STUDY OF THE INTERACTIONS BETWEEN GLYCOPROTEINS AND POLYSACCHARIDES IN CELL WALLS OF COTTON SUSPENSION CULTURE

By

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Bachelor of Science in Biochemistry

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Nanjing, China

1982

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
DOCTOR OF PHILOSOPHY
May, 1992

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ACKNOWLEDGMENTS

I would like to express my deepest appreciation to my major advisor, Dr. Andrew J. Mort, for his guidance, encouragement and friendship throughout the course of my research and the writing of this dissertation. I would like to thank the other members of my graduate committee: Dr. Earl D. Mitchell, Jr., Dr. Ulrich K. Melcher, Dr. H. Olin Spivey, and Dr. Christopher M. Adams for their suggestions and assistance.

I would like to thank Dr. Ken Jackson and his associates at the Molecular Biology Resource Facility, Saint Francis Hospital of Tulsa Medical Research Institute, Oklahoma City, OK for the amino acid sequencing, to Dr. Marcia Kielisiewski at Complex Carbohydrate Research Center, The University of Georgia for the deglycosylated tomato extensin precursors and antibodies and for the hydroxyproline glycoside profile analysis of my cell wall samples, to Dr. David Bolton at Techtronix Inc., Englewood, CO for the molecular modeling analysis, to Dr. F. McEnroe at Conoco Co., Ponca City, Oklahoma for obtaining the cross-polarization/magic angle spinning (CP/MAS) ^{13}C n.m.r. spectrum. I would like to thank Dr. Paul Geno, Mr. Paul West, and their associates, Department of Chemistry, Oklahoma State University for obtaining the Mass Spectra, to Mr. Stan Sigle, Department of Chemistry, Oklahoma State University for obtaining the NMR spectra, to Dr. Earl D. Mitchell, Jr. and Ms. Janet Rogers, Department of Biochemistry, Oklahoma State University for providing me with cotton suspension cells, to Dr. Robert Matts and his associates, Department of Biochemistry, Oklahoma State University for their help with Western Blot analysis, and to Dr. Jerry Merz, Department of Biochemistry, Oklahoma State University for his help with computer analysis of data.

I would also like to thank Dr. Padmini Komalavilas, Dr. Niels O. Maness, Dr. James D. Ryan, Dr. Jinhua An, Dr. Bruno M. Moerschbacher, Dr. Fang Qiu, and Dr. Jim Thoms for their suggestions, assistance and friendship .

Many thanks to my son, my parents and my brother and sister for their support, affection, patience and understanding, which were necessary for me to achieve this goal.

National Science Foundation, Department of Energy, and Oklahoma Agricultural Experiment Station must be likewise acknowledged for their financial support to this project.

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LIST OF ABBREVIATIONS

Ara	arabinose
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
cDNA	complementary DNA
DMSO	dimethylsulfoxide
EG	endoglucanase
ELISA	enzyme-linked immunosorbent assay
EPGase	endopolygalacturonase
Fuc	fucose
Gal	galactose
GalA	galacturonic acid
GC-MS	gas chromatography and mass spectrometry
Glc	glucose
HG	homogalacturonan
HF	hydrogen fluoride
HPLC	high performance liquid chromatography
HRGPs	hydroxyproline-rich glycoproteins
Hyp	hydroxyproline
kD	kilodaltons
IDT	isodityrosine
LSIMS	liquid secondary ion mass spectroscopy
Lys	lysine
Man	mannose
MMNO	4-methyl-morpholine-N-oxide hydrate

M.W.	molecular weight
NBA	3-nitrobenzyl alcohol
NBT	nitro blue tetrazolium
n.m.r.	nuclear magnetic resonance
PITC	phenylisothiocyanate
p.p.m.	parts per million
Rha	rhamnose
RGI	rhamnogalacturonan I
RGII	rhamnogalacturonanII
RPC	reverse phase chromatography
Ser	serine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Tyr	tyrosine
Thr	threonine
Val	valine
Vi	included volume
Vo	void volume
XG	xyloglucan
Xyl	xylose

CHAPTER I

INTRODUCTION

The plant cell wall is an extracellular layer of structural material with complex physical and chemical properties. It is mainly composed of polysaccharides, glycoproteins and phenolic compounds. The wall is considered to be a vital organelle in which various macromolecular compounds are assembled into a rigid structural complex. It is thought to be a site of metabolic activity, implying the presence of enzymes, which are catalytically active *in vivo*. Such walls with all these factors are justifiably the subject of intensive study. The study of the plant cell wall has became interesting because of its functions in the control of plant growth and morphogenesis, and its roles in plant disease resistance mechanisms (Bell, 1981), plant/plant cell recognition (Harris *et al.*, 1984; McNeil *et al.*, 1984), and signaling regulation (McNeil *et al.*, 1984; Fry, 1986a). In order to understand the molecular basis of the biological roles of the cell wall we must determine the kinds of polymers present in the wall, the special properties these macromolecules possess, and how they interact one with another to form the intricate structure of the wall.

A primary wall is one whose polysaccharide framework was deposited during growth in cell surface area. The primary wall surrounding most dicotyledonous plant cells is composed of cellulose, hemicelluloses, pectin compounds, proteins and small amounts of lignin and suberin (Fry, 1988). The cellulose in a typical primary wall accounts for 20 - 30% of the dry weight of the wall, and occupies about 15% of the volume of the wall (Fry, 1988). Hemicellulose, another cell wall component, consists of xyloglucan polymers which contribute about 20% of the dry weight of the primary wall. Pectin, a third polysaccharide component in cell wall, consists of homogalacturonans together with rhamnogalacturonan I

and II polymers. Pectin contributes about 30% of the dry weight of the wall. The neutral polysaccharides, arabinoses, galactans, and arabinogalactans, are minor components of the primary walls. The polysaccharide components of the wall have been reviewed extensively (Northcote, 1972; Preston, 1979; Darvill *et al.*, 1980; McNeil *et al.*, 1984; Varner and Lin, 1989).

In addition to polysaccharides, protein components in the wall are under intensive investigation in several laboratories. Dehydrated primary cell walls from dicotyledonous plants contain 5 -10% protein. Among the wall proteins, a family of hydroxyproline-rich glycoproteins (HRGPs), called 'extensin', are the best characterized, and perhaps the most abundant, structural proteins of dicot cell walls (Cooper *et al.*, 1987; Tierney and Varner, 1987; Cassab and Varner, 1988). The term "extensin" describes a family of special glycoproteins that is thought to play a role in cell expansion during growth (Lamport, 1965). In extensin, a small group of peptides consisting of a repeating pentapeptide units of SerHypHypHypHyp were characterized. These peptides are glycosylated by tri- and tetra-arabinosides linked to hydroxyproline (Hyp), and by galactose, linked to serine (Fry, 1988).

Considerable information has been obtained about the structures of extensins by directly extracting extensin precursors from the cell walls with a salt solution, or solubilizing the HRGPs from a cell homogenate with an acidic solution (for comprehensive reviews see Wilson and Fry, 1986; Showalter and Varner, 1987; Cassab and Varner, 1988). With the use of molecular biology techniques, Chen and Varner (1985a; 1985b) first isolated and characterized extensin cDNA and an extensin genomic clone from carrot. Subsequently, several laboratories have sequenced extensin genes or transcripts from different plants and tissues, such as tobacco, *Phaseolus vulgaris*, tomato, and soybean. The partial cDNA clone isolated from a cDNA library from wounded carrot root mRNA encodes a peptide containing SerProProProPro repeats and TyrLysTyrLys (Chen and Varner, 1985b). These sequences are found in the tomato extensin (Epstein and Lamport, 1984; Smith *et al.*, 1986)

and in six different clones of extensins from carrot genomic libraries (Chen and Varner, 1985a).

Extensins are present in the primary cell wall in widely varying quantities, making up 1-10% of the wall (Lamport, 1980a). In general, extensins share several common features (Heckman *et al.*, 1988; Varner and Jin, 1989): (1) they are rich in Hyp residues that typically are glycosylated with one to four arabinosyl residues, (2) they are basic because of their high lysine content, (3) they have a highly periodic, repeating sequence unit, (4) the side chains of amino acid residues (serine, threonine, hydroxyproline, lysine, tyrosine, and histidine) provide many sites for post-translational modification and *in muro* interaction, and (5) their procursors appear as thin, kinked rods, approximately 80 nm in length organized in a polyproline II helix.

Previous investigations involving the structure of the *Acer pseudoplatanus* cell wall polysaccharides (Albersheim, 1978) and extensins (Lamport, 1970) have provided the basic information and methods required to address the structural composition of cell walls in higher plants. Most recently, through the use of immunocytochemical studies, it has been possible to unambiguously establish the specific localization of carbohydrate and glycoprotein components in the primary cell wall, thereby providing crucial information for the development of cell wall models (Moore and Staehelin, 1988; Stafstrom and Staehelin, 1988). For instance, it has been known that cellulose microfibrils surround the protoplast in a multilayered sheath (Roland and Vian, 1979), and the cellulose layer is restrictively hydrogen-bonded with xyloglucan (Valent and Albersheim, 1974; Hayashi and MacLachlan, 1984; Moore and Staehelin, 1988). The acidic pectins rhamnogalacturonan I (RG I) and homogalacturonans are exclusively localized at middle lamella layers which are commonly shared with neighboring cells (Albersheim *et al.*, 1960; Selvendran, 1985; Moore and Staehelin, 1988). Extensin molecules are interspersed throughout the cellulose containing regions of the wall (Stafstrom, 1986; Stafstrom and Staehelin, 1988).

Several cell wall models have been proposed by different laboratories. The first model, hypothesized by Albersheim and his colleagues in 1973, suggested that cellulose microfibrils were cross-linked by the matrix polymers of the wall. In this model, xyloglucan polymers are linked to the rhamnogalacturonan by covalent bonds which consist of galactan. The reducing end of the rhamnogalacturonan is a 3,6-linked arabinogalactan which is glycosidically linked to serine residues of extensin (Keegstra *et al.*, 1973). Ten years later, Lamport and Epstein (1983) proposed the "warp and weft" model of wall organization in which cellulose microfibrils are believed to be oriented parallel to the plane of the wall where they behave as a "warp", while extensin is oriented perpendicular to the wall, as a "weft". These two elements are then interwoven through some, as yet, undefined way in a hydrophilic-pectin gel. The simplest and most general working model of the plant cell wall has been hypothesized by Cooper *et al.*(1984). Two independent wall polymer systems are characterized as xyloglucan-coated cellulose microfibrils and an extensin-pectin network in the model, and they intercalated to build the plant cell wall.

Initially, the characterization of extensins proved to be of great difficulty due to the inability to extract these proteins. Although newly synthesized extensin molecules are readily isolated from the wall with salt (Fry, 1988), no intact mature extensin has been extracted from the walls of any plant species. Previous hypotheses have suggested that extensin was insolubilized by covalent cross-links within extensin itself or between extensin and other cell wall polymers. For instance, a cross-link that isodityrosine (IDT) forms between two tyrosine residues in extensins has been proposed (Cooper and Varner, 1983; Smith *et al.*, 1984), and phenolic links between the wall protein and the lignin (O'Neill and Selvendran, 1980). Of these hypothesized linkages, only the IDT, as an intramolecular bridge within the sequence TyrLysTyr, has been detected in isolated monomers (Epstein and Lamport, 1984).

Recent progress in the characterization of extensin has come with the application of molecular cloning of the cell wall extensin genes. Information concerning the way in which

these glycoproteins interact with environments within the wall is still lacking (i.e., what other macromolecules does each extensin interact with covalently in the walls and what is the chemistry of such interactions?). The unidentified interactions, along with the structural information on extensins, should provide important clues to glycoprotein functions.

My research was conducted to determine whether or not there are covalent crosslinks between extensin and other major plant cell wall polymers, and investigate what types of these interactions need to be disrupted to liberate extensins or fragments of extensins from cotton suspension culture cell walls. Recently, the polysaccharide structures of the cotton primary cell walls have been isolated and partially characterized (Komalavilas and Mort, 1989; El Rassi *et al.*, 1991). Information concerning the structure of cotton primary cell wall extensin is still not available.

The experimental approach uses highly specific methods for cleavage of the polysaccharides and proteins in order to liberate fragments of these polymers characteristic of the way in which they exist in the cell wall. General structure analysis of the cell wall polymers includes the capillary gas-liquid chromatography (CGLC) and high performance liquid chromatography (HPLC) for very small quantities (range from pmol to nmol) of sugar and amino acids compositions, respectively. More detailed characterization of wall fragments has been greatly advanced with mass spectroscopy (MS), CGC-MS, nuclear magnetic resonance (NMR) spectrometry, 2D-NMR, as well as electrophoresis. The results of this work are organized into three major parts.

The first part reports, the isolation, purification, and partial characterization of the extensin from cotton suspension culture primary cell walls. Eighty percent of the Hyp can be solubilized by trypsin after all of the cell wall sugars have been removed by a 0°C HF treatment. Only a few major Hyp containing peptides are formed and these are now being characterized by LSIMS and sequencing by Edman degradation. Many other peptides are formed in lower abundance. Three major fragments of extensin isolated using anhydrous HF solvolysis at 0°C and trypsin digestion were purified by a reverse-phase C18

chromatography, and subsequently characterized using LSIMS and composition analysis. The sequences obtained from Edman degradation sequencing are: Ser-Hyp-Hyp-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Hyp-Lys, Ser-Hyp-Hyp-Hyp-Hyp-Val-Lys, and Ser-Hyp-Hyp-Ser-Ala-Hyp-Lys. Interestingly, there is a $(Hyp)_6$ sequence in a row instead of $(Hyp)_4$ which is normally found in a diagnostic sequence for extensins.

The second part describes the ability of a variety of selective hydrolytic methods and (combinations of them) to liberate extensin, or fragments of extensin, from cotton cell walls. These methods include: (1) endopolygalacturonase digestion of homogalacturonans, (2) cellulase digestion of cellulose and xyloglucan, (3) anhydrous hydrogen fluoride (HF) treatment at -73°C to selectively remove arabinose residues from Hyp residues of extensins, (4) anhydrous HF treatment at 0°C to completely remove sugars from the cell walls, (5) anhydrous HF treatment at -23°C to remove pectin, and (6) trypsin digestion to cleave the protein at the carboxyl side of lysine and arginine residues. None of the treatments alone liberate significant quantities of extensin as judged by release of Hyp-containing peptides. Considerations of the extent of release by combinations of treatments show that arabinose side chains on the Hyp residues are important for preventing trypsinization of the protein, and also that the presence of cellulose and pectin inhibit the action of trypsin on extensin.

In the last part, a sequential treatment of walls using methods 1, 2, 3 and 6 is described. Fifty percent of the Hyp can be solubilized by these treatments. The Hyp-containing peptides solubilized by this combination treatment are co-solubilized with sugar characteristic of RG I and some of these peptides co-elute with these sugars upon ion exchange and gel filtration chromatography, as well as upon sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

These extensin-RG I complexes, and their deglycosylated forms, cross react with the antibody against tomato cell wall extensins obtained from Dr. Lamport's group. Western blot analysis shows a dramatic increase in electrophoretic mobility of the antibody reactive material after it was deglycosylated by a 0°C HF treatment. These results give a strong

indication of a covalent linkage between the cell wall extensin and pectin. Fragments containing covalent linkage have been only partially characterized at the present. In the near future, the complete structure of these fragments will be characterized and the results will provide details regarding the whole picture of cell wall structure.

CHAPTER II

LITERATURE REVIEW

Non-Protein Polymeric Components of Plant Cell Walls

The growing cell walls of higher plants consist of a rather small number of major polymers; most of these are polysaccharides. The polysaccharides of the primary cell wall are classified as cellulose, hemicellulose, and pectin.

Cellulose

Cellulose, the best known polysaccharide of cell walls, is an unbranched polymer of D-glucopyranose residues joined by β-(1→4) linkages. The cellulose microfibrils in a typical primary wall are elliptical in cross section with axes 50-300 Å. The cellulose microfibril results from the association of individual molecules of cellulose into crystalline or near-crystalline arrays which are nearly free of water (Roelofsen, 1965). Although little is known of the enzymatic pathway for cellulose synthesis, cellulose is apparently synthesized within the plasma membrane (Herth and Hausser 1984; Delmer 1987; Wooding, 1968). Microfibril orientation upon deposition is usually transverse to the major axis of growth; however, in some primary cell walls alternate layers of microfibrils are oriented at right angles to each other (Chafe, 1970). The biological function of cellulose is suggested to be the skeletal framework, providing shape and strength to the cell wall. When cellulose synthesis is inhibited by addition of 2,6-dichlorobenzonitrile, algal cells burst under their own turgor pressure (Richmond, 1984).

Xyloglucan

Xyloglucan, a hemicellulose, is a generally rigid, rod-shaped polysaccharide which hydrogen-bonds to cellulose and can be extracted from the wall with alkali. Xyloglucan has a backbone of β-(1→4)-linked D-glucosyl residues with D-xylosyl side chains which are α-linked to the O-6 of some of the glycosyl residues. Some heterogeneity in xyloglucan's fine structure exists (Darvill *et al.*, 1980; McNeil *et al.*, 1984). Substituted residues of the xylopyranosyl polymers of xyloglucan include β-D-galactopyranosyl-(1→2)-, α-L-arabinofuranosyl-(1→2)- and α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→2)-side chains. The nonasaccharide-containing fractions, but not the heptasaccharide-containing fractions, isolated from a xyloglucan in the extracellular medium of suspension cultured sycamore (*Acer pseudoplatanus*) walls is substituted with either one or two O-acetyl groups (McNeil *et al.*, 1984). The analysis of the X-ray diffraction pattern of tamarind seed xyloglucan suggests a backbone with a flat, ribbonlike 2-fold helical conformation which would be compatible with hydrogen-bonding to the surface of cellulose chains (Taylor and Atkins, 1985). Because of these particular properties, xyloglucan molecules might mediate tight embedding of the cellulose microfibrils into the gel matrix of the wall by forming a layer on the surface of the crystalline regions of the microfibrils *in muro*. There is ambiguity as to whether or not the synthesis of polysaccharide backbone and side-chains is obligatorily coupled (Waeghe *et al.*, 1983). In the case of xyloglucan, some evidence indicates that the α-xylose residues are attached simultaneously with the elongation of the β-glucan backbone (Hayashi *et al.*, 1987). Other experiments suggest that the xylose residues, even the β-galactosyl and α-fucosyl side chains, can be added to a long, pre-formed glucan backbone (Ray *et al.*, 1980; Camirand *et al.*, 1986).

Pectins

Pectins, jelly-like polymers of the walls, are more loosely bound in the wall. Pectins can be extracted proportionately with chelating agents. The pectic polysaccharides are probably the most complex class of wall polysaccharides. The homogalacturonans, together with rhamnogalacturonans I and II, comprise the bulk of the pectins of dicots. Chemically, the homogalacturonans consist mainly of contiguous unbranched α-(1→4)-galacturonic acid residues. In addition, the homogalacturonans are apparently secreted with the uronate groups esterified to various degrees with methyl groups. The distribution of methyl ester groups is still unknown. Pectins may also carry acetyl and phenolic groups (McNeil *et al.*, 1984; Neukom *et al.*, 1980). Other sugar residues occasionally interrupt the homogalacturonan blocks (Powell *et al.*, 1982), however, the frequency and pattern of the interruption are poorly understood. Uninterrupted homogalacturonan regions, with a degree of polymerization (DP) of approximately 70, are found in pectic preparations from walls of carrot (*Daucus Carota*) suspension cultures (Konno *et al.*, 1986).

The rhamnogalacturonans I (RG I) consist of a backbone of alternating α-linked L-rhamnosyl and 4-linked D-galacturonic acid residues. A RG I from suspension cultural *Acer pseudoplatanus* walls (McNeil *et al.*, 1984; Lau *et al.*, 1985), for instance, contains up to 300 alternating residues of each sugar. Approximately half the rhamnopyranosyl residues contained in these units are glycosylated at O-4, with branches averaging about seven glycosyl residues in length in many ways. Several neutral oligosaccharides polysaccharides, which are rich in arabinosyl and galactosyl residues, are believed to be covalently attached to the RGI backbone primarily through the rhamnopyranosyl residues. These have not been characterized.

The type II rhamnogalacturonan (RG II), a very unique polysaccharide, has been isolated from walls of suspension-cultured sycamore by hydrolysis with a (1→4)-α-endopolygalacturonase (McNeil *et al.*, 1984; York *et al.*, 1985). It consists of about 60

glycosyl residues, which include many unusual glycosyl residues, e. g. 2-O-methylfucosyl, 2-O-methylxylosyl, apiosyl, 3-O-Carboxy-5-deoxy-L-xylosyl, and 3-deoxy-manno-octulosonic acid. The progress of study of the RG II structure is still quite preliminary.

Lignin, Cutin and Suberin

In addition to their high polysaccharide content, some cell walls contain certain amounts of specialized polymers. Lignins are polymers of phenylpropanoid residues that are almost exclusively derived from *p*-coumaryl, coniferyl, and sinapyl alcohols. The polymers are laid down initially in the middle lamella and primary walls of some cells which have developed secondary walls, principally vessel elements, tracheids, fibres and sclereids. The hydrophobicity, plasticity and indigestibility of lignin allows it to serve several biological roles, e. g. water proofing of the xylem, exclusion of pathogens, and provision of physical strength. From different species of cell types, lignins vary in monomeric composition, types of linkages between monomers, and organization of monomers within the macromolecule (Monties, 1985). Several types of ether (C-O-C) or carbon-carbon (C-C) linkages have been found in the joining of the monomers (Atsushi *et al.*, 1984; Scalbert *et at.*, 1985; Sharma *et al.*, 1986). These include: arylglycerol-β-aryl ethers, *p*-coumaric acid esters, ether-linked ferulic and *p*-coumaric acids. In the walls, lignin may be covalently associated with noncellulosic polysaccharides and possibly proteins.

Cutin is a hydrophobic and relatively undigestible polyester of hydroxy-fatty acids. Cutin is found in the outer epidermal walls of stems, leaves, and possibly roots (Hamilton, 1986). Suberin, another polyester, is found in certain specialized cell walls, e. g. cork, seed coats and the Casparian strip of the endodermis. Cutinized and suberinized walls contain phenolic materials, but the chemistry of the phenolics and their cross-links are poorly understood (Kolattukudy, 1981a; 1981b).

Plant Cell Wall Proteins

Proteins are important components of walls of higher plants. Primary walls contain up to 10% structural proteins. The proteins have been classified as (1) hydroxyproline-rich glycoproteins (HRGPs); (2) proline-rich proteins (PRPs); and (3) glycine-rich proteins (GRPs).

Hydroxyproline-Rich Glycoproteins (HRGPs)

The family of hydroxyproline-rich glycoproteins (HRGPs) is the best characterized family of proteins in walls. Several types of HRGPs have been found: extensins, arabinogalactan proteins (AGPs), and solanaceous hemagglutinating lectins.

Extensins are present in the primary cell walls of dicots in widely varying quantities, making up 1-10% of the weight of the wall (Lamport, 1980). They are particularly abundant in the walls of cultured cells and perhaps in epidermal cell walls. Extensin is a major structural protein which has been well characterized in walls. Details concerning extensins are discussed below.

AGPs and Solanaceae lectins are two other types of glycoproteins rich in hydoxyproline. Unlike extensins, AGPs are primarily localized in the extracellular matrix in gums, and in exudates. The matrices are suspension culture media, xylem sap, the stylar canal, and probably also the wall matrix of most tissues (Fincher *et al.*, 1983; Showalter and Varner, 1989). The polypeptide chain is rich in Ala. Most Hyp residues carry long polysaccharide-like side chains attached via a β-D-Gal*p* linkage (Strahm *et al.*, 1981). As their name implies, the large side chains are based on a highly branched backbone of β-3- or β-6-linked D-galatosyl residues with mostly L-arabinosyl residues. The unique property of AGPs is the binding activity for certain aromatic β-glycosides known as Yariv's antigens (artificial carbohydrate antigens). The antigens have been used for purifying AGPs (Anderson, 1977). AGPs are thought to be involved in cell-cell recognition rather than in a

structural role, because they are loosely associated with the cell walls and readily solubilized during tissue extraction with low-ionic-strength aqueous solutions. The N-terminal sequences of four different AGPs, three from carrot (Jermyn and Guthrie, 1985) and one from ryegrass (Gleeson *et al.*, 1985), have recently been determined. All four sequences contain Ala-Hyp repeats.

The solanaceous lectins are glycoproteins which can be distinguished from other plant lectins by their high Hyp and arabinose contents. Highly purified solanaceous lectins have been isolated and biochemically characterized from *Datura stramonium* seeds and potato tubers (Desai *et al.*, 1981; Allen *et al.*, 1978). There are at least two distinct domains in these lectins: one is rich in serine and hydroxyproline, and the other is rich in glycine and cysteine and is extensively cross-linked by disulfide bridges. The carbohydrate moieties of these lectins contain only two sugars, L-arabinose (approximately 90%) and D-galactose (approximately 10%). The glycosylation pattern of the Hyp is similar to that in the dicot extensins. The cellular localization of the solanaceous lectins is unclear. They may have both an intra- and extra-cellular distribution. A recent subcellular fractionation study of potato tuber disks by Casalongne and Pont Lezica (1985) indicated that most of the lectin is tightly, but covalently, bound to the cell wall. Basically, it is thought that lectin's ability to bind sugars is somehow involved with their function. Consequently, one of the most frequently suggested biological roles for the solanaceous lectins is their various forms of cell-cell interaction.

Proline-Rich Proteins (PRPs)

Proline-rich proteins (PRPs) represent another class of cell wall structural proteins. Several soybean PRPs and a carrot PRP, P33, have been localized to the cell wall (Averyhart-Fullard *et al.*, 1988; Tierney *et al.*, 1988; Kleis-San Francisco and Tierney, 1990). Among these proteins, two PRPs purified from soybean contain approximately equal levels of proline and hydroxyproline (Datta *et al.*, 1989; Kleis-San Francisco and

Tierney, 1990). To date, the PRPs studied have been shown to contain many copies of repeating units which differ from the units in extensins. For instance, sequencing of soybean cDNA clones has shown that encoded proteins contain the repeat Pro-Pro-Val-Tyr-Lys (SbPRP1, Hong *et al.*, 1987; 1A10-1, Averyhart-Fullard *et al.*, 1988). PRPs genes are expressed in response to wounding in carrot roots and soybean hypocotyls (Chen and Varner, 1985b; Kleis-San Francisco and Tierney, 1990; Tierney *et al.*, 1988) and during the early stages of soybean and pea root nodule formation (Franssen *et al.*, 1987; Scheres *et al.*, 1990). Moreover, expression of three PRP genes in soybeen showed distinct, individual patterns in different organs and at different developmental stages (Hong *et al.*, 1989).

Glycine-Rich Proteins (GRPs)

Wall structural proteins, other than extensins and PRPs, have been reported by Brown and Kimmins (1981) in bean (*Phaseolus vulgaris*). They are low in Hyp, but like extensins are also glycosylated. More recently, a cDNA from *Petunia hybrida* encoding a 314 amino acid polypeptide which contains 67% glycine has been characterized (Condit and Meagher, 1987). The result indicates this cDNA for a glycine-rich, wall structural protein. The cDNA encodes the bulk of the sequence consists of $(Gly\text{-}X)_n$ units in which X is frequently glycine. Two similar genes occur in *Phaseolus vulgaris*, one of the encoded proteins is associated with the walls of cells in the vascular tissues (Keller *et al.*, 1988).

