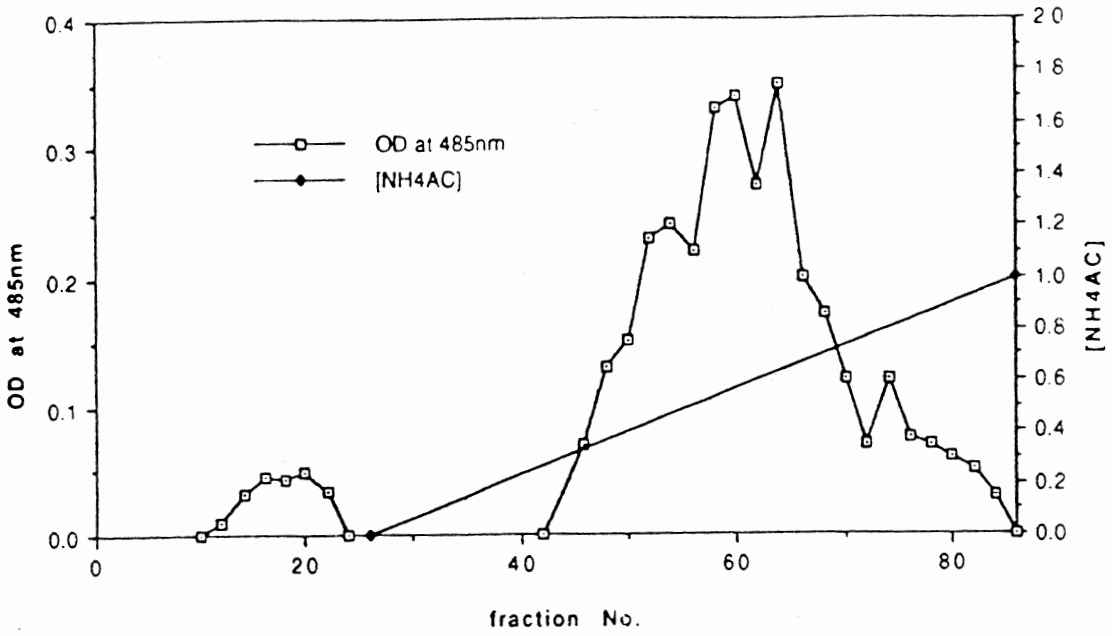


Figure 30. Chromatography on a DEAE-Sephadex Anion Exchange Column (1.2X30 cm) of RGI Isolated by Method 2. Tubes 11-24 were pooled into RGIn, tubes 43-57 into fraction 1, tubes 58-71 into fraction 2, and tubes 72-86 into fraction 3.

TABLE 16

GLYCOSYL COMPOSITIONS (MOLE%) OF DEAE-SEPHADEX ANION EXCHANGE FRACTIONS OF THE RGI ISOLATED BY METHOD 2

Residue	Fra 1	Fra 2	Fra 3	Fra 4
Ara	10.1	22.0	12.0	10.3
Rha	trace	13.4	27.6	27.3
Fuc	-	-	-	-
Xyl	7.5	12.2	7.5	9.2
GalA	9.0	33.3	38.4	28.7
Man	-	4.2	1.9	8.0
Gal	3.5	7.7	9.5	8.5
Glc	68.8	7.2	3.0	7.7

with higher DOM would bind to the column less firmly than the RGI with lower DOM. In addition, more neutral sugars such as xylose and arabinose present in fraction 1 than in fraction 2 may also be a big contribution to the looser binding of fraction 1 to the column. These results suggested that there might be some methylesterified homogalacturonans co-isolated with RGI.

CarboPac PA-1 Ion Exchange Chromatography

In order to determine if there are methylesterified homogalacturonans co-isolated with RGI together, the methylesterified RGI (Me-RGI), and the de-methylesterified RGI (De-Me-RGI) were applied on a CarboPac PA-1 HPLC anion exchange column (Figure 31). Sugar compositions of the fractions of the Me-RGI and De-Me-RGI from the chromatography are presented in Tables 17 and 18. The elution patterns of the Me-RGI and the De-Me-RGI are very similar, except the De-Me-RGI was eluted a little later than the Me-RGI. The fact that fraction 2 for Me-RGI contained a ratio of GalA/Rha of 2.33 : 1 compared to the ratio of 1.4 : 1 in the fraction 2 for De-Me-RGI may be due to the result that some RGI containing more methylesterified GalA bound more firmly to the column after demethylesterification. This is further explained by the observation that fraction 3 for Me-RGI had the ratio of GalA/Rha 2 : 1, smaller than that (2.3 : 1) of fraction 3 for De-Me-RGI.

DOM of fraction 1, 2, 3, and 4 for Me-RGI were determined (Table 19). A decrease in DOM from fraction 2 to fraction 4 is consistent with the explanation that the lower the DOM, the tighter the binding of the RGI (or HG) to the column.

To answer the question as to whether there are really methylesterified homogalacturonans which co-isolated with RGI, a commercial non-methylesterified pectic acid (HG) from Aldrich Chemical Company was subjected as a control to the same CarboPac PA-1 ion exchange chromatography under the same conditions that were used for Me-RGI and De-Me-RGI (Figure 31). The HG was found to be eluted much later, in

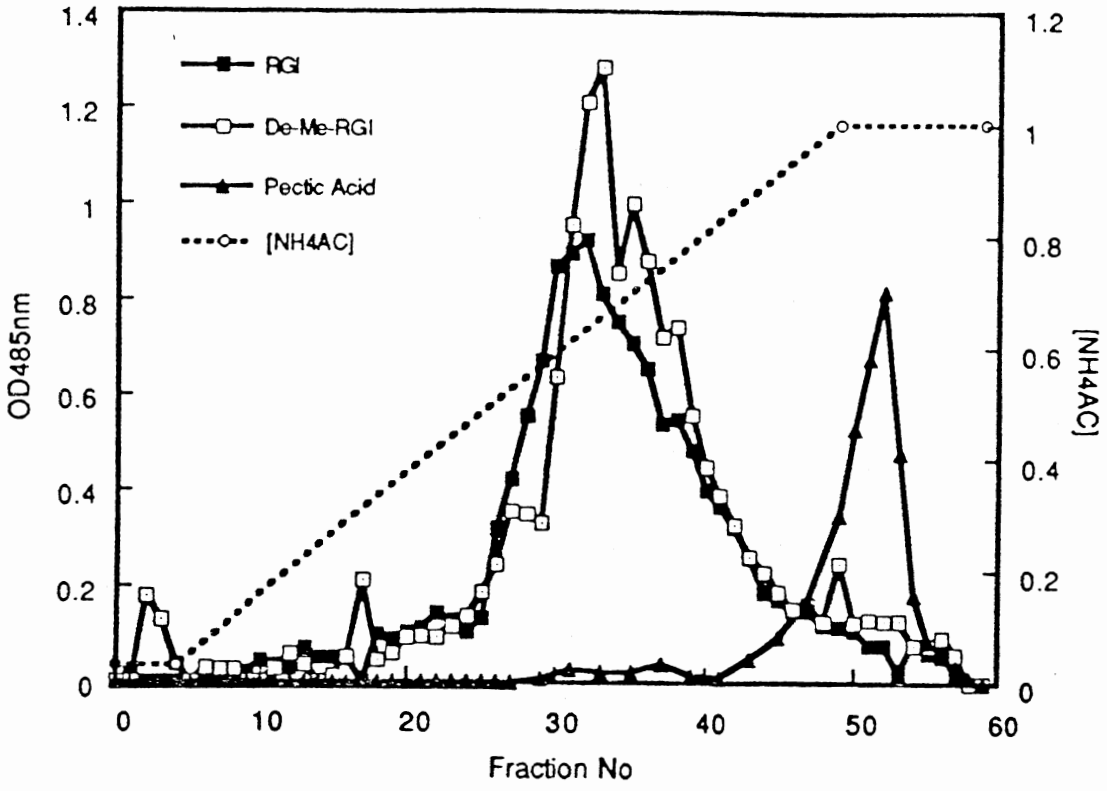


Figure 31. Chromatography on a Dionex CarboPac PA-1 HPLC Ion Exchange Column of the Methylesterified RGI, Demethylesterified RGI Isolated by Method 2 and a Non-methylesterified Homogalacturonan (Pectic Acid) from Aldrich Chemical Company. Tubes 15-24 were pooled into fraction 1, 25-30 into fraction 2, 31-35 into fraction 3, 36-44 into fraction 4 and 45-60 into fraction 5 for both De-Me-RGI and Me-RGI.

TABLE 17

GLYCOSYL COMPOSITIONS OF THE FRACTIONS FROM DIONEX PA1 ION EXCHANGE CHROMATOGRAPHY OF THE RGI ISOLATED BY METHOD 2

(Relative Molar Ratio Normalized to That of Rha)

Residue	Fra 1	Fra 2	Fra 3	Fra 4	Fra 5
Ara	0.6	0.7	0.3	0.3	0.2
Rha	1.0	1.0	1.0	1.0	1.0
Xyl	1.4	0.5	0.3	0.6	0.3
GalA	2.6	2.3	2.0	1.6	1.5
Man	0.3	-	-	-	-
Gal	0.8	0.4	0.4	0.4	0.3
Glc	0.5	0.1	0.1	0.3	0.2

TABLE 18

GLYCOSYL COMPOSITIONS OF THE FRACTIONS FROM DIONEX PA1 ION EXCHANGE CHROMATOGRAPHY OF THE DE-METHYLESTERIFIED RGI ISOLATED BY METHOD 2

(Relative Molar Ratio Normalized to That of Rha)

Residue	Fra 1	Fra 2	Fra 3	Fra 4	Fra 5
Ara	0.4	0.4	0.4	0.3	0.2
Rha	1.0	1.0	1.0	1.0	1.0
Xyl	0.3	0.2	0.4	0.3	0.3
GalA	1.9	1.4	2.3	1.8	1.6
Man	0.3	-	-	-	-
Gal	0.6	0.4	0.5	0.3	0.3
Glc	0.3	0.1	0.2	0.1	0.2

TABLE 19

DEGREE OF METHYLESTERIFICATION OF THE FRACTIONS FROM DIONEX PA1 HPLC ION EXCHANGE CHROMATOGRAPHY OF THE RGI ISOLATED BY METHOD 2

	Fra 1	Fra 2	Fra 3	Fra 4
RGI*	15%	13%	11%	5%
HG**	21%	33%	27%	15%

* This refers to all the GalA in the RGI.

** This refers to the GalA excluding the ones in the repeating unit of $[GalA\text{-}Rha]_n$.

other words, eluted with much higher concentration of ammonium acetate buffer than the de-methylesterified RGI from the column. This indicates that there is neither methylesterified nor non-methylesterified homogalacturonans co-isolated with RGI, otherwise they should have separated from the RGI in this ion exchange column and eluted in a similar position to the commercial HG. Another approach taken to confirm the above conclusion was to apply an apple pectin (mainly homogalacturonan) with 30-40% methylesterification, a gift from Dr. Benjamin of the Campbell Soup Company, to the PA-1 column under the same conditions (Figure 32). This pectin came out of the column in a position between the cotton RGI and the commercial homogalacturonan, and further indicated that if there were any HG with 20 -40% of methylesterification (Table 19), it should have a similar eluting behavior to this pectin and have been eluted much later than the RGI.

Therefore it is concluded that some 20-40% methylesterified homogalacturonans, accounting for 10-20% of the RGI molecules with regard to sugar compositions, must be covalently linked to the RGI molecules. Considering the large content of these extra GalA in the RGI and the big molecular weight of the RGI, it is assumed that they are linked to the RGI backbone either as a long "tail" or as short oligomers. The latter would have to be interspersed within the backbone. Indeed, according to the sugar compositions of the fractions from the PA-1 chromatography, from the DEAE-Sephadex chromatography, and from the result of the TSK400 chromatography (Figure 24), it is apparent that the RGI molecules in cotton suspension culture cell walls are not very homogeneous either in their structures or in their sizes.

HF Treatment of Rhamnogalacturonan I

To test the above hypothesis, Me-RGI isolated by method 2 was treated with liquid hydrogen fluoride (HF) at -23^{0}C for 30 min. A little water, representing 1% of the liquid HF used, was added in the reaction to prevent the cleavage of homogalacturonans by the

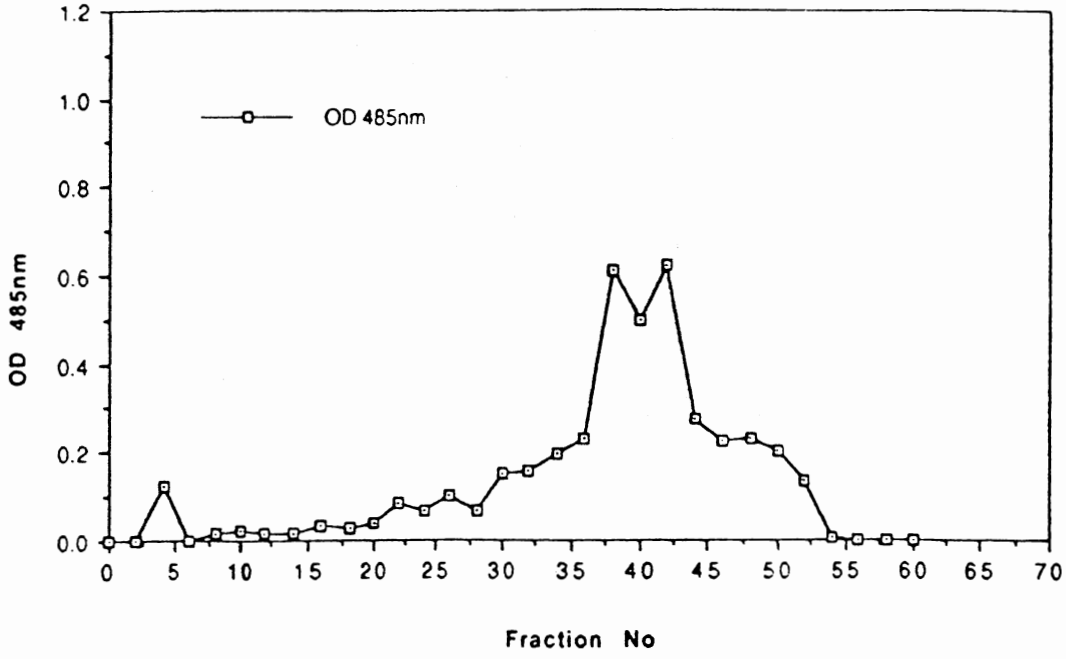


Figure 32. Chromatography on a Dionex CarboPac PA-1 HPLC Ion Exchange Column of an Apple Pectin with 30-40% Methylesterification.

reagent. According to previous experiments with cotton suspension culture cell walls, it was clear that this treatment could specifically cleave the glycosyl linkages of neutral sugars such as arabinose, xylose, galactose, glucose and rhamnose residues, but not those of galacturonosyl residues. Therefore, the homogalacturonans or oligogalacturonans linked to the RGI molecules should stay polymeric after this treatment . Whereas the repeating disaccharides backbone, i.e. [GalA-Rha]$_n$, should be cleaved into the disaccharide GalA-Rha, and the other neutral sugars should be cleaved into monomers.

Application of the HF-treated Me-RGI to Fracto-Gel HW40S HPLC gel filtration chromatography gave rise to 8 fractions (Figure 33). The sugar compositions of these fractions are presented as molar ratios in Table 20. Taking into account the sugar compositions and the elution patterns of these fractions during the chromatography, it is reasonable to state that the result is very consistent with what was expected. Fraction 1, having approximately a 13 : 1 molar ratio of GalA/Rha, probably contains a mixture of oligogalacturonans with a rhamnosyl residue linked to their reducing end, and the average length of these oligomers is about 14 residues. Application of this fraction to a Bio-Sil TSK400 gel filtration column demonstrated that the sugars in this fraction eluted close to the included volume, suggesting a M.W. of <5000 by pullulan standard. And this result eliminated the possibility that the GalA were linked to the RGI backbone as long homogalacturonans. Fraction 5, 6, and 7, which have a 1 : 1 molar ratio of GalA/Rha, probably are the disaccharides of GalA-Rha. Whereas fraction 8, eluted in the included volume, represents all the neutral sugar monomers. However fractions 2, 3, and 4, which eluted earlier than the assumed dimers, could not be explained at this point. Further analysis with LSIMS and n.m.r. spectroscopy solved this problem.

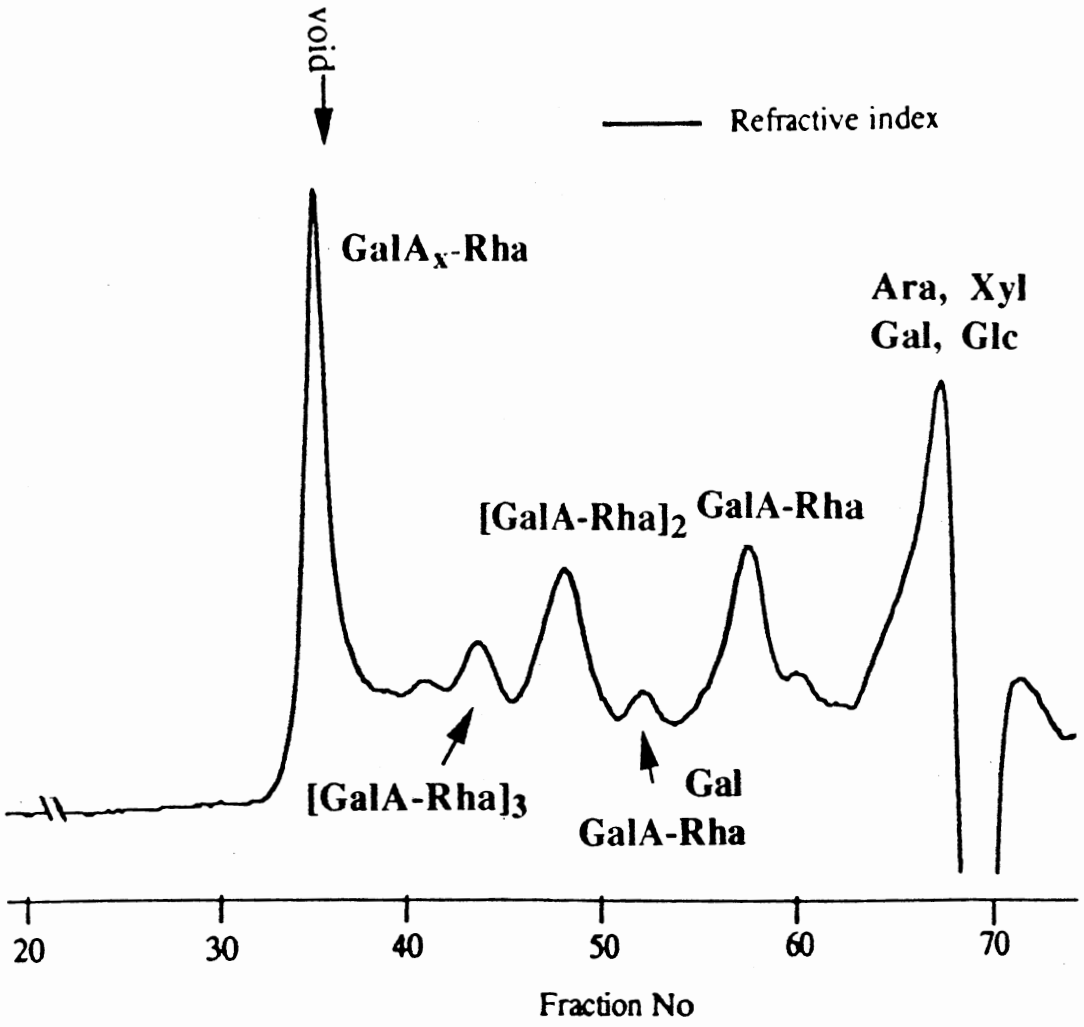


Figure 33. Chromatography on a Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. Tubes 33-38 were pooled into fraction 1, 39-42 into fraction 2, 43-45 into fraction 3, 46-50 into fraction 4, 51-53 into fraction 5, 54-59 into fraction 6, 60-62 into fraction 7, and 63-75 into fraction 8. No sugars were detected from tubes 76-82.