Other Wall Proteins

In addition to the structural proteins, thionin is a major soluble protein with antifungal activity in the cell walls of barley leaves (Bohlmann *et al.*, 1988). Thionin is basic protein and rich in cysteine (17%). The protein encoded by a complex multigene family containing 50-100 members per haploid genome. Cell walls also contain many enzymes, such as peroxidases, phosphatases, proteases, pectin methylesterases, polygalacturonase, invertases, etc.

Extensins

In the 1950s, Steward *et al.* (1954) discovered hydroxyproline-containing proteins in plants. Shortly thereafter, the bulk of the peptidyl hydroxyproline were determined in plant cell walls from tissue cultures (Lamport and Northcote, 1960; Dougall and Shimbayashi, 1960). Since then, a number of subsequent investigations, including the recent immunoelectron microscopy of the cell wall HRGPs in carrot root tissue (Stafstrom and Staehelin, 1988), have elucidated clearly that a family of HRGPs (often called extensins) constitute the major structural protein component of cell walls. These HRGPs are especially abundant in dicotyledonous plants and certain green algae (Gotelli and Cleland, 1968).

Biochemical and Physical Properties

Extensin is a generic name because it is involved in extension growth of cell walls. For several years, extensin was just known from the characterization of a series of hydroxyproline-rich glycopeptides and peptides obtained by partial hydrolysis and enzymatic digestion of tomato cell walls (Lamport 1967; 1969). The protein is rich in Hyp along with a number of other amino acids (valine, serine, threonine, lysine, and tyrosine). Attached to the protein are carbohydrate side chains, which make up about 50-60% of the weight of extensin. Most of the Hyp residues typically are glycosylated with one to four arabinosyl residues on 4-OH of the Hyp, and the serine residues are also O-substituted with galactose (Lamport, 1973). The first amino acid sequences characteristic of extensin were the sequences of three peptides resulting from a tryptic digestion of cell walls: Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys, Ser-Hyp-Hyp-Hyp-Hyp-Lys, and Ser-Hyp-Hyp-Hyp-Hyp-Val-Tyr-Lys-Lys (Lamport, 1974). Two precursors of extensins, P1 and P2, accumulate in the walls of tomato cells in suspension culture and have been partially

characterized by Smith *et al.* (1986). One of abundant repeating peptides in P1 is: Ser-$(Hyp)_4$-Val-Tyr-Lys-Tyr-Lys.

Extensins occur as extended rodlike molecules, largely due to their polyproline II helical conformations (Lamport, 1977; Van Holst and Varner, 1984). The secondary structure of soluble carrot, tomato, and sycamore-maple extensins have been studied using electron microscope and circular dichroism. Upon electron microscopy, extensins appear as thin, kinked and rod-shaped molecules of approximately 80 nm in length (Lamport 1980; Stafstorm and Staehelin, 1986a, b). Circular dichroism analysis indicates that cell wall extensins exist in a left-handed helix with 3 residues per turn and a pitch of 9.36 Å (Van Holst and Varner, 1984). The deglycosylation of extensins with anhydrous hydrogen fluoride results in a distortion or unwinding of the helix. Therefore, Van Holst and Varner suggested that the carbohydrate moiety of this glycoprotein serves a role in stabilizing the helical conformation of extensin, presumably through intramolecular hydrogen bonding. Heckman and his colleagues (1988) have demonstrated the deglycosylated extensin monomers to be extended, discernable rods using transmission electron microscopy. Additionally, this deglycosylated precursors are highly retained on a gel filtration column. This retaining force is due to ionic interacting between the deglycosylated precursors and the gel filtration column.

Molecular Biology Study

A significant advance in our understanding of the structure of extensin was achieved with molecular biology techniques. Chen and Varner (1985a) obtained cDNA and genomic clones for a wound-induced extensin from carrot. One of the genes encoding carrot extensin has been sequenced (Chen and Varner, 1985b). It codes for 25 repeats of pentapeptide Ser-Pro-Pro-Pro-Pro. Repeats of Try-Lys-Tyr-Lys and Thr-Pro-Val are also well represented. In addition, cDNA clones for extensins from petunia (*Petunia Sp.*), tomato (*Lycopersicon esculentum*), and potato (*Solanum tuberosum*) also show numerous

Ser-Pro-Pro-Pro-Pro repeats (Showalter and Varner, 1989, Showalter *et al.* 1991). Three extensin transcript sequences of *Phaseolus vulgaris* have been recently reported (Corbin *et al.*, 1987). Two of these transcripts code for proteins rich in repeats of Tyr-Tyr-Tyr-Lys-Ser-Pro-Pro-Pro-Pro-Ser-Pro-Ser-Pro-Pro-Pro-Pro, the third for repeats of Tyr-Tyr-Tyr-His-Ser-Pro-Pro-Pro-Pro-Lys-His-Ser-Pro-Pro-Pro-Pro. These results confirm the remarkable repetition that typifies extensins, but do not distinguish between hydroxyproline and proline residues since Hyp is formed by a post-translational modification of proline.

Biosynthesis and Insolubilization

Newly synthesized extensin molecules are readily isolated from some walls with salt. The first salt-extractable Hyp-containing protein, which might be the precursor to the covalently bound cell wall extensin, was identified in carrot roots by Chrispeels (1969). Several laboratories have purified extensin from different plants and tissues, such as sycamore tissue-culture cells (Heath and Northcote, 1971), runner bean (O'Neill and Selvendran, 1980), carrot root (Stuart and Varner, 1980), potato tuber (Leach *et al.*, 1982), tobacco callus (Mellon and Helgeson, 1982), tomato cell suspension cultures (Smith *et al.*, 1984), soybean seed coats (Cassab *et al.*, 1985), melon callus (Mazau *et al.*, 1988), and maize cell cultures (Hood *et al.*, 1988; Kielisewski and Lamport, 1987). The true molecular weight of extensin in carrot and tobacco was reported to be approximately 90 kD.

Cell wall biosynthesis has been studied using immunological techniques with polyclonal antibodies raised against cell wall fractions from high plants (Huber and Nevins, 1981) and a synthetic peptide (Smith, 1981). Monoclonal antibodies raised against *Chlamydomonas* wall HRGPs (Smith *et al.*, 1984) and *Fucus* cell wall carbohydrates (Vreeland *et al.*, 1984) have also been used for this study. The availability of polyclonal antibodies against extensin monomers provides the possibility of immunological approaches for detection, assay, cytochemical localization and manipulation of cell wall extensin. The properties and cross-reactivities of polyclonal rabbit antibodies raised against

two extensin precursors P1 and P2 of tomato and their HF-deglycosylated polypeptides dP1 and dP2 have been determined using enzyme-linked immunosorbent assay (Kieliszemski and Lamport, 1986). Three types of antigenic determinants (epitopes) are identified in each antigenic precursor: (1) glycosylated epitopes; (2) nonglycosylated epitopes of the intact glycoprotein; and (3) epitopes exposed only after deglycosylation.

Although the subcellular localization of some of the reactions is still controversial, the general biosynthetic pathway for wall extensins is fairly well established (Sadana and Chrispeels, 1978; Robinson *et al.*, 1984; Wilson and Fry 1986; Delmer and Stone, 1988; Showalter and Varner 1989). The extensin precursors are first synthesized on the rough endoplasmic reticulum and then undergo extensive multiple-step post-translational modifications. Many studies with carrot discs (Weinecke *et al.*, 1982; Robinson and Glass 1982; Chen and Varner, 1985a) indicated that the major HRGPs were synthesized in the endoplasmic reticulum and then transported to the Golgi prior to secretion into the wall. The hydroxylation of proline residues in the polypeptide are carried out by peptidyl proline hydroxylase (Chrispeels, 1970). The distribution of the plant prolyl hydroxylase has been documented from maize and rye grass endosperm cells (Sawer and Robinson, 1985; Cohen *et al.*, 1983). The report indicated that the enzyme is localized both in the endoplasmic reticulum and Golgi. Following hydroxylation, the second type of post-translational modification occurs in the Golgi, namely glycosylation. To date, only the mechanism of attachment of arabinose to hydroxyproline has been reported. At least three different arabinosyl transferases are involved in the arabinosylation of endogenous protein (Bolwell, 1984; Karr, 1973; Owers and Northcote, 1981). The glycosylated protein that could be salt extracted is then secreted into the walls. Later, a soluble form of extensin is slowly incorporated into the walls and becomes unextractable (Stuart and Varner, 1980; Cassab, 1986; Cooper and Varner 1983, 1984). The insolubilization of precursor to form insoluble extensin in the wall is reviewed by Wilson and Fry (1986) and Delmer and Stone (1988).

Biologic and Physiologic Roles

Extensins have been characterized chemically and physically as the major protein component of the primary wall of plant cells. However, the roles of extensins in plant cell walls are not understood. For several years, it was suggested that the distribution of extensin in plant cell is tissue specific. Thus, it is necessary to know the localization of extensins in different plant tissues in order to assign various functions of extensin in cell walls. For instance, immunogold-silver localization of soybean seed coat extensin revealed marked deposition of the glycoprotein in the walls of both palisade and hourglass cells (Cassab and Varner, 1987). Moreover, the distribution of extensin among the different cells of the seed coat changes during seed development. A recently developed tissue-printing technique allows us to immunolocalize extensin in different plant tissues as well as plant species (Cassab and Varner 1987). Tissue-printing of developing soybean seeds on nitrocellulose paper indicates that extensin is mainly localized in the sclerenchyma cells of the vascular regions of the seed. Sclerenchyma cells were known to act as the skeletal elements of the plant body (Haberlant, 1914). The fact that extensin is a major component of sclerenchyma cell walls suggests that the extensin could be related to the mechanical function of this tissue in the plant. Extensin has also been immunolocalized in cell walls of carrot roots (Stafstrom and Staehelin, 1988). Results from these studies suggest that : (a) newly synthesized extensin is added to the wall by intercalating with new particles among those existing in the wall; (b) extensin cannot cross the middle lamella; and (c) incorporations of extensin is a late event in the development of carrot phloem parenchyma walls.

Some other studies suggest a role for extensin in plant growth. The accumulation of extensin occurs coincidentally with the cessation of cell elongation in the wall of growing pea epicotyls (Sadava, 1973). Moreover, Klis (1976) reported that the content of Hyp and Hyp-arabinosides of the wall in elongating tissue of pea stems increase, and these changed

only slightly once elongation was complete. It is interesting to note that the rate of elongation at a given point on stems of intact etiolated peas is inversely proportional to the accumulation of hydroxyproline content. This may be because extensin stiffens the wall during growth.

One of the important biological roles of extensin is the possible involvement of the disease resistance response in the general defense reaction of plants. Mazau and Esquerre-Tugaye (1986) reported that HRGPs concentrations increase in some dicotyledonous plants by between 50% and 900% after infection by fungi, bacteria and viruses, and this accumulation occurs earlier and reaches higher levels in resistant plants. Most recently, from the same group O'Connell *et al.* (1990) using immunocytochemistry found that the accumulated extensins are highly localized at sites where the growth of bacteria and fungi is arrested. Several other observations also support this proposed role of extensin in disease resistance. Extensins accumulated in plant papillae, which may present a physical barrier to penetration by fungi (Mazau *et al.*, 1987). Cell wall Hyp levels increase more rapidly in resistant than in susceptible cultivars of cucumber infected with the fungus *Cladosporium cucumerinum* (Hammerschmid *et al.*, 1984). Furthermore, there is rapid accumulation of extensin mRNAs in response to fungi infection (Showalter *et al.* 1985), elicitor treatment (Roby *et al.*, 1985) and ethylene (Ecker and Davis, 1987). It has been suggested that ethylene produced in response to biological stress is a signal for plants to activate defense mechanisms against invading pathogens.

Interactions Between Polymers in Walls

Intrinsically, the individual polymers of the cell walls, except cellulose, are water soluble, since most of them are poly-ionic, and hydrophilic, and poly-hydroxy. These polymers are highly hydrated in the living plant, yet the wall matrix is water-insoluble and very coherent (Fry, 1986b). If water-soluble polymers in the walls cannot be extracted by water, the implication is that macromolecules are somehow associated with each other

through physical (non-covalent) or chemical (covalent) bonds, or both. Indeed, all components interact with each other forming a fabric of great strength, coupled with elasticity and plasticity, resistance to digestion, and an ability to adhere to neighboring walls. In other words, extensibility, digestibility, and adherence are important properties of cell walls, dictated by wall polymer interaction *in muro*. Such wall cross-links are becoming interesting to plant scientists.

Cellulose-Xyloglucan Associations

It is generally accepted that much of the xyloglucan of the dicot wall is firmly bound to cellulose microfibrils by hydrogen bonds (Hayashi *et al.*, 1987). Hemicelluloses can self-assemble (Wada and Ray, 1978) and xyloglucan binds to cellulose via hydrogen-bonds (Bauer *et al.*, 1973) *in vitro*. Inter-polymer hydrogen bonds can be disrupted by chaotropic agents, e.g. 8 M urea; guanidinium thiocyanate; 4-methyl-morpholine-N-oxide hydrate (MMNO) (Joseleau *et al.*, 1981). Xyloglucan might also interact with other polysaccharides which are not so strongly associated with cellulose microfibrils, and such interactions between other polysaccharides and xyloglucan-coated cellulose microfibrils might form a gel to prevent protoplasmic blow out. The gel properties of wall polysaccharides *in vitro* have been reviewed recently (Bacic *et al.*, 1987; Fincher and Stone, 1986).

Pectin-Pectin and Pectin-Xyloglucan Associations

The analysis of cross-linking in pectins is sophisticated because of the diversity of potential sites from their composition mentioned above. Proposed cross-links include Ca^{2+} bridges (Jarvis, 1982), other ionic bonds (Smith *et al.*, 1984), glycosidic bonds (Mort and Lamport, 1977) , ester bonds (Fry, 1984), and phenolic coupling (Fry, 1985).

Certain pectins will cross-link *in vitro* in the presence of Ca^{2+} owing to the formation of Ca^{2+} bridge (Thom *et al.*, 1982; Yamaoka *et al.*, 1983; Baydown and Brett,

1984). Thus, strong cross-links can be formed between pectin molecules, probably in a well-defined arrangement known as the 'egg-box' model (Powell *et al.*, 1982; Rees, 1982; Jarvis, 1984).

A pectin-xyloglucan hybrid fragment isolated from sycamore culture cell walls was reported and formed the basis of a widely accepted wall model (Keegstra, *et al.*, 1973; Albersheim, 1978). Although later work failed to characterize a putative cross-linkage between xyloglucan and pectin (Monro, *et al.*, 1976; McNeil, *et al.*, 1979), the possibility of a small proportion of glycosidic bonds between two polysaccharides is not excluded (Stevens and Selvendran, 1984; Jarvis, 1984; O'Neill and Selvendran, 1985; Selvendran *et al.*, 1985).

Polysacharides-Phenolic Compounds Associations

The possibility that polysaccharide hydroxyls could be bonded to lignin phenyl-propanoid residues through ether linkage has been postulated on the basis of model compounds (Lai and Sarkanen, 1971; Leary *et al.*, 1983). The evidence for ether linkages between polysaccharides and lignin in black spruce (*Picea mariana*) has been presented by Eriksson *et al.* (1980). Such a linkage should be formed by dehydrogenation in the presence of H_2O_2 and peroxidase.

Pectins from spinach culture cell walls contained the cinnamate derivatives ferulate and *p*-coumarate (Fry, 1979; 1980), ester-linked through their COOH groups to pectins (Fry, 1983). There are about 10 feruloyl groups per 1000 pectic sugar residues, and the feruloylated sugar residues are nonreducing ends of pectic side-chains. $NaClO_2$, which would cleave cross-linked cinnamate derivatives (e.g. diferulate), solubilized some pectin (approximately 12%) from sycamore culture cell walls and kale cell walls (Fry, 1986b). These results taken together raise the possibility that the pectin molecules are cross-linked by oxidative coupling of their phenolic substitutes. It has also been speculated that ester bonds help to hold pectin in the wall. Cold Na_2CO_3 solution solubilized some CDTA-

insoluble wall pectins by breaking ester bonds (Jarvis *et al.*, 1981). These esters could be diferuloyl and related bridges (Fry, 1979) or ester bonds between uronic acids and neutral sugars (Lamport, 1970; Mares and Stone, 1973; Wada and Ray, 1978; Das *et al.*, 1981; Fry, 1985).

Extensin-Polysaccharides Associations

For a long time, it was believed that extensin is secreted as a salt-extractable wall HRGPs, and becomes insolubilized by cross-linking to wall polymers. The side-chains of amino acid residues in extensins, such as serine, threonine, hydroxyproline, lysine, tyrosine, and histidine, provide many sites for post-translational modification and *in muro* interaction (Varner and Lin, 1989; Cooper *et al.*, 1984). Extensins are positively-charged glycoproteins because of their high lysine content, and the lysine residues are regularly spaced along the extended peptide backbone. Therefore, it seems inevitable that basic extensins are ionically bound to the block acidic regions of pectin in the wall (Smith *et al.*, 1984; Fry, 1986b). An electrovalent zippering up of opposite charges might well occur when the polycationic wall extensins and the polyanionic pectins form the complex. Lowering the pH or increasing $[Ca^{2+}]$ could unzipper the complex. Such a reversible assembly of wall proteins and polysaccharides could be very useful in later positioning wall components for irreversible cross-linking to stop cell expansion. A reversible covalent binding of protein to polysaccharides might form through reaction of the amino groups of the lysine with the reducing ends of the polysaccharides (Schiff's base). The discovery and characterization of an oxohexuromic acid (5-hexosulopyranosidic acid) by Painter (1983) in the walls of several plant species opens the possibility for extensins to cross-link polysaccharides by a Schiff's base linkage.

Covalent associations of wall protein and non-cellulosic polysaccharides in the walls of a number of dicotyledons have been proposed on the basis of co-elution by Selvendran and colleagues (1985). Moreover, there is also tentative evidence for complexes between

proteins and pectic polysaccharides. The proteins are hydroxyproline poor. It is conceivable that extensin and polysaccharides could be directly crosslinked by ester bonds formed between peptidyl carboxyl groups (aspartate and/or glutamate) and sugar hydroxyl groups.

Extensin-Extensin Associations

It was proposed that extensin was insolubilized by interacting with wall polysaccharides. However, removal of wall polysaccharides with the deglycosylation reagent (e. g. liquid hydrogen fluoride) did not solubilize extensin, and left an insoluble residue (Mort and Lamport 1977). A tyrosine derivative present in some of the extensin peptides remained identified for more than a decade. In 1982, Fry identified the tyrosine derivative as isodityrosine (IDT), a diphenyl ether linked bityrosine which has the molecular properties of a protein-protein crosslink. In the wall, extensin is linked intramolecularly through IDT bridges (Epstein and Lamport, 1984). However, no information exists concerning the intermolecular isodityrosine cross bridges between extensin molecules. Nevertheless, extensin can be solubilized with acid chlorite which cleaves IDT bridges (O'Neill and Selvendran, 1980). Most likely, such intermolecular linking would render the extensin molecule insoluble.

In addition, the histidine residues contained in some of the extensins can be attacked by oxygen-free radicals to open the imidazole ring and generate an aldehyde compound (Rivett, 1986). This would permit for a reversible polymerization of extensin molecules through Schiff's base linkages.

Extensin-Lignin Associations

Protein is commonly found in preparation of Klason lignin obtained by treating wood with sulfuric acid, but wall proteins are partially degraded by this treatment. Dill *et al* (1984) reported that Klason lignin from beech (*Fagus sylvatica*) contains significant levels

of protein-bound amino acids, including hydroxyproline. Based on a comparison of the amino acid composition of proteins complexed with thioglycolic acid-lignin in a wall preparation from *Pinus elliottii* callus, Whitmore (1978, 1982) suggested that lignin interacts with hydroxyproline-rich glycoproteins in walls. In addition, the solubilization of both lignin and protein by acid chlorite treatment of walls indicates that the phenolic linkages are disrupted between the wall proteins and the lignins (O'Neill and Selvendran, 1980).

Although there is a wealth of information about the chemistry of wall polymers, a fundamental gap in our knowledge exists in the molecular organization and interaction of these wall components. Different cell types in the plant have different cell wall compositions, and these compositions are determined by developmental stage. It seems that cell wall components are capable of interacting with each other in a variety of ways, The challenge is now to find out exactly what these interactions and crosslinks are *in muro*.

CHAPTER III

MATERIALS AND METHODS

Preparation of Cell Walls from Cotton Suspension Cultures

Cell walls were obtained from suspension-cultured cotton plant cells (*Gossypium hirsutum L.*). Explant tissue for callus initiation was obtained from 30- to 90-day old plants. Callus cultures from fully-expanded leaves of Acala 44 were established in 1985 (Janet Rogers and Earl D. Mitchell, Jr., Department of Biochemistry, Oklahoma State University, OK) using a modification of the method of Ruyack *et al.* (1979). Cotton suspension cells were initiated from these cultures after 3 to 4 weeks by transferring 3 to 4 grams callus (late log phase) from SH (Schenk and Hildebrandt) agar medium to 125 ml culture flasks containing 50 ml of liquid SH medium. About two-week-old suspension cultured cells in late log phase were used for cell wall preparation. Cell walls were prepared using a method described by Komalavilas and Mort (1989). Cultured cells were collected on a coarse scintered glass funnel and were washed once with 100 mM potassium phosphate (pH 7) four times to remove extracellular debris. Cells were collected using Nitex-Nylon Mes HL3-15 (Tetko, Inc., Precision Woven Screening Media) after each washing. The washed cells were then resuspended in one volume of 500 mM phosphate buffer and homogenized with a polytron (Brinkmann Instruments, Inc., Westbury, NY) set at its maximum speed in 4 bursts of 3 minutes each for a total of 12 minutes. The polytron was allowed to cool for 2 minutes between bursts. The sample was kept in cold (in ice) during the grinding procedure. Preparations were microscopically examined to show that cell breakage was complete. The suspension of broken cells was then placed in a coarse scintered glass funnel using the same washing procedure mentioned above. It was then

suspending in five volumes of the 500 mM phosphate buffer, and the pellet washed five times with distilled water. The washed cell wall pellet was suspended by vigorous stirring in 5 volumes of 1:1 chloroform:methanol (v/v) and placed in a coarse scintered glass funnel. The organic solvent was removed by applying gentle suction to the funnel. The cell walls were exhaustively washed with acetone to remove water. Then, the cell walls were air-dried in a desiccator.

After air-drying, the cell wall preparation were treated with α-amylase (Bacillus type II-A, from Sigma chemical Company) as described by York *et al.* (1986) to remove residual starch. 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 gently stirred for 48 hours at 25°C. The procedure was repeated until microscopic examination indicated that there is no starch bound to the cell walls. The enzyme treated cell walls were then washed extensively with distilled water followed by acetone and air-dried. The prepared cell walls were stored in a desiccator ready for use.

Preparation of Endopolygalacturonase (EPGase)

Source of EPGase

An *Escherichia coli* clone (HB101/pAKC 213:: Tn5-2) expressing a *peh* gene from *Erwinia carotovora subsp. carotovora*, kindly provided by Dr. A. K. Chatterjee, was used as a source of endopolygalacturonase (EPGase) enzyme (Willis *et al.*, 1987). Bacterial cultures, subcultured from colonies grown on L-agar supplemented with the antibiotics tetracycline (10 μg/mL), kanamycin (50 μg/mL), and ampicillin (50 μg/mL), were grown in L-broth at 30°C for 60 hours. Periplasmic shock fluids, containing greater than 80% of the total enzyme activity, were prepared as described by Witholt *et al.* Typically, 800 mL of periplasmic shock fluids were prepared from 2 liters of bacterial culture.

Purification of EPGase

Endopolygalacturonase (EPGase) was purified as described by Maness and Mort (1989). Purification was carried out in a two-step procedure with crosslinked polypectate as an affinity absorbant. The crosslinked polypectate was prepared from apple pectin (Sigma Chemical Company) using a procedure described by Barash *et al.*, (1984). In the first step, 1 liter periplasmic shock fluid was added to 50 g (dry weight) crosslinked polypectate previously equilibrated with 100 mM Tris, pH 8.0. This mixture was stirred slowly for 5 minutes to allow affinity binding to occur and the supernatant discarded after the polypectate was allowed to settle. The bound enzyme was eluted from affinity absorbant 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 ultrafilter (10,000 Mr cutoff; Amicon Corporation, Cambridge, MA). After the initial step, the partially purified enzyme was applied 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 bound enzyme was eluted from the column with a 300 mL linear gradient from 0 to 1.0 M NaCl in 100 mM Tris buffer (pH 8.0). The fractions containing EPGase activity were combined and concentrated as before to 1 or 2 mL, and then stored in 10% (v/v) glycerol at -70°C.

Assay of EPGase

Enzyme sample plus buffer (0.1 mL) were mixed with a 0.4 mL assay stock solution containing pectic acid (Aldrich) and incubated at room temperature for 15 minutes. The reaction was stopped by adding 0.5 mL Nelson-Somogyi reagent and heating at 100°C for 10 minutes. After cooling, 1 mL of arsenomolybdate reagent was added and the samples were vortexed. If necessary, the unreacted pectate could be removed by centrifugation and the optical densities were measured at 500 nm. Enzyme-dependent pectic

acid hydrolysis was determined by comparing absorbance values to standards prepared with 10-100 µL of a 2.5 mM galacturonic acid stock solution. Enzyme activity units were defined as the amount of EPGase which released 1.0 µmole reducing sugar per minute.

Cell Wall Composition Analyses

Determination of Carbohydrate Composition

Carbohydrate compositions were determined for whole cell walls and other samples by gas chromatographic analysis of the trimethylsilyl glycosides. Methanolysis and derivatization were performed by a modification of the protocol of Chaplin (1982). About 50 µg of dry sugars were weighed on a CAHN 29 electrobalance and placed in screw-cap glass vials fitted with Teflon-lined lids containing 100 nmoles of myo-inositol as an internal standard. One hundred µL of 1.5 M methanolic HCl and 25 µL of methylacetate were added to the samples, after which the vials were tightly sealed and incubated in a 80°C heating block overnight (at least 3 hours). The vials were removed the following morning, and 3 to 6 drops of t-butanol were added to each vial. The samples were evaporated to dryness under a stream of argon. Trimethylsilylating reagent was prepared fresh in an exhaust hood by mixing 1 part of Tril-Sil concentrate (Pierce Chemical Company, Rockford, IL) with 3 parts of dry pyridine. Twenty-five µL of the reagent was added to the dried samples and allowed to incubate at room temperature for at least 15 minutes to derivatize. The derivatized samples were then evaporated slowly under a stream of argon just to dryness, and redissolved in 50 to 200 µL of isooctane. A 1 µL aliquot was injected into a fused silica capillary column (see Section on Analytical method, Capillary Gas-Liquid Chromatography) installed in a Varian 3300 gas-liquid chromotograph. Peak integration was performed using a Varian 4290 Integrator.

Cellulosic glucose was determined for samples treated with anhydrous hydrogen fluoride (HF) at 0°C for 15 minutes before methanolysis and derivatization.

Determination of Amino Acid Composition

Approximately 500 pmoles (on an amino acid basis) of dry protein samples were hydrolyzed in 200 µL of 6 N HCl constant boiling (sequanal grade Pierce) at 110°C for 18 to 24 hours in sealed, screwed-cap glass vials fitted with Teflon-lined lids. The resulting amino acids were evaporated to complete dryness. All the necessary drying steps were carried out in a Speed Vac concentrator (Savant). The hydrolyzed samples were than analyzed with a method of Seferiadis et al (1987). One hundred µL phenylisothiocyanate (PITC) derivatization coupling buffer (acetonitrile : pyridine : triethylamine : distilled water, 16:5:2:3 v/v/v/v) was used to dilute and remove the remaining acid through successive drying steps. Coupling conditions were essentially as described by Heinriksona and Meredith (1984).