TABLE 20

RELATIVE SUGAR MOLAR RATIOS* AND WEIGHT PERCENT OF THE FRACTIONS FROM HW40SHPLC GEL FILTRATION OF THE HF-TREATED RGI ISOLATED BY METHOD 2

Residue	Fra 1	Fra 2	Fra 3	Fra 4	Fra 5	Fra 6	Fra 7	Fra 8
Ara	0.1	-	-	-	-	-	0.1	7.3
Rha	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Xyl	0.7	-	-	-	0.1	-	-	7.5
GalA	13.0	2.1	1.4	1.3	1.3	1.0	1.0	0.1
Man	0.2	-	-	-	-	-	-	0.1
Gal	0.2	0.1	0.2	0.2	1.1	0.3	0.5	4.8
Glc	0.3	-	0.2	0.2	0.4	0.2	0.2	2.8
GlcA	0.5	0.1	0.1	0.1	0.4	0.1	0.1	-
wt%	17%	8%	5%	12%	4%	12%	7%	35%

* Normalized to that of rhamnosyl residues.

Analysis of the RGI Fragments Generated by HF Treatment

Identification of the RGI Fragments by LSIMS and NMR Spectroscopy

The RGI oligosaccharides generated by HF treatment of RGI were identified and analyzed by LSIMS and n.m.r. spectroscopy as described previously.

The LSIMS mass spectra of both fraction 6 (Figure 34) and fraction 7 (Figure 36) contain peaks with masses consistent with the molecular weights of four types of GalA-Rha disaccharides. Masses of 341 and 383 are consistent with $[M+H^+]$ of the non-acetylated and the acetylated disaccharides, respectively. Masses of 323 and 365 are consistent with $[M+H^+]$ of the non-acetylated and the acetylated disaccharides which were internally cyclized through the linkage of Rha-β-(1-2)-GalA (reducing-end cyclization), respectively. The cyclization is an artifact of the HF reaction due to the high reactivity of the rhamnosyl fluorides formed initially. The acetylation was found to be at position 3 of the galacturonic acid of the disaccharides (Komalavilas and Mort, 1989). ^{1}H-n.m.r. spectra of fraction 6 (Figure 35) and fraction 7 (Figure 37) gave the information consistent with the LSIMS results. Both spectra showed two sets of signals. One was from the disaccharide with the acetate ester and the other was from the disaccharide without an acetate ester. Fraction 7, however, contained predominantly acetylated disaccharides (77%) and fraction 6 contained predominantly non-acetylated disaccharides (85%). This was also reflected by their LSIMS spectra in which the signals for acetylated disaccharide were more intense than that for non-acetylated disaccharide in the spectrum of fraction 7. The reason why the acetylated disaccharides were eluted later than the non-acetylated ones from HW40S HPLC gel filtration column is not known .

The mass spectrum of fraction 5 (Figure 38) contained two groups of peaks. Group one, consisting of several peaks with masses of 498.4, 542.4, 586.5, 630.5, 674.5 and 718.6, a series differing by 44 atomic mass units, was not identified. Group two, consisting of peaks with masses of 751.3, 771.3, 793.3, 813.3, 835.3 and 855.3, was

identified to be from a reducing-end cyclized tetrasaccharide of GalA-Rha-GalA-Rha with 2 to 4 acetyl esters attached (these are the major components found in fraction 4). Masses of 751.3, 771.3, 793.3, 813.3, 835.3 and 855.3 are consistent with $[M+H^+]$, $[M+Na^+]$ of the tetramer with 2 acetyl esters, $[M+H^+]$, $[M+Na^+]$ of the tetramer with 3 acetyl esters, and $[M+H^+]$, $[M+Na^+]$ of the tetramer with 4 acetyl esters, respectively. Since this fraction contains significant amounts of galactosyl, glucosyl, xylosyl and arabinosyl residues other than galacturonosyl and rhamnosyl residues (see Table 20), there must be some other sorts of oligosaccharides present in this fraction which were not shown or not identified from the mass spectrum. ^{1}H-n.m.r. spectrum (Figure 39) of this fraction did show many signals which were most likely from these residues. A 2D-^{1}H-n.m.r (Cosy) spectrum of this fraction (Figure 40) helped us identify some of those signals, such as the signals for the β-galactosyl residue. The ratios of acetylated-GalA-linked Rha to non-acetylated-GalA-linked Rha in fraction 5, fraction 4 and fraction 3 was determined from relative intensities of the signals at 1.15 ppm and 1.25 ppm which were assigned as the CH_3 of rhamnose linked to acetylated GalA and the CH_3 of rhamnose linked to non-acetylated GalA. Apparently the ratios in fraction 3 and 4 are about 2 : 1, whereas in fraction 5 the ratio is about 0.3 : 1, suggesting the presence of some unidentified oligomers consisting of rhamnose and other sugar residues in this fraction. In addition, very low intensity of the signal for H-3 of acetylated GalA at 5.75 ppm and very high intensity of the signal for acetyl esters at 2.05 ppm were observed in the n.m.r. spectrum, demonstrating that certain acetylation must be present on sugar residues other than GalA residues. Some signals in this spectrum were tentatively identified as the acetylated galactosyl residue. For instance, the signals at 5.3 ppm may arise from H-4 of 2-linked β-galactose with acetylation at position 4, and the signals at 4.6 ppm may arise from H-1 of 2-linked β-galactose acetylated at position 6. Further analysis of this fraction may provide information for both acetylation and sidechain characteristics of cotton cell wall RGI.

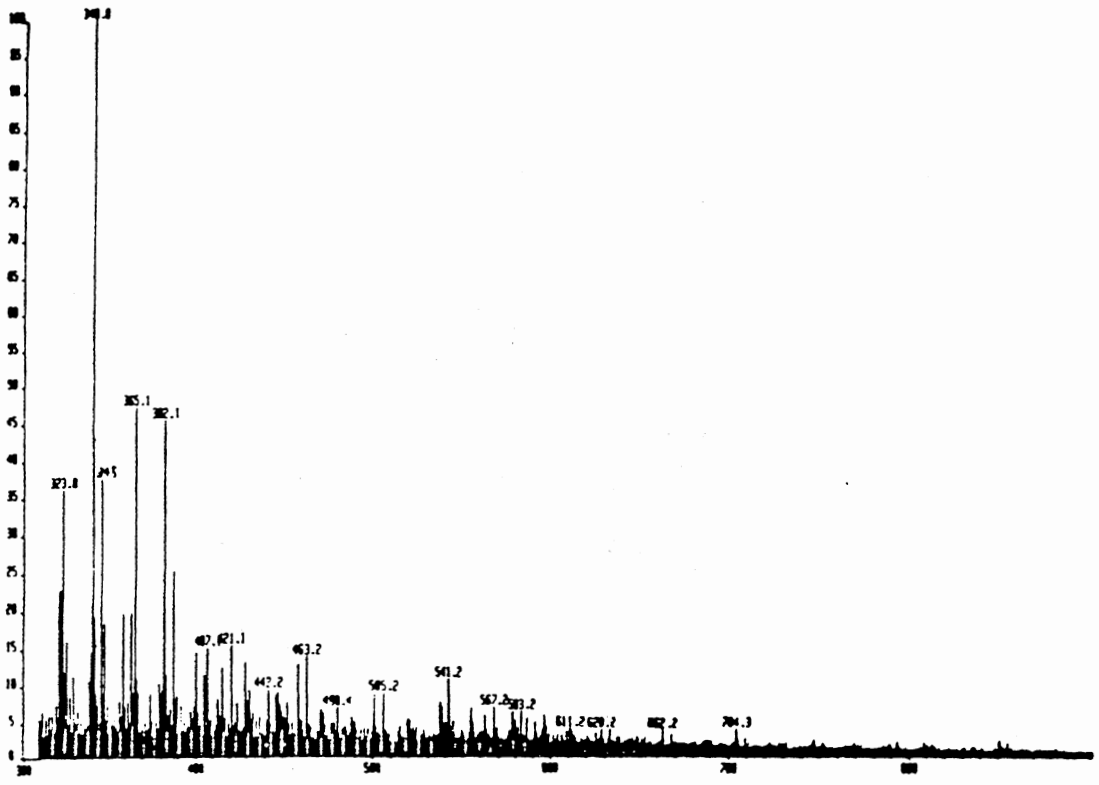


Figure 34. LSIMS Spectrum of the Fraction 6 Obtained by Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. Masses of 323 and 340 were assigned as [$M+H^+$] of the cyclized and non-cyclized disaccharide of GalA-Rha, and masses of 365.1 and 382.1 were assigned as [$M+H^+$] of the cyclized and non- cyclized disaccharide of GalA-Rha with one acetyl group attached, respectively.

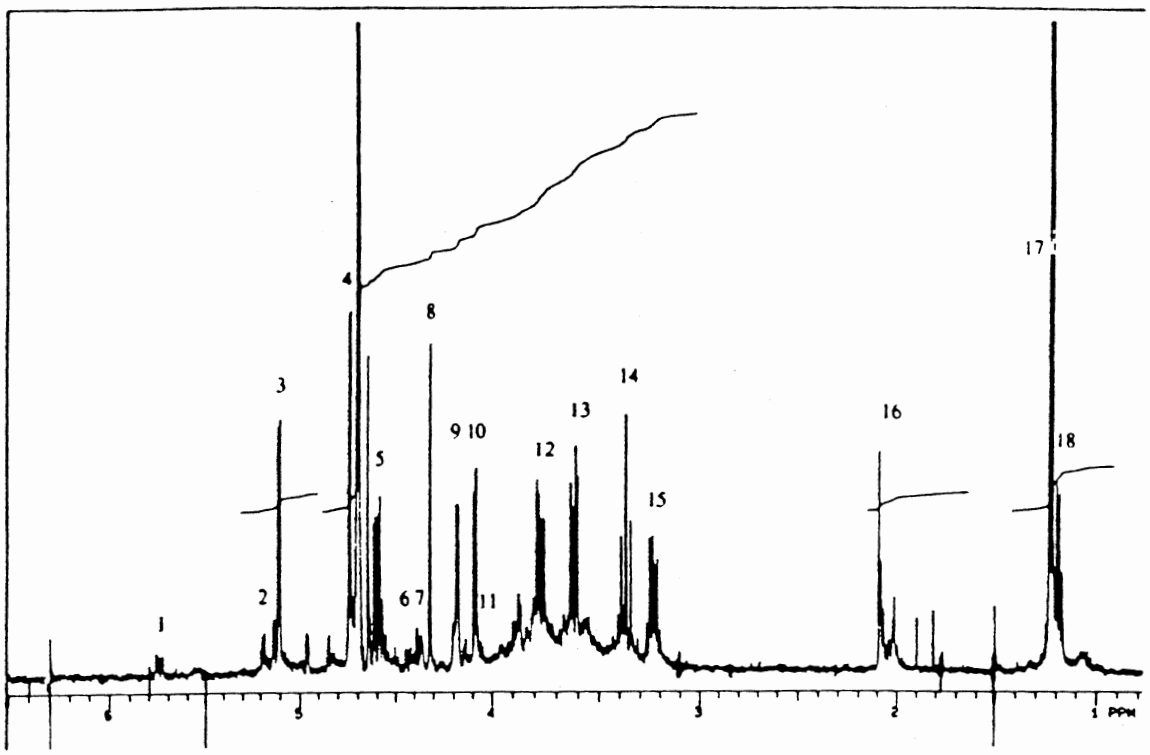


Figure 35. ^{1}H-n.m.r. Spectrum of Fraction 6 Fractionated on Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25^{0}C in D_2O. Peaks are assigned as: 1) H-3 of acetylated and cyclized (A.C) GalA , 2) H-1 of GalA(A.C), 3) H-1 of GalA(C), 4) H-1 of Rha, 5) H-5 of GalA(C), 6) H-5 of GalA(A.C), 7) H-4 of GalA(A.C), 8) H-5 of GalA(C), 9)H-4 of GalA(C), 10) H-2 of Rha, 11) H-2 of GalA(A.C), 12) H-2 of GalA(C), 13) H-3 of Rha, 14) H-4 of Rha, 15) H-5 of Rha, 16) CH_3 of acetate, 17) CH_3 of Rha, 18) CH_3 of Rha(A).

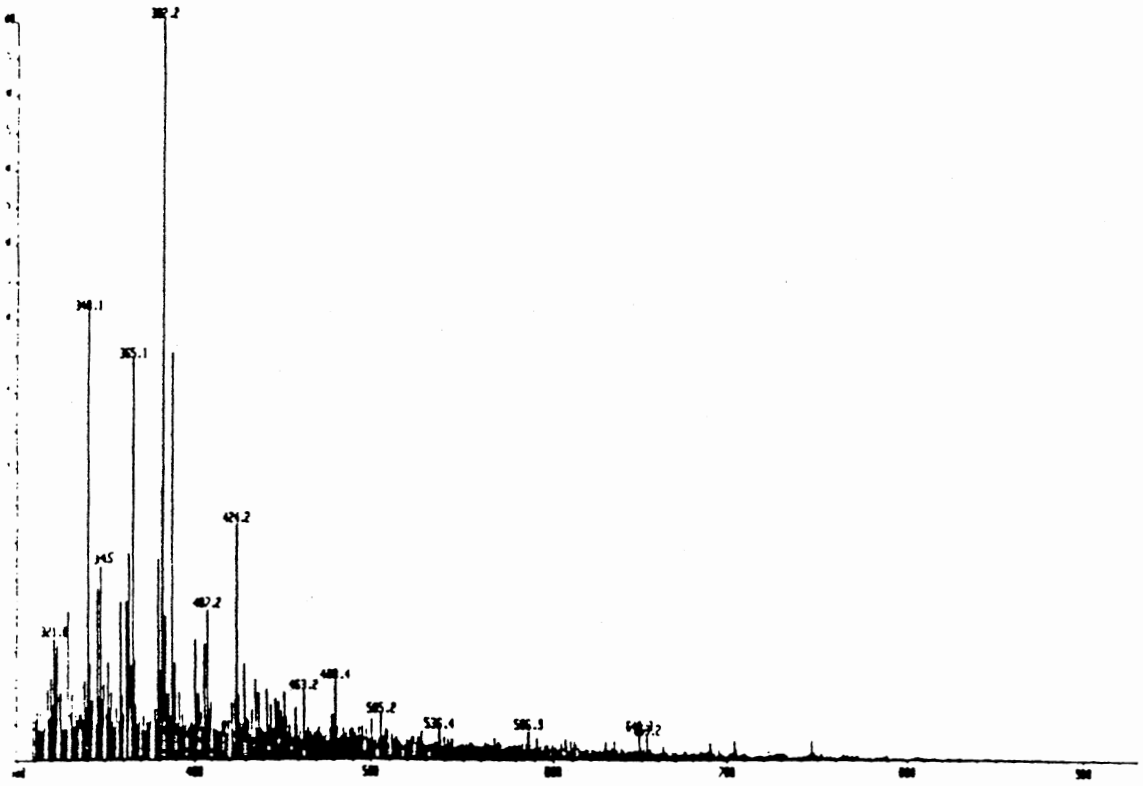


Figure 36. LSIMS Spectrum of the Fraction 7 Obtained by Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. Mass of 340.1 were assigned as [$M+H^+$] of the non-cyclized disaccharide of GalA-Rha, and masses of 365.1 and 382.2 were assigned as [$M+H^+$]of the cyclized and non-cyclized disaccharide of GalA-Rha with one acetyl group attached, respectively.

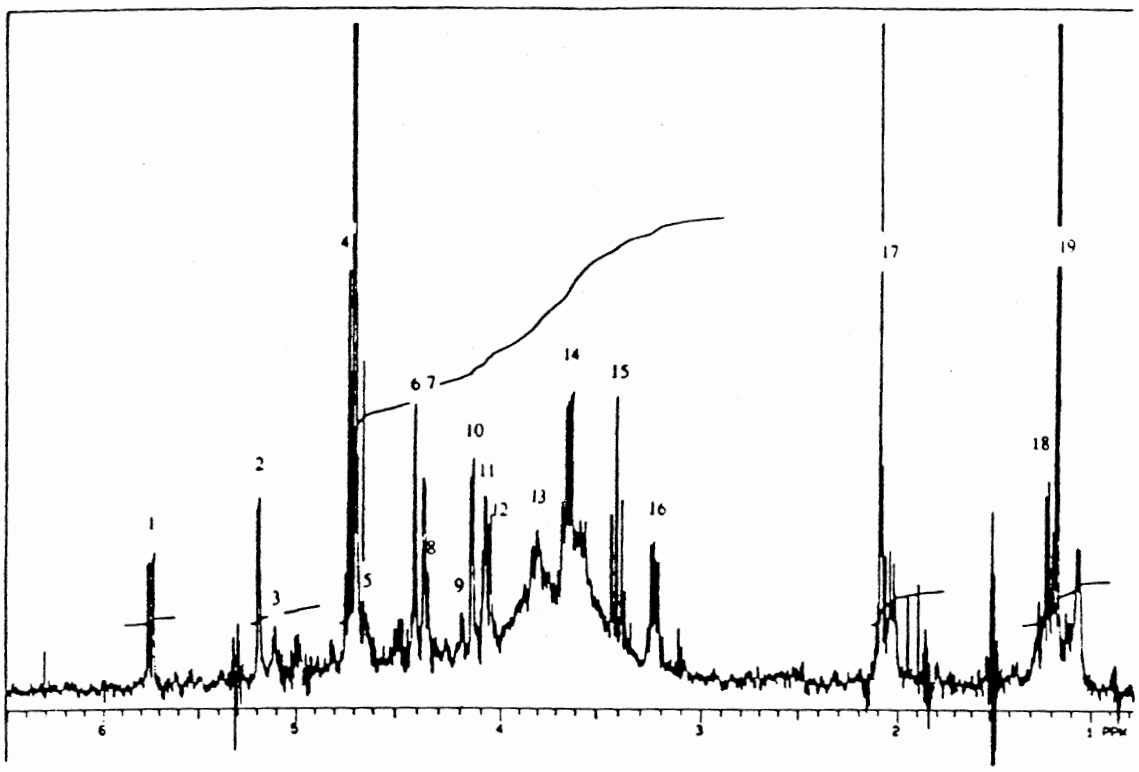


Figure 37. ^{1}H-n.m.r. Spectrum of Fraction 7 Fractionated on Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25^{0}C in D_2O. Peaks are assigned as: 1) H-3 of acetylated and cyclized (A.C) GalA, 2) H-1 of GalA(A.C), 3) H-1 of GalA(C), 4) H-1 of Rha, 5) H-5 of GalA(C), 6) H-5 of GalA(A.C), 7) H-4 of GalA(A.C), 8) H-5 of GalA(C), 9) H-4 of GalA(C), 10) H-2 of Rha(A), 11) H-2 of Rha, 12) H-2 of GalA(AC), 13) H-2 of GalA(C), 14) H-3 of Rha, 15) H-4 of Rha, 16) H-5 of Rha, 17) CH_3 of acetate, 18) CH_3 of Rha, 19) CH_3 of Rha(A).

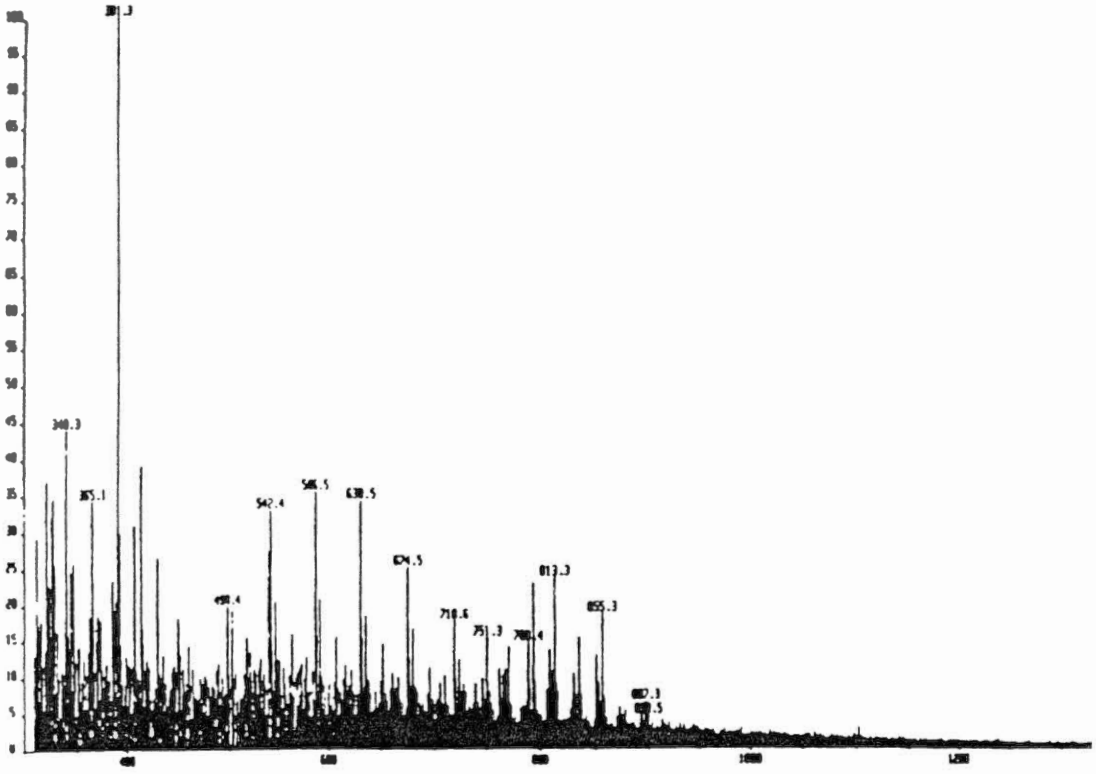


Figure 38. LSIMS Spectrum of the Fraction 5 Obtained by Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. Masses of 751.3 were identified as [$M+Na^+$] of the reducing-end cyclized tetramer of GalA-Rha-GalA-Rha with two acetate esters(AC), 771.3 and 793.3 as [$M+H^+$] and [$M+Na^+$] of the tetramer with 3 AC, 813.3 and 835.3 as [$M+H^+$] and [$M+Na^+$] of the tetramer with 4 AC, and 855.3 as [$M+H^+$] of the tetramer with 5 AC.

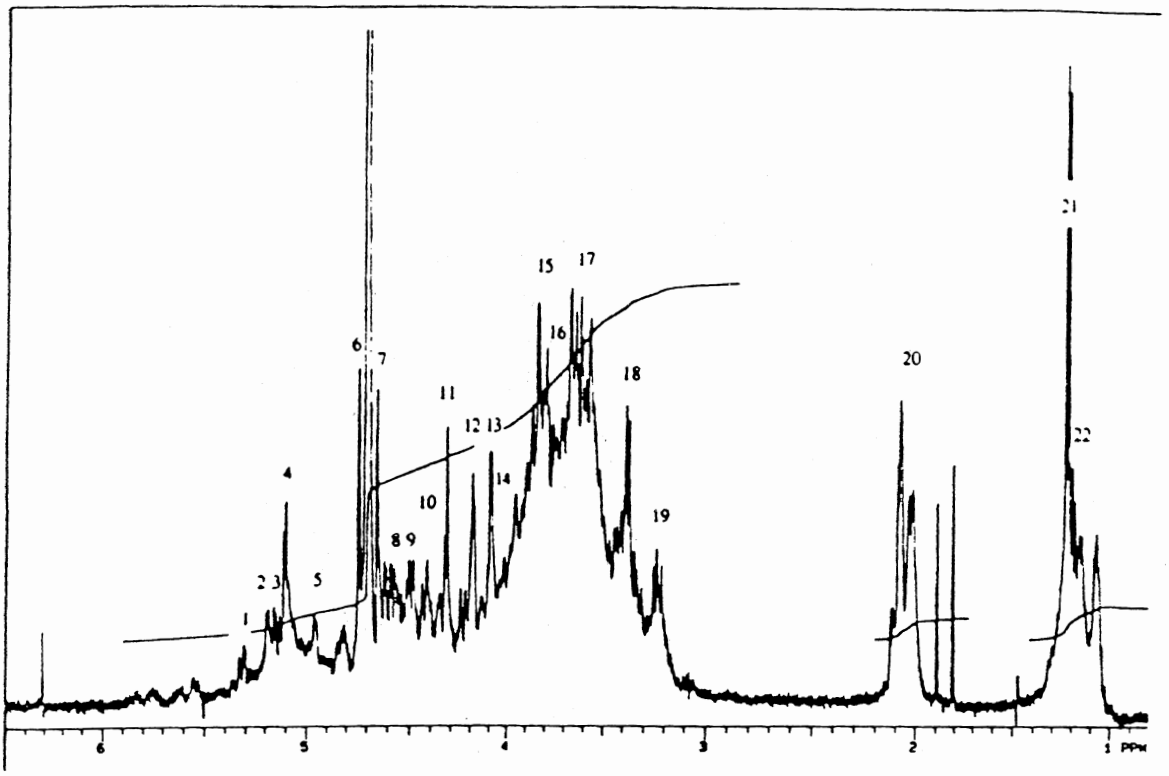


Figure 39. ^{1}H-n.m.r. Spectrum of Fraction 5 Fractionated on Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25^{0}C in D_2O. Peaks are assigned as follows: 1) H-4 of 2-β-Gal with AC at C4? 2) H-1 of GalA(A.C), 3) H-1 of α-Rha,4) H-1 of GalA(C), 5) H-1 of GalA, 6) H-1 of β-Rha, 7) H-3 of GalA(C), 8) H-1 of β-Gal with AC at C6, H-5 of GalA, 9) H-1 of β-Gal, 10) H-5 of GalA(C), 11) H-4 of GalA(C), 12) H-4 of GalA, 13) H-2 of β-Rha, 14) H-2 of α-Rha, 15) H-2 of GalA(C), H-3 of GalA, H-4 of β-Gal ,16) H-3 of α-Rha, H-6 of β-Gal, 17) H-3 of β-Rha, H-5 of β-Gal, H-3 of β-Gal, H-2 of β-Gal, 18) H-4 of β-Rha, 19) H-5 of β-Rha, 20) CH_3 of acetate, 21) CH_3 of Rha, 22) CH_3 of Rha(A).

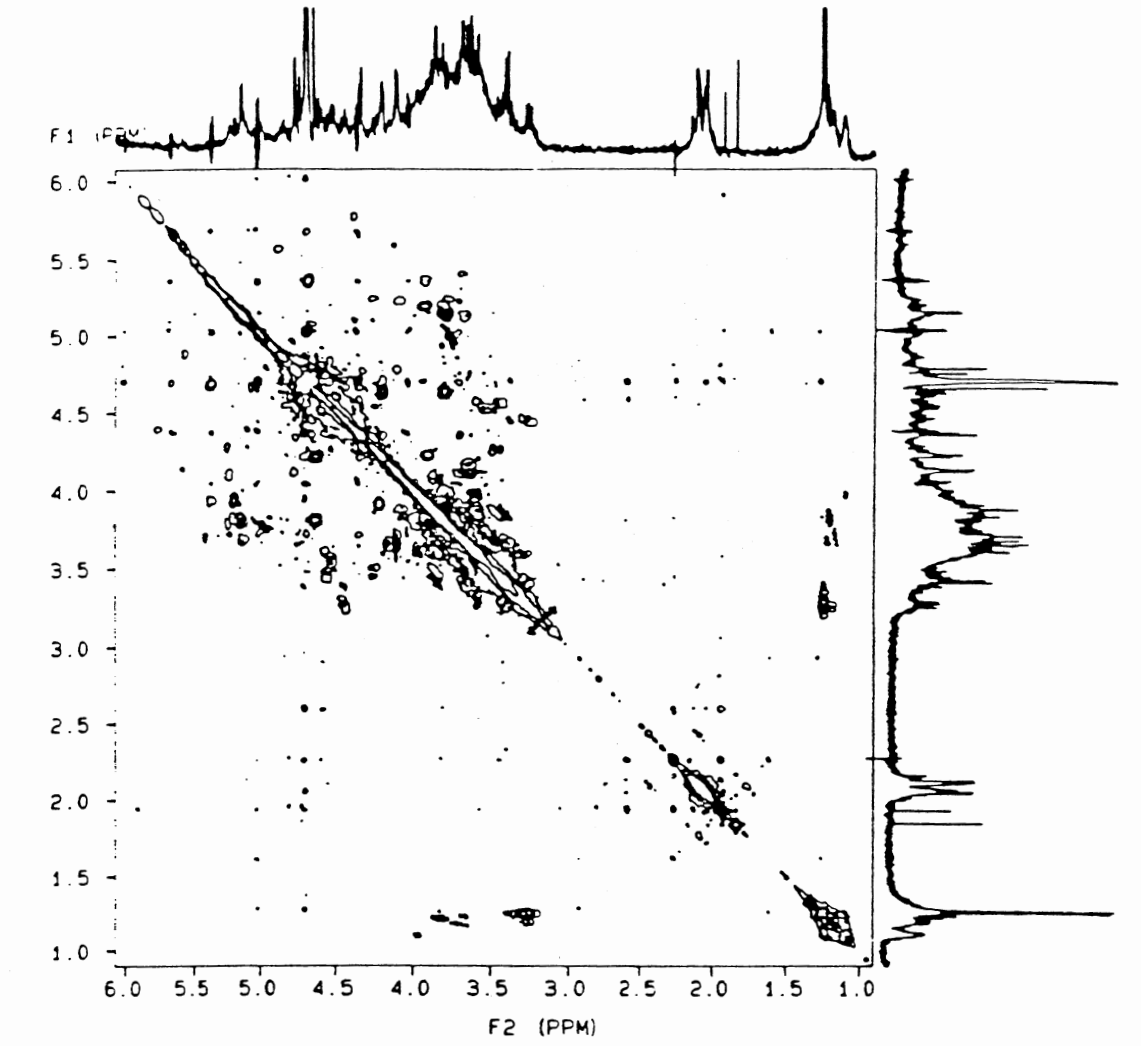


Figure 40. 2D-^{1}H-n.m.r. Spectrum (Cosy) of Fraction 5 Fractionated on Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25^0C in D_2O.

Fraction 4 was identified by LSIMS spectroscopy (Figure 41) to be composed of a mixture of reducing-end-cyclized tetrasaccharides (GalA-Rha-GalA-Rha) with 0 to 5 acetyl groups attached. Masses of 667.4, 709.4, 751.4, 793.5, 835.4, and 877.5 in the spectrum are consistent with [$M+Na^+$] of the reducing-end-cyclized tetrasaccharide with 0, 1, 2, 3, 4, and 5 acetate esters attached, respectively. This was further confirmed by 1D-^1H n.m.r (Figure 42) and 2D-^1H n.m.r spectroscopy (Figure 43).

Fraction 3 has a very similar ^1H-n.m.r. spectrum (Figure 44) to that of fraction 4 except that the signals for the non-cyclized GalA are relatively abundant in fraction 3 compared to that in fraction 4. This is because the fact that fraction 3 is composed of a mixture of reducing-end cyclized hexasaccharides of GalA-Rha-GalA-Rha-GalA-Rha with 0 to 6 acetate esters attached, and this was identified by LSIMS (Figure 45). In other wards, hexasaccharides in fraction 3 contain more GalA residues which were not involved in the internal cyclization than the tetraners in fraction 4 .

Fraction 2, eluted close to the void volume of HW40S column, contains a mixture of bigger oligosaccharides. LSIMS spectrum (Figure 46) of this fraction shows two groups of peaks with masses consistent with the molecular weights of reducing-end cyclized octasaccharides with the degree of acetylation from 0 to 9, and masses consistent with the molecular weights of the reducing-end cyclized decasaccharides with degrees of acetylation from 6 to 11. Based on the signal intensity in the spectrum, most abundant oligomers in this fraction are the octasaccharide with 6 acetyl groups attached and the decasaccharide with 7 acetyl groups attached. The 2 : 1 molar ratio of GalA/Rha in this fraction suggests that some oligogalacturonans are likely present in the fraction. Two peaks with masses of 1767.6 and 1809.7 in the fraction do coincide with ($M+Na^{+}$) of the reducing-end cyclized Me-$(GalA)_8$-GalA-Rha, and 4Me-$(GalA)_8$-GalA-Rha, respectively, even though the intensity of the peaks are not very high.

According to the results presented here, acetylation occurs in all kinds of oligomers of the repeating disaccharide unit. But the degree of acetylation varies greatly, depending

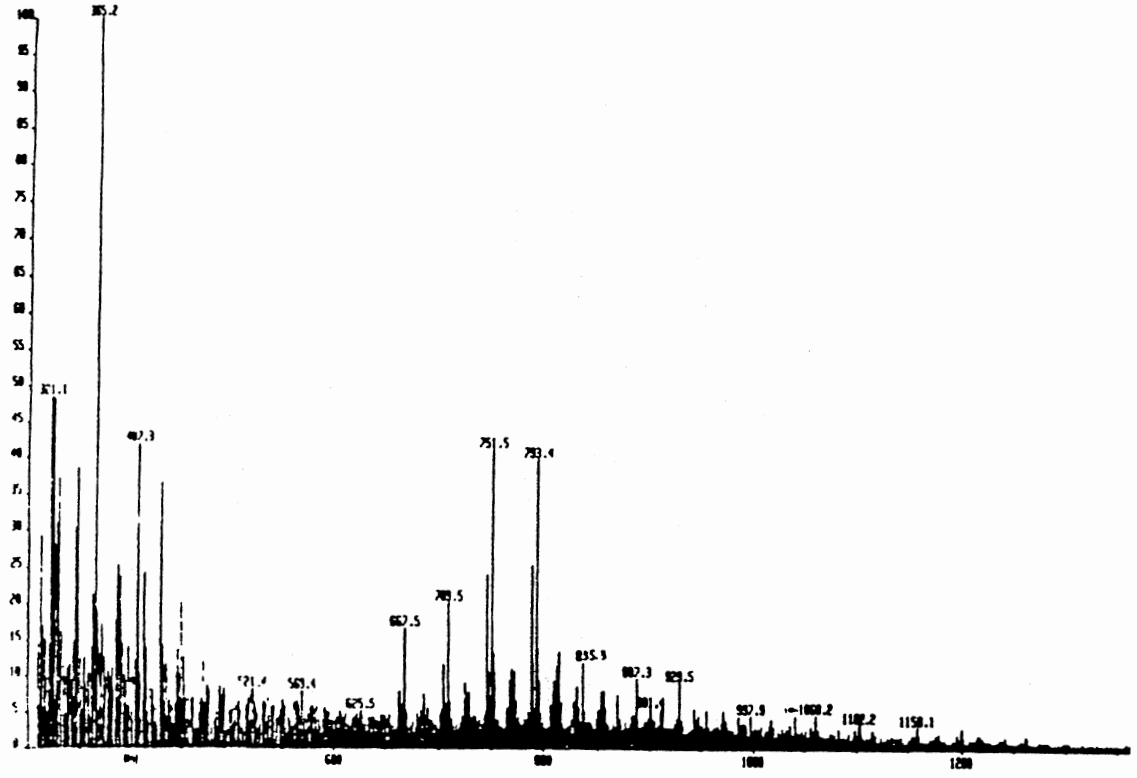


Figure 41. LSIMS Spectrum of the Fraction 4 Obtained by Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. Masses of 667.4, 709.4, 751.4, 793.5, 835.4, and 877.5 were identified as [$M+Na^+$] of the reducing-end cyclized tetrasaccharide of GalA-Rha-GalA-Rha with 0, 1, 2, 3, 4 and 5 AC, respectively.

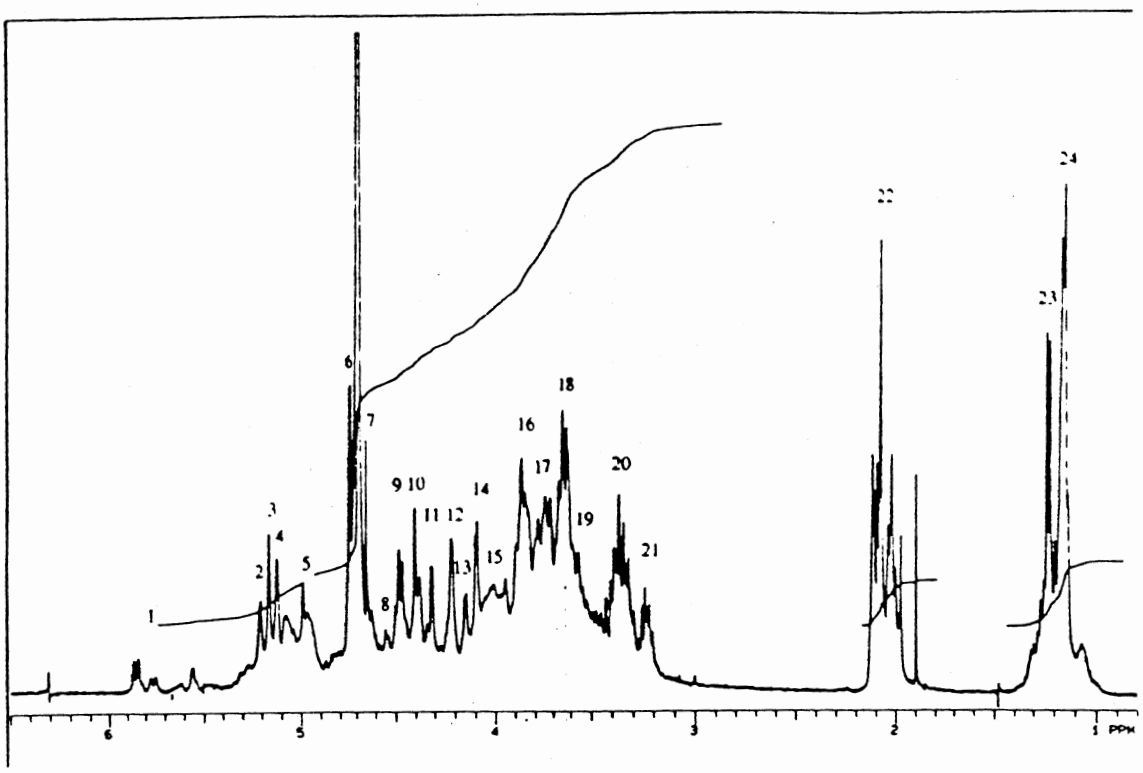


Figure 42. ^{1}H-n.m.r. Spectrum of Fraction 4 Fractionated on Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25^{0}C in D_2O. Peaks are assigned as follows: 1) H-3 of GalA(A.C), 2) H-1 of GalA(A.C), 3) H-1 of α-Rha, 4) H-1 of GalA(C), 5) H-1 of GalA, 6) H-1 of β-Rha, 7) H-3 of GalA(C), 8) H-5 of GalA, 9) H-1 of β-Gal, 10) H-5 of GalA(C), 11)H-4 of GalA(C), 12) H-4 of GalA, 13) and 14) H-2 of β-Rha, 15) H-2 of α-Rha, 16) H-2 of GalA(C), H-3 of GalA, H-4 of β-Gal ,17) H-3 of α-Rha, H-6 of β-Gal, 18) H-3 of β-Rha, H-5 of β-Gal, H-3 of β-Gal, 19) H-2 of β-Gal, 20) H-4 of β-Rha, 21) H-5 of β-Rha, 22) CH_3 of acetate, 23) CH_3 of Rha, 24) CH_3 of Rha(A).