Five µL of PITC was added along with 100 µL of coupling buffer. The reaction time was 5 minutes for the amino acid standards (Pierce-H) and hydroxypoline standard and 20 minutes for unknown protein or peptide hydrolysates. Amino butyric acid was used as the internal standard.

Prior to high performance liquid chromatography (HPLC), the PTC-derivatized samples were dissolved in acetonitrile/water (1:1 v/v) and diluted in the same volume of solvent A. The eluent consisted of solvent A; 0.05 M ammonium acetate, pH 6.0 and solvent B; 0.1 M ammonium acetate in acetonitrile/methanol/water (44:10:46 v/v/v).

Analyses were performed using a Beckman HPLC system consisting of two model 110B pumps, a model 421 controller and a model 163 variable wavelength detector. The column was an Lichrosorb, C_{18}, 5-micron, 4.0 X 250 mm (Hibar), preceded by a guard column, 5-micron, 4.6 X 20 mm, C_{18} (Alltech). The column and the guard column were covered by a glass jacket and held at 52°C by a model FS recirculating water bath (Haake). Peaks were recorded and integrated with a auto-ranging date logger system (Merz, in draft 1991).

Determination of Hydroxyproline Content

The hydroxyproline content was determined colorimetrically at 560 nm by the procedure of Drozdz *et al.* in 1976. Between 150 μg and 500 μg dry wall samples or other samples were hydrolyzed in 6 N HCl at 110°C for 24 hours in screw-cap glass vials fitted with Teflon-lined lids The hydrolyzed samples were reacted with chloramine T which is a oxidizing reagent, and hydroxyprolines in the samples were converted to compounds related to pyrrole. Formation of chromophore is based on the reaction of *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) with pyrrole derivatives of hydroxyprolines. The chromophore can be easily detected spectrophotometrically (Stegemann, 1958).

Methylation Analysis

Methylation

Methylation of polysaccharides was performed mainly as described by Kvernheim (1987). Between two hundred and five hundred micrograms of each sample was flushed with an argon stream for 2 minutes in a 10-mL test tube sealed by a rubber cap. Four hundred μL of DMSO was added using a glass syringe with argon flushing. The tube was then sonicated and heated at 50°C in a heating block for 3-5 hours in order to dissolve the sample. 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 hours. The reaction mixtures were cooled 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 minutes.

Extraction of Methylated Sugars

Extraction of methylated sugars was conducted with Sep-Pack C_{18} Cartridge (Waters Associates Inc. Milford, MA) as described by Mort *et al.* in 1983. The Cartridge

was prewashed with (1) 5 mL of ethyl acetate; (2) 5 mL of methanol; and (3) 15 mL of water before the sample was applied. The methylated mixtures were diluted with 5 mL of water to ensure adequate solvent polarity for the sugars to be absorbed and applied slowly to the preconditioned Sep-Pak with a 10 mL glass syringe. The Sep-Pak was next washed with 15 mL of water, and the methylated sugars were eluted with 3 mL of water-methanol (1:1 v/v); 3 mL of methanol and 3 mL of methanol-chloroform (1:1 v/v), respectively, depending on the molecular sizes of the derivatives. The eluted compounds were evaporated to dryness under a stream of nitrogen.

Hydrolysis

The dry methylated sugars were hydrolyzed in 88% formic acid (100 µL) at 100°C for one hour (this step was omitted for oligosaccharides with less than 4 residues). After drying the hydrolysates under a nitrogen stream, 100 µL of 2 N trifluoroacetic acid was added, and hydrolysis was continued for 1.5 hours at 121°C. After cooling, 250 nmoles of inositol were added and the acid was evaporated as before.

Reduction

Ammonium hydroxide (1 M, 10 µL) was added to the samples from the previous step, followed by addition of 100 µL of freshly prepared 0.3 M potassium borohydride in dimethyl sulfoxide. The mixture allowed to reacted at 40°C for 1.5 hours. After the reaction, excess potassium borohydride was decomposed by addition of 10 µL of glacial acetic acid (Harris *et al.*, 1984).

Acetylation

The reduced samples were acetylated using 1-methylimidazole (20 µL) and acetic anhydride (200 µL) (Blakeney *et al.*, 1983). The reaction was allowed to procede for 10 minutes at room temperature. Excess acetic anhydride was decomposed by adding 5 mL of

distilled water. Preconditioned Sep-Pak C_{18} cartridges were used to purify the alditol acetates. Alditol acetates were eluted with 2-3 mL methylene chloride. The water layer was sucked out with a pasteur pipet, and the remaining water was absorbed with a few milligrams of sodium sulfate. After evaporation of methylene chloride with a nitrogen stream, the samples were dissolved in isooctane for gas chromatography-mass spectrometry analysis.

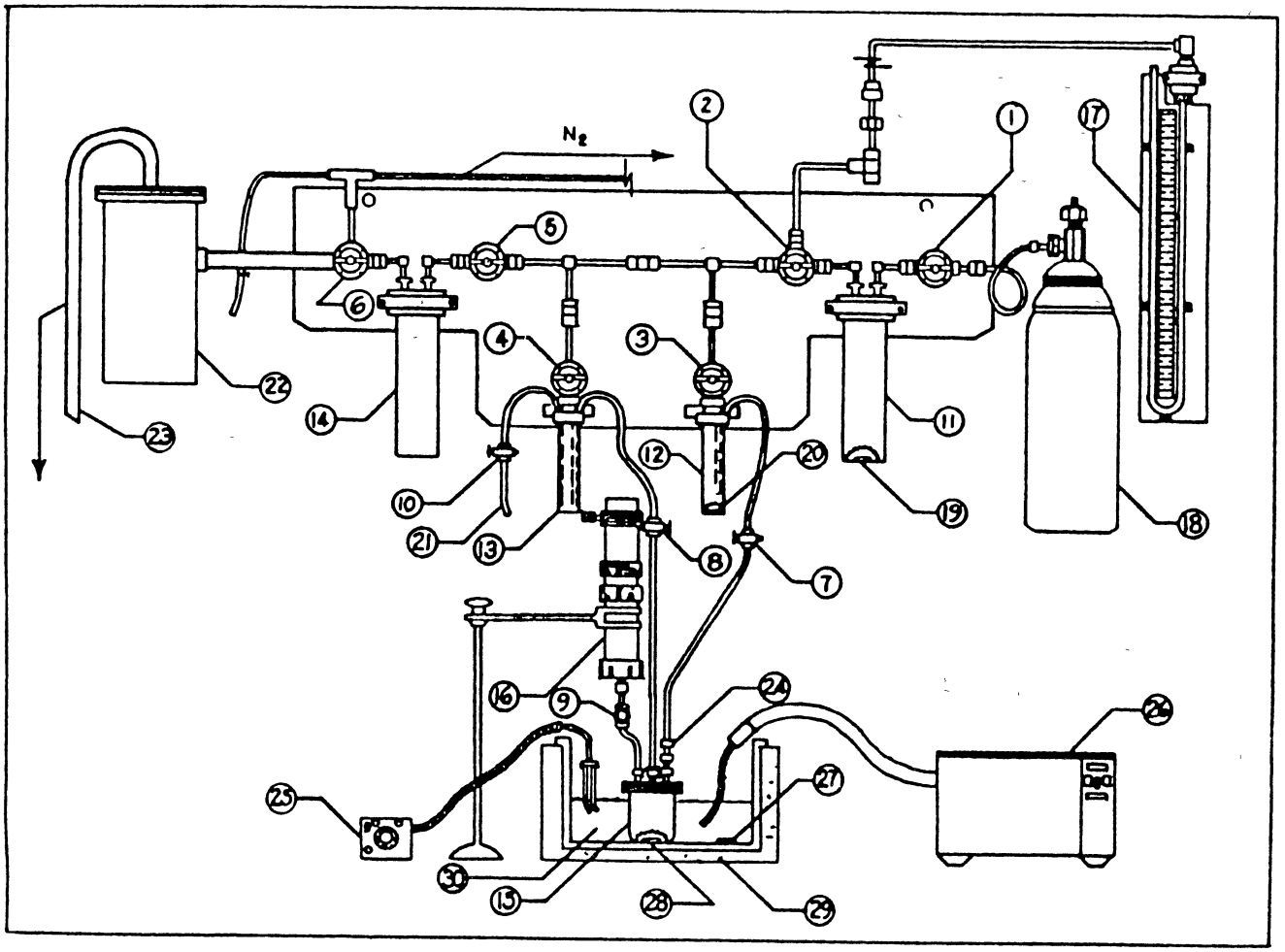
Selective Hydrogen Fluoride (HF) Solvolysis

HF Solvolysis and Apparatus

Hydrogen fluoride (HF) is a fuming, volatile liquid at room temperature. These fumes are extremely reactive, toxic to living tissues, and corrosive to glass and many metals. To safely handle HF, a special apparatus that is closed to the atmosphere and constructed of Teflon with which is inert to HF was designed (Mort, 1983). The apparatus is shown in Figure 1 (Mort, *et al.*, 1989). In a typical experiment, dry cotton cell walls and their residues (500 mg) were placed in reaction vessel 15, along with a stirrer bar. The whole apparatus was evacuated, and leaks in any part of the system were checked and sealed. Approximately 20 mL of HF was then transferred from the reservoir 11 to the HF holding vessel 12. This was done by cooling vessel 12 with dry ice in acetone and allowing the HF to distill from reservoir 11. Both vessel 12 and 15 were next allowed to reach an appropriate temperature equilibrium through the use of a cooling bath and the temperature maintained during solvolysis. HF was transfered from vessel 12 to the reaction vessel 15 using a positive nitrogen pressure. The reaction in vessel 15 was allowed to continue for 30 minutes with stirring. The reaction was then stopped by freezing the reaction mixtures in a liquid nitrogen bath. About 300 mL of cold ether (cooled by adding dry ice) was added to the frozen samples from vessel 16. The liquid nitrogen bath was removed, and the quenched mixture was allowed to warm. The mixture was then stirred at room temperature

Figure 1. Schematic Representation of the Hydrogen Fluoride Solvolysis Apparatus.

1-8, 10, stopcocks; 9, Teflon needle valve; 11-16, Teflon and Kel-F vessel; 17, manometer; 18, hydrogen fluoride tank; 19, 20, stirrer bars; 21, exit to the sink for pressure release, if necessary; 22, calcium oxide trap; 23, connection to the vacuum pump; 24, 3 mm to 6 mm adaptor; 25, heater/regulator; 26, immersion cooler; 27, 28, stirrer bars; 29, insulated container; 30, 95% ethanol. Reprodued with permission from Springer-Verlag.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for 3 h. Then filtered using a Teflon filter (50 mm diameter, fine grade, Savillex Corporation). Sugars from the filtrate (HF/ether/sugar mixture) were recovered by evacuation under vacuum into a liquid nitrogen trap of HF/ether complex, dissolved in water and freeze dried. The residue in the filter unit was extracted with water three times. The aqueous extract and the water insoluble residue were freeze dried. Alternatively, the quenched reaction mixture (HF/ether solution) from the HF solvolysis of a small quantities of sample (a few milligrams) was evacuated under vacuum to dryness without filtering. The remaining materials were dissolved in water, or 50% acetic acid, then freeze dried.

HF Treatment at Different Temperatures for Different Samples

Acala 44 cotton suspension culture cell walls were treated with anhydrous hydrogen fluoride (HF) either at 0°C by using an ice bath or at -23°C with 95% ethanol in a cooling system. The enzyme treated cell wall residues were treated with HF at temperature -73°C through the use of dry ice in acetone.

Three different temperatures selected for the reaction were 0°C, -23°C, and -73°C. For the 0°C reaction, the cooling bath was ice in water; for the -23°C reaction, the bath was 95% ethanol, and the temperature was maintained using immersion cooler offset by a regulated immersion heater; and for the -73°C, the bath was dry ice in acetone. All reactions were carried out for 30 minutes and subsequent treatment of the reaction mixtures was performed as described above.

Enzymatic Treatments

Digestion of Cell Walls with Endopolygalacturonase

Dry cell walls were suspended in 50 mM ammonium acetate buffer, pH 5.2 (approximately 10 mg/mL), and subjected to a NAPCO vacuum oven at room temperature for 30 minutes to hydrate the walls completely. A EPGase solution (0.1 units/mg walls)

was then added in each sample and incubated at room temperature with gentle stirring overnight. A few drops of toluene were added to the solution to prevent bacteria growth. After digestion, the reaction solution was filtered using a concentrator with NYLON 66 membrane (Alltech, 0.45 micron) under nitrogen pressure. The residue was washed with 3 volumes of water and lyophilized. The filtrates were collected and lyophilized.

Digestion of Cell Walls or Their Residues with Cellulase

Dry cell walls or their residues (EPGase-treated, HF treated) were digested with chromatographically purified cellulase (Worthington Biochemical Company, Freedhold, NJ) by suspending in ammonium acetate buffer (50 mM pH 5.2, approximately 10 mg wall materials/mL), and incubating with the enzyme (2% w/w) overnight at room temperature. A few drops of toluene were added. After incubation, the digestion mixture was filtered and washed. Filtrates were combined. Combined filtrates and residue were freeze-dried.

Digestion of Wall Protein-Containing Samples with Trypsin

Dry samples (cotton cell walls, cell wall residues, or deglycosylated Hyp-containing protein or peptides) were treated with trypsin prepared from porcine pancreas (2% w/w, Sigma, type IX) in ammonium bicarbonate buffer (100 mM, pH 7.6, 10 mg wall materials/mL) at room temperature and gently stirred overnight. The resulting mixture was subsequently filtered and subjected to the same procedure as described for the above samples. If the samples were solubilized in buffer, the reaction mixture was freeze-dried without filtering.

Digestion of Cellulase-Treated Residue with Esterase or α-Chymotrypsin

Cellulase-treated residue was digested with esterase (1 unit/mg) or α-chymotrypsin (2%, w/w) in ammonium bicarbonate buffer (100 mM, pH 7.6, 10 mg/mL) at room temperature and gently stirred overnight. The resulting mixture was then filtered and freeze dried as described above.

Mild Alkaline Treatment of Extensin-RG I Complexes A

Preparation of a Solution of Cadmium Acetate-EDTA-Na_2

A mixture of solutions of 0.1 M EDTA-Na_2 (4.5 mL) and cadmium acetate (23 mg/mL, 4.5 mL) was titrated with 0.5 M NaOH (~1.7 mL) to pH 7-7.5.

Alkaline Reaction of Extensin-RG I Complexes A

Reactions were performed in Pyrex glass test-tubes (1.8 x 20 cm) covered with Parafilm. Extensin-RG I complexes A (3.2 mg) were dissolved in 2.4 mL distilled water, and cadmium acetate-EDTA-Na_2 (0.43 mL), 0.5 M NaOH (0.17 mL) and $NaBH_4$ (228 mg) were added to the mixture. Butanol (5%, v/v) was added to the reaction mixtures containing $NaBH_4$ to prevent foaming. The mixture was then incubated at 50°C. After 16 h incubation, the reaction mixture was cooled , diluted with water (3.5 mL), and acidified with acetic acid to pH 6. The resulting solution was freeze dried, and could be directly fractionated on an HW 50(S) gel filtration column.

Isolation of Extensin Peptides

Acala 44 cotton cell walls were treated with HF at O°C for 30 min. After ether and ammonium acetate buffer (50 mM, pH 5.2) extractions, the residues were digested with

trypsin. The tryptic peptides were then separated on a reverse phase C_{18} column, and further chromatographed on a gel filtration HW 40(S) column.

Isolation of Extensin-RG I Complexes

Ac 44 cotton cell walls were treated sequentially with EPGase, cellulase, HF at -73°C, ammonium bicarbonate buffer (100 mM, pH 7.6), and trypsin. The final soluble mixture was separated on a gel filtration HW 50(S) column. Fractions were collected, and sugar and amino acid compositions of fractions were analyzed.

All filtration steps after each treatment were conducted with a NYLON 66 membrane filter (Alltech, 0.45 micron, 47 mm) using a concentrator under argon pressure.

Analytical Methods

Capillary Gas-Liquid Chromatography

The trimethylsilyl 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. After one minute, the temperature was raised to 160°C at the rate of 10°C/min and held for 4 minutes, and then raised 1°C/min to 200°C. The temperature was immediately raised to 240°C at the rate of 10°C/min and held there for 10 minutes to clean the column. Peaks were integrated using a Varian 4290 integrator.

Gel Filtration Liquid Chromatography

The cellulase-solubilized fractions from EPGase-treated wall residue were separated on Fracto-gel HW40(S) gel filtration column (50 X 1 cm) from Supelco Inc., Bellefonte, PA. The elution buffer was 50 mM ammonium acetate buffer, pH 5.2, and the flow rate was

0.5 mL/min. Sugars were detected by a refractive index (RI) monitor and collected in 0.5 mL fractions. The molecular weight standards used were Pullulan (Polymer Laboratories Technical Center, Amherst, MA).

The Extensin-RG I complex containing fractions from the sequential treatments (EPGase, cellulase, HF -73°C, ammonium bicarbonate buffer extraction, trypsin) were fractionated on Fracto-gel HW 50(S) gel filtration column (50 X 1 cm) from Supelco Inc., Bellefonte, PA. The column was equilibrated with 0.1% trifluoroacetic acid; pH 2.5, and 50 mM ammonium acetate buffer, pH 5.2 with a flow rate of 0.5 mL/min. The peptides were detected by a UV monitor at either 215 nm or 280 nm.

Reverse Phase Chromatography

The tryptic peptides were mapped using a procedure modified from the method described by Aebersold *et al.* (1987). A Bakerbond wide-pore (300 Å) octadecyl C_{18} (5 μm) reversed phase column (4.6 X 250 mm from J. T. Baker Inc.) was used in this experiment. Solvent A was 0.1% (v/v) of Trifluoroacetic acid and solvent B was a mixture of 37.5% solvent A and 62.5% acetonitrile. The cotton extensin peptides were eluted with a gradient of consecutive linear segments of increasing acetonitrile concentration: Solvent B from 0 to 30% (v/v) in 60 minutes, and from 30 to 100% (v/v) in 5 minutes, followed by elution at 100% solvent B for 10 minutes. Peaks were detected by ultraviolet absorption at 214 nm, and interesting ones were collected. The peptide mappings were performed using the Beckman HPLC system, as described in section "Determination of Amino Acid Composition".

Ion Exchange Liquid Chromatography

The RGI-extensin complexes were 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 60 minutes followed by a steady

elution of 1.0 M for 10 more minutes was used to elute the sample. Flow rate was 1.0 mL/min, and 1.0 mL fractions were collected. The peaks were analyzed using gas chromatographic analysis of their trimethylsilyl glycosides after methanolysis as described above or phenol-sulfuric acid colorimetric analysis. The phenol-sulfuric acid method was reported by Ashwell (1966). Two hundreds microliters of the fractions were mixed with 300 μL water, and 12.5 μL of phenol reagent (80%), followed by 1.25 mL of concentrated sulfuric acid. Samples were vortex-mixed and the absorption measured at 485 nm after 30 minutes.

The deglycosylated extensins and mild alkaline (pH 11-12) treated extensin-RG I complexes were also subject to the same chromatography. Isolated and purified RG I fragments from cotton cell walls was used as "standard" samples to elute on the PA-1 column under the conditions described above.

Liquid Secondary Ion Mass Spectrometry (LSIMS)

The molecular masses of the peptides and oligosaccharide fragments generated by EPGase and/or cellulase, and the peptides produced by trypsin were determined by LSIMS. The dried sample was dissolved in 2 to 5 μL of either water or methanol, and 1 μL of the solution was mixed with 1 μL of thioglycerol or 3-nitrobenzyl alcohol (NBA) on a stainless steel target before determination. 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 extensin-RG I complexes from cotton suspension culture cell walls were subjected to GC-MS to identify their glycosyl-linkage compositions. One μL of sample dissolved in isooctane was injected into a

Hewlett-Packard 5890 gas chromatography, which was connected to a TS-250 Trisector tandem mass spectrometer (VG Tritech, Manchester, UK) with EBE geometry. The program of GC temperature raising was the same as that described in determination of carbohydrate composition. Linkages of the different sugar residues were identified based on their mass fragmentation patterns.

Nuclear Magnetic Resonance (n.m.r.) 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) at 25°C. To exchange O-linked hydrogen atoms with deuterium atoms, samples (1-20 mg) were dissolved in 98% D_2O) (Aldrich Chemical Company) and lyophilized. The samples were then dissolved in 99.9 % D_2O (Aldrich Chemical Company) and freeze-dried again. Before n.m.r. recording, the samples were redissolved in 99.9% D_2O. Acetic acid (2.05 p.p.m.) and sodium 2, 2, 3, 3-tetra deuterio-4, 4-dimethyl-4-silapentanotate (TSP) (0.0 p.p.m.) were used as internal standards.

Both ^{1}H-^{1}H homonuclear and ^{13}C-^{1}H heteronuclear two dimensional n.m.r. spectroscopy were recorded on a Varian XL-400 NMR spectrometer at 25°C using 20-40 mg samples. O-linked hydrogen atoms were exchanged for deuterium as described above. These experiments were performed as described by Gray (1983). CP/MAS (cross-polarization/magic angle spinning) n.m.r. spectra of dry cotton cell walls and extracted walls were recorded on an IBM WP-100 NMR spectrometer (25 MHz) by Dr. F. McEnroe of Conoco Company, Ponca City, OK.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis in the presence of SDS was carried out as described by Laemmli (1970). The acrylamide concentrations in the running gel were 7.5, 10, 12 and 14%. The samples (5 μg - 10 μg by total weight/20 μL extensin-RG I complex

mixtures deglycosylated extensin fragments) were run at 200 volts for about 30 minutes using a mini-Protean II Dual Slab Cell system (Bio-Rad Laboratories, Richmond, CA). The proteins in the gel were stained using the Bio-Rad silver staining method for proteins (Bio-Rad Laboratories, Richmond, CA) or Coomassie blue in 50% methanol/10% acetic acid. The prestained low molecular weight standards (Bio-Rad Laboratories) were run as follows: phosphorylase b, 130,000 MW; bovine serum albumin (BSA), 75,000 MW; ovalbumin 50,000 MW; carbonic anhydrase, 39,000 MW; soybean trypsin inhibitor, 27,000 MW; lysozyme, 17,000 MW.

The carbohydrates in the gels were stained with Schiff reagent as described by Mitchell (1976). The gels were washed with 40% methanol and 7% acetic acid for 24 h. After rinsing with 7% acetic acid, the gels were incubated with 1% sodium periodate in 7% acetic acid at 4°C in dark for 1 h. The gels were then washed twice with 7% acetic acid for 12 h to remove sodium periodate. The gels were stained with Schiff reagent in the dark at room temperature until the red color bands on the gels appeared. The Schiff reagent was prepared by mixing 16 g sodium metabisulfite, 21 mL concentrated HCl and 8 g basic fuchsin in 1 liter distilled water. After gentle stirring for 2 h, a small amount of Darco charcoal granule (G-60) were added to the solution to decolorize it, and the solution was filtered within 15 min. The reagent appeared light pink in color and was stored in a refrigerator at 4°C for a maximum of two months.

Western Blot Analysis

Western blot analyses were performed using a method of Tombin *et al.* (1979). Samples separated by electrophoresis in SDS-PAGE (37.5:1 acrylamide:bis), 7.5, 10, 12, and 14% gels were used for the analysis of the distribution of Extensin-RG I Complexes fractions A and B. Glycoproteins in the gels were electro-transferred at a constant current density of 2.5 mA/cm for 15 to 20 minutes in buffer (pH 8.3) containing 20 mM Tris; 150 mM glycine and 20% methanol using an ABN polyblot apparatus (Model SBD-1000) as

described in the American Bionetics instruction manual. The Immobilon-P blot papers were soaked in methanol to wet and were rinsed with distilled water before blotting. The papers were rinsed with tris buffered saline (TBS) buffer containing 20 mM Tris-HCl (pH 7.6) and 150 mM NaCl and soaked for 45 minutes at room temperature in TBS/5% N-ethylmaleimide (NEM) solution containing 5% (w/v) non-fat dry milk in TBS buffer. After blocking, blots were rinsed twice with TBS, followed by incubation overnight at 4°C with 50 mL TBS containing antibodies to deglycosylated tomato extensin precursor dP1 (description in Kieliszewski and Lamport, 1986) diluted 1:500. Blots were washed consecutively for 10 minutes each with TBS, TBS/0.5% Tween 20 (polyoxyethylene sorbitan monolaurate), TBS and TBS/5% NEM, followed by incubation for 2 hours at room temperature with 50 mL TBS/0.05% NEM containing anti-rabbit immunoglobulin G alkaline phosphatase conjugate (ICN Immunobiologicals) diluted 1:100. Blots were again washed as described above. Bands are then visualized at 35°C by using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP). The BCIP substrate solution (30 mL) consists of 10 mg of nitro blue tetrazolium (NBT), 5 mg of BCIP in AP buffer (pH 9.5) containing 100 mM Tris HCl, 100 mM NaCl and 100 mM $MgCl_2$, prepared freshly prior to use. The reaction was stopped immediately after bands showed up by soaking blots in distilled water. Western blots were dried with paper towels and photographed to make a permanent record.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cross-reactivities of extensin-RG I complexes as well as their deglycosylated forms with polyclonal antibodies raised against glycosylated and deglycosylated tomato extensin precursors (P2 and dP2) obtained from Kieliszemski and Lamport (1986) (Department of Energy Plant Research Laboratory, and Department of Biochemistry, Michigan State University, East Lansing, MI) were determined by the indirect ELISA method of Voller *et al.* (1980). Each test well of the 96 well Microtest Tissue Culture Plate (CMS) was coated with antigen samples (2-10 μg by total weight/mL, Extensin-RG I complexes or their

deglycosylated forms) in 200 µL Tris buffer (pH 8.8) for 1 hour and room temperature, then overnight at 4°C. The plates were washed three times with PBS/Tween (50 mM sodium potassium phosphate, pH 7.4; 150 mM sodium chloride; 0.02% potassium chloride; 0.05% Tween-20). Remaining protein binding sites were blocked by adding 200 µL 1% (w/w) bovine serum albumin (BSA) in PBS/Tween for 30 minutes at room temperature, followed by washing three times again with PBS/Tween. Two hundred µL of the antibodies (P1, P2, dP1 and dP2) diluted 1:500 were added to each sample well and incubated 2 hours at room temperature. After incubation, the plate was washed three times with PBS/Tween. Two hundred µL of the conjugate, goat anti-rabbit Ig G-alkaline phosphatase was added to each well, allowed to incubate 2 hours at room temperature. The plate was washed again with PBS/Tween. Two hundred µL of the substrate, *p*-nitrophenylphosphate (1 mg/mL) in 0.1 M glycine, 1 mM $MgCl_2$, 1 mM $ZnCl_2$ (pH 10.4), was added to each well and incubated for 30 minutes at room temperature. The reaction was stopped by the addition of 50 µL of 3 N NaOH to each well. The plate was then scanned by a Model EL 309 of automated microplate reader (BIO-TEK Instruments, Inc., Winooski, Vermont).

Amino Acid Sequencing

Approximately 200 pmoles of extensin peptides from Ac 44 cotton cell walls were sequenced commercially on a Model 473A amino acids sequencer (Applied Biosystems, Inc.) by Dr. Ken Jackson, the Molecular Biology Resource Facility, Saint Francis Hospital of Tulsa Medical Research Institute, Oklahoma City, Oklahoma.