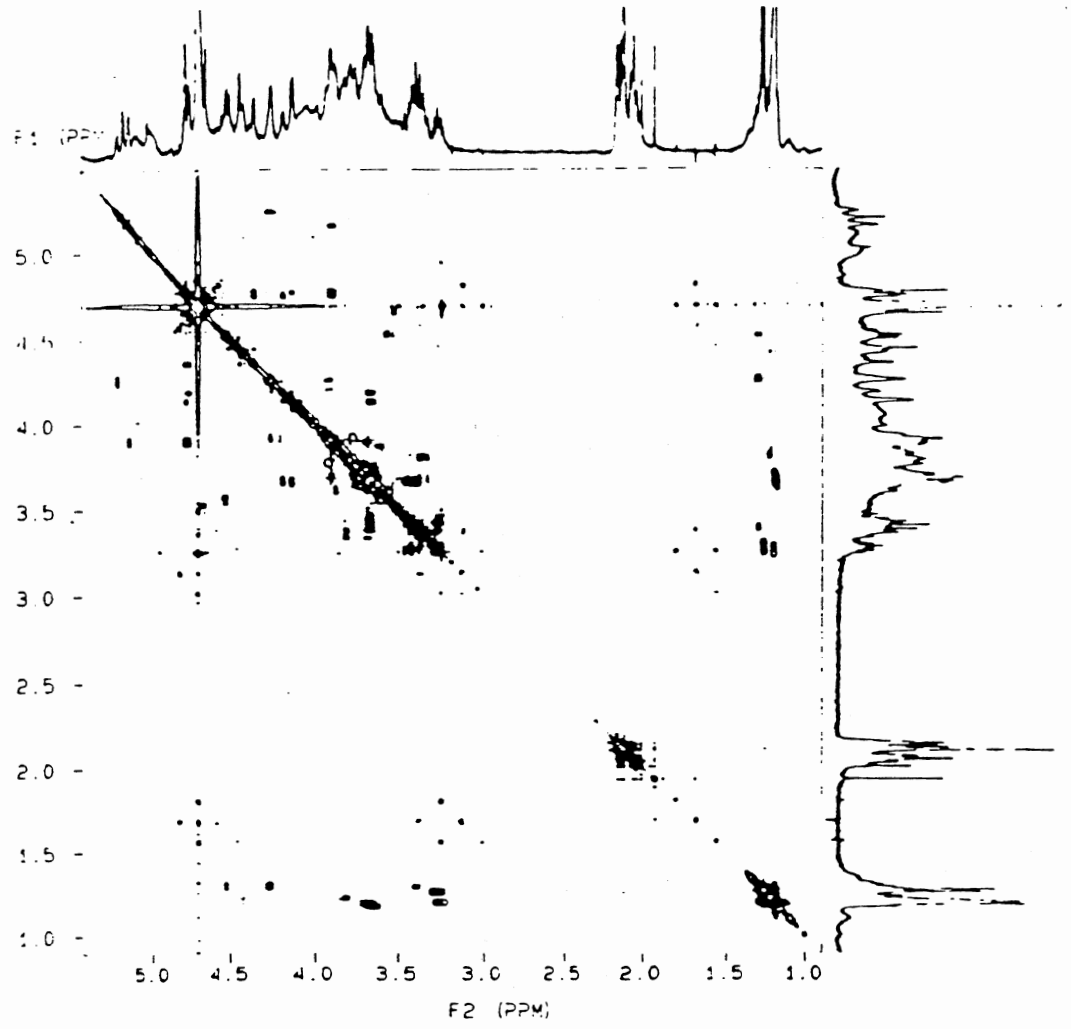


Figure 43. 2D-^{1}H-n.m.r. Spectrum (Cosy) of Fraction 4 Fractionated on Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25^{0}C in D_2O.

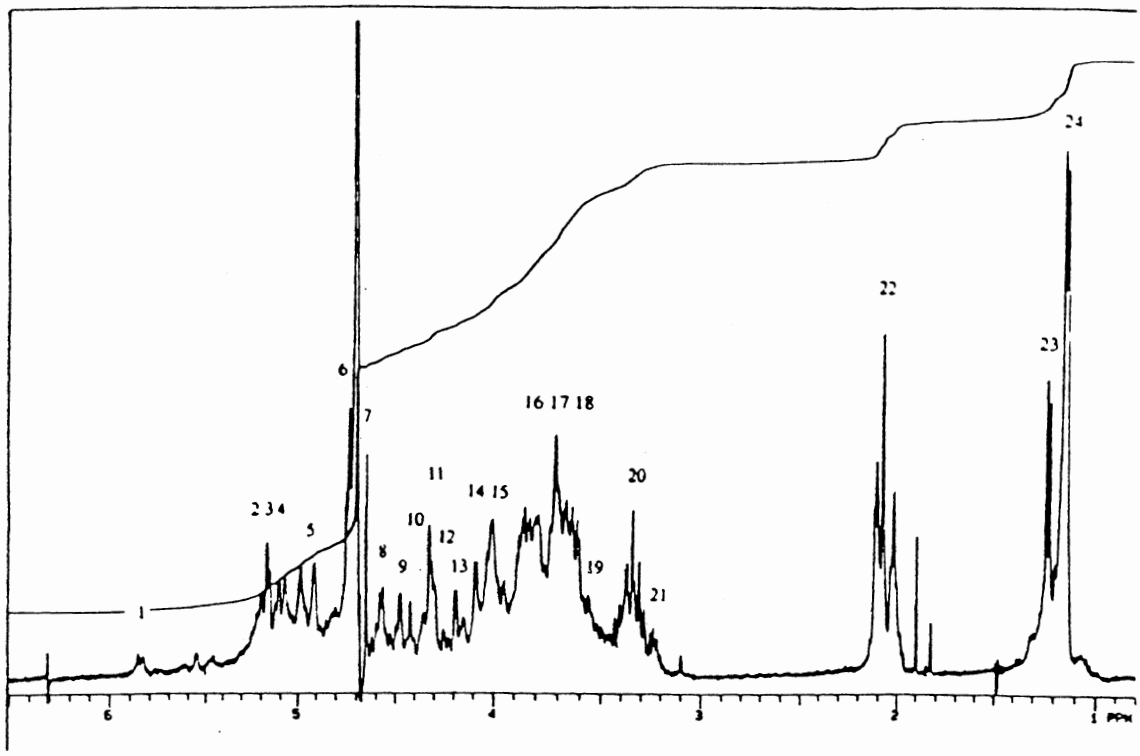


Figure 44. ^{1}H-n.m.r. Spectrum of Fraction 3 Fractionated on Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25^{0}C in D_2O. Peaks are assigned as follows: 1) H-3 of GalA(A.C), 2) H-1 of GalA(A.C), 3) H-1 of α-Rha,4) H-1 of GalA(C), 5) H-1 of GalA, 6) H-1 of β-Rha, 7) H-3 of GalA(C), 8) H-5 of GalA, 9) H-1 of β-Gal, 10) H-5 of GalA(C), 11)H-4 of GalA(C), 12) H-4 of GalA, 13) and 14) H-2 of β-Rha, 15) H-2 of α-Rha, 16) H-2 of GalA(C), H-3 of GalA, H-4 of β-Gal ,17) H-3 of α-Rha, H-6 of β-Gal, 18) H-3 of β-Rha, H-5 of β-Gal, H-3 of β-Gal, 19) H-2 of β-Gal, 20) H-4 of β-Rha, 21) H-5 of β-Rha, 22) CH_3 of acetate, 23) $CH_3$of Rha, 24) CH_3 of Rha(A).

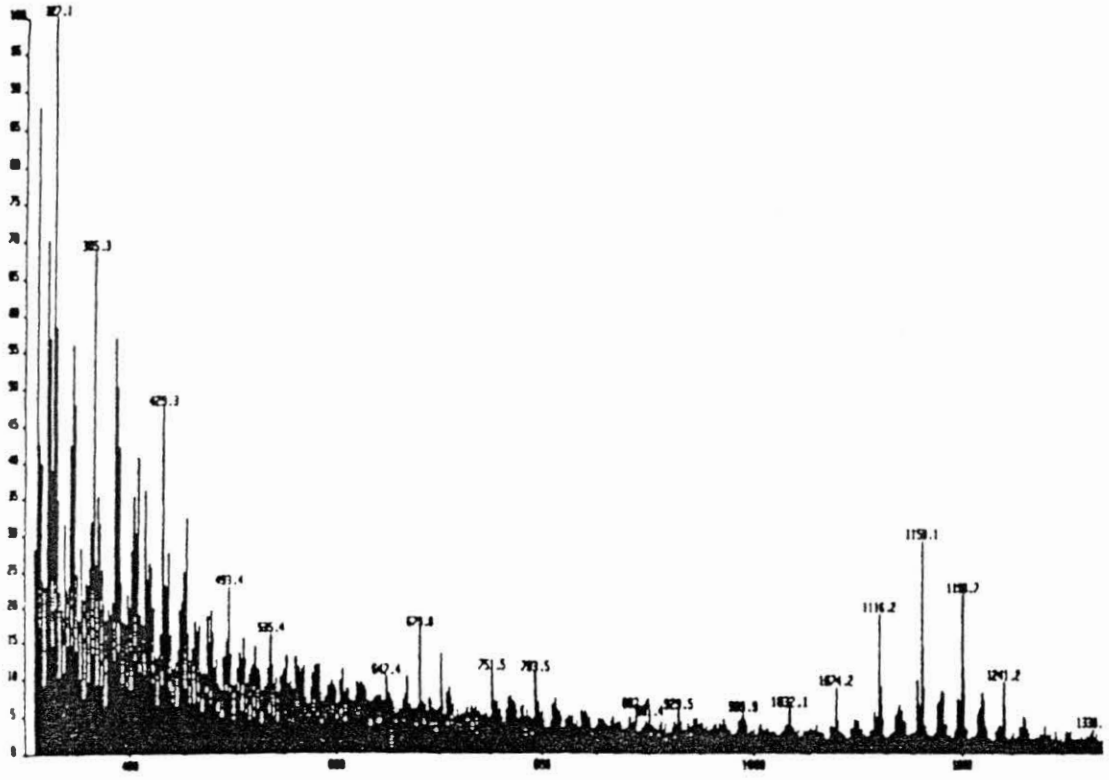


Figure 45. LSIMS Spectrum of the Fraction 3 Obtained by Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. Masses of 989.9, 1032.1, 1074.2, 1116.2, 1158.1, 1199.7, and 1241.2 were identified as[$M+Na^+$] of the reducing-end cyclized hexasaccharide of GalA-Rha-GalA-Rha-GalA-Rha with 0, 1, 2, 3, 4, 5 and 6 AC, respectively.

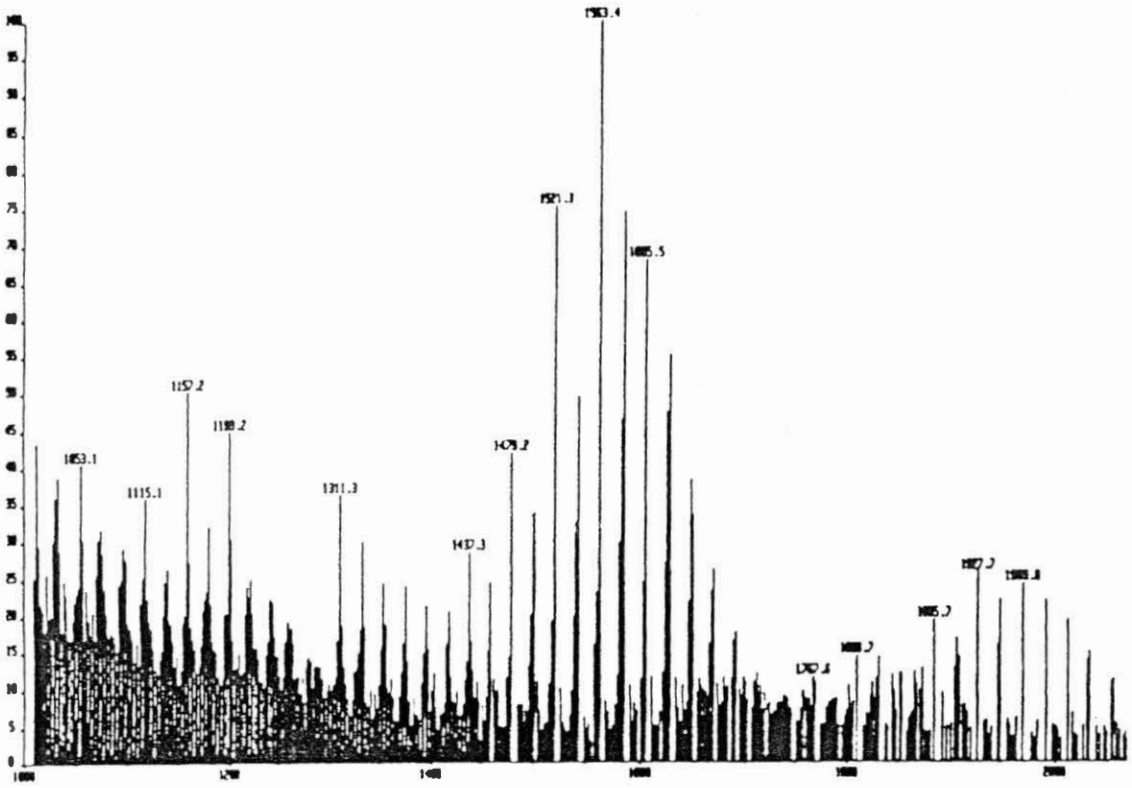


Figure 46. LSIMS Spectrum of the Fraction 2 Obtained by Fracto-Gel HW40S HPLC Gel Filtration Chromatography of the HF-treated RGI Isolated by Method 2. Masses of 1311.3, 1353.3, 1395.3, 1437.3, 1479.3, 1521.3, 1563.4, 1605.5, and 1647.5 were identified as $[M+Na^+]$ of the reducing-end cyclized octasaccharide of GalA-Rha-GalA-Rha-GalA-Rha-GalA-Rha with 0, 1, 2, 3, 4, 5, 6, 7, and 8 AC, respectively. Masses of 1885.7, 1927.7, 1969.8, 2011.8, 2032.9 and 2075.0 were identified as $[M+Na^+]$ of the reducing-end cyclized decasaccharide of GalA-Rha-GalA-Rha-GalA-Rha-GalA-Rha-GalA-Rha with 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 AC, respectively. Masses of 1767.6 and 1809.7 were identified as $[M+Na^+]$ of the reducing-end cyclized $(GalA)_9$-Rha with 1 Me, and $(GalA)_9$-Rha with 4 Me, respectively

on the different oligomers. Disagreement between the degree of acetylation of some oligomers and the positions available for acetylation on the GalA residues of these oligomers indicates that a certain degree of acetylation is on Rha residues of the oligomers. For instance, the available acetylation positions on the two GalA of the tetrasaccharide are only 3, while tetramers with up to 5 acetyl groups have been identified by LSIMS.

Fraction 1, containing a molar ratio of GalA/Rha of 13 : 1, and being expected to consist of oligogalacturonans with a rhamnose attached at their reducing ends, was also subjected to LSIMS and ^{1}H-n.m.r. spectroscopy analysis. ^{1}H-n.m.r. spectrum (Figure 47) of the fraction shows an intense signal at 3.7 ppm for methyl ester, indicating that a high degree of methylesterification is present on the expected Rha-terminated oligogalacturonans. Splitting pattern of the signal for H-4 of GalA suggests that about 40-50% of GalA are methylesterified, and this was further confirmed by quantitative DOM determination with selective reduction method which showed a DOM of ~40% for this fraction. The n.m.r. spectrum also shows a considerable degree of acetylation present in this fraction.

LSIMS spectrum (Figure 48) of this fraction shows a number of peaks, and most of them could not be identified because of the large masses. A few of them, such as masses of 1505.4, 1681.7, 1798.1 and 2059.4 were consistent with the molecular weights of reducing-end cyclized $(GalA)_6$-GalA-Rha with 2 AC and 3 Me attached; $(GalA)_7$-GalA-Rha with 2 AC and 3 Me attached; $(GalA)_8$-GalA-Rha with 2 Me attached; $(GalA)_8$-GalA-Rha with 1 AC and 3 Me attached, respectively. A mass spectrum of this fraction from one of the preparations (Figure 49) exhibits several interesting peaks with masses consistent with the expected polysaccharides. For instance, mass of 2709 was consistent with $[M+H^+]$ of reducing-end cyclized $(GalA)_{13}$-GalA-Rha with 4 Me; mass of 3131 was consistent with $[M+H^+]$ of reducing-end cyclized $(GalA)_{15}$-GalA-Rha with 12 Me; mass of 3391 was consistent with $[M+H^+]$ of reducing-end cyclized $(GalA)_{16}$-GalA-Rha with 12 Me and 2 AC; mass of 3531 was consistent with $[M+H^+]$ of reducing-end cyclized

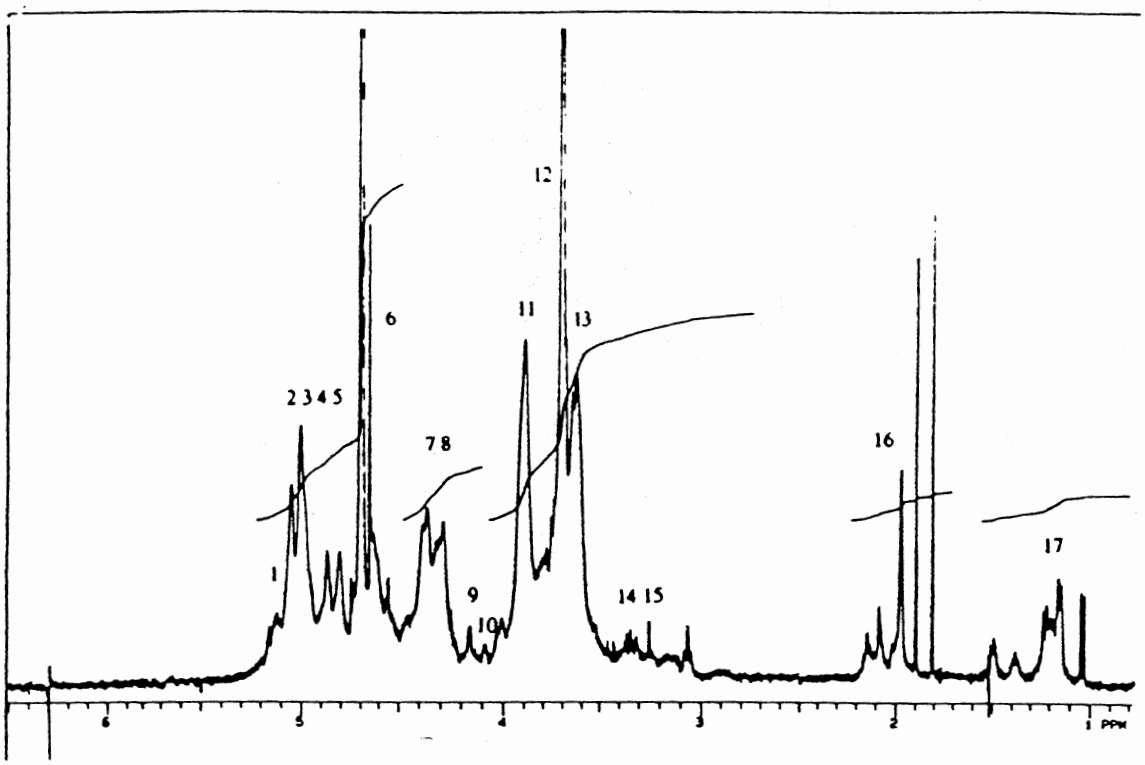


Figure 47. ^{1}H-n.m.r. Spectrum of Fraction 1 Fractionated on Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 2. The spectrum was recorded on a 400 MHz n.m.r.spectrometer at 25^{0}C in D_2O. Peaks are assigned as: 1) H-1 of GalA(C), 2,3,4,5) H-1 of GalA, 6) H-5 of GalA and H_2O, 7,8) H-4 of GalA, 9) H-4 of GalA(C), 10) H-2 of β-Rha, 11) H-3 of GalA, 12) methyl ester of GalA, 13) H-2 of GalA, 14) H-4 of β-Rha, 15) H-5 of β-Rha, 16) CH_3 of acetate, 17) CH_3 of Rha.