Hydroxyproline Glycoside Profile Determination

Ac 44 cotton cell walls (50 mg) were placed in screw-caped glass vials fitted with Teflon-lined lids. Three microliters of 0.2 M barium hydroxide solution were added into each sample, and the vials well sealed. The alkaline hydrolysis was carried out in a heating

block at 105°C for 18 hours. After cooling, concentrated sulfuric acid (8 μL for each 1 mL barium hydroxide) was added into each vial to adjust the solution to pH 7-8. The samples were centrifuged 15 minutes at 10,000g and the supernatant were freeze dried. The dry samples were redissolved in water (200 μL) and analyzed by Dr. Marcia Kieliszewski, Michigan State University. The method was described by Lamport and Miller (1971). Using the same procedure, the fraction A and B of Extensin-RG I complexes from sequential treatments were analyzed for their hydroxyproline-glycoside profile.

Light Microscopy

Dry cotton cell walls and their residues were suspended in water, and were viewed using a Nikon (UFX-II) light microscope.

Molecular Modeling Analysis

Energy-minimized computer model of peptide PI was generated using the CAChe worksystem (Augmented/MM 2, Mol. Mech.) on an Enhanced Macintosh 2FX computer by Dr. David. Bolton, Computer Aided Chemistry Group (CAChe) Scientific, Tektronix Inc., 393 Inverness Dr. So., Englewood, CO.

CHAPTER IV

RESULTS AND DISCUSSION

Characterization of Cotton Cell Wall Extensin

Solubilization of Deglycosylated Extensin Fragments from Cotton Cell Walls

A preliminary compositional analysis of cotton cell walls provides information on the amount and the nature of the polymers such as cellulose, hemicellulose, pectin, and structural protein contained in the walls. Based on the compositional analysis, cotton cell wall contains about 39 weight percent non-cellulosic sugars and 7.1 weight percent proteins. The gas chromatogram of the trimethylsilyl derivatives of the methyl glycosides of standard sugars is shown in Figure 2. The HPLC profile of the standard amino acid derivatives is shown in Figure 3.

To solubilize most of the extensin fragments, the cell walls were treated with anhydrous hydrogen fluoride (HF) at 0°C to remove more than 95% of the sugars followed by trypsin digestion. As shown in Figure 4, one gram of Acala 44 cotton suspension cultured cell walls was treated with 20 mL of liquid HF at 0°C for 30 min. This treatment breaks all of the glycosidic linkages present except for those of amino sugars (which are in very low concentration), and some of the non-methyl esterified homogalacturonans. The peptide bond is stable under this condition (Mort and Lamport, 1977). The mole percent and weight percent of the sugars and Hyp in the extract and residue resulting from the HF treatment are shown in Table 1. After HF solvolysis, the insoluble residue from cotton suspension cultures contains more then 98% of the Hyp and 55% of the galacturonic

Figure 2. Gas Chromatogram of the Trimethyl Silyl Derivatives of the Methyl Glycosides of Standard Sugars.

Peaks at retention time (minute) 15.23, 15.60 and 16.84 were identified as Ara; 16.44 and 16.92 as Rha; 16.57, 17.44 and 18.30 as Fuc; 19.24 and 20.09 as Xyl; 23.17, 24.59, 27.80 and 28.25 as GalA; 25.29 and 26.70 as Man; 25.38, 26.98 and 28.47 as Gal; 29.65 and 30.69 as Glc; 22.59, 29.83 and 30.28 as GlcA; 21.35 as reagent; 39.30 as inositol, the internal standard.

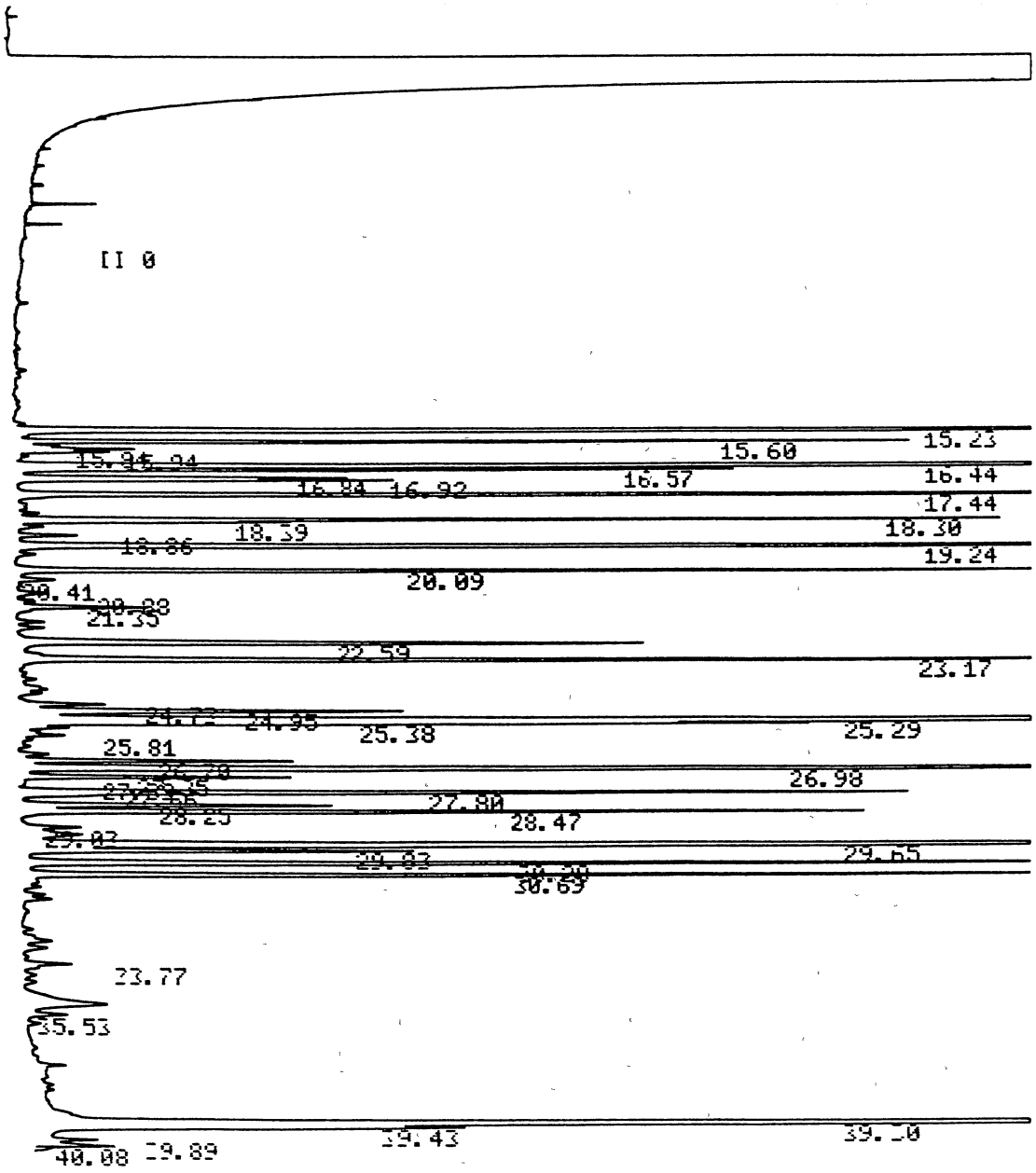
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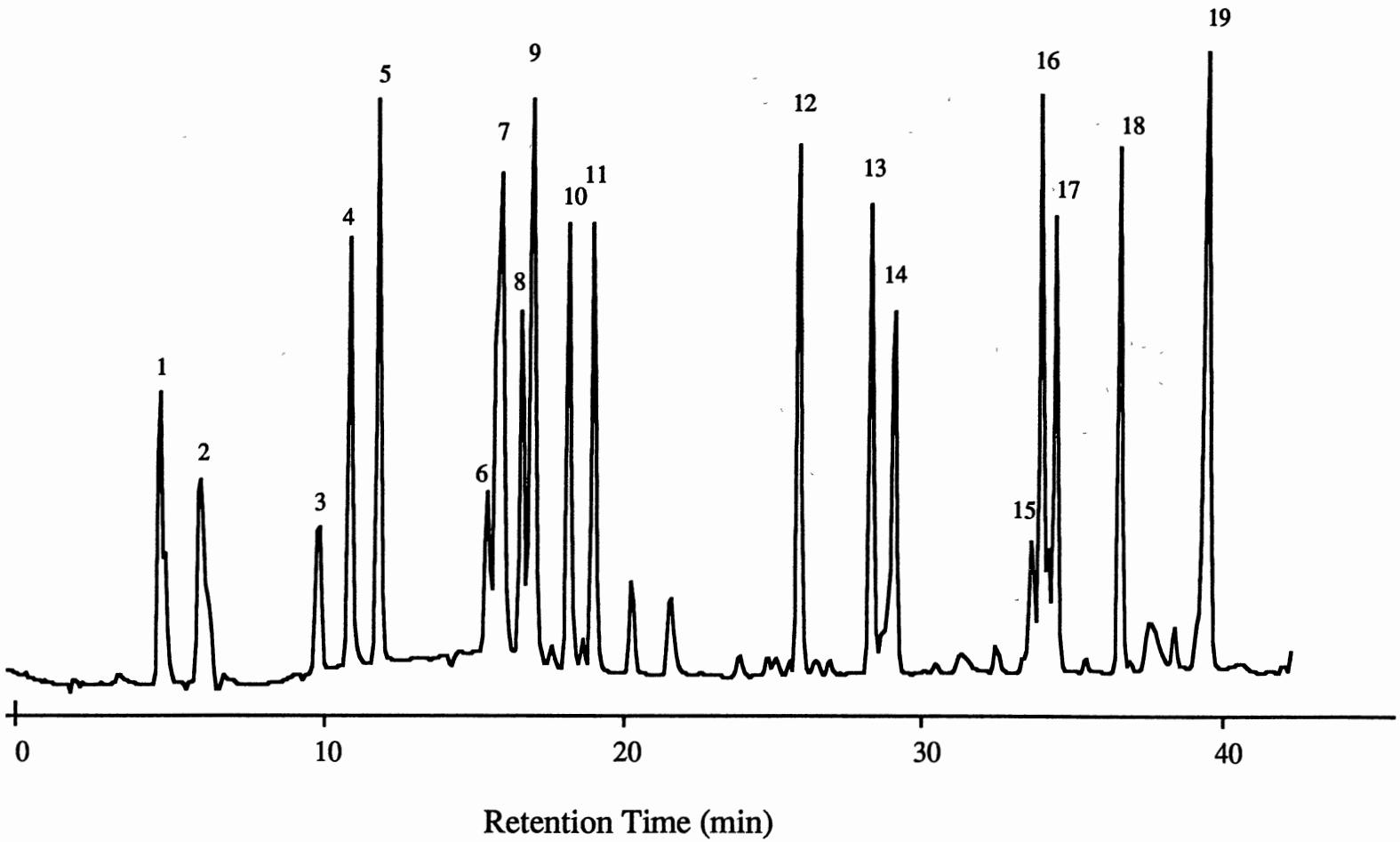
Figure 3. Reverse Phase HPLC Trace of the Standard Amino Acid Derivatives.

625 pmoles of standard amino acid (312.5 pmoles of Hyp and Cys standards) phenylthiocarbamyl (PTC) derivatives were injected on to a C_{18} column and eluted with a gradient of 5-15% buffer B in one minute, 15-30% B in 10 minutes, 30-50% B in 10 minutes, 50% B for 10 minutes and 50-100% in one minutes and 100% B for 10 minutes, B was 0.1 M ammonium acetate (pH 6.0) in acetonitrile/methanol/water (44:10:46). Buffer A was 0.05 M ammonium acetate. Flow rate was 1 mL/min, detection was by UV absorbance at 254 nm, sensitivity was 0.1. Peaks were identified as follows: 1, Asx; 2, Glx; 3, Hyp; 4, Ser; 5, Gly; 6, His; 7, Reagent; 8, Thr; 9, Arg; 10, Ala; 11, Pro; 12, Tyr; 13, Val; 14, Met; 15, Cys; 16, Ile; 17, Leu; 18, Phe; 19, Lys.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Retention Time (min)

Figure 4. Flow Chart of Solubilization of Fragments Containing Hyp-Rich Extensin Peptides from Cotton Suspension Cultures.

Cotton Cell Wall was solvolyzed with liquid HF at 0°C for 30 minutes followed by ether and water extractions to remove most of the sugars. After extraction with 50 mM ammonium acetate buffer overnight at room temperature to remove some homogalacturonan fragments, the resulting residue was digested with trypsin overnight at room temperature. Eighty percent of hyp was solubilized. The determination of sample content (%) was based on a total weight percent of the dry cell wall. The value given in parentheses represents the weight of each fraction recovered after lyophilization and the percentage of total cell walls.

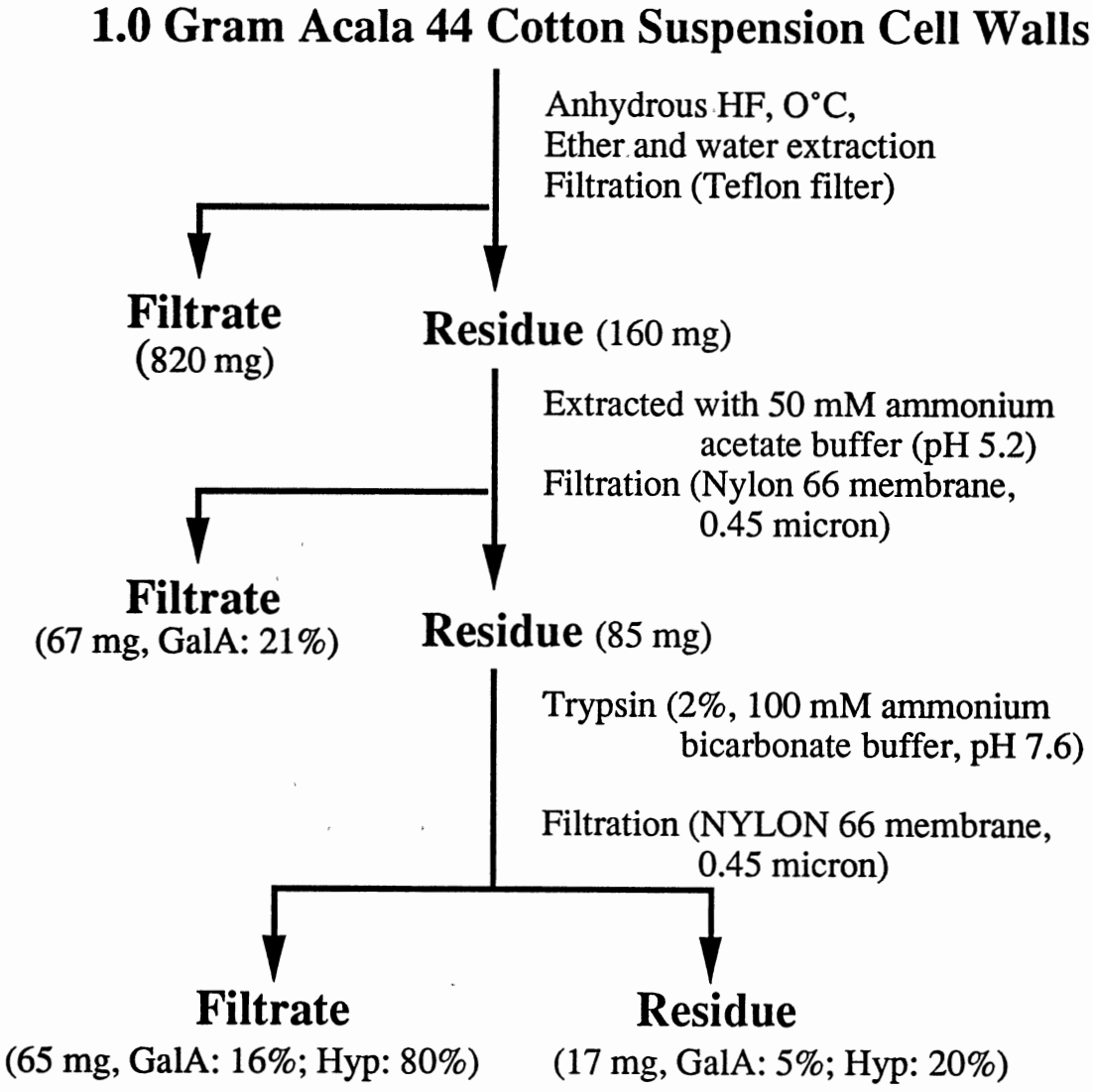

1.0 Gram Acala 44 Cotton Suspension Cell Walls
Anhydrous HF, O°C,
Ether and water extraction
Filtration (Teflon filter)
Filtrate
(820 mg)
Residue (160 mg)
Extracted with 50 mM ammonium acetate buffer (pH 5.2)
Filtration (Nylon 66 membrane, 0.45 micron)
Filtrate
(67 mg, GalA: 21%)
Residue (85 mg)
Trypsin (2%, 100 mM ammonium bicarbonate buffer, pH 7.6)
Filtration (NYLON 66 membrane, 0.45 micron)
Filtrate
(65 mg, GalA: 16%; Hyp: 80%)
Residue
(17 mg, GalA: 5%; Hyp: 20%)

TABLE 1

MOLE PERCENT OF SUGARS IN COTTON SUSPENSION CULTURE CELL WALLS AND IN THE SOLUBLE AND THE INSOLUBLE PORTIONS OF THE WALLS AFTER HF SOLVOLYSIS AT 0°C FOLLOWED BY AMMONIUM ACETATE BUFFER EXTRACTION AND TRYPSIN DIGESTION

Material	Ara	Rha	Fuc	Xyl	GalA	Gal	Glc	wt%*	wt (mg)
Intact Walls (Acala 44 Cotton)	17.7	12.4	1.9	17.8	33.0	10.3	6.5	39.0	1,000
Ether Extract (HF, 0°C)	10.3	5.6	2.0	17.5	4.7	6.8	52.7	—	—
Water Extract (HF, 0°C)	4.6	3.8	—	7.4	17.6	6.8	59.3	64.0	320
Residue (HF, 0°C)	0.9	0.6	—	1.8	91.8	—	4.9	18.9	160
Filtrate (NH_4Ac Buffer)	0.5	0.4	—	1.0	95.3	—	2.7	22.7	67
Residue (NH_4Ac Buffer)	11.0	—	—	10.5	41.5	—	36.9	7.8	85
Filtrate (Trypsin)	2.2	1.4	—	5.3	75.5	—	15.6	6.2	65
Residue (Trypsin)	5.1	1.8	—	11.3	53.0	—	28.9	5.4	17

* wt% (weight percent sugar) was calculated from the weight of sugar detected by GLC for known weight of sample.

acid (GalA) present in the initial sample. Removal of some of the remaining polygalacturonic acid by extraction with 50 mM ammonium acetate buffer (pH 5.2) overnight at room temperature left a residue containing about 20% of the original GalA. However, cotton extensins were still insolubilized in the buffer extracted-residue. Eighty five milligrams of this residue were digested with trypsin in 100 mM ammonium bicarbonate buffer (pH 7.6) overnight. This treatment allowed solubilization of 80 % of the Hyp content from the original sample.

The trypsin treated filtrate, accounting for about 6.5% by weight of the starting material, contains predominantly proteins, a small amount of GalA (16% of that in the initial sample), and a small amount of unidentified constituents (probably phenolic compounds). These galacturonic acids which are present as homogalacturonan (HG) oligomers may ionicly interact with extensin proteins (Fry, 1986), therefore, be co-solubilized by trypsinization. Five percent of the GalA remained insoluble in the trypsin-treated residue along with 20% of the Hyp in the original sample. A question may be asked as to what is keeping the remaining Hyp in the cell wall insoluble, since the amino acid composition of the residue (see Table 2) showed that the proportion of Hyp in terms of amino acids in the insoluble residue is lower than that in the original walls. Perhaps, Hyp is high in extensin which keeps it from being too extensively crosslinked. The Hyp-containing proteins remaining in the residue were not liberated with trypsin, indicating that there may be direct covalent cross-links between extensin molecules or extensin and phenolic compounds.

Analysis of Tryptic Peptides

After filtration through a NYLON 66 (0.45 micron) membrane using a concentrator under nitrogen pressure, the soluble material containing extensin peptides from the trypsin treatment was fractionated on a reverse phase HPLC, giving rise to two major fractions (I and II) and many minor ones (see Figure 5). From the amino acid composition analysis, which is shown in Table 3, both of the major fractions are rich in Hyp. Fraction I contains

TABLE 2

MOLE PERCENT AND TOTAL WEIGHT PERCENT OF AMINO ACID RESIDUES IN COTTON SUSPENSION CULTURE CELL WALLS AND IN THE SOLUBLE AND THE INSOLUBLE PORTIONS OF THE WALLS DIGESTED WITH TRYPSIN AFTER HF SOLVOLYSISAT 0°C AND AMMONIUM ACETATE BUFFER EXTRACTION

Amino	Acala 44		Trypsin Digestion			
Acid	Intact Walls		Filtrate Fraction		Residue Fraction	
Residue	Mole %	wt %	Mole %	wt%	Mole %	wt%
Asx	6.9	0.5	5.9	1.6	2.2	1.6
Glx	6.2	0.5	5.6	1.7	2.0	1.6
Hyp	7.8	0.5	22.3	5.9	8.3	5.8
Ser	8.5	0.5	10.9	2.2	7.3	3.9
Gly	7.3	0.3	9.7	1.3	11.9	4.2
His	1.9	0.2	—	—	—	—
Thr	7.8	0.8	3.9	0.9	7.8	7.5
Arg	5.3	0.3	0.9	0.3	3.4	2.1
Ala	5.6	0.3	1.6	0.3	3.8	1.7
Pro	7.6	0.5	7.9	1.8	10.3	6.2
Tyr	3.9	0.4	1.3	0.5	2.7	2.7
Val	7.6	0.5	7.1	1.6	9.3	5.7
Met	5.3	0.4	—	—	—	—
Ile	3.3	0.2	4.4	1.2	8.8	6.2
Leu	7.3	0.5	8.4	2.2	11.4	8.0
Phe	3.7	0.4	3.5	1.2	5.3	4.8
Lys	4.1	0.3	6.5	1.9	5.5	4.3
Total	100.0	7.1	100.0	24.6	100.0	66.3

Figure 5. The HPLC Chromatogram of the Filtrate Solubilized by Trypsin Digestion in Figure 4 on Reverse Phase C_{18} Column.

The elution buffer consisted of solvent A; 0.1% trifluoroacetic acid (pH ~2), solvent B; 0.1% trifluoroacetic acid/acetonitrile (37.5:62.5). The gradient composition was 0-30% solvent B in 60 minutes, 30-100% B in 5 minutes and 100% B for 10 minutes. The flow rate was 1 mL/min. Detection was by UV monitor at 214 nm. The sample was dissolved in 0.1% trifluoroacetic acid and injected. Tubes 18-20 were pooled in to fraction I, and 33-35 in fraction II.

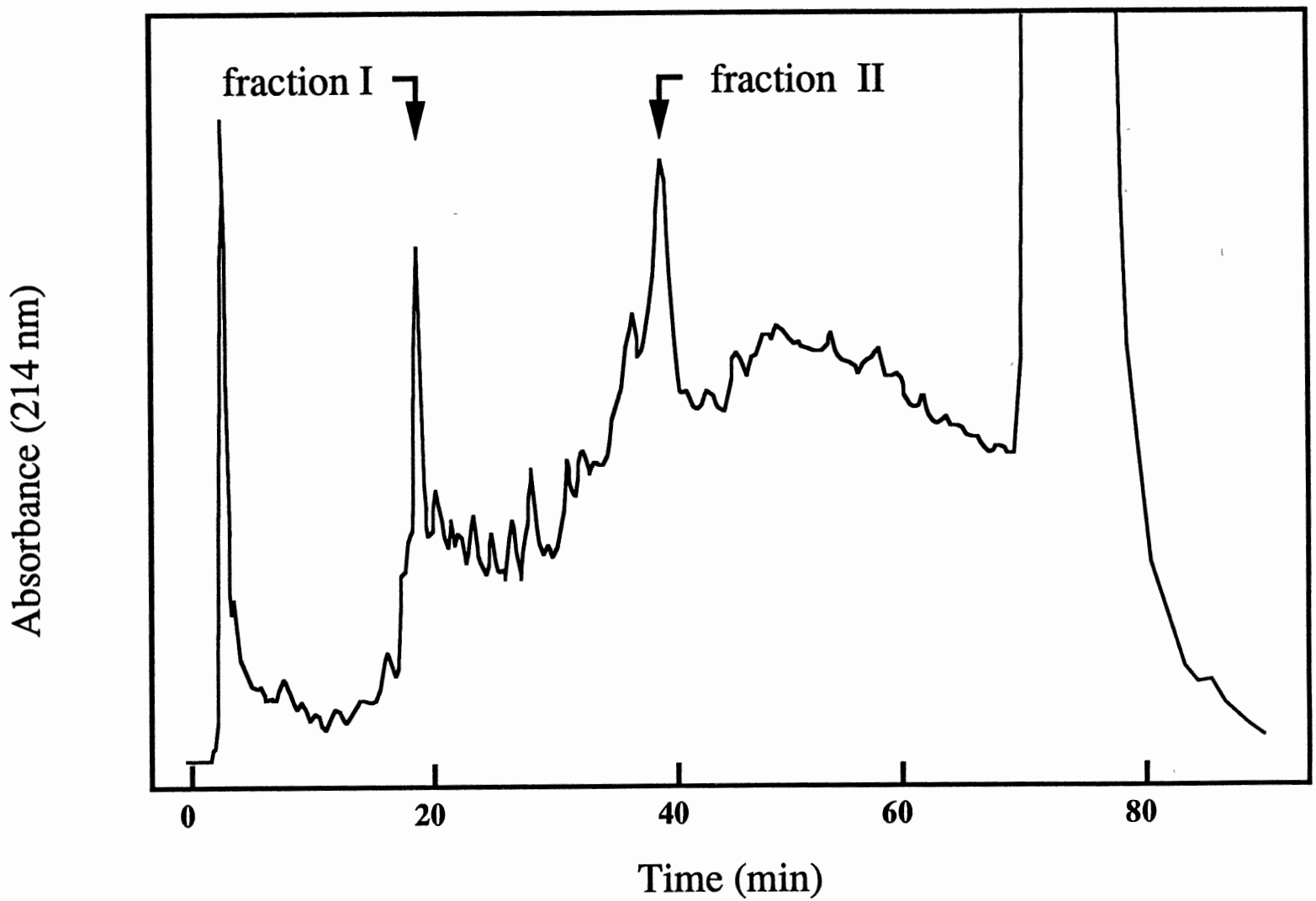

fraction I
fraction II
Absorbance (214 nm)
0
20
40
60
80
Time (min)

TABLE 3

MOLE PERCENT OF AMINO ACIDS IN THE FRACTIONS OF RP C18 AND HW 40(S) HPLC CHROMATOGRAPHY FROM FILTRATE OF COTTON SUSPENSION CULTURE CELL WALLS DIGESTED WITH TRYPSIN AFTER HF SOLVOLYSIS AT 0°C AND AMMONIUM ACETATE BUFFER EXTRACTION

Amino Acid Residue	RP C18 HPLC					HW 40 HPLC	
	Fractions (#3-17)	Fraction I(#18,19)	Fractions (#20-37)	Fraction II(#38,39)	Fractions (#40-75)	Peak 1	Peak 2
Asx	2.8	—	3.7	—	5.9	—	—
Glx	3.9	—	4.1	—	5.3	—	—
Hyp	1.9	25.0	15.9	34.4	7.7	58.6	35.5
Ser	11.9	10.7	9.2	10.8	8.4	14.8	19.2
Gly	20.2	11.5	9.0	6.0	9.1	5.0	7.6
His	—	3.9	—	6.6	1.5	—	—
Thr	11.4	3.1	5.3	2.2	11.1	3.7	5.5
Arg	6.3	1.8	3.8	1.6	4.7	—	—
Ala	36.1	8.1	7.5	3.1	2.1	6.7	5.3
Pro	5.6	4.0	7.4	4.1	7.3	3.4	3.4
Tyr	—	2.1	2.8	3.7	2.8	—	—
Val	—	6.8	7.0	4.8	8.5	—	15.9
Met	—	—	—	—	1.2	—	—
Ile	—	7.2	4.1	6.4	5.4	—	—
Leu	—	7.9	8.8	3.9	10.6	—	—
Phe	—	3.3	3.1	6.3	4.9	—	—
Lys	—	4.6	8.2	6.0	3.5	7.8	7.6
Hyp%*	2	23	13	19	18	18	5

* Hyp% was presented as weight percent of total hyp in original cell walls.

23% of the Hyp present in initial sample, and fraction II contains 19% of the Hyp. This may imply that the cell wall contains only one or a few extensins composed of many repetitive Hyp rich sequences, and an array of minor Hyp poor or non-hydroxyproline-containing proteins. Fraction I and II were further fractionated by gel filtration chromatography on a Fracto-Gel HW40(S) column. The peptides contained in fraction I were purified by the gel filtration chromatography which is shown in Figure 6. These peptides account for about 23% of the Hyp in the cell wall and must, therefore, be repeated several times within the cotton extensins.

These peptides were sequenced and found to be quite similar to peptides already isolated from extensins in other plant species. In Figure 6, peak 1 contains a peptide 1 (P1) which is an undecamer with the sequence: Ser-Hyp-Hyp-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Hyp-Lys. Peak 2 contains two heptapeptides named peptide 2a (P2a) and peptide 2b (P2b). P2a was sequenced as: Ser-Hyp-Hyp-Hyp-Hyp-Val-Lys; and P2b sequence as: Ser-Hyp-Hyp-Ser-Ala-Hyp-Lys. P1 contains about 18% of the total Hyp in the original wall. Interestingly, the most abundant peptide in this fraction contained the sequence $Ser(Hyp)_6$, which is two sequential hydroxyproline residues longer than the most commonly encounted $Ser(Hyp)_4$ peptide in extensins of the other plants. From the amino acid composition analysis of peak 1 in Table 3, the molar ratio of Hyp:Ser:Lys is 7.5:1.9:1.0 which is close to the ratio found in the sequence of peptide 1.

It has been suggested that extensin molecules are mostly in a polyproline type II (ppII) *trans* helix conformation (Van Holst and Varner, 1984), but also contain some non-helical sections which could allow the glycoprotein to be bent (Wilson and Fry, 1986; Stafstrom and Staehelin, 1986). Figure 7 shows a model of peptide P1 with a helical form. Perhaps, the $Ser(Hyp)_6$ repeat sequence found in cotton extensin has more ppII helical form than other plant species. In addition, two more Hyp residue in a repeat sequence provide extra potential glycosylation sites for arabinose side chain. The carbohydrate moieties stabilize the glycoprotein conformation (Stafstrom and Staehelin, 1986).

Figure 6. Chromatography on an HW 40(S) Gel Filtration Column of Fraction I of Figure 5.

Sample was applied in and eluted with 0.1% trifluoroacetic acid. The flow rate was 0.5 mL/fraction/min. Detection was by UV monitor at 215 nm. Fractions 29-34 were pooled into peak 1 (P1), and fractions 36-39 were pooled into peak 2 (P2).

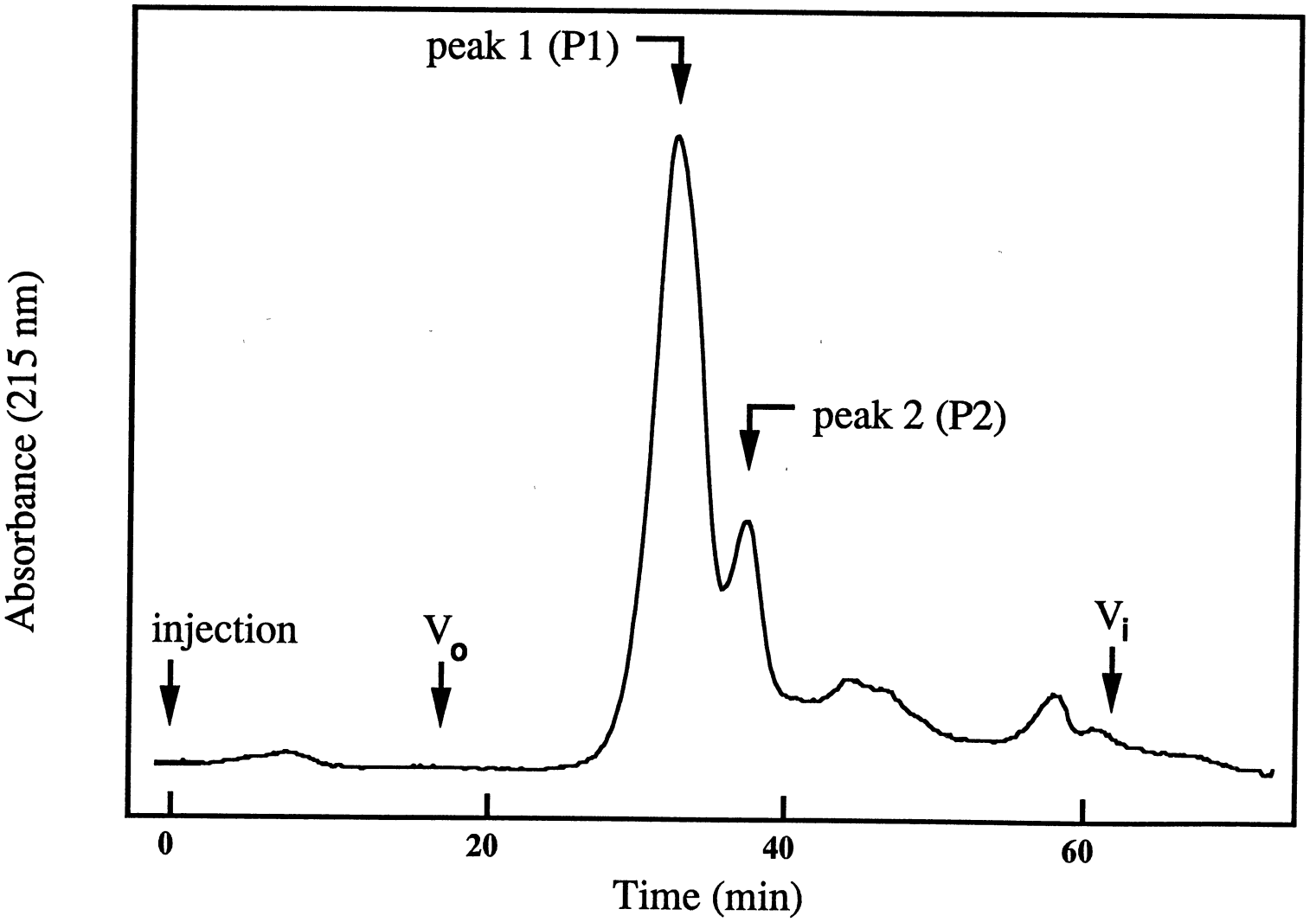
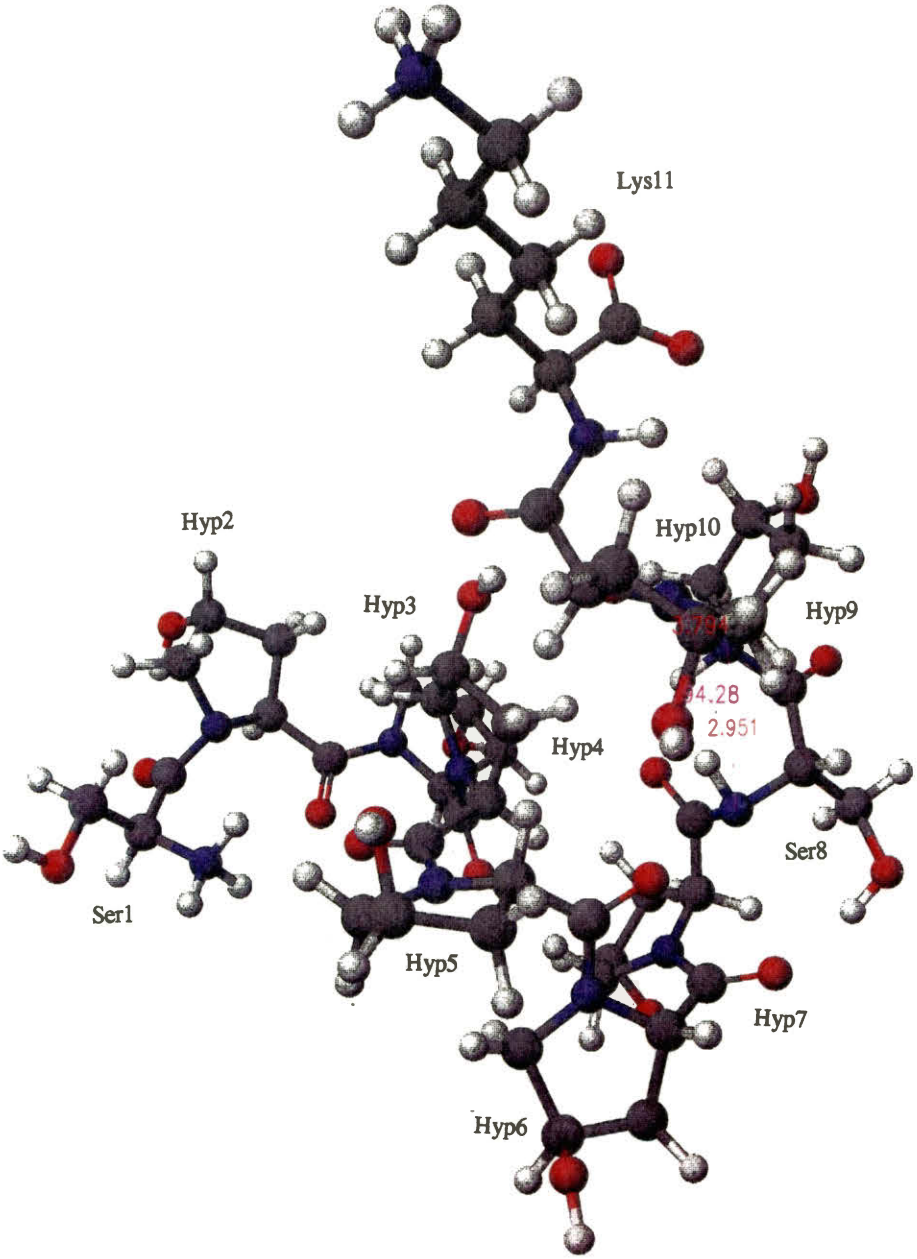

peak 1 (P1)
peak 2 (P2)
injection
V_o
V_i
Absorbance (215 nm)
0
20
40
60
Time (min)

Figure 7. Energy-Minimized Computer Model of a Polyproline-Like Helical Form of Peptide P1.

The sequence shown here is Ser-Hyp-Hyp-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Hyp-Lys. The model was generated using the CAChe Worksystem (Augmented/MM2 Mol. Mech.) from Tektronix Inc. by Dr. David Bolton.


Lys11
Hyp2
Hyp10
Hyp3
Hyp9
4.28
2.951
Hyp4
Ser8
Ser1
Hyp5
Hyp7
Hyp6

The second major fraction (fraction II) from the reverse phase column was intractable. Its amino acid composition was similar to that of the fraction I except that it contained more tyrosine, phenylalanine and histidine (see Table 3). Upon chromatography on the Fracto-Gel HW40(S) gel filtration column, several broad peaks of 280 nm absorption were obtained (see Figure 8). An attempt to sequence fraction II indicated a broad range of N-termini. Alternatively, the fractionation of fraction II on an HW40(S) gel filtration column with 0.1 % trifluoroacetic acid (pH 1) was monitored by a diode-array detector instead of a regular UV detector. A maximum of UV absorbance at 273 nm was observed for several fractions (see Figure 9). The UV spectrum of fraction II which was not observed in fraction I may be related to the specific UV spectrum of isodityrosine (a intermolecular crosslinkage within extensin), or other unidentified cross-liking structures. The UV spectra of isodityrosine was reported by Epstein and Lamport (1984) and is shown in Figure 10, (A). The UV spectra of fraction II in 0.1 M HCl (pH 1.1) and 0.1 M NaOH (pH 13) are shown in Figure 10, (B) and (C). The spectral (λ_{max}) shift of fraction II in acid and base from 273 nm to 297 nm are similar to that of isodityrosine. The spectral (λ_{max}) shift of tyrosine in 0.1 M HCl (pH 1.1) and 0.1 M NaOH (pH 13) is from 275 nm to 293 nm. (Mihalyi, 1968).

From the results described above, it appears that all cell wall proteins (whether or not they contain Hyp) are crosslinked into a network after 0°C HF solvolysis. However, some of the network may be an artifact of the HF treatment (Mort, 1978).

N.m.r. Analyses of Fractions I and II

It is known that extensins of primary cell walls are rich in *trans* 4-L-hydroxyproline (Lamport, 1967). Therefore, poly-L-hydroxyproline (Sigma) is used as standard sample to assign the proton peaks of the *trans* 4-L-hydroxyproline residue in the peptide by ^{1}H-^{1}H homonuclear (COSY) n.m.r. spectroscopy. The spectrum is shown in Figure 11. The signal at 4.85 p.p.m. represents the α-H of Hyp ; the other signals are γ-H of Hyp at 4.65

Figure 8. Chromatography on an HW 40(S) Gel Filtration Column of Fraction II of Figure 5.

Sample was applied in and eluted with 0.1% trifluoroacetic acid. The flow rate was 0.5 mL/fraction/min. Detection was by UV monitor at 215 nm. Fractions 27 to 60 were scanned in the wavelength range between 200 nm and 600 nm using a HP 1040A photodiode array HPLC detector.

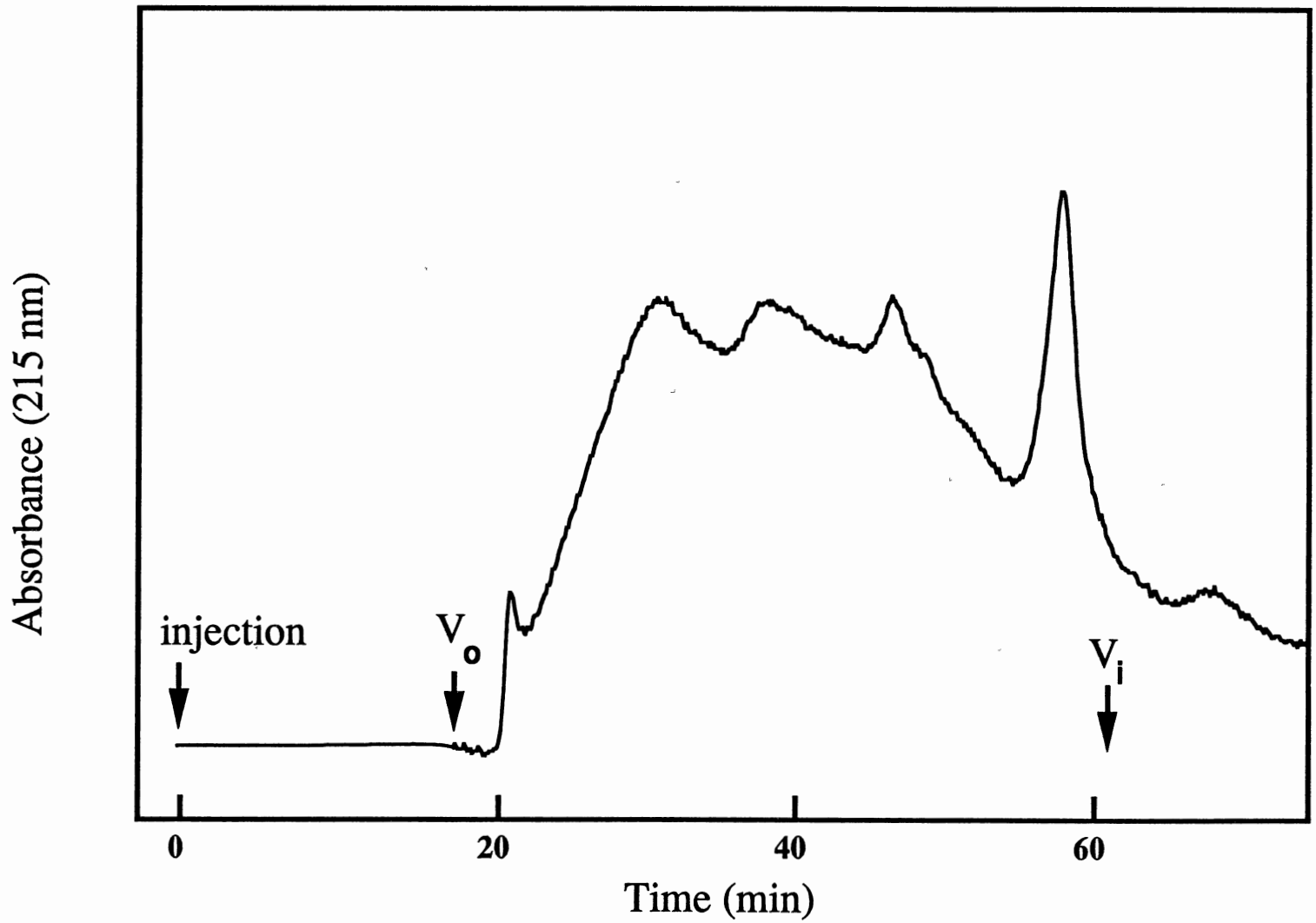

Absorbance (215 nm)
injection
V_o
V_i
0
20
40
60
Time (min)

Figure 9. Scanning Spectra of HW 40(S) Gel Filtration Fractions 27, 29, 31, and 33 of Fraction II.

The spectra were recorded on a HP 1040A photodiode array HPLC detector. The scanning wavelength range was from 200 nm to 600 nm. The maximum absorbances of fraction 27, 29 are 220 nm and 206 nm, respectively. The maximum absorbance of fraction 31 is same as fraction 33 at 204 nm.

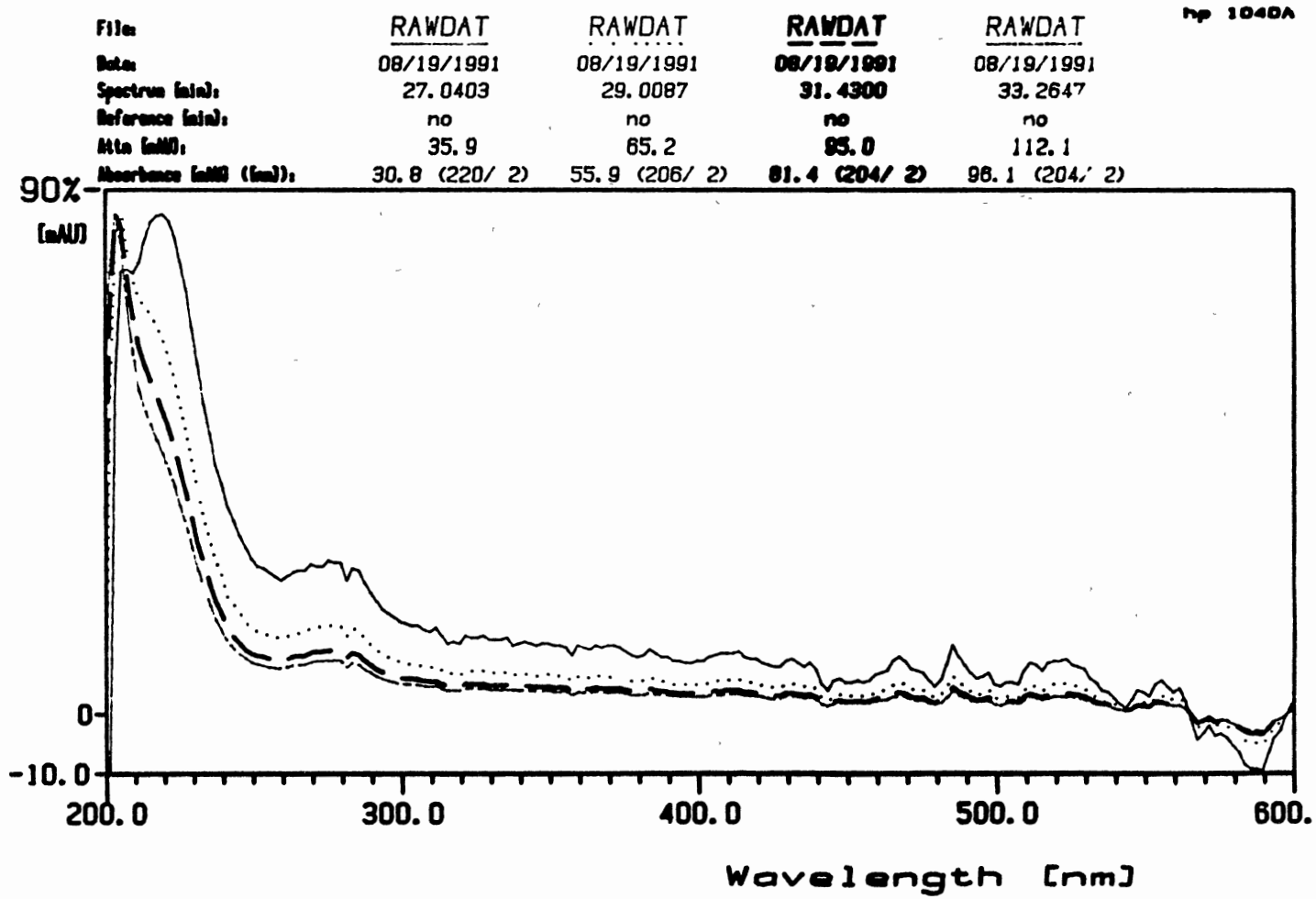
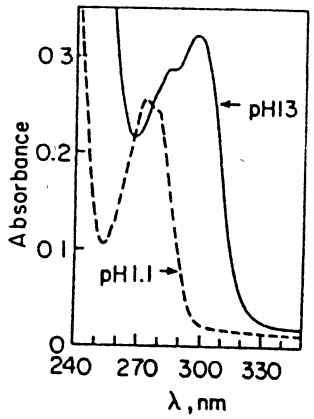

hp 1040A
File: RAWDAT RAWDAT RAWDAT RAWDAT
Date: 08/19/1991 08/19/1991 08/19/1991 08/19/1991
Spectrum (min): 27.0403 29.0087 31.4300 33.2647
Reference (min): no no no no
Min (mAU): 35.9 65.2 85.0 112.1
Absorbance (mAU) (nm): 30.8 (220/ 2) 55.9 (206/ 2) 81.4 (204/ 2) 98.1 (204/ 2)
90%
[mAU]
0
-10.0
200.0
300.0
400.0
500.0
600.
Wavelength [nm]

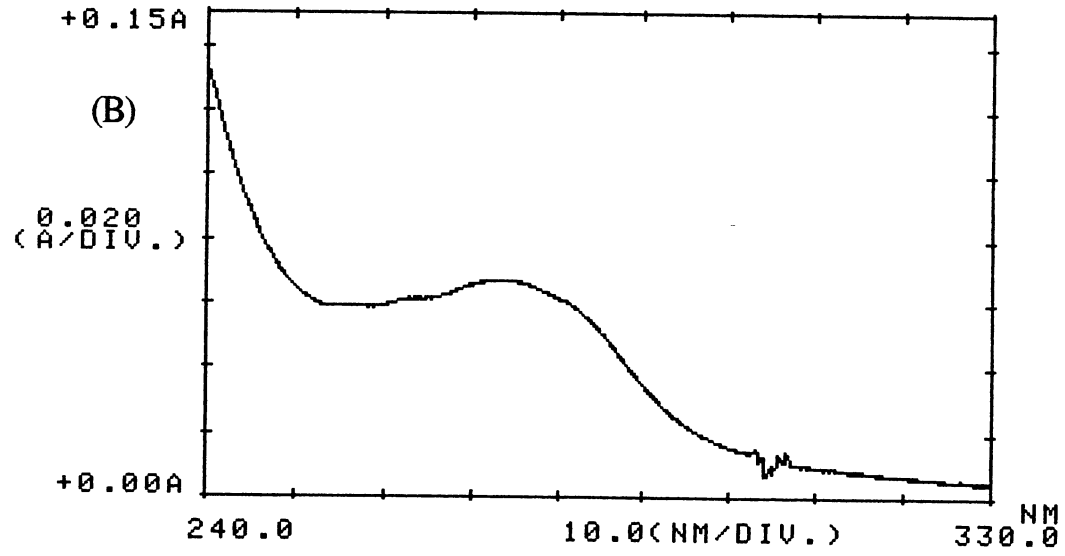
Figure 10. The UV Spectra of Isodityrosine and Fraction II in 0.1 M HCl and 0.1 M NaOH.

(A) The UV spectra of isodityrosine in 0.1 M HCl (pH 1.1) and 0.1 M NaOH (pH 13) (Epstein and Lamport, 1984). (B) The spectrum of fraction II in 0.1 M HCl (pH 1.1). (C) The spectrum of fraction II in 0.1 M NaOH (pH 13).