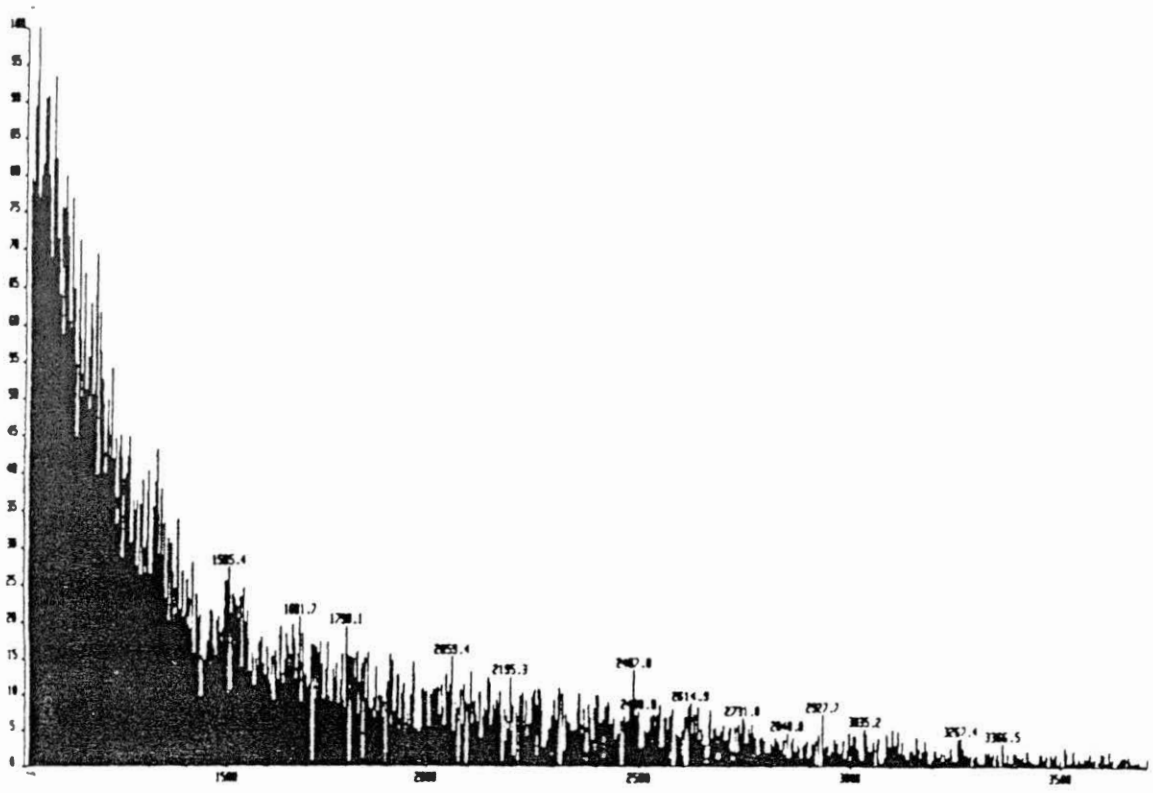


Figure 48. LSIMS Spectrum of the Fraction 1 Obtained by Fracto-Gel HW40S HPLC Gel Filtration Chromatography of the HF-treated RGI Isolated by Method 2. Masses of 1505.4, 1681.7, 1798.1 and 2059.4 were consistent with the molecular weights of the reducing end cyclized $(GalA)_6$-GalA-Rha with 2 AC and 3 Me attached, $(GalA)_7$-GalA-Rha with 2 AC and 3 Me attached, $(GalA)_8$-GalA-Rhawith 2 Me attached, and $(GalA)_8$-GalA-Rha with 1 AC and 3 Me attached, respectively.

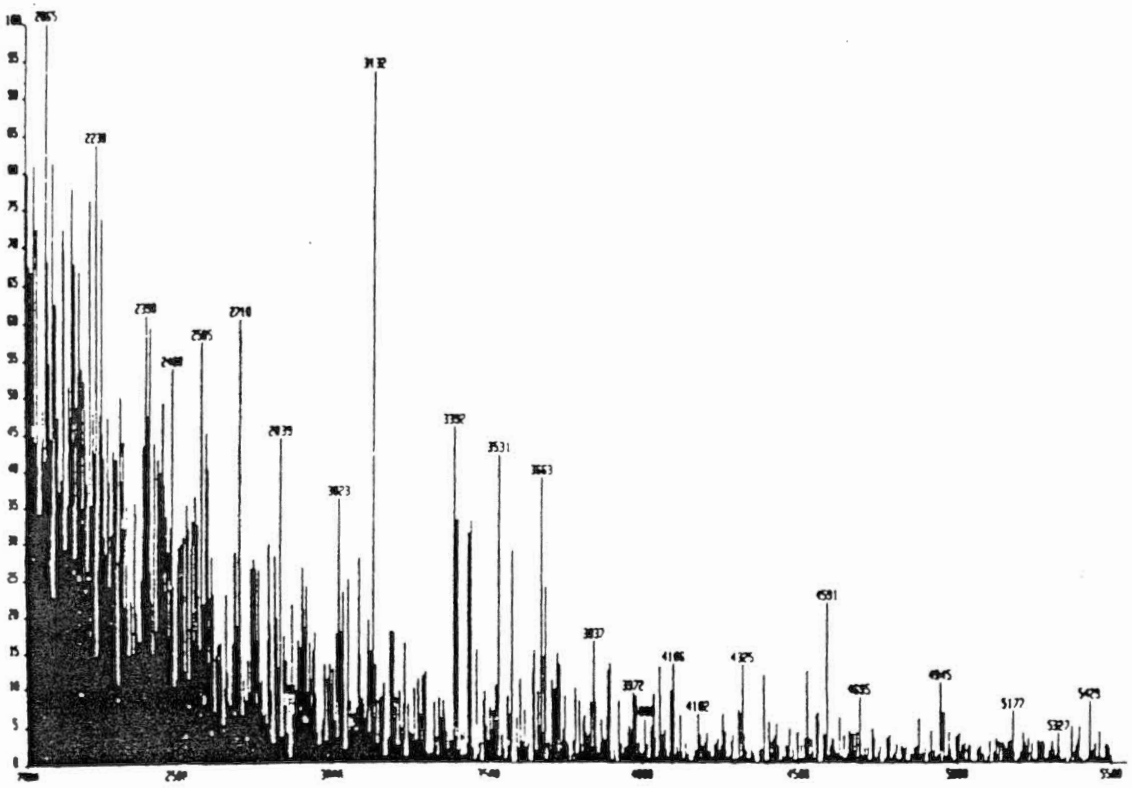


Figure 49. LSIMS Spectrum of the Fraction 1 Obtained by Fracto-Gel HW40S HPLC Gel Filtration Chromatography of the HF-treated RGI Isolated by Method 2. Masses of 1505.4, 1681.7, and 2059.4 were consistent with the M.W. of the reducing end-cyclized $(GalA)_6$-GalA-Rha with 2 AC and 3 Me, $(GalA)_7$-GalA-Rha with 2 AC and 3 Me, and $(GalA)_8$-GalA-Rha with 1 AC and 3 Me, respectively.

$(GalA)_{17}$-GalA-Rha with 6 Me and 1 Xyl linked; mass of 3663 was consistent with $[M+H^+]$ of reducing-end cyclized $(GalA)_{17}$-GalA-Rha with 6 Me and 2 Xyl linked; and mass of 3837 was consistent with $[M+H^+]$ of reducing-end cyclized $(GalA)_{17}$-GalA-Rha with 9 Me and 3 Xyl linked, respectively

For comparison, RGI isolated by method 1 (strong alkali extracted, and any methyl and acetate esters cleaved off) was also treated with HF at -23^0C and then fractionated on Fracto-Gel HW40S HPLC gel filtration column (Figure 50). The pattern of this chromatography was almost identical to that of RGI isolated by method 2, except for a relatively lower sugar content in the fraction 1 (Vo) and a relative higher sugar content in fraction 6. The molar ratio of GalA/Rha in fraction 1 was also close to 13 : 1, suggesting that the same Rha-terminated oligogalacturonans are present in this fraction as in fraction 1 from RGI isolated by method 2. LSIMS spectra of fraction 3, 4, 5, and 6 from Figure 49 confirmed the identification of the RGI oligomers obtained from the corresponding fractions in Figure 42 by the results presented as follows. The mass spectrum of fraction 3 contained two significant peaks, one with mass of 667.1, and the other with mass of 989.2, representing the tetrasaccharide and hexasaccharide, respectively. The mass spectrum of fraction 4 contained a very intense peak with mass of 667.1, representing the tetrasaccharide. The mass spectrum of fraction 5 contained a number of peaks, two of which with masses of 667.1 and 507.1 are consistent with $[M+Na^+]$ of the tetramer and of a trimer of GalA-Rha-Gal that also appeared in the spectrum of fraction 4, respectively. The mass spectrum of fraction 6, as expected, contained two big peaks with masses of 323 and 342, representing the cyclized and the non-cyclized disaccharide, respectively.

To learn more about the size distribution of the Rha-terminated oligogalacturonans in fraction 1, which accounted for about 17% of the weight of the HF-treated RGI isolated by method 2, the fraction was de-methylesterified with 0.05 M NaOH at room temperature for 15 min and then applied to a Dionex CarboPac PA-1 HPLC ion exchange column (Figure 51). The chromatogram showed many peaks, but the significant ones, about 13,

were eluted with retention times of 17 to 40 min. By comparison with the standard chromatogram which was created by application of a mixture of non-methylesterified galacturonides (Aldrich Chemical Company Inc.) to the same column under the same conditions (Figure 52), it is concluded that these Rha-linked oligogalacturonans have sizes from 5 to 15 residues long, assuming that they performed in the chromatography similarly to the commercial galacturonides. Indeed, peaks of these Rha-terminated oligogalacturonans were found to be eluted a little earlier (~1.0 min) than the corresponding peaks of oligogalacturonides, and this behavior may have resulted from the rhamnosyl residue, a neutral sugar, linked at the reducing end of every oligogalacturonans. Some peaks, such as peaks 4, 6, 8, 10, and 12, which eluted at somewhat irregular retention times, might have been Rha-terminated oligogalacturonans which had some other sugars attached. The candidates for these sugars could be xylose, galactose, glucose and even glucuronic acid which were present in this fraction in significant amounts based on sugar composition analysis (Table 20).

Glycosyl composition analysis revealed that peaks 1, 2, and 3 from Figure 51 have a molar ratio of GalA/Rha of 4 : 1, 5 : 1, and 6 : 1, respectively, confirming the above structural elucidation for those Rha-terminated oligogalacturonans.

Fraction 3 to 8 from the HW40S chromatography of HF-treated RGI were also chromatographed on Dionex CarboPac PA-1 HPLC column with the following elution program: Inject in 0.1 M NaOH, after 10 min a linear increase in NaAcetate from 0 to 0.25 M (0.1 M NaOH) over 10 min followed by a linear increase to 0.5 M NaAcetate (0.1 M NaOH) over 30 min and another linear increase up to 0.75 M NaActate (0.1 M NaOH) over next 20 min. The oligosaccharides in these fractions separated very well and coincided with the above results of LSIMS and n.m.r. analysis. In other words, in this chromatography fraction 8 showed several big peaks which are neutral monosaccharides; fraction 7 and 6 showed a major peak of dimer of GalA-Rha; fraction 5 showed two major peaks which are neither dimer nor tetramer of GalA-Rha repecting units and could not be identified yet;

fraction 4 showed three major peaks which were di-, tetra-, and hexamers, respectively; and fraction 3 showed five major peaks which were di-, tetra-, hexa-, octa-, and decamers, respectively (Figure 53). Other smaller peaks with different retention times may be from the oligomers with one or more neutral sugars attached.

The results presented here indicate that the specific HF reaction cleaved almost all the RGI sidechain sugars into monomers which made up the fraction 8, and cleaved the RGI backbone at rhamnosyl linkages to produce both GalA-Rha repeating unit fragments which ranged from dimer to decamer (mainly di- and tetramer) and Rha-terminated oligogalacturonans (some of them are likely attached to other sugars) most of which were in the range from pentamer to 15-mer in length.

It is thus reasonably proposed that the backbone of the RGI isolated by method 2 is composed not only of GalA-Rha repeating units but also of oligogalacturonans. Sugar composition analysis of the RGI showed that neutral sugars largely in sidechains accounted for about 40%; Rha and GalA making up disaccharide repeating units of backbone accounted for about another 40%; and the extra GalA residues which were probably in the form of oligogalacturonans linked to the backbone accounted for the remaining 20% of RGI. Fraction 1, consisting of Rha-linked oligogalacturonans from HW40S HPLC gel filtration chromatography of the HF-treated RGI, represented 17% of the applied material by weight, closely coinciding with the above result of sugar compositions. It is thus concluded that the RGI backbone is composed of the GalA-Rha repeating units which are interrupted by oligogalacturonans in about every 10 units (20 residues in length). The average length of these interspersed oligogalacturonans is about 10 residues. In other words, the RGI backbone is composed of alternating oligo-GalA-Rha repeating fragments and oligogalacturonans. A proposed structure of the cotton suspension culture cell wall RGI backbone is exhibited in Figure 54.

The biggest GalA-Rha repeating fragment identified by LSIMS was 10 residues in length and this was due to the fact that most of the rhamnosyl glycosidic bonds had been

cleaved by HF in the employed reaction condition. In addition, bigger fragments of the repeating unit might have existed in small amount in the HW40S fractions of the HF-treated RGI but were not identifed by the above techniques (LSIMS, n.m.r. and HPLC) due to their small quantities.

The cellulase-pretreated residue which still contained RGI-like polysaccharide in addition to proteins was also treated with HF at -23^{0}C to yield a whole picture of cotton cell wall rhamnogalacturonan I. HW40S gel filtration chromatography of the treatment-solubilized material was very similar to that in Figures 33 and 50. The corresponding fraction 1, containing only about 40% sugar by weight and having a significant UV absorbance, may have contained both polysaccharide and other UV-absorbed components like phenolic compounds, but no Hyp was detected in this fraction. The GalA in this fraction was 40% methylesterified. The ratio of GalA/Rha in this fraction was about 4 : 1, suggesting that there were smaller and/or fewer oligogalacturonans present in the RGI isolated in this way, and this was different from the RGI isolated by method 2 discussed previously. The corresponding fractions 3, 4, and 5 from the chromatography, having a1: 1 ratio of GalA/Rha, were mainly from RGI backbone. Therefore it could be proposed that two types of RGI backbone are present in cotton suspension cultured cell walls: one consists of the GalA-Rha repeating units which are interspersed by oligogalacturonans; and the other consists of the GalA-Rha repeating units without or with shorter or fewer oligogalacturonans interspersed. The latter is likely cross-linked with phenolic components and glycoproteins. This proposal is also consistent with the result obtained by treating the alkali-extracted RGI with HF followed by HW40S HPLC fractionation, where the oligogalacturonan fraction accounted for only about 10% by weight of the total treated RGI instead of 17%. In other words, only about half of the RGI backbone had the structure described in Figure 54.

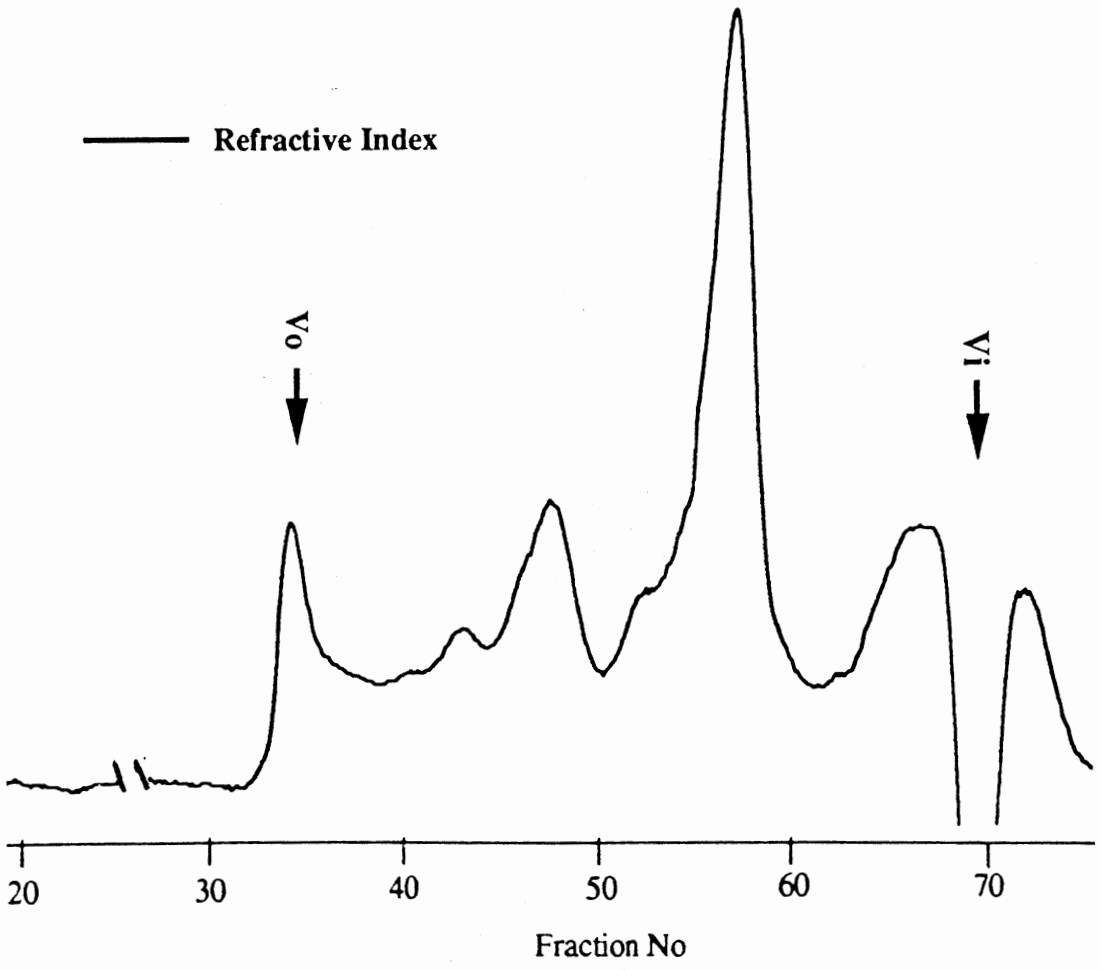


Figure 50. Chromatography on a Fracto-Gel HW40S HPLC Gel Filtration Column of the HF-treated RGI Isolated by Method 1. Tubes 33-37 were pooled into fraction 1, 38-41 into fraction 2, 42-44 into fraction 3, 45-50 into fraction 4, 51-53 into fraction 5, 54-59 into fraction 6, 60-62 into fraction 7, and 63-75 into fraction 8. No sugars were detected in tubes 76-82.