(A)


0.3
0.2
0.1
0
Absorbance
pH13
pH1.1
240 270 300 330
λ, nm

(B)


+0.15A
0.020
(A/DIV.)
+0.00A
240.0
10.0(NM/DIV.)
330.0
NM

(C)

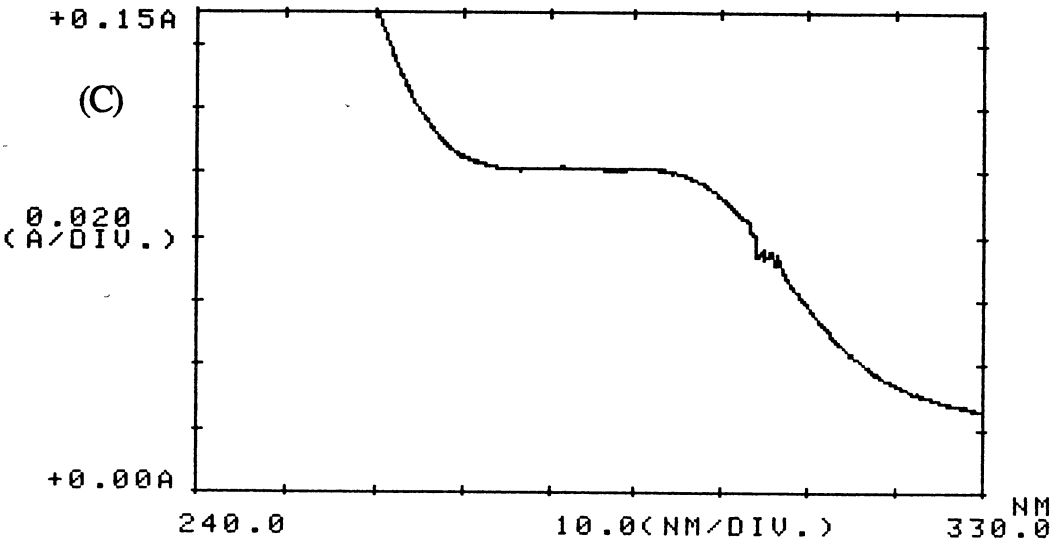
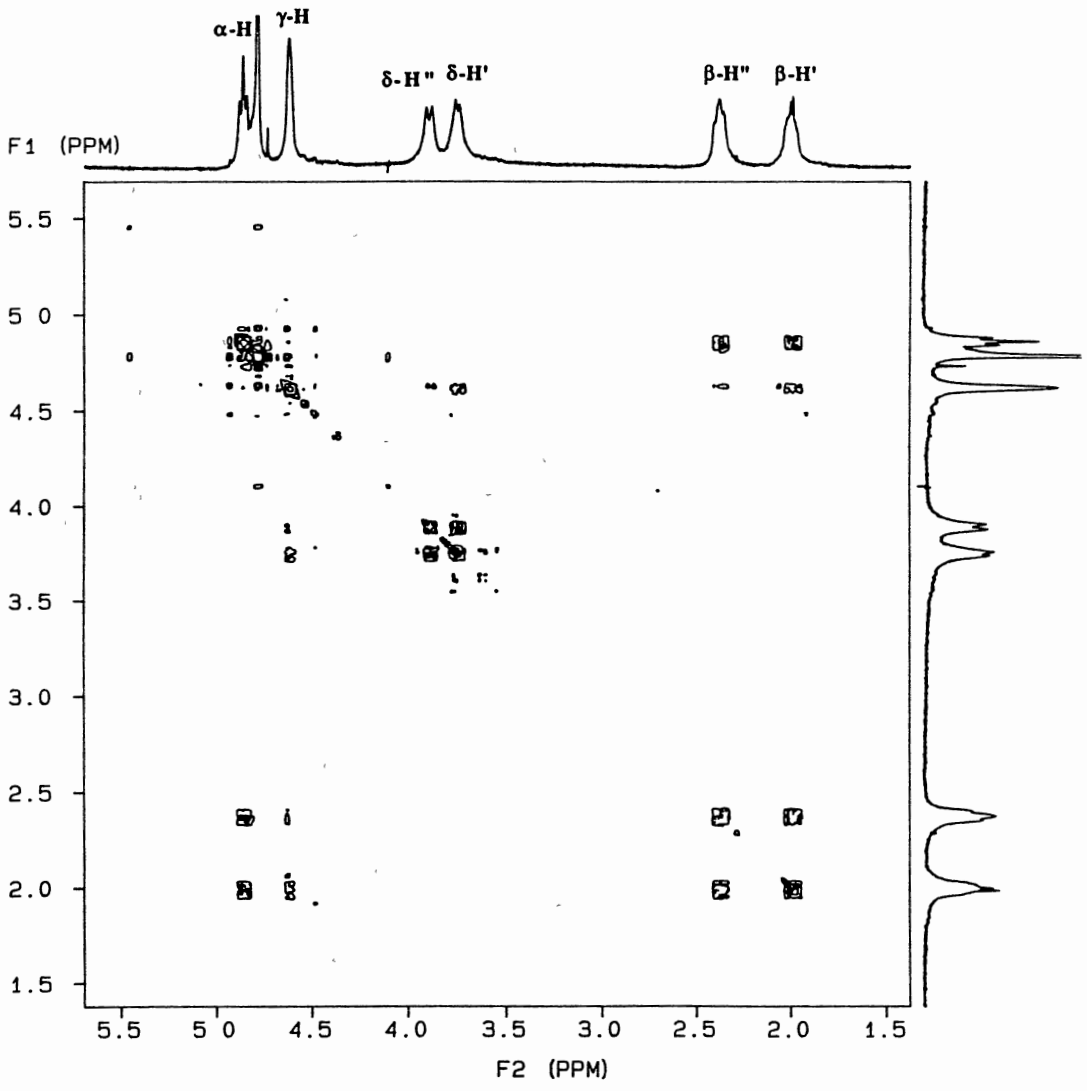
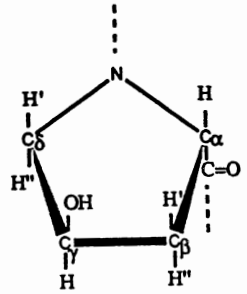

+0.15A
0.020
(A/DIV.)
+0.00A
240.0
10.0(NM/DIV.)
330.0
NM

Figure 11. ^{1}H-^{1}H Homonuclear (COSY) n.m.r. Spectrum of Poly-L-Hydroxyproline.

The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are assigned as follows: α-H of Hyp at 4.85 p.p.m.; γ-H of Hyp at 4.65 p.p.m.; δ-H" of Hyp at 3.9 p.p.m.; δ-H' of Hyp at 3.75 p.p.m.; β-H" of Hyp at 2.4 p.p.m.; β-H' of Hyp at 2.0 p.p.m.





trans 4-L- hydroxyproline residue in peptide

p.p.m.; δ-H" of Hyp at 3.9 p.p.m.; δ-H' of Hyp at 3.75 p.p.m.; β-H" of Hyp at 2.4 p.p.m.; β-H' of Hyp at 2.0 p.p.m. The ^{1}H n.m.r. spectrum of fraction I is shown in Figure 12. The ^{1}H n.m.r. spectrum and ^{1}H-^{1}H n.m.r. spectrum of fraction II are shown in Figure 13 and 14. These experiments were done to obtain additional evidence of the compositions of fractions I and II. The presence of Hyp, Ser, Lys, Ala, Val, Gly, Ile, Leu, and Tyr would readily be apparent in the ^{1}H n.m.r. spectra (Figures 12 and 13). This determination is quite generally useful for the detection of certain specific amino acid residues rather than for a complete analysis of the amino acid composition (Wuthrich, 1976). By comparing the ^{1}H chemical shifts of the Hyp residue assigned in poly-L-hydroxyproline of Figure 11 and the ^{1}H chemical shifts of other amino acid residues in peptides reported by Jardetzky and Roberts (1981), most of the protons in the major amino acid residues found in fraction I and II from the amino acid analysis were identified as in Figure 12, 13 and 14. In addition, the Hyp residues present in cotton extensin are in the *trans* 4-L-hydroxyproline form since they have the same ^{1}H chemical shifts as those Hyp residues in poly-L-hydroxyproline (compare Figure 11 to Figure 13). Several peaks, however, were not assigned because of the lack of some information. For example, the unassigned peaks at 6.7-7.4 p.p.m. may result from aromatic groups of unidentified components (suspected as cell wall phenolic compounds) in the fraction II (see Figure 13). Moreover, some of the peaks, in both fractions I and II, at 0.8-1.1 ppm would only be assigned as Val, Ile and Leu, but some of them may result from aliphatic compounds (such as cell wall lipids) other than amino acid residues.

Identification of Peptides P1, P2a, and P2b by LSIMS Spectroscopy

The exact amino acid stoichiometry of the peptides P1, P2a and P2b were verified using LSIMS. The calculated molecular weight of P1, P2a and P2b are 1224.6, 784.4 and 730.3. The LSIMS spectra of P1, P2a and P2b are shown in Figure 15. Pseudomolecular ions [M+H$^+$] and [M+Na$^+$] of P1, P2a and P2b were seen at 1225.8, 786.0, 731.8,

Figure 12. ^{1}H-n.m.r. Spectrum of Fraction I.

The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25°C in D_2O. Peaks are assigned as follows: 1) 2,6-H of Tyr; 2) 3,5-H of Tyr; 3) γ-H of Hyp; 4) α-H of Ser; 5) α-H of Lys and α-H of Ala; 6) α-H of Val; 7) δ-H of Hyp, β-H of Ser and α-H of Gly; 8) ε-H of of Lys; 9) β-H of Hyp; 10) β-H of Hyp; 11) β-H of Lys; 12) δ-H of Lys; 13) γ-H of Lys and β-H of Ala; 14) γ-H of Val, δ–H of Ile and Leu.

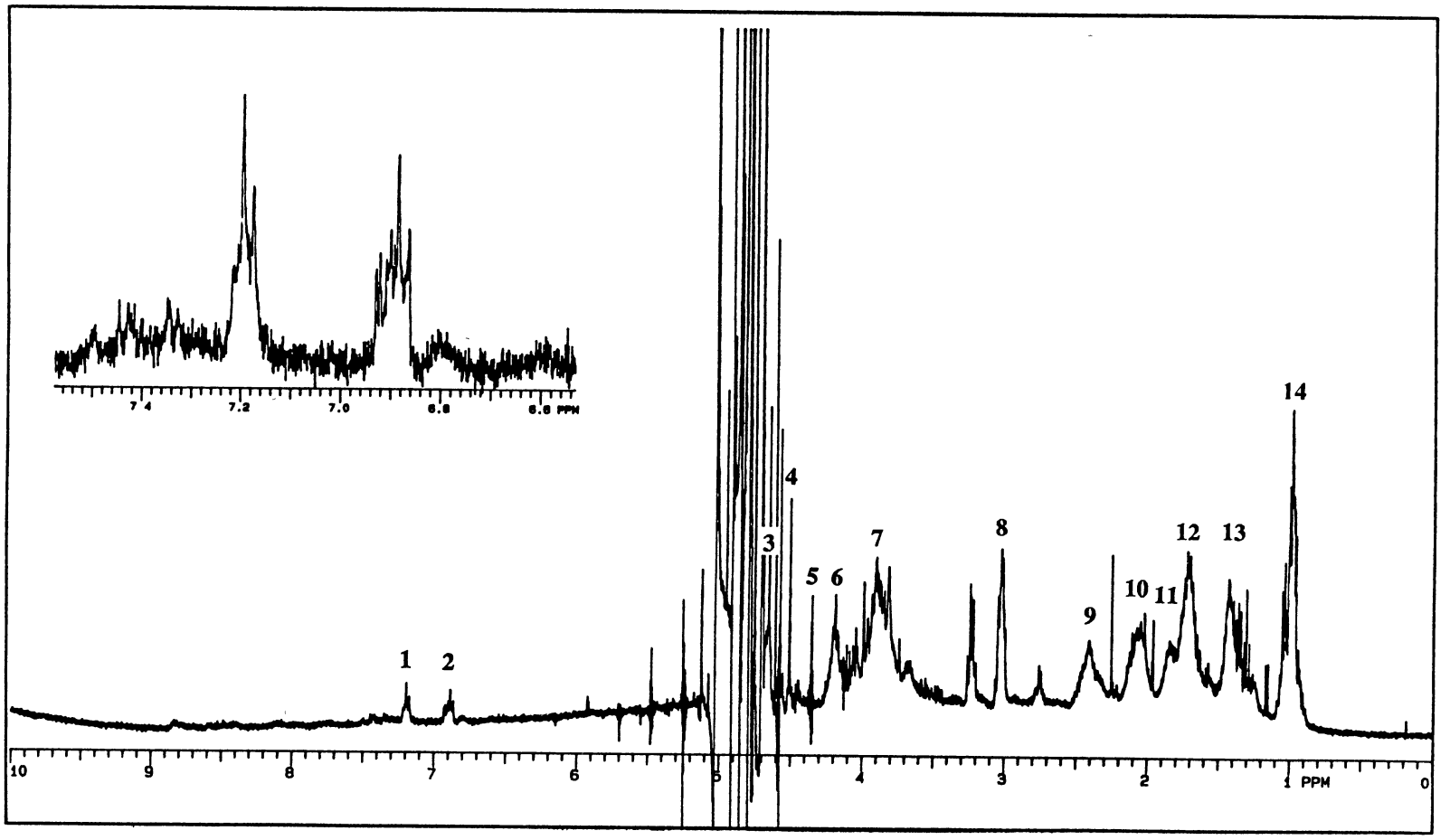

1
2
3
4
5
6
7
8
9
10
11
12
13
14
10
9
8
7
6
5
4
3
2
1 PPM
0
7.4
7.2
7.0
6.8
6.6 PPM

Figure 13. ^{1}H-n.m.r. Spectrum of Fraction II.

The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25°C in D_2O. Peaks are assigned as follows: 1) 2,6-H of Tyr; 2) 3,5-H of Tyr; 3) γ-H of Hyp, α-H of Tyr, α-H of Phe; 4) α-H of Ser; 5) α-H of Val; 6) δ-H of Hyp, β-H of Ser and α-H of Gly; 7) ε-H of of Lys and β-H of Tyr; 8) β-H of Phe; 9) β-H" of Hyp; 10) β-H' of Hyp; 11) β-H and δ-H of Lys; 12) γ-H of Lys; 13) γ-H of Val, δ–H of Ile and Leu.

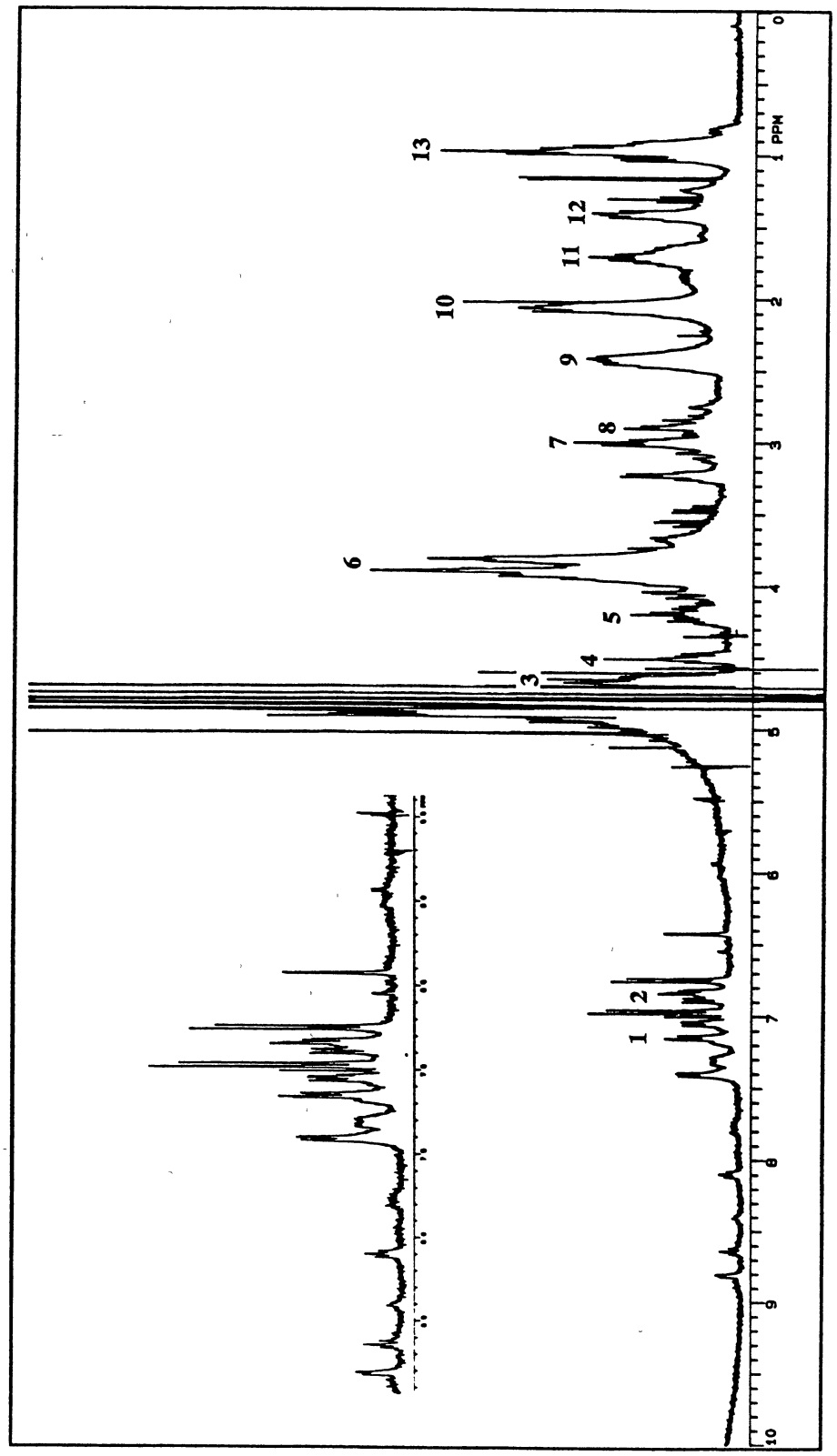

1
2
3
4
5
6
7
8
9
10
11
12
13
PPM
0
1
2
3
4
5
6
7
8
9
10

Figure 14. ^{1}H-^{1}H Homonuclear (COSY) n.m.r. Spectrum of Fraction II.

The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25°C in D_2O using TSP as internal standard at 0.00 p.p.m. Peaks are assigned as follows: 1) 2,6-H of Tyr; 2) 3,5-H of Tyr; 3) α-H of Hyp; 4) γ-H of Hyp; 5) α-H of Tyr; 6) α-H of Lys; 7) α-H of Ser; 8) α-H of Ala; 9) α-H of Val; 10) β-H of Ser; 11) δ-H of Hyp; 12) β-H of Tyr; 13) ε-H of of Lys; 14) β-H of Hyp; 15) β-H of Ala.

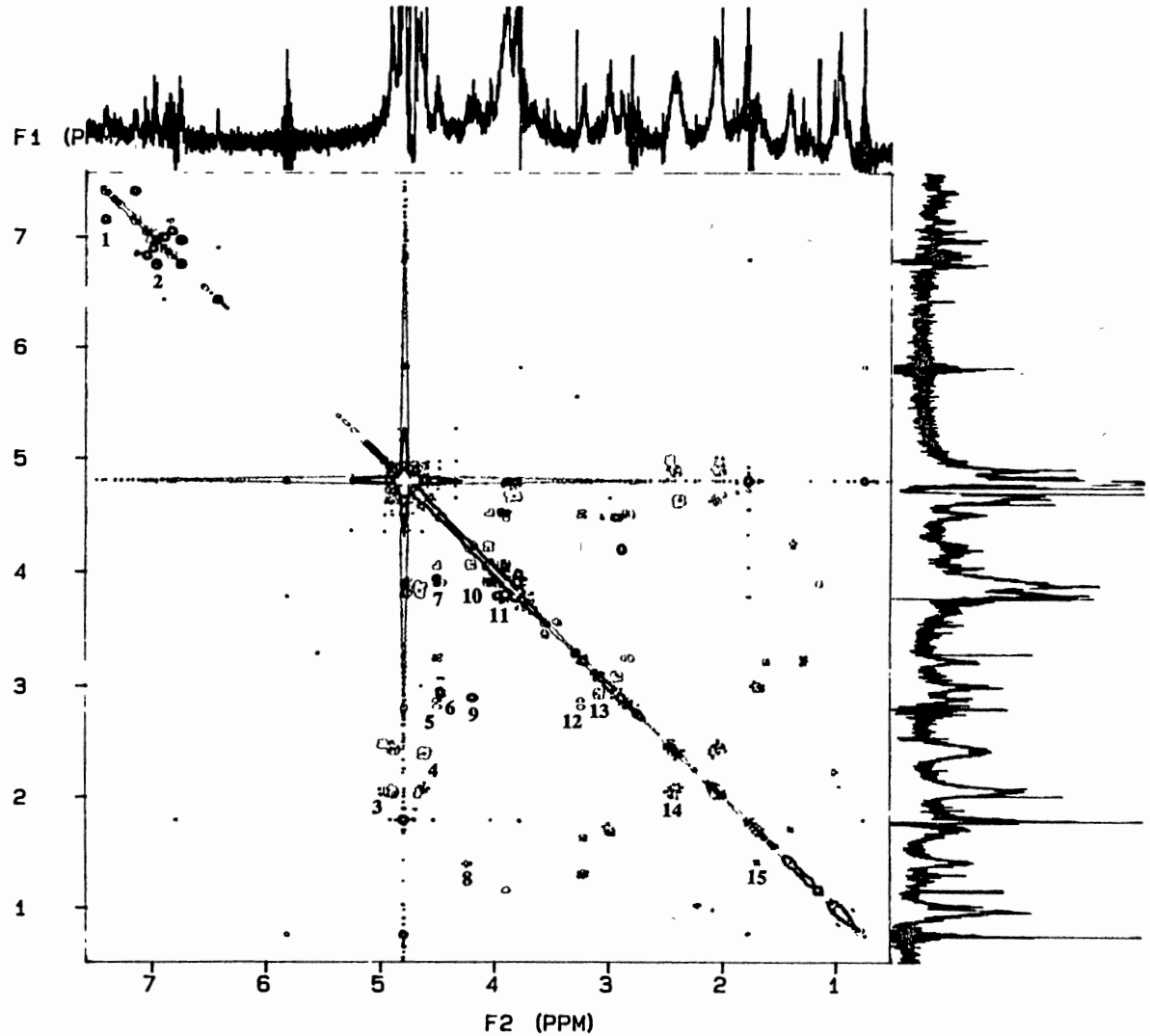

F1 (P
7
6
5
4
3
2
1
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
7
6
5
4
3
2
1
F2 (PPM)

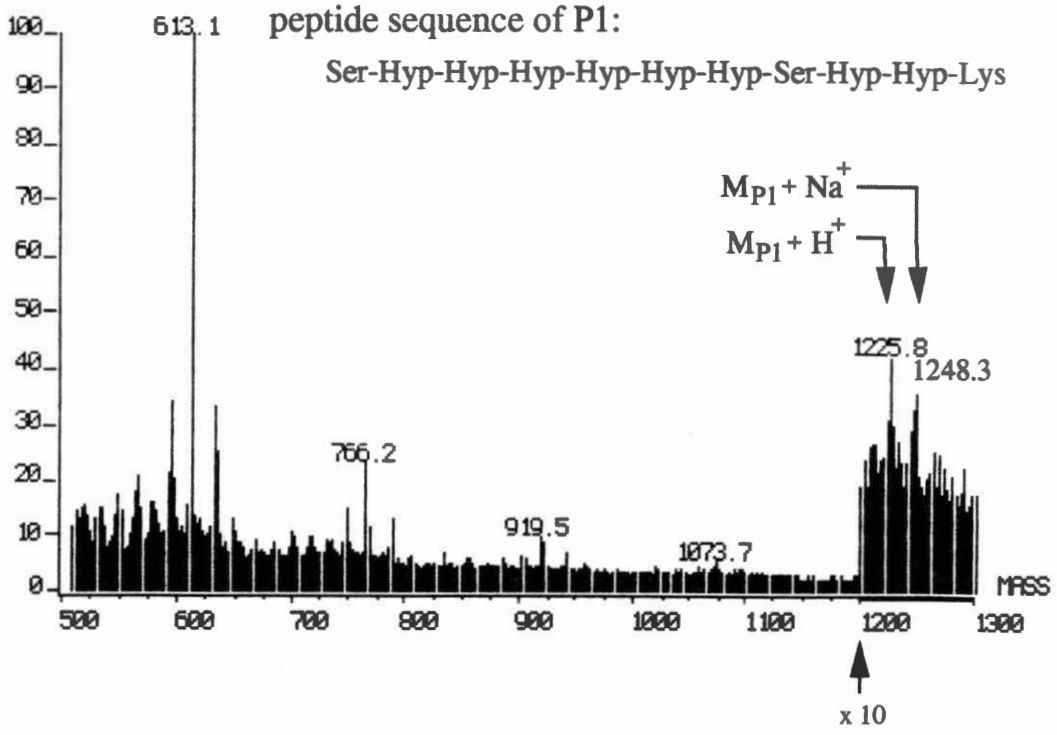
Figure 15. Mass Spectra of Peptide 1 in Peak 1(P1), Peptides 2a and 2b in Peak 2 (P2) of Figure 6.

The spectra were recorded on a ZAB-2SE spectrometer. (A) Mass spectrum of peptide P1 in peak 1. Peaks at m/z of 1225.8 and 1248.3 correspond to the $[M+H^+]$ and $[M+Na^+]$ of P1. Peaks at m/z of 1073.7, 919.5, 766.2 and 613.1 correspond to NBA matrix. (B) Mass spectrum of peptides P2a and P2b in peak 2. Peaks at m/z of 786.0, 807.9; 731.8, and 753.9 correspond to the $[M+H^+]$ and $[M+Na^+]$ of P2a and P2b, respectively. Thioglycerol was used as matrix.

(A) The mass spectrum of peak 1

peptide sequence of P1:

Ser-Hyp-Hyp-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Hyp-Lys

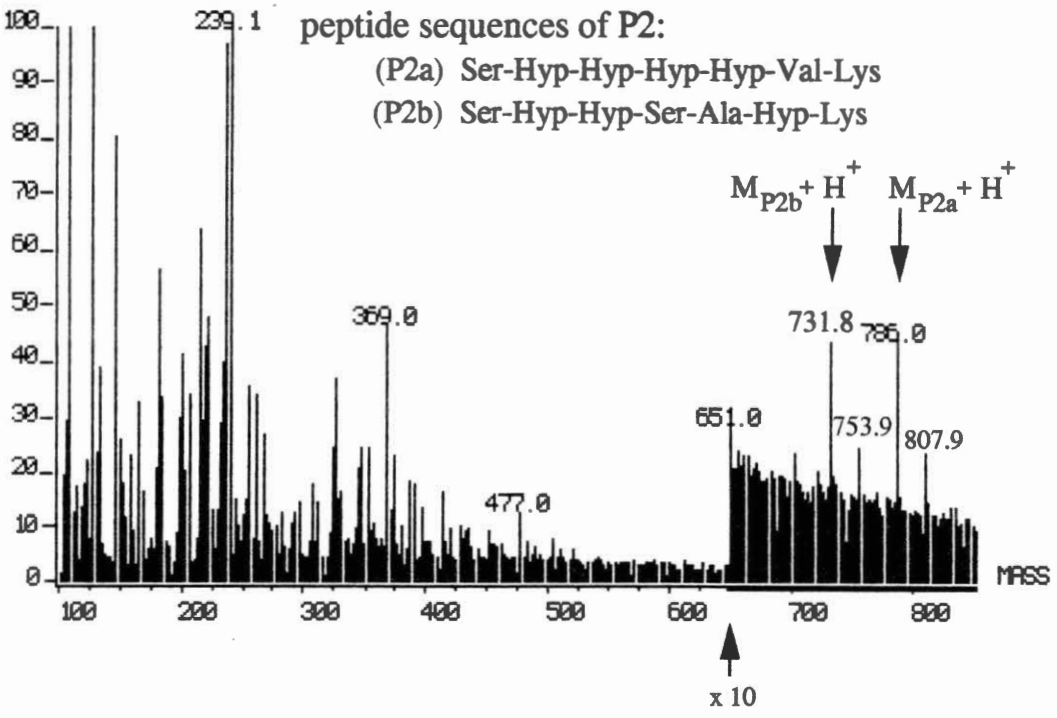


(B) The mass spectrum of peak 2

peptide sequences of P2:

(P2a) Ser-Hyp-Hyp-Hyp-Hyp-Val-Lys

(P2b) Ser-Hyp-Hyp-Ser-Ala-Hyp-Lys



and 1248.3, 807.9, 753.9, respectively. Due to the low sensitivity of hydrophilic peptides, the scale of LSIMS spectra in the mass range of interest needed to be expanded by a factor of 10.

Sequencing of the peptides by mass/mass spectrometry (MS/MS) was unsuccessful, perhaps because of the extreme hydrophilicity of the peptides which caused very low ionization efficiency and sample peak intensity.

Determination of Interactions between Extensin and Other Major Wall Polymers by Liberation of Cotton Cell Wall Extensins

It is known that the hydroxyproline-rich glycoprotein, extensin, becomes insolubilized into cell walls (Cooper *et al.*, 1984). In order to learn about the nature of the insolubilization we have tested the ability of a variety of selective hydrolytic methods (or combinations of them) to extract extensin fragments from cell walls of cotton suspension cultures.