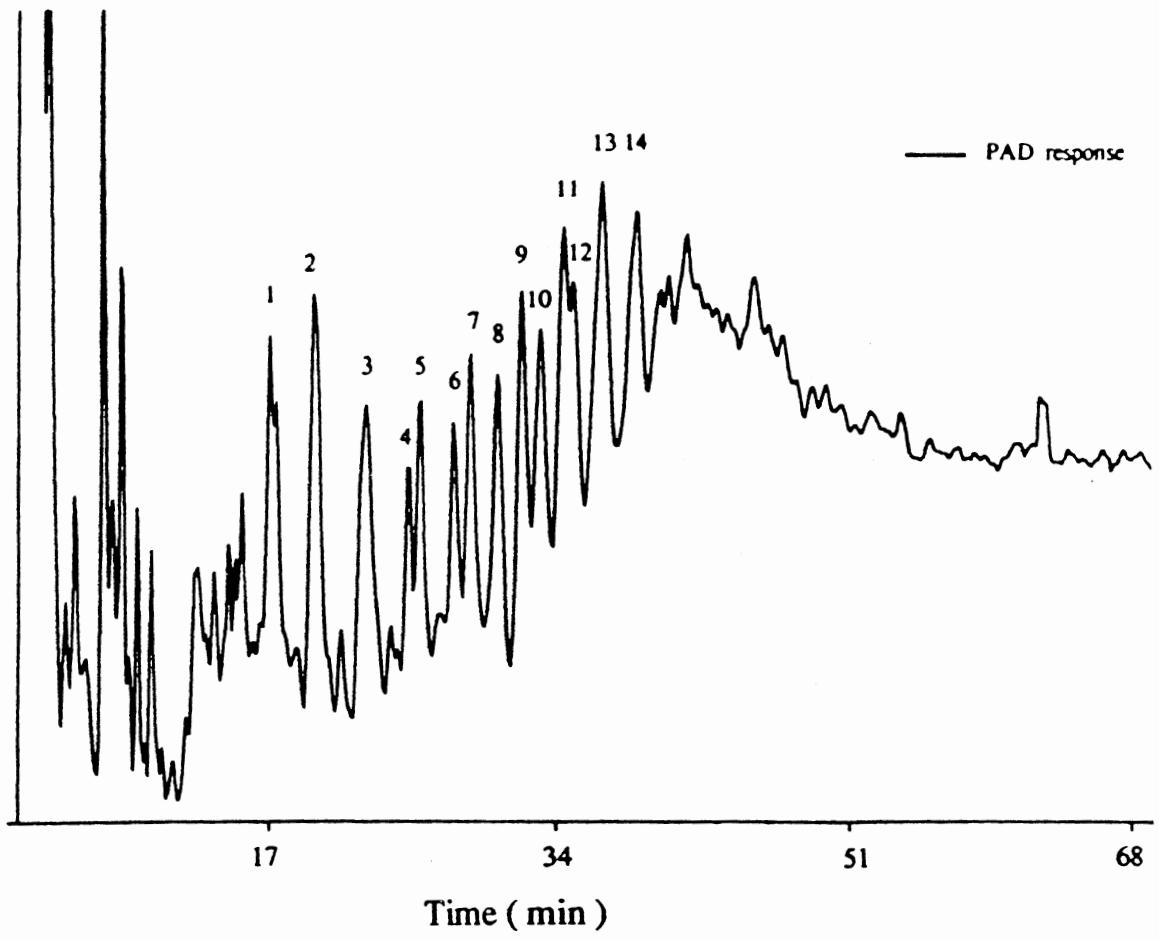


Figure 51. Chromatography on a Dionex CarboPac PA-1 HPLC Ion Exchange Column of HW40S Fraction 1 of the HF-treated RGI Isolated by Method 2.

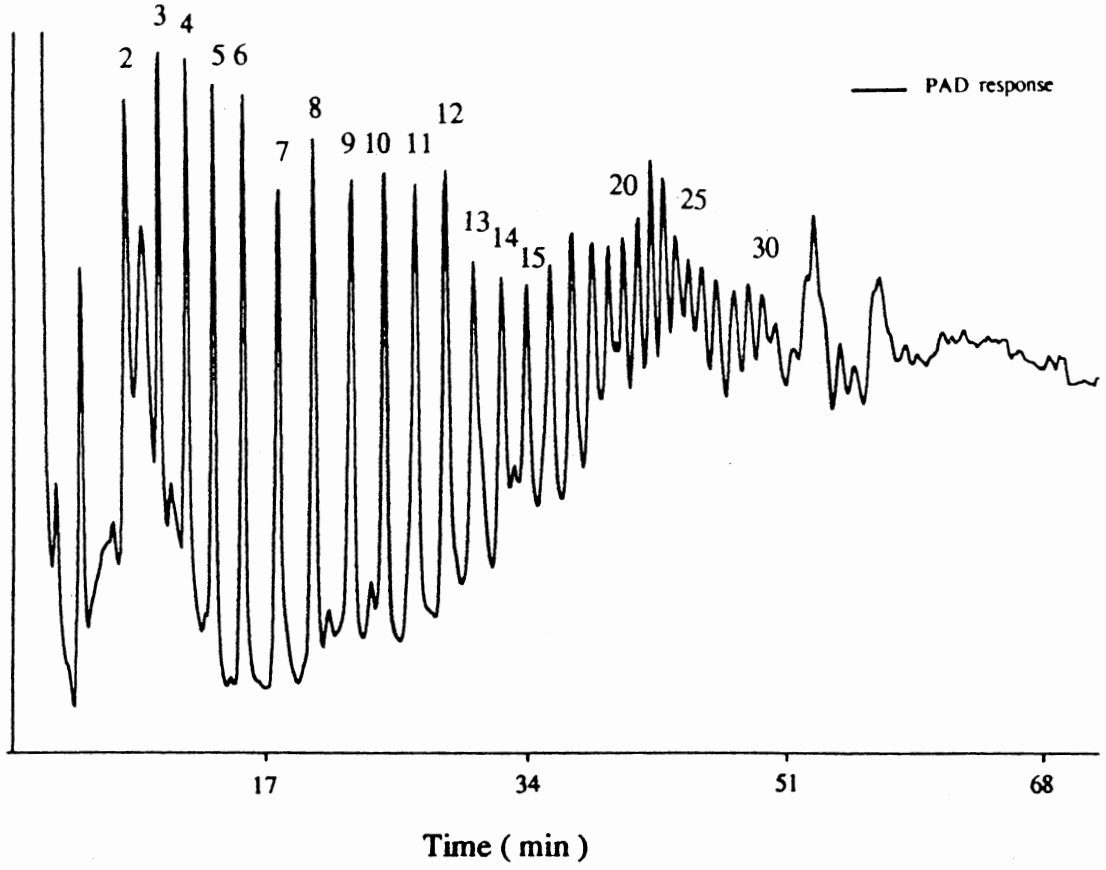


Figure 52. Chromatography on a Dionex CarboPac PA-1 HPLC Ion Exchange Column of a Mixture of Non-methylesterified Galacturonides from Aldrich Chemical Company Inc.. Peaks 2-30 were identified as dimer to thirtymers of the galacturonides.

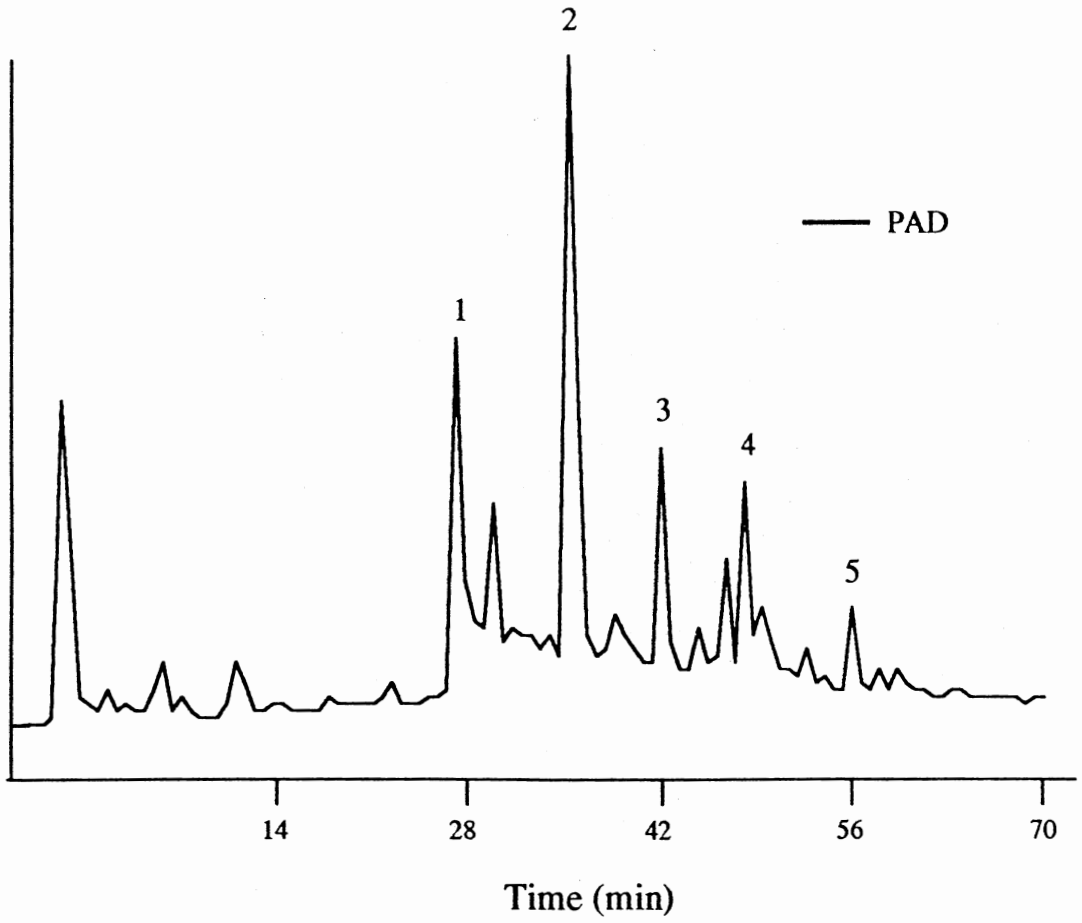


Figure 53. Chromatography on a Dionex CarboPac PA-1 HPLC Ion Exchange Column of the Fraction 3 of the HF-treated RGI Isolated by Method 2. Peaks 1 to 5 were identified as di-, tetra-, hexa-, octa-, and decamers of the [GalA-Rha] repeating units in RGI backbone, respectively.

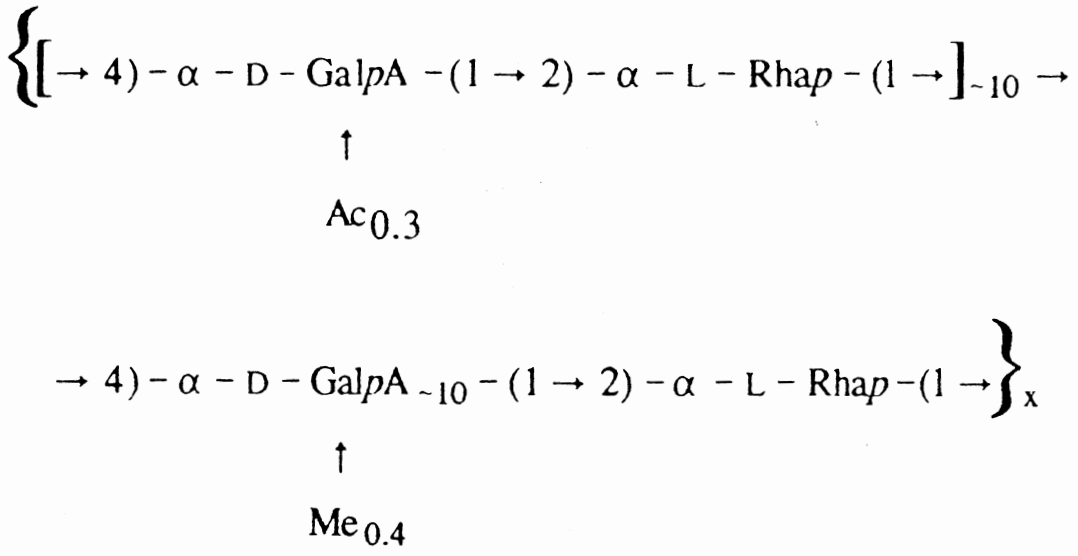


Figure 54. Proposed Structure of Cotton Suspension Culture Cell Wall RGI Backbone.

Isolation of Rhamnogalacturonan II from Cotton Cell Walls

RGII, one of the pectic polysaccharides already found in the cell walls of suspension cultured sycamore cell, suspension cultured douglas fir and rice cells, and in Pectinol-AC (Darvill *et. al.*, 1978; Thomas *et. al.*, 1987 and 1988; Spellman *et. al.*, 1983), was also isolated from cotton suspension culture cell walls. Treatment of cotton suspension culture cell walls (both Im 216 and Acala 44 strains) with endopolygalacturonase solubilized RGII together with homogalacturonan in the form of tri- and tetragalacturonides as described earlier. Application of the EPG-solubilized sugar mixture on a DEAE-Sephadex anion exchange column separated RGII-containing polysaccharides, which eluted at higher concentrations in the NaCl gradient, from the tri- and tetramers of galacturonic acid (Figure 1 and Table 2). In order to further purify the RGII from the RGII-containing fraction, the fraction was chromatographed on a Bio-Gel A-5m gel filtration column (Figure 55). The sugar compositions of the fractions from the chromatography are shown in Table 21. Two small fractions (Fra 1 and 2), which eluted earlier, had sugar compositions very similar to that of RGI discussed earlier. In contrast, fraction 3, the major one, which eluted close to the included volume, contained glucuronic acid, rhamnose, fucose and some unusual sugars which have not been found in RGI, showing the glycosyl composition characteristic of RGII (Darvill *et. al.*, 1978).

Partial Analysis of the Rhamnogalacturonan II

Size Estimation of the RGII

The size distribution of the fractionated RGII was determined by a chromatography of Bio-Sil TSK2000SW HPLC gel filtration standardized by pullulans (Figure 56). Five fractions were collected and the sugar compositions of these fractions are shown in Table 22. From the chromatography it was found that the size of the isolated RGII was not

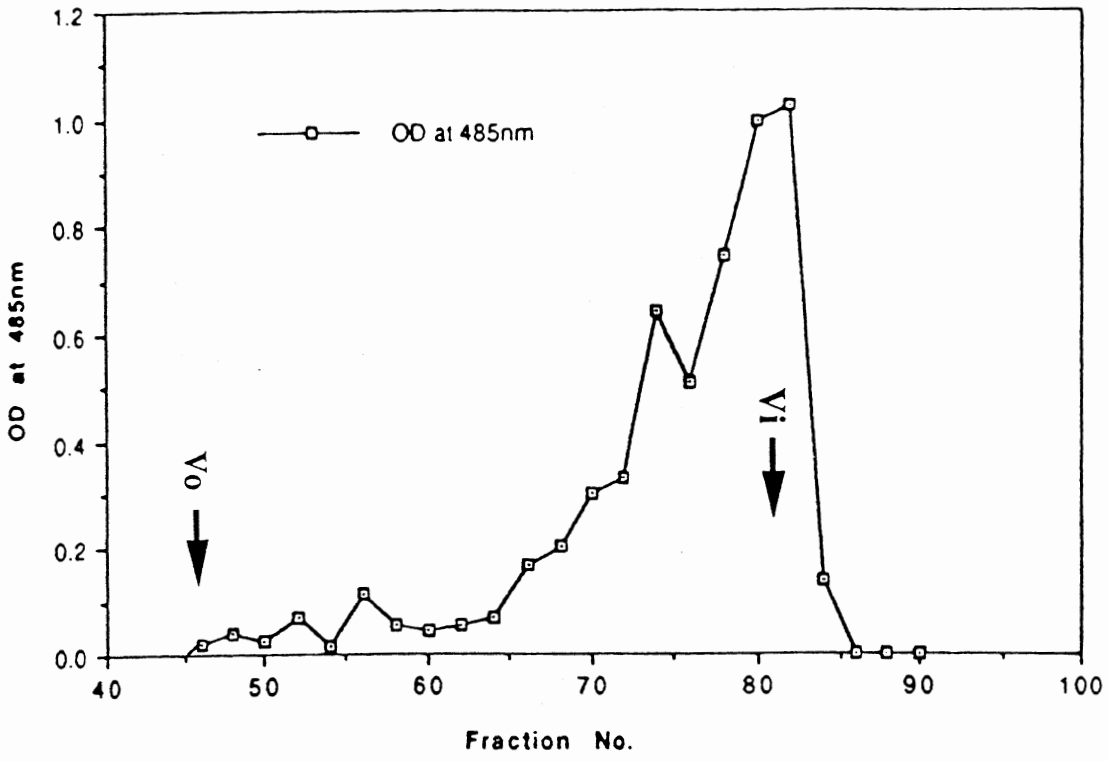


Figure 55. Chromatography on a Bio-Gel A-5m Gel Filtration Column of the Fraction 4 from DEAE-Sephadex Anion Exchange Chromatography of the Fraction Extracted by EPG from Acala 44 Cotton Cell Walls. Tubes 45-60 were pooled into fraction 1, 61-71 into fraction 2, and 72-86 into fraction 3 (RGII).

TABLE 21

GLYCOSYL COMPOSITIONS (MOLE%) OF BIO-GEL A-5m GEL FILTRATION OF THE FRACTION 4 FROM DEAE-SEPHADEX ION EXCHANGE CHROMATOGRAPHY OF THE EPG EXTRACT FROM COTTON CELL WALLS

Residue	Fra 1	Fra 2	Fra 3
Ara	8.2	6.1	9.4
Rha	18.8	22.4	11.3
Fuc	-	-	1.7
Xyl	7.4	4.0	6.7
GalA	37.8	50.8	62.3
Man	2.8	-	-
Gal	20.3	13.0	6.7
Glc	4.7	3.7	trace
GlcA	-	-	1.6
Unusual Sugars	-	-	yes

TABLE 22

GLYCOSYL COMPOSITIONS (MOLE%) OF SW2000 HPLC GEL FILTRATION FRACTIONS OF FRACTION 3 FROM BIO-GEL A-5m OF FRACTION 4 FRACTIONATED BY DEAE-SEPHADEX OF EPG-TREATED CELL WALLS

Residue	Fra 1	Fra 2	Fra 3	Fra 4	Fra 5	Fra 6
Ara	6.8	7.1	11.7	10.7	6.0	0.7
Rha	15.3	8.9	13.5	13.5	7.6	0.8
Fuc	2.0	1.5	2.9	2.7	1.6	-
Xyl	10.7	9.0	4.4	3.9	5.2	6.8
GalA	54.9	65.2	59.8	60.7	70.2	90.4
Man	-	-	-	-	1.4	1.8
Gal	7.7	6.2	7.8	.8.7	5.7	-
Glc	2.6	2.1	trace	trace	2.6	1.7
GlcA	trace	trace	trace	trace	-	-
Unusual Sugars	yes	yes	yes	yes	yes	no

uniform, ranging from 5,800 to 23,700 in molecular weights according to pullulan standard. The major fraction, fraction 3, had a molecular weight between 12,200 and 5,800 by pullulan standard, and was quite consistent with the reported molecular weight of RGII from sycamore suspension culture cell walls.

Glycosyl Compositions of the RGII

The sugar compositions of the five RGII fractions from Bio-Sil TSK2000SW column were very similar. Due to the lack of standard, however, some small peaks in the gas chromatogram which almost undoubtedly represented the derivatized unusual sugars such as aceric acid, apiose, KDO and DHA, were not identified. Comparing the peak areas of these unidentified sugars with the identified ones, the relative proportions of these sugars were assumed, and thereby a molar ratio of the sugars in the RGII was deduced as normalized to that of the xylosyl residue (Table 23). Comparing these data with that for RGII from sycamore cell walls (Stevenson *et. al.*, 1988), it was found that the sugar molar ratios of RGII from cotton and sycamore cell walls are very similar except that the RGII from cotton contains much more galacturonic acid. Some homogalacturonans, most likely short oligogalacturonans, may be linked to the RGII molecule. Some oligogalcturonans, containing up to 8 galacturonic acid residues were reported to be released from sycamore RGII by mild hydrolysis with acid (Stevenson *et al.*, 1988).

DOM Determination of the RGII

The degree of methylesterification of the isolated RGII was determined by reducing the sample (fraction 3 from Bio-Sil TSK2000SW chromatography) with $NaBH_4$ to convert the any methylesterified galacturonic acid into galactose. Comparison of the sugar compositions of the $NaBH_4$-reduced RGII with the control indicated that there was no methylesterified uronic acid present in the RGII. This suggests that the oligogalacturonans linked to the RGII as assumed are probably either branched or less than five residues long

TABLE 23

COMPARISON OF GLYCOSYL COMPOSITIONS IN MOLAR RATIO* BETWEEN COTTON AND SYCAMORE CELL WALL RGII OF SUSPENSION CULTURE

Residue	Cotton RGII	Sycamore RGII	Residue	Cotton RGII	Sycamore RGII
GalA	17	8	Ara	3	3
Rha	4	4	GlcA	1	1
Xyl	1	1	Apiose	?	3
Fuc	-	-	DHA	?	1
2-0-Me-Fuc	1	1	KDO	?	1
2-0-Me-Xyl	1	1	Aceric Acid	?	1
Gal	2	2			

* Normalized to that of xylose residue.

since the substrate specificity activity of the endopolygalacturonase probably needs more than 5 consecutive unbranched GalA residues for activity.

^{1}H-n.m.r. Analysis of the RGII

^{1}H-n.m.r. spectrum of the isolated RGII (fraction 3 from Bio-Sil TSK2000SW chromatography) is shown in Figure 57. Five signals with the chemical shifts expected for protons of galacturonic acid were identified from the spectrum. Signals 1 through 5 were assigned as H-1, H-5, H-4, H-3, and H-2 of galacturonic acid residue, respectively. Signal 6 with a chemical shift of 3.52 p.p.m. was assigned as the CH_3 of the 2-0-methyl fucose and 2-0-methyl xylose residues. Signal 7, the most upfield one, was identified as the methyl group from both rhamnosyl and fucosyl residues as described previously in the ^{1}H-n.m.r. spectra of xyloglucan and rhamnogalcturonan I.

Absence of the signals for acetate ester in the spectrum indicates that there is no acetylation present in these RGII molecules.

Identification of 2-0-Me-Xyl and 2-0-Me-Fuc by GC-MS

In order to further confirm the presence of 2-0-Me-xylose and 2-0-Me-fucose in the cotton cell wall RGII, the isolated RGII was analyzed by GC-MS of partially acetylated alditol derivatives. The gas chromatogram of the analysis is illustrated in Figure 58. Peak 518 has a mass spectral fragmentation pattern corresponding to that of the derivatized 2-0-methyl fucose, and thus was assigned as 2-0-linked fucosyl residue. Peak 558 has a mass spectral fragmentation pattern corresponding to that of the derivatized 2-0-lined xylose, and thus was assigned as 2-0-linked xylose.

Glycosyl-linkage Compositions Analysis of the RGII

Glycosyl-linkage compositions of the RGII were determined by GC-MS of partially methylated alditol acetate derivatives. A gas chromatogram of the derivatized RGII is

shown in Figure 59. Peak 86 was identified as terminal arabinofuranose, peak 118 as terminal xylose, peak 122 as terminal rhamnose, peak 146 as terminal fucose, peak 211 as 2-linked rhamnose, peak 242 as 3-linked rhamnose, peak 282 as terminal glactose, and peak 498 as 2, 6-linked galactose, respectively. Peak 288 is likely, but not certain, to be 3'-linked apiose from its fragmentation pattern that gives a big fragment of 117.

The glycosyl-linkage compositions and molar proportions of cotton cell wall RGII identified here are very similar to those of sycamore RGII (Table 24). However, the glycosyl linkages of 2,3,4-linked rhamnose, 3,4-linked fucose and 2-linked arabinopyranose which were found in sycamore RGII were not identified in cotton cell wall RGII. Possibly, the peaks which have not been identified were derived from these residues. Another difference is that cotton RGII contains much more 2-linked rhamnose than sycamore RGII, suggesting that some kind of structural difference may exist between cotton and sycamore RGIIs.

Analysis of the Mild Acid-hydrolyzed RGII Fragments

The isolated RGII was partially hydrolyzed with mild acid of 0.1M TFA for 24 h at 50^0C. This hydrolysis cleaved the acid-labile glycosidic bonds of apiosyl residues, releasing oligosaccharides having an apiose residue at their reducing terminus (Thomas *et al.*, 1989). LSIMS spectrum of the hydrolate (Figure 60) exhibited a few significant peaks with masses of 1055.2, 1187.2 and 1305.3. A mass of 1055.2 is consistent with the M.W. of the heptasaccharide (1) identified from sycamore suspension culture cell wall RGII, and 1187.2 is consistent with the M.W. of the heptasaccharide (1) plus a pentose. 1305.3 and 1327.3 are likely the signals for [$M+H^+$] and [$M+Na^+$] of another heptasaccharide (2) identified from sycamore RGII although the masses differ in about 2. Fractionation of the resulting oligosaccharide mixture produced by the mild acid hydrolysis with Fracto-Gel HW40S HPLC gel filtration chromatography gave rise to four fractions. LSIMS spectrum

of one of the fractions does contain a significant peak with a mass of 1329.4, which is consistent with [M+Na$^+$] of the heptasaccharide (2).

The results presented here, of course, provide only preliminary information about the structural characteristics of RGII from cotton suspension culture cell walls. More work needs to be done to reveal the detailed structure of this complex cell wall polysaccharide, and thus to facilitate the understanding of both its biological function and its relationship with other polymers in the walls.

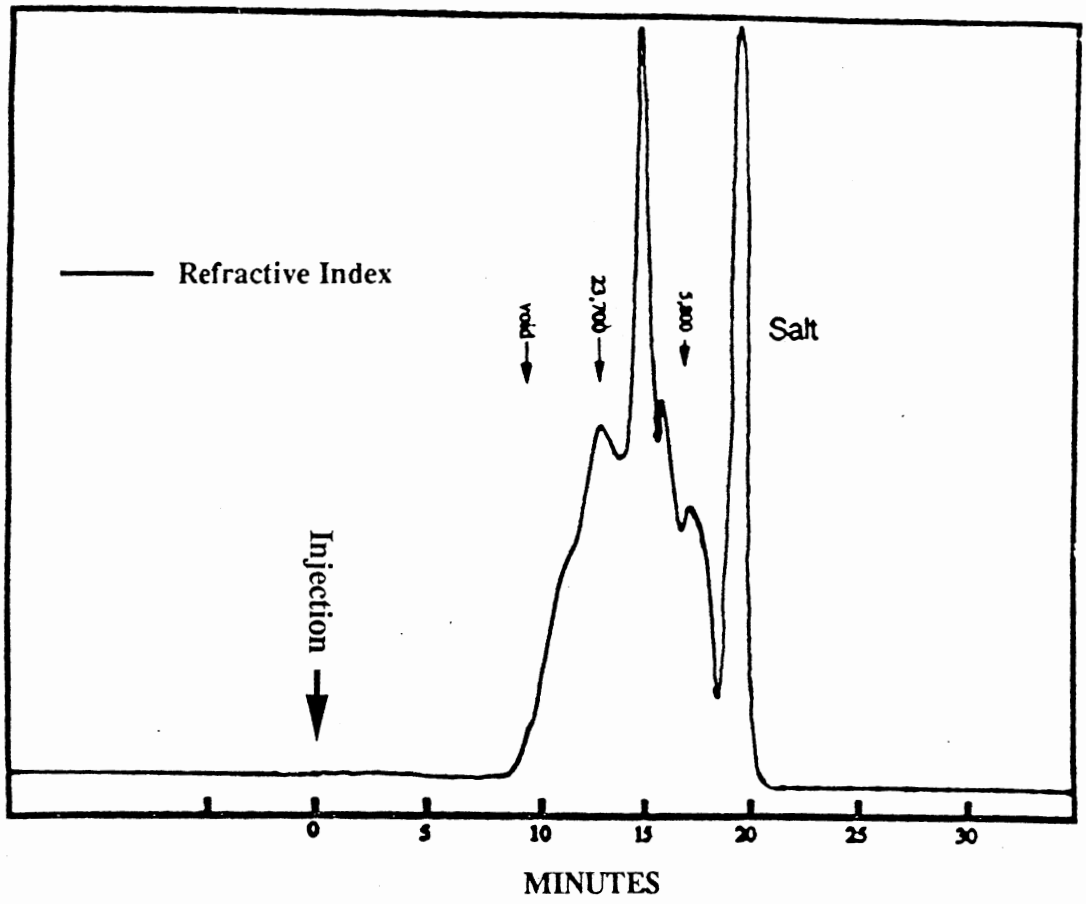


Figure 56. Chromatography on a Bio-Sil TSK 2000SW HPLC Gel Filtration Column of the Fraction 3 from Bio-Gel A-5m Chromatography. Tubes 9-11 were pooled into fraction 1, 12-14 into fraction 2, 15-16 into fraction 3, 17 into fraction 4, 18-19 into fraction 5 and almost no sugars were detected in tubes 20-21.

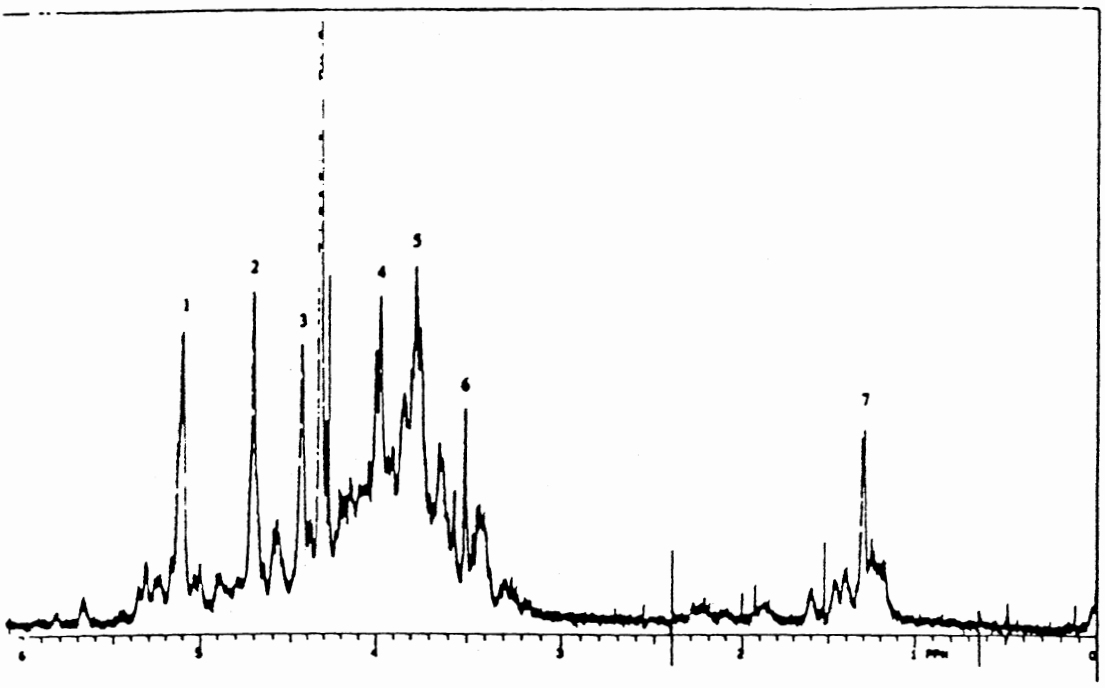


Figure 57. ^{1}H-n.m.r. Spectrum of the RGII (Fraction 3 of TSK 2000SW HPLC Chromatography) Isolated by EPG from Cotton Suspension Culture Cell Walls. Peak 1 was identified as H-1 of GalA, peak 2 as H-5, peak 3 as H-4, peak 4 as H-3, peak 5 as H-2 of GalA, respectively. Peak 6 is the signal of the methyl ester of the 2-0-Me-Xyl, and 2-0-Me-Fuc, and peak 7 is the signal for CH_3 of Rha and Fuc.

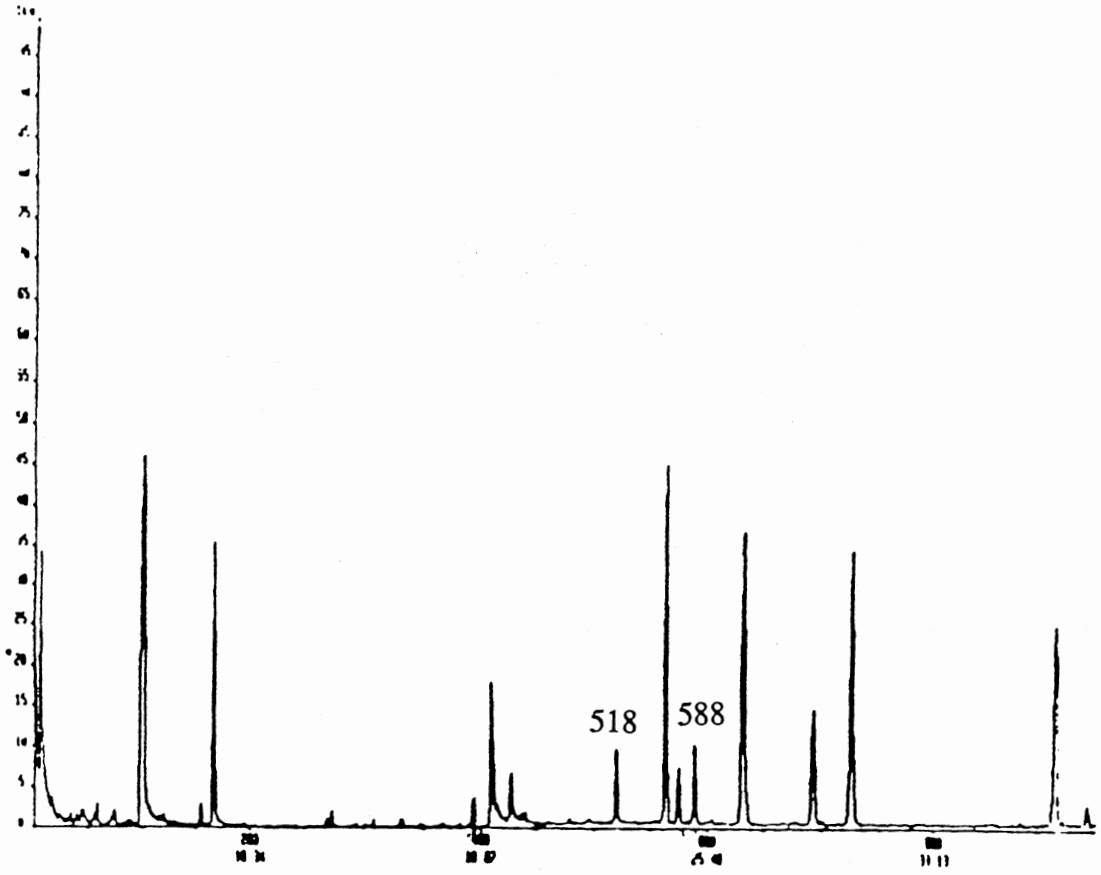


Figure 58. Gas Chromatogram of the Partially Acetylated RGII (Fraction 3 of TSK2000SW HPLC Chromatography) Isolated by EPG from Cotton Suspension Culture Cell Walls. Peaks were identified from the mass spectra of these signals. 518: 2-0-linked methyl fucose, and 588: 2-0-linked methyl xylose.

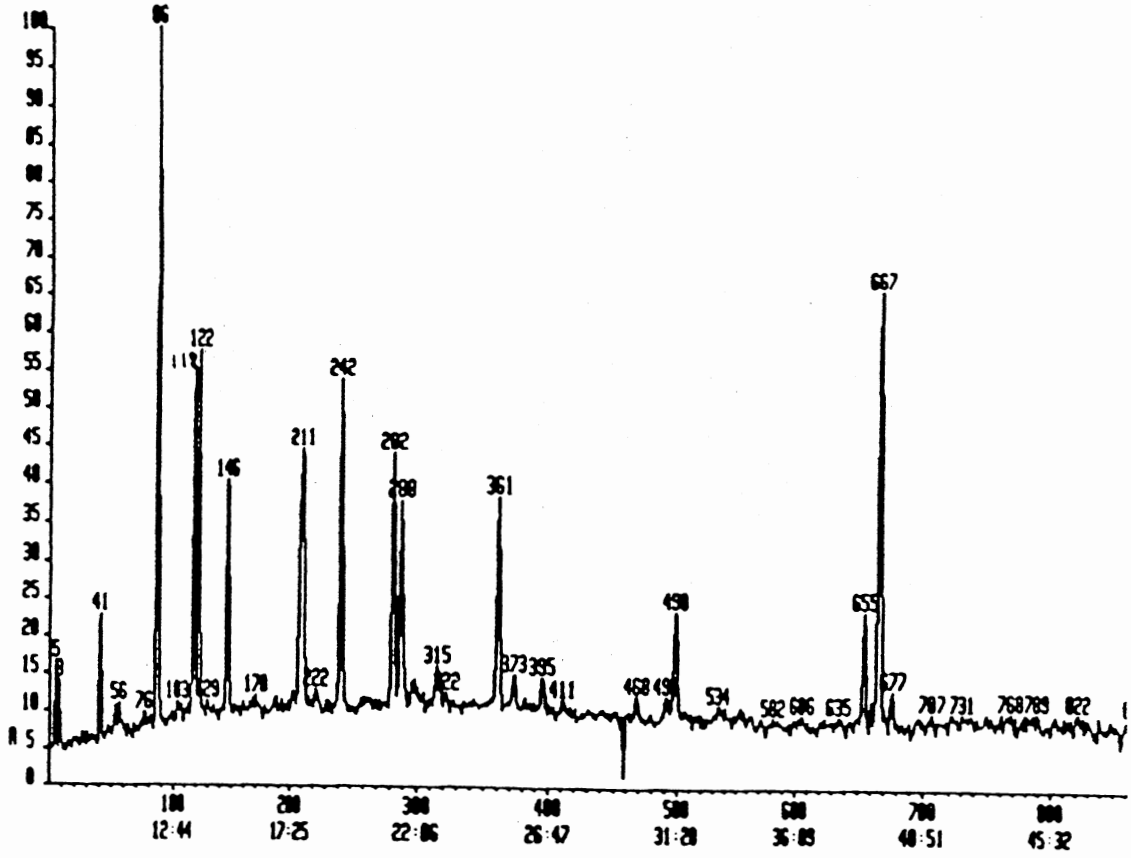


Figure 59. Gas Chromatogram of the Partially Methylated Alditol Acetate Derivatives of the RGII Solubilized by EPG from Cotton Cell Walls. Peaks were identified from the mass spectra of these signals. 86: t-Ara, 118: t-Xyl, 122: t-Rha, 146: t-Fuc, 211: 2-Rha, 242: 3-Rha, 282: t-Gal, 498: 2,6-Gal, and 288: 3'-apiose?

TABLE 24

COMPARISON OF GLYCOSYL-LINKAGE COMPOSITIONS BETWEEN COTTON AND SYCAMORE RGII OF SUSPENSION CULTURE

Residue	Deduced Linkage	Cotton Cell Wall RGII Molar Ratio*	Sycamore Cell Wall RGII Molar Ratio
Rha	Terminal	1.0	1.0
	2-linked	1.5	trace
	3-linked	1.0	1.0
	2,3,4-linked	ND**	1.0
Ara	Terminal	2.0	1.0
	2-linked	ND	1.0
Xyl	Terminal	1.0	1.0
Fuc	Terminal	1.0	1.0
	3,4-linked	ND	1.0
Gal	Terminal	0.5	1.0
	2,4-linked	0.5	1.0
Apiose	3'-linked	0.5?	2.0

* Normalized to that of the terminal xylose residue.