Liberation of Cotton Extensin Fragments with Trypsin

Cotton suspension culture cell walls were treated with trypsin (2% w/w) in 100 mM ammonium bicarbonate buffer (pH 7.6) overnight at room temperature. This treatment only solubilized 7% of the total wall weight percent (see Figure 16). During our investigation, the liberation of cotton extensin fragments was judged by the release of Hyp content. Five percent of the Hyp from the original sample was released by trypsin digestion. A small amount of sugars was also solubilized, and the mole and weight percent of sugars in the filtrate are shown in Table 4. This experiment demonstrated that cotton extensin was somehow prevented from trypsin digestion by some other wall components. Obviously, removal of the polymers protecting extensin is necessary in order to be able release more

Figure 16. Flow Chart of Liberation of Cotton Extensin Fragments with Trypsin.

Cotton cell wall was digested with trypsin in 100 mM ammonium bicarbonate (pH 7.6) at room temperature overnight. Five percent of hyp was liberated. The determination of sample content (%) was based on a total weight percent of the dry cell wall. The value given in parentheses represents the weight of each fraction recovered after lyophilization and the percentage of total cell walls.

1.0 Gram Acala 44 Cotton Suspension Cell Walls

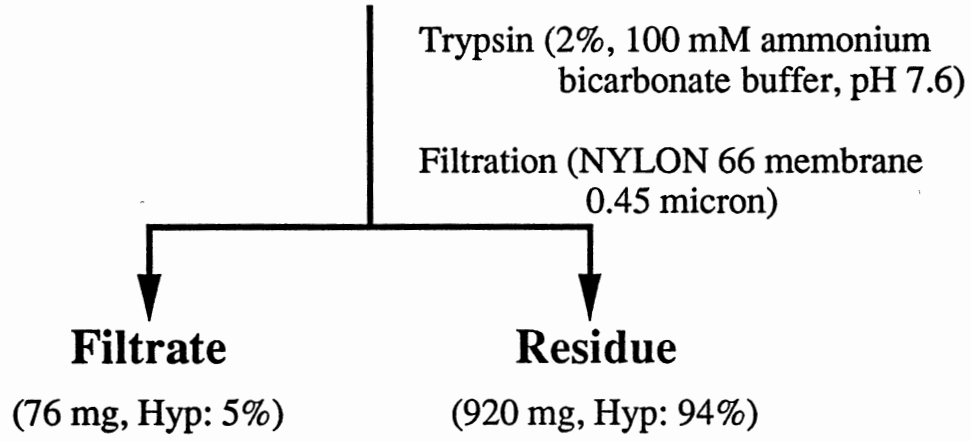


TABLE 4

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

Material	Ara	Rha	Fuc	Xyl	GalA	Gal	Glc	wt%*	wt (mg)
Intact Walls (Acala 44 Cotton)	17.7	12.4	1.9	17.8	33.0	10.3	6.5	38.8	1,000
Filtrate (Trypsin)	15.0	9.0	—	5.2	51.2	6.0	13.6	8.5	76
Residue (Trypsin)	16.0	10.3	—	23.6	30.3	11.3	8.5	39.4	920

* Same as indicated in Table 1.

extensin fragments. Several specific chemical and enzymatic methods, therefore, were used to selectively remove these contents prior to trypsin treatment.

Liberation of Cotton Extensin Fragments with EPGase
Followed by Trypsin Digestion

EPGase is a bacterial pectinase which specifically cleaves the α (1→4) galacturonic acid linkage. Cotton cell walls were treated with EPGase in 50 mM ammonium acetate buffer (pH 5.2) at room temperature overnight to remove about 50% of the galacturonic acid. The EPGase-treated residue was then digested with trypsin (2% w/w) at pH 7.6 overnight at room temperature. As shown in Figure 17, about the same amount (5%) of Hyp was released as was in the trypsin treatment alone. The sugar contents in either the filtrates or residues after both treatments are shown in Table 5. The result indicates that removal of homogalacturonan does not allow trypsin greater access to extensin. In other words, homogalacturonan may not contribute to the prevention of extensin digestion by trypsin.

Liberation of Cotton Extensin Fragments with Cellulase
Followed by Trypsin Digestion

Cotton cell walls were treated with cellulase (2% w/w) in 50 mM ammonium acetate buffer (pH 5.2) overnight at room temperature, and the remaining residue was then digested with trypsin (2% w/w) in 100 mM ammonium bicarbonate buffer (pH 7.6) overnight at room temperature as shown in Figure 18. The mole and weight percent of sugars in the filtrates and residues after cellulase or trypsin treatment are given in Table 6. Most of the sugars solubilized are Xyl, Glc and GalA in cellulase treated-filtrate. Most of the hemicellulose and cellulose fragments were removed from the cotton cell wall by cellulase. Also, 2% of the Hyp was detectable in the filtrate by colorimetric method. The Hyp may be released by proteases contained in the commercial cellulase preparation. More Hyp (9%)

Figure 17. Flow Chart of Liberation of Cotton Extensin Fragments with EPGase Followed by Trypsin Digestion.

Cotton cell wall was digested with EPGase in 50 mM ammonium acetate buffer (pH 5.2) at room temperature overnight to cleave non-methylesterified homogalacturonan. The resulting residue was then digested with trypsin in 100 mM ammonium bicarbonate buffer (pH 7.6) overnight at room temperature. Five percent of hyp was released. The determination of sample content (%) was based on a total weight percent of the dry cell wall. The value given in parentheses represents the weight of each fraction recovered after lyophilization and the percentage of total cell walls.

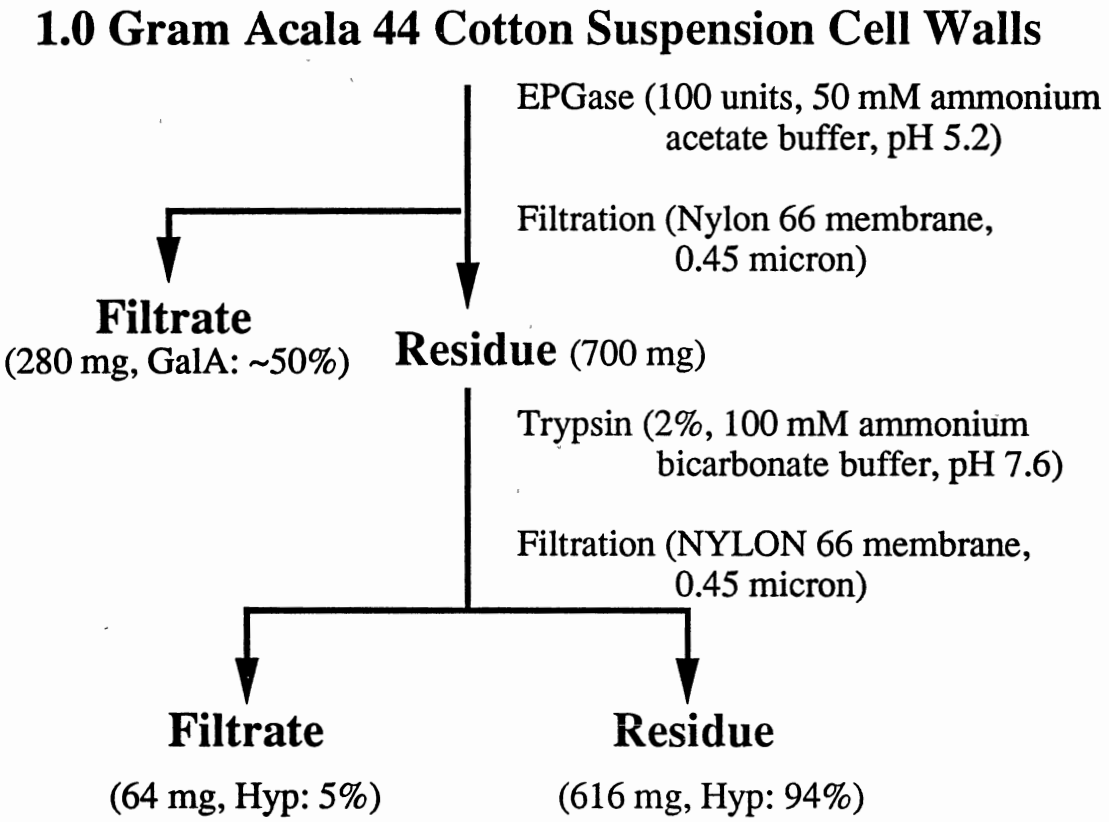

1.0 Gram Acala 44 Cotton Suspension Cell Walls
EPGase (100 units, 50 mM ammonium acetate buffer, pH 5.2)
Filtration (Nylon 66 membrane, 0.45 micron)
Filtrate
(280 mg, GalA: ~50%)
Residue (700 mg)
Trypsin (2%, 100 mM ammonium bicarbonate buffer, pH 7.6)
Filtration (NYLON 66 membrane, 0.45 micron)
Filtrate
(64 mg, Hyp: 5%)
Residue
(616 mg, Hyp: 94%)

TABLE 5

MOLE PERCENT OF SUGARS IN COTTON SUSPENSION CULTURE CELL WALLS AND IN THE SOLUBLE AND THE INSOLUBLE PORTIONS OF THE WALLS TREATED WITH ENDOPOLYGALACTURONASE FOLLOWED BY TRYPSIN DIGESTION

Material	Ara	Rha	Fuc	Xyl	GalA	Gal	Glc	wt%*	wt (mg)
Intact Walls (Acala 44 Cotton)	17.7	12.4	1.9	17.8	33.0	10.3	6.5	38.8	1,000
Filtrate (EPGase)	3.8	3.8	—	3.5	85.7	2.1	1.1	40.9	280
Residue (EPGase)	24.0	11.4	—	21.3	12.6	13.3	17.3	33.5	700
Filtrate (Trypsin)	15.4	11.7	—	11.5	26.2	8.6	23.6	8.1	64
Residue (Trypsin)	19.5	10.1	4.0	29.0	14.8	14.0	7.8	25.9	616

* Same as indicated in Table 1.

Figure 18. Flow Chart of Liberation of Cotton Extensin Fragment with Cellulase Followed by Trypsin Digestion.

Cotton cell wall was digested with cellulase in 50 mM ammonium acetate buffer (pH 5.2) at room temperature overnight to degraded xyloglucan and cellulose. The resulting residue was then digested with trypsin in 100 mM ammonium bicarbonate buffer (pH 7.6) at room temperature overnight. Nine percent of hyp was solubilized. The determination of sample content (%) was based on a total weight percent of the dry cell wall. The value given in parentheses represents the weight of each fraction recovered after lyophilization and the percentage of total cell walls.

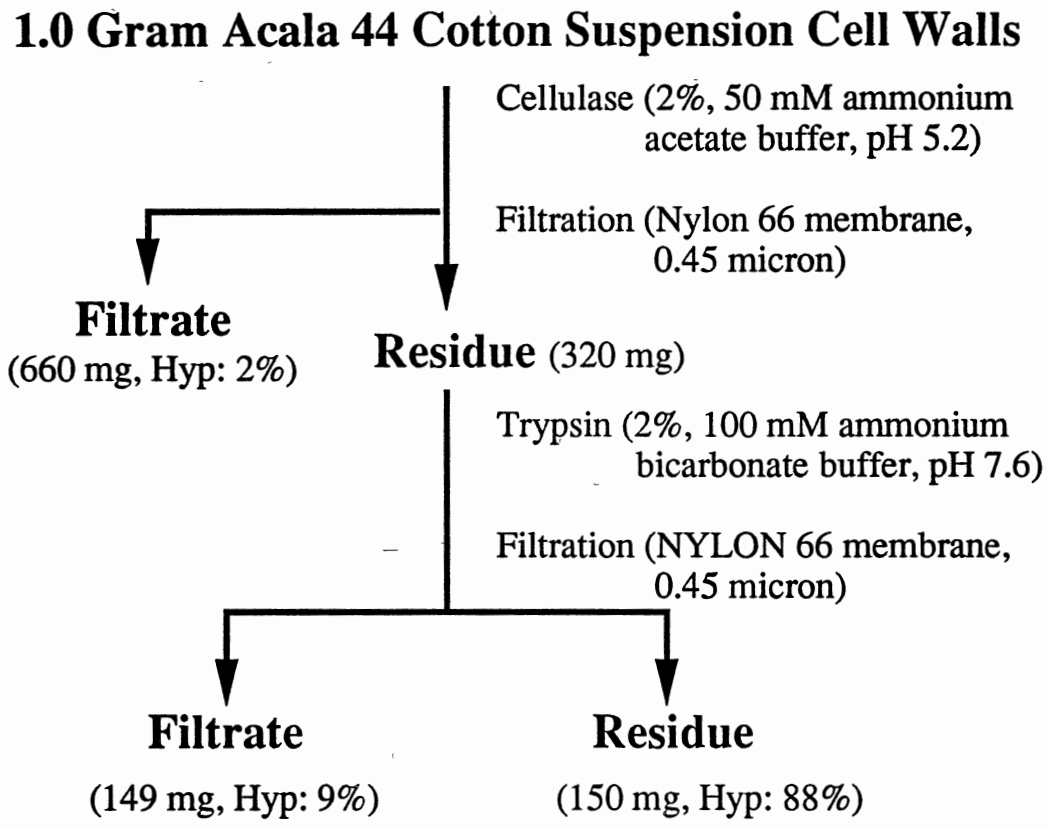

1.0 Gram Acala 44 Cotton Suspension Cell Walls
Cellulase (2%, 50 mM ammonium acetate buffer, pH 5.2)
Filtration (Nylon 66 membrane, 0.45 micron)
Filtrate
(660 mg, Hyp: 2%)
Residue (320 mg)
Trypsin (2%, 100 mM ammonium bicarbonate buffer, pH 7.6)
Filtration (NYLON 66 membrane, 0.45 micron)
Filtrate
(149 mg, Hyp: 9%)
Residue
(150 mg, Hyp: 88%)

TABLE 6

MOLE PERCENT OF SUGARS IN COTTON SUSPENSION CULTURE CELL WALLS AND IN THE SOLUBLE AND THE INSOLUBLE PORTIONS OF THE WALLS TREATED WITH CELLULASE FOLLOWED BY TRYPSIN DIGESTION

Material	Ara	Rha	Fuc	Xyl	GalA	Gal	Glc	wt%*	wt (mg)
Intact Walls (Acala 44 Cotton)	17.7	12.4	1.9	17.8	33.0	10.3	6.5	38.8	1,000
Filtrate (Cellulase)	3.4	1.9	1.6	13.8	29.2	4.4	45.1	58.5	660
Residue (Cellulase)	25.0	20.7	—	7.1	30.4	7.5	8.8	44.6	320
Filtrate (Trypsin)	13.0	21.9	—	6.8	44.1	9.5	4.3	38.0	149
Residue (Trypsin)	35.5	17.3	—	5.9	23.1	10.1	7.3	44.3	150

* Same as indicated in Table 1.

was liberated after trypsin treatment than the amount of Hyp released by trypsin digestion alone or by both EPGase and trypsin treatments. An explanation of this experimental result is that interaction of cellulose fibrils and extensin prevents trypsin access to the proteins to a certain extent. Therefore, the following experiment was conducted to verify this explanation.

Liberation of Cotton Extensin Fragments with HF at -23°C

Followed by Trypsin or Cellulase and Trypsin Digestions

Cotton cell walls were solvolyzed with liquid HF at -23°C followed by ether, water, EDTA solution, and imidazole solution extractions as described by Komalavilas (1988). In addition to cell wall proteins, the extracted residue contains large amounts of cellulosic sugars and small amounts of other sugars. The residue was then treated in two different ways, as shown in Figure 19. When the residue was digested with trypsin, about nineteen percent of Hyp in the original sample was liberated. While fifty percent of the Hyp was liberated by trypsin treatment after removal of most cellulosic sugars by cellulase digestion. The mole and weight percent of sugars in each fraction from all these treatments are compared in Table 7. Apparently these results, together with the previous experiment (see Figure 18), indicate that cellulose fibrils prevent trypsin access to the cotton extensin molecules. This may be because cellulose microfibrils intercalate with extensin molecules (Cooper *et al.*, 1984).

Fifty percent of the Hyp was released by trypsin after both HF -23°C and cellulase treatments, whereas only 9% of the Hyp was solubilized by trypsin after cellulase without HF solvolysis. Actually four times more Hyp was liberated in HF -23°C, cellulase, trypsin sequential treatment than was liberated using the cellulase and trypsin treatments. In addition to the cellulosic sugars, another cell component must also contribute significantly to protecting extensin proteins from trypsin digestion. This content was removed by HF

Figure 19. Flow Chart of Liberation of Cotton Extensin Fragments with Anhydrous HF Solvolysis at -23°C Followed by Trypsin or Cellulase and Trypsin Digestions.

Cotton cell wall was solvolyzed with liquid HF at -23°C for 30 minutes followed by ether, water, 0.5 M imidazole buffer (pH 7), 0.1 M EDTA extractions. The resulting residue containing most wall proteins and cellulose was digested with trypsin or cellulase and trypsin as described in Figure 15 and 17. The former treatment liberated 19% of hyp content, while later treatments liberated 50% of hyp. The determination of sample content (%) was based on a total weight percent of the dry cell wall. The value given in parentheses represents the weight of each fraction recovered after lyophilization and the percentage of total cell walls.

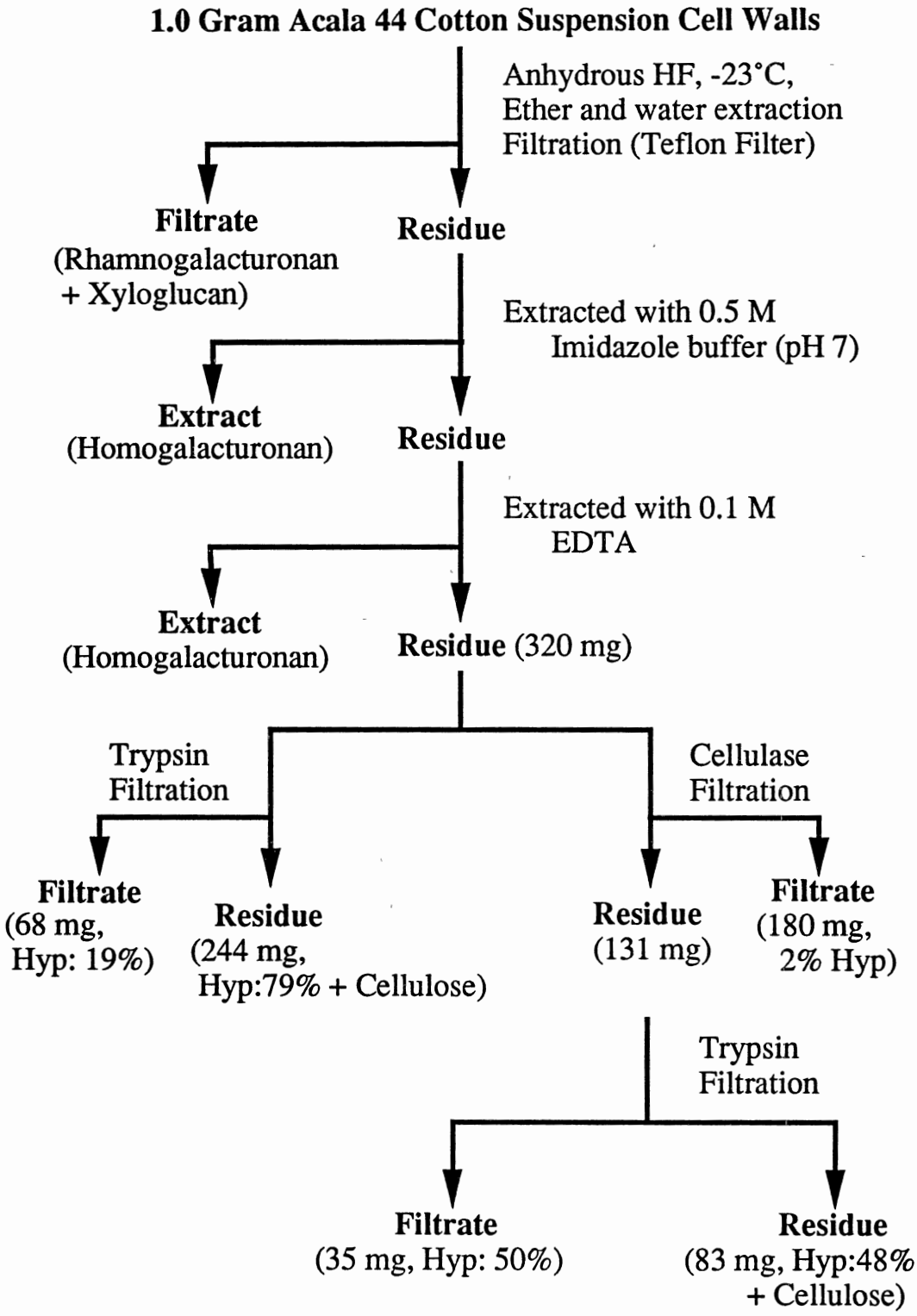

1.0 Gram Acala 44 Cotton Suspension Cell Walls
Anhydrous HF, -23°C,
Ether and water extraction
Filtration (Teflon Filter)
Filtrate
(Rhamnogalacturonan
+ Xyloglucan)
Residue
Extracted with 0.5 M
Imidazole buffer (pH 7)
Extract
(Homogalacturonan)
Residue
Extracted with 0.1 M
EDTA
Extract
(Homogalacturonan)
Residue (320 mg)
Trypsin
Filtration
Cellulase
Filtration
Filtrate
(68 mg,
Hyp: 19%)
Residue
(244 mg,
Hyp:79% + Cellulose)
Residue
(131 mg)
Filtrate
(180 mg,
2% Hyp)
Trypsin
Filtration
Filtrate
(35 mg, Hyp: 50%)
Residue
(83 mg, Hyp:48%
+ Cellulose)

TABLE 7

MOLE PERCENT OF SUGARS IN COTTON SUSPENSION CULTURE CELL WALLS AND IN THE SOLUBLE AND THE INSOLUBLE PORTIONS OF THE WALLS AFTER HF SOLVOLYSIS AT -23°C FOLLOWED BY TRYPSIN DIGESTION OR CELLULASE AND TRYPSIN DIGESTIONS

Material	Ara	Rha	Fuc	Xyl	GalA	Gal	Glc	Glc#	wt%*	wt (mg)
Intact Walls (Acala 44 Cotton)	17.7	12.4	1.9	17.8	33.0	10.3	6.5	—	39.0	1,000
Residue (HF at -23°C and Extrations)	3.2	1.2	—	3.4	32.7	3.5	56.1		12.4 or	320
	—	—	—	—	22.5	—		77.5	42.4	
Method 1										
Filtrate (Trypsin)	6.1	2.2	—	4.9	68.1	5.8	7.0	—	5.0	68
Residue (Trypsin)	5.0	1.5	—	4.7	26.2	4.6	58.0		12.3 or	244
	—	—	—	—	—	—		100.0	37.7	
Method 2										
Filtrate (Cellulase)	1.4	0.9	—	2.3	35.5	1.1	56.2	—	58.4	180
Residue (Cellulase)	7.0	2.4	—	6.3	12.8	8.5	59.5		7.1 or	131
	—	—	—	—	—	—		100.0	27.5	
Filtrate (Trypsin)	6.3	1.9	—	5.8	19.8	8.1	50.7	—	4.4	35
Residue (Trypsin)	6.5	1.7	—	6.5	11.5	7.5	64.9		9.5 or	83
	—	—	—	—	—	—		100.0	41.8	

Glucose content was determined after HF solvolysis at 0°C.
* Same as indicated in Table 1.

treatment at -23°C prior to trypsin digestion. The next experiment was conducted to investigate this possibility.

Liberation of Cotton Extensin Fragments with HF at -73°C Followed by Trypsin Digestion

From previous research, the arabinosides attached to Hyp residue in extensin proteins are thought to contribute to the stability of the polyproline II polypeptide helix (Van Holst *et al.*, 1986). It has also been proposed that another role of the arabinose side chains on extensin is to protect the wall glycoprotein from proteolysis (Wilson and Fry, 1986). Liquid HF solvolysis at -73°C was selected to remove most of the arabinose residues from the cotton extensin proteins. As reported in Table 8, if HF treatment was carried out at -73°C, only arabinose furanose glycosidic linkages are broken (Mort *et al.*, 1989).

Cotton cell walls were treated with liquid HF at -73°C for 30 minutes as outlined in Figure 20. Approximately 23% of the walls became soluble in the HF/ether mixture and water extracted fraction. The residue left behind contained about 76% of the original weight. Analysis of the HF/ether soluble sugar fraction showed that it contained about 80% arabinose indicating that the main linkage broken during this treatment was the arabinosyl bond (see Table 9). This fraction also had small amounts of Xyl and Glu. The sugar composition of the water extracted fraction is shown in Table 9. This fraction contains mostly hemicellulosic and pectic sugars. The HF treated residue was digested with trypsin to liberate significant amounts of Hyp. Twenty percent of the Hyp was trypsinized after removal of most of the arabinose residues from the extensin. This result indicated that arabinose side chains of extensin protected the wall glycoproteins from trypsin digestion. The possible mechanism of deprotecting extensin by removal of the arabinose side chains is suggested as shown in Figure 21.

TABLE 8

EXPECTED LABILITY OF THE VARIOUS CELL-WALL SUGAR LINKAGES TO HF SOLVOLYTSIS AT SELECTED TEMPERATURES

Temperature °C	α-Araf	Arap	α-Fucp	β-Galp	α-GalpA	α-GalpA Me	α-Glcp	α-GlcpA	β-Glcp	β-GlcpA	β-Manp	α-Rhap	α-Xylp	b-Xylp
RT	+	?	+	+	+	+	+	?	+	?	+	+	+	+
O	+		+	+	±	+	+	?	+	?	+	+	+	+
− 6	+		+	+	−	+	+	?	+	?	+	+	+	+
−23	+		+	+	−	−	+	−	±	−	?	+	+	+
−40	+		+	±	−	−	±	−	−			±	+	±
−73	+		−	−	−	−	−	−	−			−	−	−

+ Labile
− Not labile
RT Room temperature.
? Not tested.
± Depends on additional factors.

Figure 20. Flow Chart of Liberation of Cotton Extensin Fragments with Anhydrous HF at -73°C Followed by Trypsin Digestion.

Cotton cell wall was solvolyzed with liquid HF at -73°C for 30 minutes followed by ether and water extractions to remove approximately 80% of arabinose residues. Then, the resulting residue was digested with trypsin as described in Figure 2. Twenty percent of hyp was liberated. The determination of sample content (%) was based on a total weight percent of the dry cell wall. The value given in parentheses represents the weight of each fraction recovered after lyophilization and the percentage of total cell walls.