** Not identified.

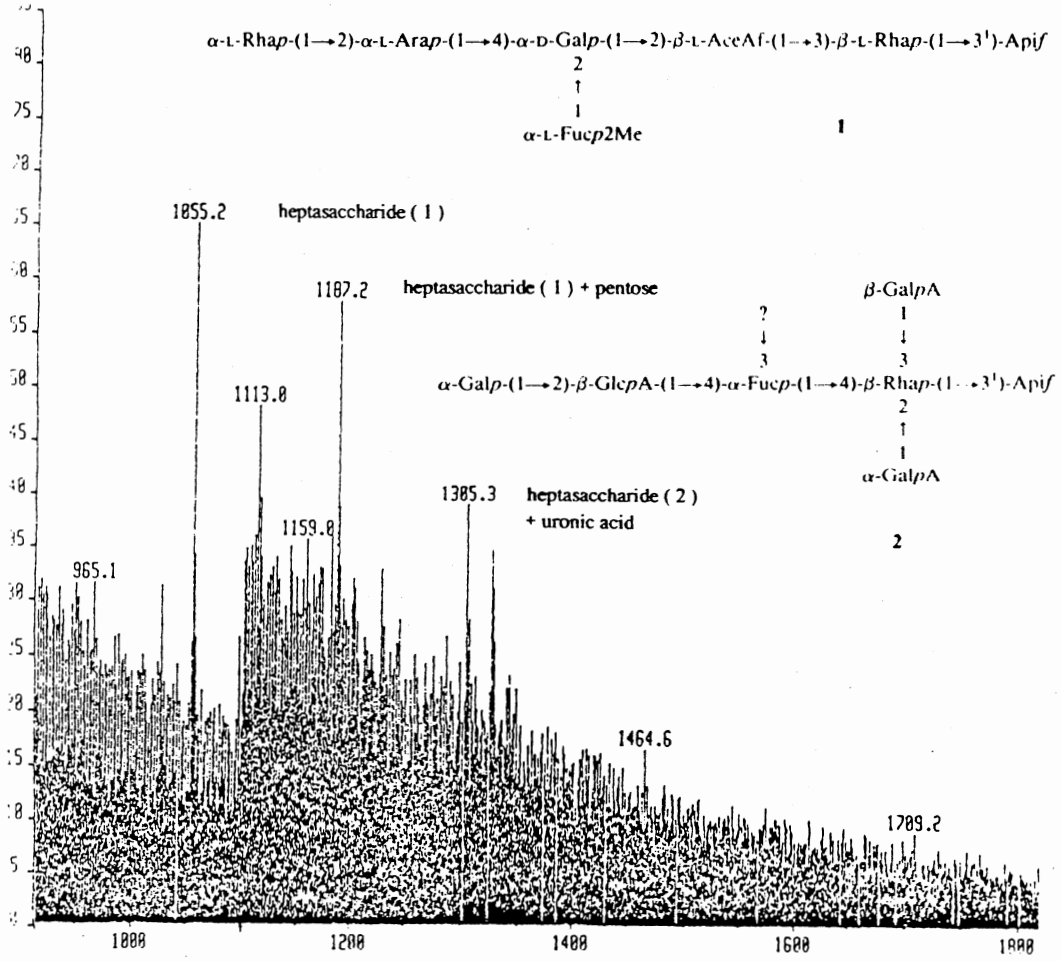


Figure 60. The LSIMS Spectrum of the Mild Acid-hydrolyzed RGII Oligosaccharides. Peak with mass of 1055.2 was consistent with the M.W. of heptasaccharide 1, 1187.2 consistent with heptasaccharide 1 + a pentose, and 1305.3 was putatively assigned as heptasaccharide 2 + a uronic acid.

CHAPTER V

SUMMARY AND CONCLUSION

Xyloglucan and pectic polysaccharides are major components of plant primary cell walls. A detailed characterization of their structures and their structural relationship is undoubtedly essential for understanding their physiological and pathological functions in plant cell walls. This research was undertaken to isolate and characterize xyloglucan and pectic polysaccharides, especially rhamnogalacturonan I, from cotton suspension culture cell walls.

Xyloglucan was isolated from cotton suspension culture cell walls (including Acala 44 and Im 216 strains) in two different ways using both chemical and enzymatic methods. Strong alkali (24% KOH-0.1% $NaBH_4$) extracted more than 95% of xyloglucan along with rhamnogalacturonan I from the endopolygalacturonase pre-treated cotton cell walls. The xyloglucan and RGI extracted in this way could not be separated by DEAE-Sephadex ion exchange chromatography but were separated by digesting the xyloglucan with a "purified" cellulase into oligosaccharides in this fraction (XG-RGI complex), followed by Fracto-Gel HW40S HPLC gel filtration chromatography. Xyloglucan was also extracted from the endopolygalacturonase pre-treated cotton cell walls by directly treating with the "purified" cellulase. About 40% of rhamnogalacturonan I, in this way, was solubilized from the walls along with the cellulase digested xyloglucan fragments.

The xyloglucan fragments isolated by these two methods showed the same elution patterns on HW40S HPLC gel filtration chromatography and had almost the same sugar compositions in their corresponding fractions of the chromatography. The fractions from HW40S chromatography containing different sizes of xyloglucan oligosaccharides were

subjected to liquid secondary ion mass spectroscopy (LSIMS) analysis, from which nine different xyloglucan oligosaccharides were identified. These oligosaccharides include nona-, acetylated nona- (isolated by method II), octa-, acetylated octa- (isolated by method II), hepta-, hexa-, penta-, tetra-, and isopentamers. Eight different kinds of glycosyl linkages for xylose, galactose, fucose and glucose residues were identified by GC-MS and quantitatively analyzed in molar ratio. This result is consistent with what was expected. ^{1}H n.m.r. analysis further confirmed the existence of acetate esters on galactose residues. Three acetate ester signals found in the n.m.r. spectrum suggested that more than one position of galactose residue was acetylated.

Application of reverse phase chromatography (RPC) of HPLC enabled us to separate the 2-AP-derivatized xyloglucan fragments isolated by the two methods from cotton cell walls individually. This method was proven to give resolution much superior to the reported ones used in separating xyloglucan fragments, such as Bio-Gel P-2 or paper chromatography. In addition, since each xyloglucan oligosaccharide, no matter what size it has, was linked with only one 2-aminopyridine molecule at its reducing end, the molar ratios of these xyloglucan oligomers therefore could be expressed by their peak area ratios which were determined exclusively by the UV absorbance of the attached 2-AP. The separated xyloglucan oligosaccharides were further identified by LSIMS, and a map of the xyloglucan oligosaccharide distribution and proportion in cotton cell walls was therefore constituted according to their relative peak areas in the chromatography. It is concluded that cotton suspension culture cell wall xyloglucan is mainly composed of nonasaccharide (~55% acetylated on its galactosyl residue), heptasaccharide, decasaccharide and octasaccharide in addition to small amount of other oligomers. The molar percent of these oligosaccharides is as follows: nona-: 34%; hepta-: 29%; octa-: 10%; deca-: 10%; tetra-: 4%; penta-: 4%; hexa-: 2%; isopenta-: 4%; and isohepta-: 3%, respectively. This result is quite in agreement with reported proportions of xyloglucan oligosaccharides isolated by

Streptomyces endoglucanase from cotton fiber cell walls and similar to that for most dicot cell wall xyloglucans.

Preliminary evidence for covalent crosslinking between xyloglucan and rhamnogalacturonan I was obtained during the isolation and the characterization of these two polysaccharides. First, RGI which was only slightly extracted by endopolygalacturonase was always solubilized together with xyloglucan either by method 1 or by method 2. Second, the RGI and the xyloglucan isolated by method 1 could not be separated by DEAE-Sephadex anion exchange chromatography, and were washed together through the column. But the RGI itself, after separating from the XG by cellulase treatment , did bind the column very firmly. Third, after digestion of the XG-RGI complex isolated by method 1 with cellulase, the size of RGI was reduced obviously on TSK400 HPLC gel filtration chromatography compared to the untreated complex. Finally, the significant amount of xylose and glucose present in the RGI extracted by both methods suggested that the xylosyl and/or glucosyl residues may have been shared by both RGI and xyloglucan molecules in native cell walls. In other words, these xylose and glucose residues may be the crosslinking points between the two polysaccharides.

Rhamnogalacturonan I was isolated from cotton suspension culture cell walls along with xyloglucan in two different ways as described previously. TSK400 HPLC gel filtration chromatography was used to separate RGI, the polysaccharide with a large molecular weight of about 100,000 - 200,000 based on pullulan standard, from the small xyloglucan fragments and other components.

Sugar composition analysis of the RGI demonstrated that the amounts of the RGI sidechain sugar residues like galactose and arabinose relative to rhamnose residues is much less than those in sycamore cell wall RGI. Glycosyl linkage composition analysis of the RGI showed that the molar ratio of 2-linked rhamnose to 2,4-linked rhamnose is about 1 : 1, and this is similar to that in sycamore cell wall RGI. These results indicated that the

sidechains of cotton RGI, on average, were shorter than that of sycamore RGI, and most of them should be only one or two residues in length.

^{1}H n.m.r spectrum of the RGI showed three intense signals for acetate esters, demonstrating the presence of a high degree of acetylation and variation with regard to different positions and different sugar residues. DOM analysis indicated that about 13% of galacturonic acid residues in the RGI were methylesterified.

To explain the fact that the molar ratio of GalA to Rha in the RGI isolated by the two methods was about 2 : 1 and about 1.5 : 1, respectively, whereas the generally reported other plant cell wall RGI backbone consisting of GalA-Rha repeats has the ratio of 1: 1, methylesterified RGI, de-methylesterified RGI, commercial non-methylesterified homogalacturonan, and 30-40% methylesterified homogalacturonan were subjected to an ion exchange chromatography on a Dionex CarboPac PA-1 HPLC column. Based on the analysis of their elution patterns, sugar compositions, degrees of methyl esterification, it was concluded that there were no free homogalacturonans or methylesterified homogalacturonans which were co-isolated with the RGI, and the extra galacturonic acid in the RGI must be covalently linked to it either in the form of long homogalacturonans somehow as a "tail", or in the form of interspersed short oligogalacturonans.

Treatment of the methylesterified RGI with HF at -23^{0}C followed by HW40S HPLC gel filtration gave rise to eight fractions with different sizes. LSIMS, ^{1}H n.m.r. and CarboPac PA-1 HPLC analyses demonstrated that the assumed RGI backbone consisting of GalA-Rha repeating units was cleaved into di-, tetra-, hexa-, octa- and decasaccharides, and most of them were di-, and tetramers. Degrees of acetylation of these oligomers varied from 0 to 11, and this was more than the available positions for acetylation on GalA residues of the oligomers, indicating that some acetylation occurs on rhamnosyl residues in addition to galacturonic acid residues.

Fraction 1, which eluted in the void volume and accounted for 17% by weight of the HF-treated RGI, had a molar ratio of GalA : Rha : Xyl of about 13 : 1: 0.5. DOM of

the GalA in this fraction was determined to be about 40% by the selective reduction method and n.m.r. spectroscopy. Molecular weight of the sugars in this fraction was estimated to be less than 5,000 from TSK400 HPLC gel filtration chromatography standardized with pullulan. After de-methylesterification, this fraction was applied to a PA-1 HPLC ion exchange column to separate the mixture into individual oligosaccharides. These oligogalacturonans with a rhamnose residue attached at their reducing end (some of them might also be linked with neutral sugar residues such as xylose) showed sizes mostly from 5 to 15 sugar residues in length according to the standard chromatogram made with fragments of Aldrich pectic acid. Based on sugar compositions of the RGI, and weight percent of this fraction in the RGI, the backbone of the cotton cell wall RGI (~40%) is proposed to be composed of short segments (~10 units) of the GalA-Rha repeat interspersed with short oligogalacturonan segments of ~10 GalA residues of which about 40% are methylesterified. In other words, the RGI backbone consists of alternating segments of GalA-Rha disaccharide repeat and oligogalacturonans. The structural feature of RGI backbone proposed here has not been reported to be present in other plant cell wall pectic polysaccharides except that a similar pectin backbone structure was proposed from apple juice (Schols *et al.*, 1990).

The oligogalacturonides interspersed within the RGI backbone may be the good candidates for eliciting phytoalexins in resistance reactions if there is an enzyme which degrades the RGI backbone to release these oligomers. A rhamnogalacturonase that could cleave galacturonic-rhamnosyl linkages in the hairy regons of apple juice pectins has been isolated and characterized from a fungus, *Aspergillus aculeatus* (Schols *et al.*, 1990).

Rhamnogalacturonan II was solubilized by endopolygalacturonase from cotton suspension culture cell walls, and isolated by a series of chromatographies. Size of the RGII was estimated to be about 6,000 to 20,000 based on the HPLC gel filtration chromatography which was standardized with pullulans. The sugar composition of the RGII is very similar to that of the RGII isolated from sycamore suspension culture cell

walls, except that the RGII isolated from cotton cell walls contains much more galacturonic acid than the RGII from sycamore cell walls, suggesting that more or longer homogalacturonans are linked to cotton RGII. Unlike RGI, there was neither methylesterification nor acetylation found in the RGII molecules isolated in this way.

2-0-Me-xylose and 2-0-Me-fucose were identified from the RGII by GC-MS of partially acetylated alditol derivatives. Glycosyl-linkage compositions of the RGII were determined by GC-MS of partially methylated alditol acetate derivatives. Two major heptasaccharides isolated and characterized from sycamore cell wall RGII were also identified by LSIMS from cotton cell wall RGII, suggesting the structural similarity between cotton and sycamore cell wall RGIIs.

During the whole process of isolation and characterization of xyloglucan, rhamnogalacturonan I and rhamnogalacturonan II, the polysaccharide constitution of cotton suspension culture cell walls has been quantitatively determined (Figure 61 and Figure 62). Cellulose (determined as described by Updegraff, 1969), xyloglucan, homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II account for approximately 24%, 12%, 16%, 15%, and 6% of the walls by weight, respectively. Glycoproteins and other cell wall components comprise the remaining 27% of the cell walls.

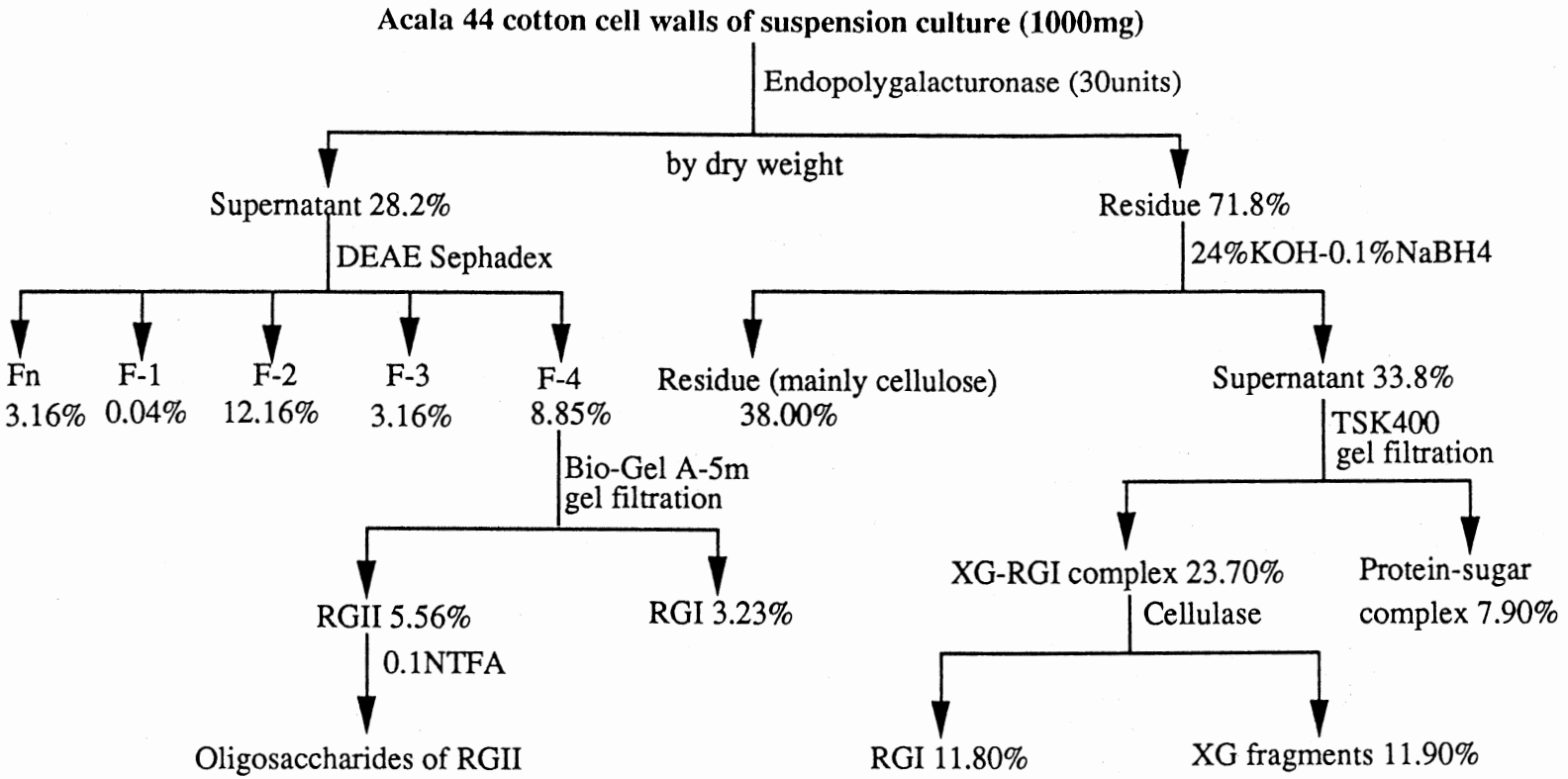


Figure 61. Flow Chart I of the Experiment with Quantitative Analysis of the Different Polysaccharides.

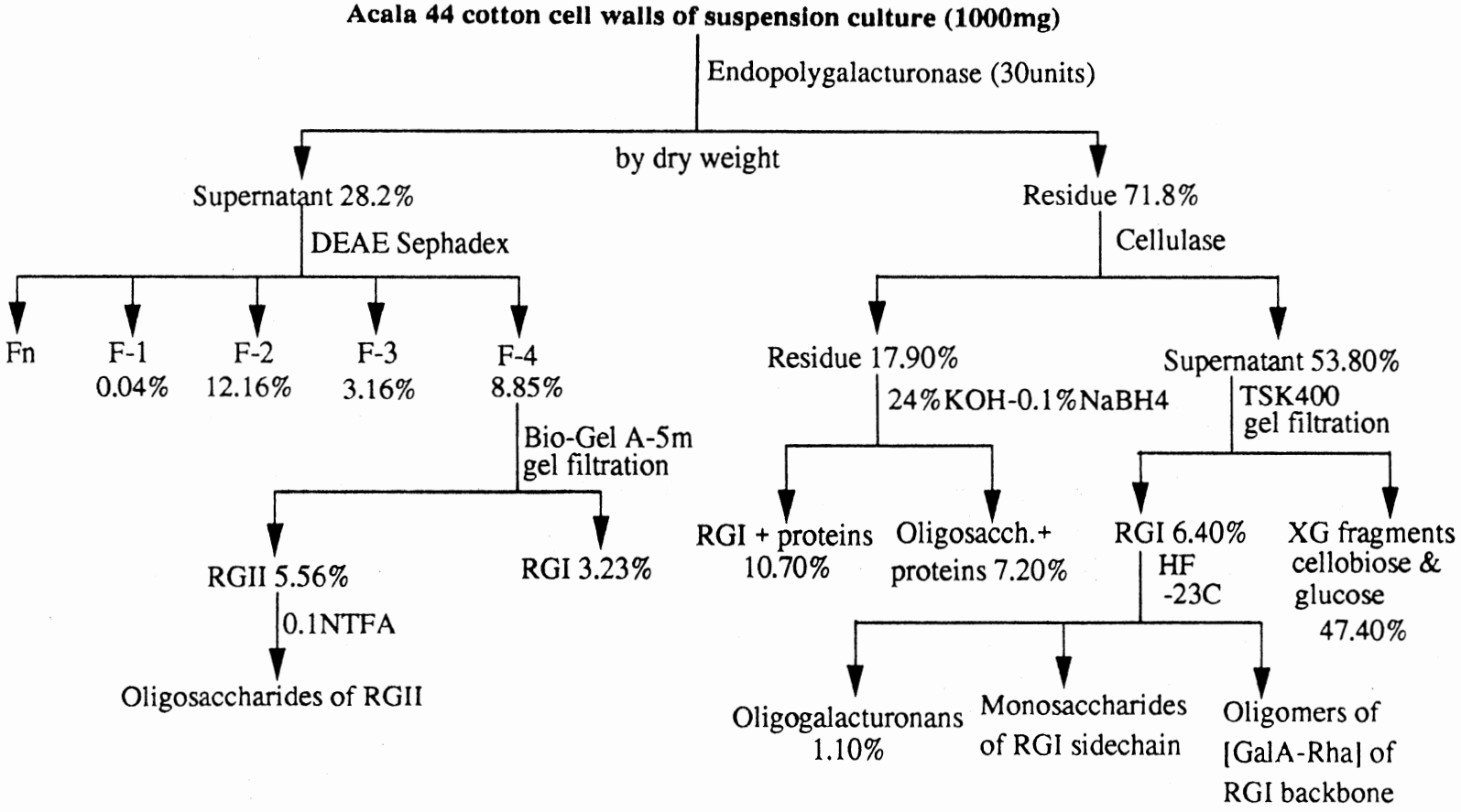


Figure 62. Flow Chart II of the Experiment with quantitative Analysis of the Different Polysaccharides.

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