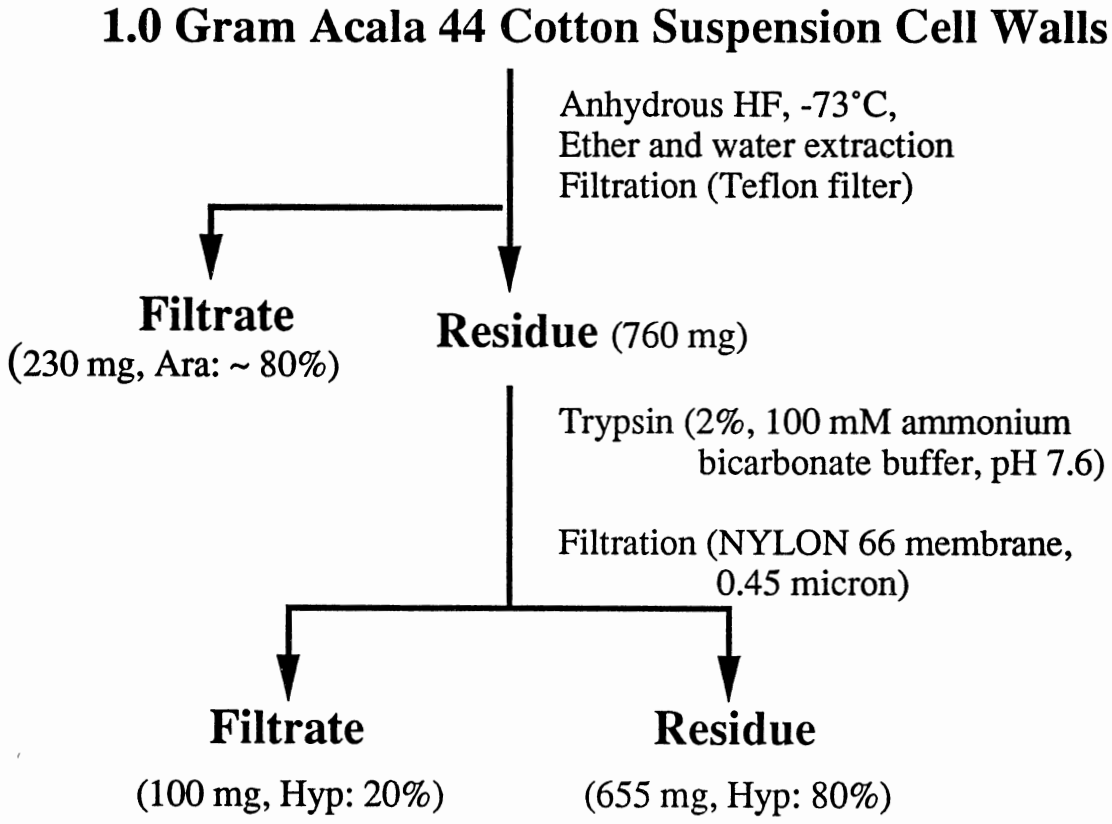

1.0 Gram Acala 44 Cotton Suspension Cell Walls
Anhydrous HF, -73°C,
Ether and water extraction
Filtration (Teflon filter)
Filtrate
(230 mg, Ara: ~ 80%)
Residue (760 mg)
Trypsin (2%, 100 mM ammonium
bicarbonate buffer, pH 7.6)
Filtration (NYLON 66 membrane,
0.45 micron)
Filtrate
(100 mg, Hyp: 20%)
Residue
(655 mg, Hyp: 80%)

TABLE 9

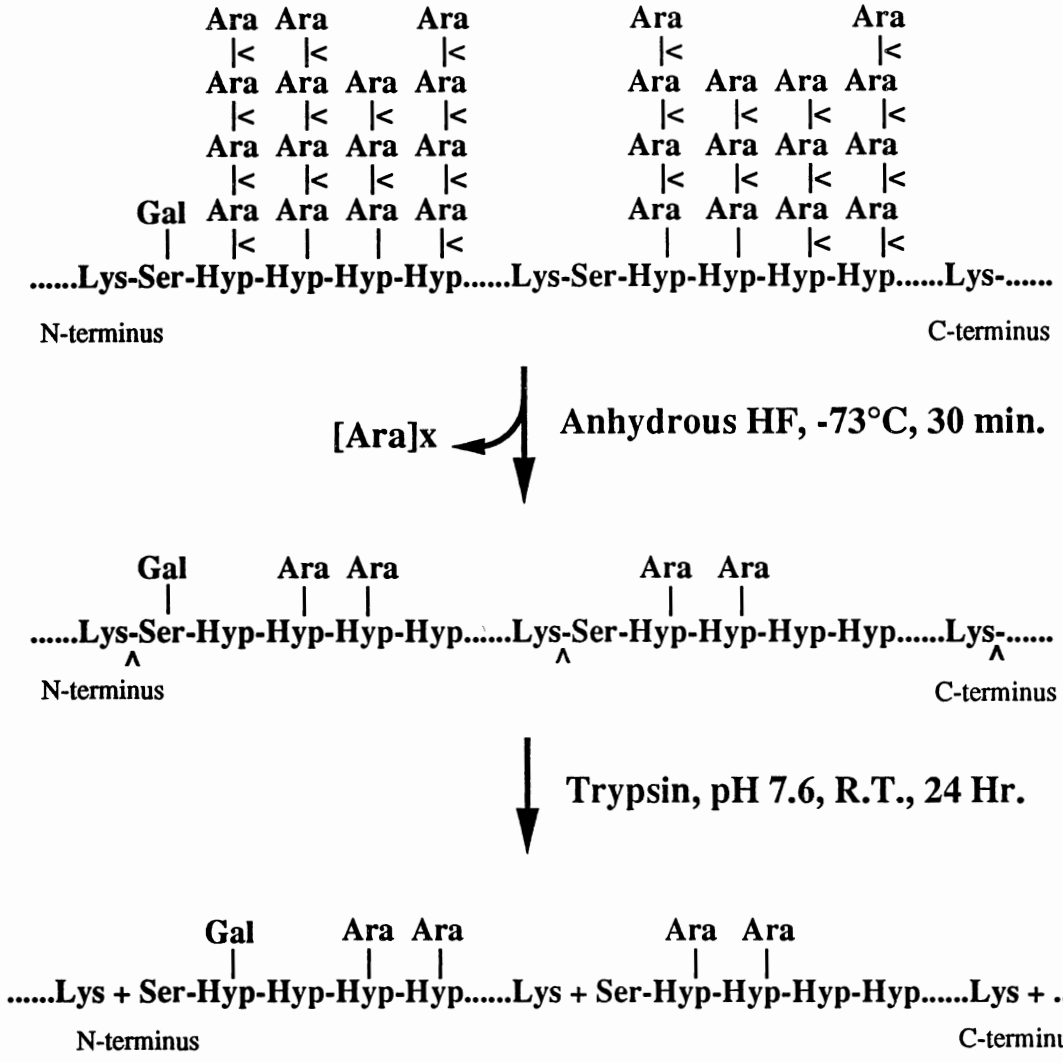
MOLE PERCENT OF SUGARS IN COTTON SUSPENSION CULTURE CELL WALLS AND IN THE SOLUBLE AND THE INSOLUBLE PORTIONS OF THE WALLS AFTER HF SOLVOLYSIS AT -73°C FOLLOWED BY TRYPSIN DIGESTION

Material	Ara	Rha	Fuc	Xyl	GalA	Gal	Glc	wt%*	wt (mg)
Intact Walls (Acala 44 Cotton)	17.7	12.4	1.9	17.8	33.0	10.3	6.5	39.0	1,000
Ether Extract (HF, -73°C)	79.8	6.6	4.2	7.0	0.0	2.6	0.0	—	—
Water Extract (HF, -73°C)	18.8	10.0	2.5	33.1	9.1	14.0	12.5	27.5	94
Residue (HF, -73°C)	5.1	12.2	1.5	17.0	42.8	10.1	11.4	38.5	760
Filtrate (Trypsin)	7.5	8.3	—	11.3	51.1	7.5	14.3	38.7	100
Residue (Trypsin)	4.9	12.3	1.5	19.2	35.1	12.3	14.7	47.3	655

* Same as indicated in Table 1.

Figure 21. Diagram of Liberation of Cotton Extensin Fragments by Trypsin after HF Solvolysis at -73°C.

The possible mechanism of deprotecting of extensin by removal of arabinose side chains suggested in this figure. (>) indicates the site where HF or trypsin cleave.


Ara Ara Ara Ara Ara Ara
Ara Ara Ara Ara Ara Ara Ara Ara
Ara Ara Ara Ara Ara Ara Ara Ara
Gal Ara Ara Ara Ara Ara Ara Ara Ara
.....Lys-Ser-Hyp-Hyp-Hyp-Hyp.....Lys-Ser-Hyp-Hyp-Hyp-Hyp.....Lys-.....
N-terminus
C-terminus
[Ara]x
Anhydrous HF, -73°C, 30 min.
Gal Ara Ara Ara Ara
.....Lys-Ser-Hyp-Hyp-Hyp-Hyp.....Lys-Ser-Hyp-Hyp-Hyp-Hyp.....Lys-.....
N-terminus
C-terminus
Trypsin, pH 7.6, R.T., 24 Hr.
Gal Ara Ara Ara Ara
.....Lys + Ser-Hyp-Hyp-Hyp-Hyp.....Lys + Ser-Hyp-Hyp-Hyp-Hyp.....Lys + ...
N-terminus
C-terminus

Digestions of Cotton Suspension Cell Wall with Cellulase Followed by Esterase or α-Chymotrypsin

The trypsin used in the preceding experiments catalyzes the hydrolysis of proteins as well as numerous esters (Neurath and Schwert, 1950). Therefore, I wish to test the effects of an esterase alone. A pig liver esterase preparation was used for this purpose. Also I wish to test the effects of a protease with different specificity. Chymotrypsin, which cleaves peptide bonds involving aromatic amino acids, and bonds of leucyl, methionyl, asparaginyl, and glutamyl residues. In both cases, the residue treated with the esterase or α-chymotrypsin was that left after cellulase treatment of the cell walls to remove cellulose and xyloglucan (see Figure 22). Almost no Hyp was detected in both soluble materials (filtrates). However, sugar composition analysis of both filtrate fractions showed that the filtrates contained large amount of GalA and Rha, and small amounts of Ara, Gal, Xyl and Glc (see Table 10). Obviously, some RG I and polygalacturonan fragments were solubilized by esterase or α-chymotrypsin from the cellulase-treated residue. The explanation of these results is not known.

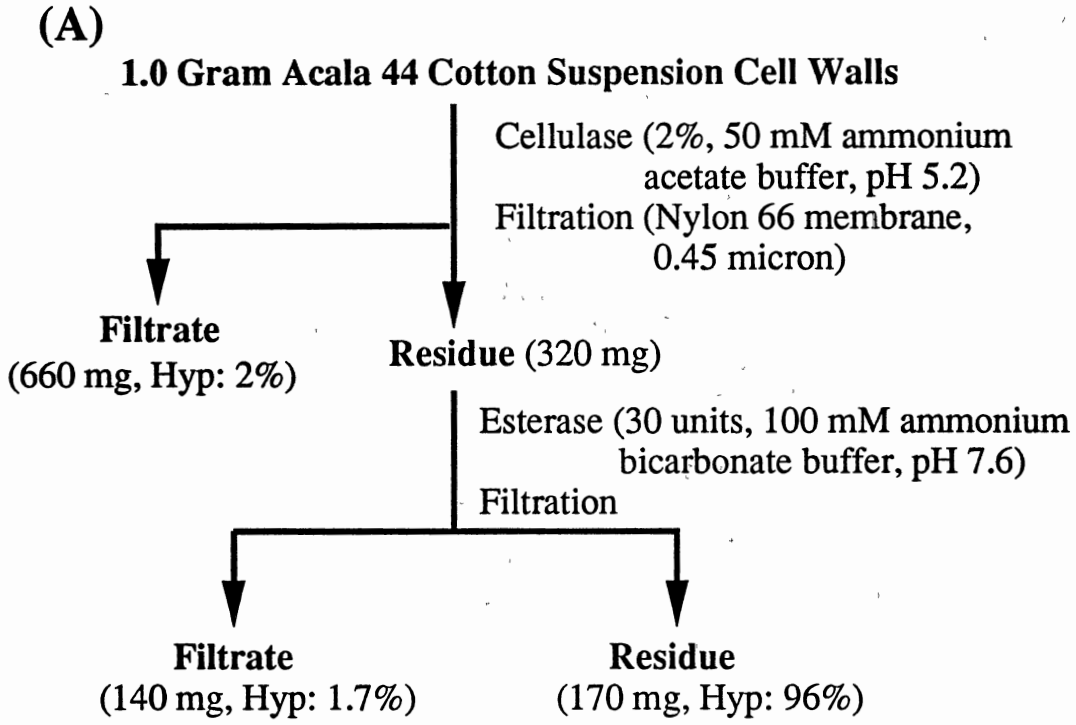
Partial Characterization of Extensin-RG I Complexes from Cotton Cell Walls

Isolation of Extensin-RG I Complexes from Cotton Cell Walls Using Sequential Specific Cleavages

The primary focus of this research is to determine whether or not there are covalent cross-links between extensin molecules and the other major plant cell wall polymers. The approach is to use as many highly specific methods for cleavage of the polysaccharides and proteins as are needed to solubilize still recognizable fragments of the polymers, and look for fragments which contain amino acid and/or sugars characteristic of two polymer types.

Figure 22. Flow Charts of Digestions of Cotton Suspension Cell Wall with Cellulase Followed by Esterase or α-Chymotrypsin.

(A) Cotton cell wall was digested with cellulase in 50 mM ammonium acetate buffer (pH 5.2) at room temperature overnight to degraded xyloglucan and cellulose. The resulting residue was then digested with esterase in 100 mM ammonium bicarbonate buffer (pH 7.6) at room temperature overnight. Less than two percent of hyp was released. The determination of sample content (%) was based on a total weight percent of the dry cell wall. (B) Cotton cell wall was digested with cellulase as described in (A). The cellulase-treated residue was treated with α-chymotrypsin in 100 mM ammonium bicarbonate buffer (pH 7.6) at room temperature overnight. No hyp was detected in the filtrate. The value given in parentheses represents the weight of each fraction recovered after lyophilization and the percentage of total cell walls.


(A)
1.0 Gram Acala 44 Cotton Suspension Cell Walls
Cellulase (2%, 50 mM ammonium acetate buffer, pH 5.2)
Filtration (Nylon 66 membrane, 0.45 micron)
Filtrate
(660 mg, Hyp: 2%)
Residue (320 mg)
Esterase (30 units, 100 mM ammonium bicarbonate buffer, pH 7.6)
Filtration
Filtrate
(140 mg, Hyp: 1.7%)
Residue
(170 mg, Hyp: 96%)

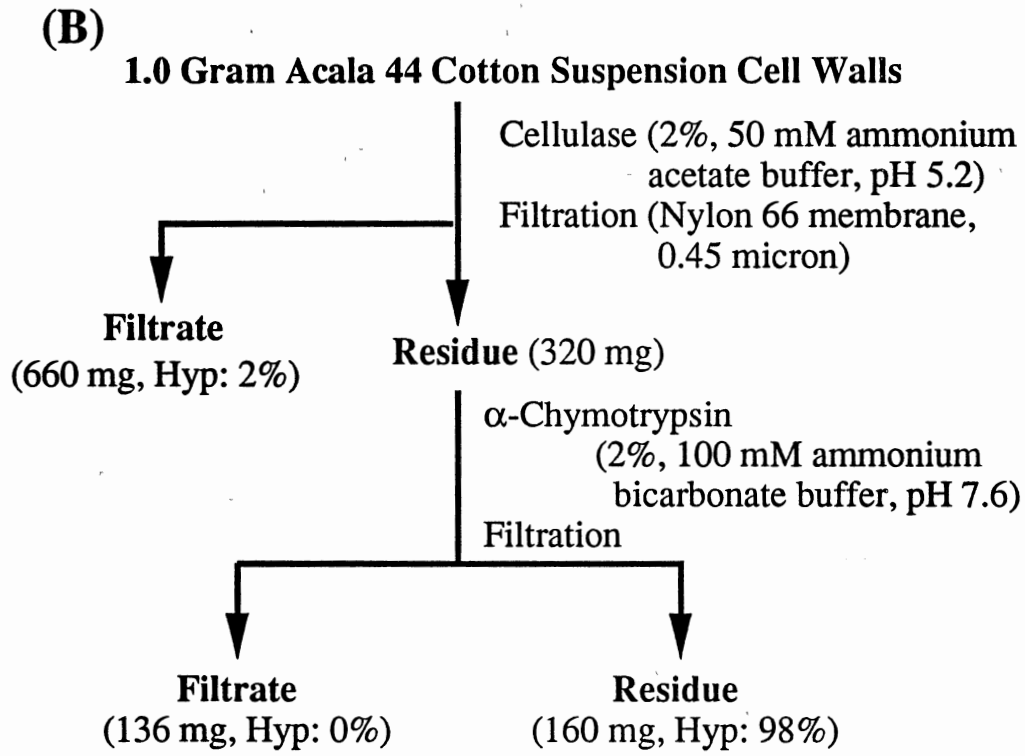

(B)
1.0 Gram Acala 44 Cotton Suspension Cell Walls
Cellulase (2%, 50 mM ammonium acetate buffer, pH 5.2)
Filtration (Nylon 66 membrane, 0.45 micron)
Filtrate
(660 mg, Hyp: 2%)
Residue (320 mg)
α-Chymotrypsin (2%, 100 mM ammonium bicarbonate buffer, pH 7.6)
Filtration
Filtrate
(136 mg, Hyp: 0%)
Residue
(160 mg, Hyp: 98%)

TABLE 10

MOLE PERCENT OF SUGARS IN COTTON SUSPENSION CULTURE CELL WALLS AND IN THE SOLUBLE AND THE INSOLUBLE PORTIONS OF THE WALLS DIGESTED WITH CELLULASE FOLLOWED BY ESTERASE OR α-CHYMOTRYPSIN DIGESTIONS

Material	Ara	Rha	Fuc	Xyl	GalA	Gal	Glc	wt%*	wt (mg)
Intact Walls (Acala 44 Cotton)	17.7	12.4	1.9	17.8	33.0	10.3	6.5	39.0	1,000
Residue (Cellulase)	25.0	20.7	—	7.1	30.4	7.5	8.8	44.6	320
Method 1									
Filtrate (Esterase)	11.7	23.2	—	6.5	44.6	9.5	4.4	42.4	140
Residue (Esterase)	37.8	15.7	—	3.7 —	21.2	10.3	11.3	41.3	170
Method 2									
Filtrate (α-Chymotrypsin)	12.4	22.4	—	6.7	43.2	10.0	5.4	43.6	136
Residue (α-Chymotrypsin)	39.4	18.7	—	4.1	19.0	8.6	10.3	42.5	160

* Same as indicated in Table 1.

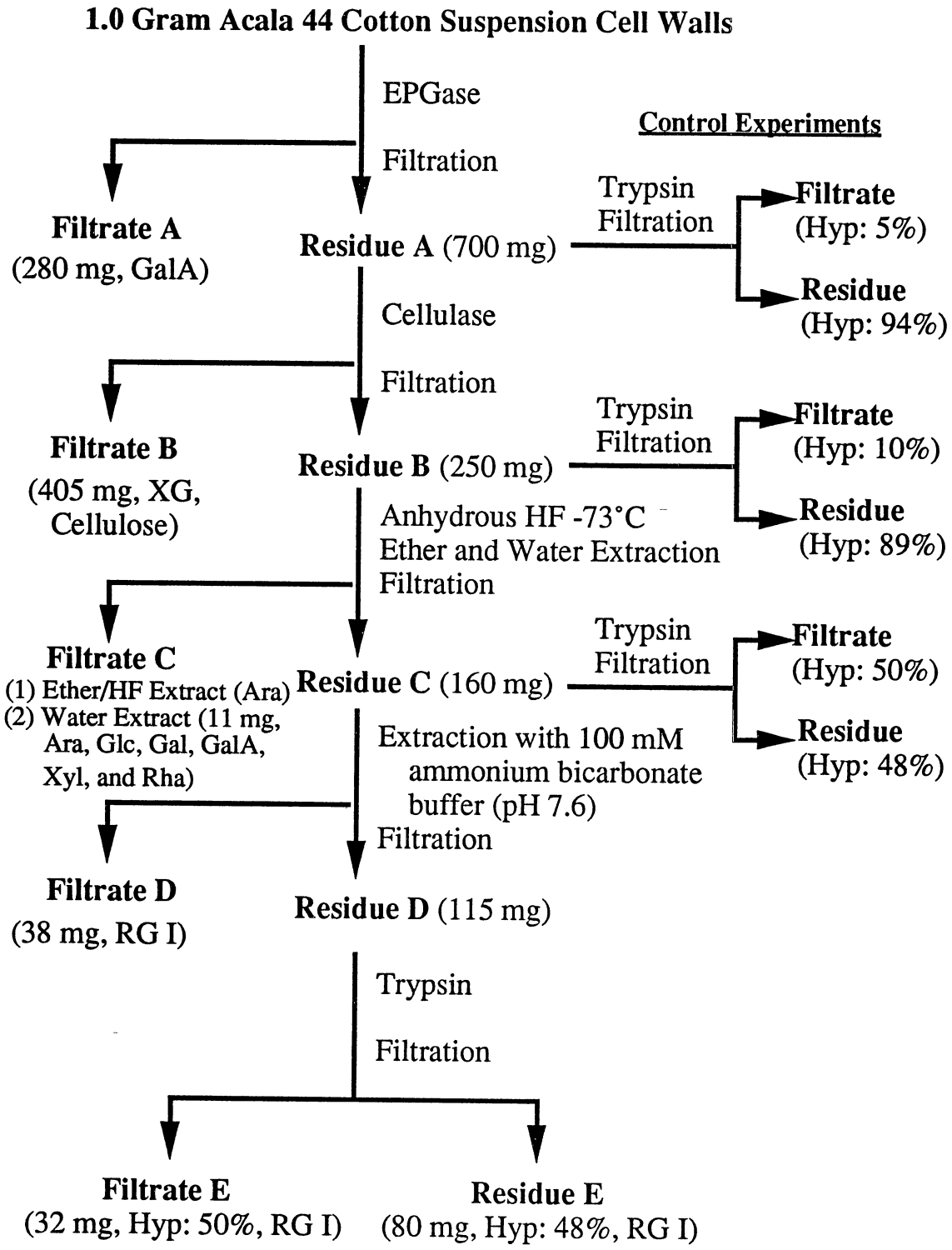
The methods used were as gentle as possible in the attempt, to isolate such crosslinking fragments. In the previous section, various permutations of methods have been used to determine which types of glycosyl and amide bonds need to be cleaved to liberate the cell wall proteins as assessed by the solubilization of hydroxyproline (Hyp). Among these methods, enzymatic (EPGase, cellulase, trypsin) and HF (-73°C) treatments were selected because these cleavage reactions are highly selective, more gentle and largely expose extensin to trypsin.

Fifty percent of the Hyp in the original sample was solubilized by the sequence of (1) EPGase digestion to remove the homogalacturonic acid; (2) cellulase digestion to degrade the cellulose, xyloglucan; (3) HF solvolysis at -73°C to specifically cleave arabinose residues from the Hyp residues in extensin; (4) ammonium bicarbonate buffer extraction to remove the noncovalently linked RG I-like sugars; (5) trypsin treatment to liberate the Hyp-containing proteins or peptides by cleavage of the peptide bond at C-termini of lysine and arginine residues. The details of the procedure are shown in Figure 23. None of the treatments during the sequential steps liberated significant quantities of extensins except when followed by treatment with trypsin.

During control experiments in which the residues after each step were subjected to trypsin digestion, only 5% of the Hyp was solubilized by trypsin after EPGase treatment; twice as much Hyp (10%) was solubilized by trypsin after the cellulase treatment; the highest amounts of Hyp (50%) was digested by trypsin after HF treatment at -73°C. It is likely that the original covalent crosslinks between the wall polymers, if they exist, should remain intact after the sequential treatment unless they involve arabinofuranoses. Thus, it is possible to define such crosslinks in the wall by chemical characterization of the fragments solubilized from each treatment.

Figure 23. Flow Chart of the Isolation of Cotton Extensin-RGI Complexes with a Sequential Treatment.

Cotton cell wall was treated with the combination of 1) EPGase digestion 2) cellulase digestion 3) HF solvolysis at -73°C 4) 100 mM ammonium bicarbonate buffer (pH 7.6) extraction 5) trypsin digestion as described in Figures 15, 16, 17, and 19 to isolate extensin-RG I complexes. Fifty percent of hyp content along with RG I-like sugars were solubilized. The determination of sample content (%) was based on a total weight percent of the dry cell wall. The value given in parentheses represents the weight of each fraction recovered after lyophilization and the percentage of total cell walls.


1.0 Gram Acala 44 Cotton Suspension Cell Walls
EPGase
Filtration
Filtrate A
(280 mg, GalA)
Residue A (700 mg)
Control Experiments
Trypsin
Filtration
Filtrate
(Hyp: 5%)
Residue
(Hyp: 94%)
Cellulase
Filtration
Filtrate B
(405 mg, XG,
Cellulose)
Residue B (250 mg)
Trypsin
Filtration
Filtrate
(Hyp: 10%)
Residue
(Hyp: 89%)
Anhydrous HF -73°C
Ether and Water Extraction
Filtration
Filtrate C
(1) Ether/HF Extract (Ara)
(2) Water Extract (11 mg,
Ara, Glc, Gal, GalA,
Xyl, and Rha)
Residue C (160 mg)
Trypsin
Filtration
Filtrate
(Hyp: 50%)
Residue
(Hyp: 48%)
Extraction with 100 mM
ammonium bicarbonate
buffer (pH 7.6)
Filtration
Filtrate D
(38 mg, RG I)
Residue D (115 mg)
Trypsin
Filtration
Filtrate E
(32 mg, Hyp: 50%, RG I)
Residue E
(80 mg, Hyp: 48%, RG I)

Analysis of EPGase-Treated Filtrate A and Residue A

One gram wall of Acala 44 Cotton suspension cultured cells was treated with 50 units of endopolygalacturonase purified from E. Coli (gene cloned from *E. carotovara*) in a volatile buffer of 50 mM ammonium acetate, pH 5.2. The solubilized materials were filtered through a 0.45 micron NYLON 66 membrane held in an Amicon concentrator, and the residue was washed three times with distilled water and then freeze dried. The filtrate and washing solutions were combined and freeze dried. The mole percent and weight percent of the sugars in the combined filtrate (filtrate A) and residue A are listed in Table 5. The filtrate, accounting for about 28% of by weight of the treated walls, contains predominantly galacturonic acid (~35% of the weight of the filtrate representing approximately 40% of the total GalA present in cotton cell wall), arabinose (1.6% of the weight of the filtrate), rhamnose (1.6%), xylose (1.4%). and galactose (0.9%). Analysis of filtrate A on a PA1 anion exchange column using the Dionex HPLC demonstrated that the filtrate mainly consists of digalacturonides and trigalacturonides (see Figure 24). The trigalacturonic acid was identified by ^{1}H n.m.r. spectroscopy (see Figure 25). The GalA residues in the filtrate had previously been determined to be mostly nonmethylesterified by An (1991). In filtrate A, the homogalacturonic acid eluting last from the PA1 column was determined by ^{1}H n.m.r. spectroscopy to be almost completely nonmethyesterified as shown in Figure 26.

Five percent of the Hyp along with a very small amount of sugars could be solubilized by trypsin after the EPGase treatment. The data of the sugar analyses are given in Table 5.

Analysis of Cellulase-Treated Filtrate B and Residue B

Approximately 40% of the weight of the cell wall material was solubilized from the EPGase-treated residue (residue A) by digestion with the chromatographically purified

Figure 24. Chromatography on a Dionex CarboPac PA-1 HPLC Ion Exchange Column of the Filtrate A of Figure 23.

A linear gradient of ammonium acetate from 0.03 to 1.00 M over 60 minutes followed by a steady elution of 1.00 M for 10 minutes was applied to elute the sample. The peaks were determined by the phenol-sulfuric acid method at 485 nm. The sugar composition contained in each peak was analyzed using a gas chromatography. The oligomers of galacturonic acid, RG I polymer and pectic acid were also used as "standards", eluted on this column under the same conditions (chromatograms are not presented).

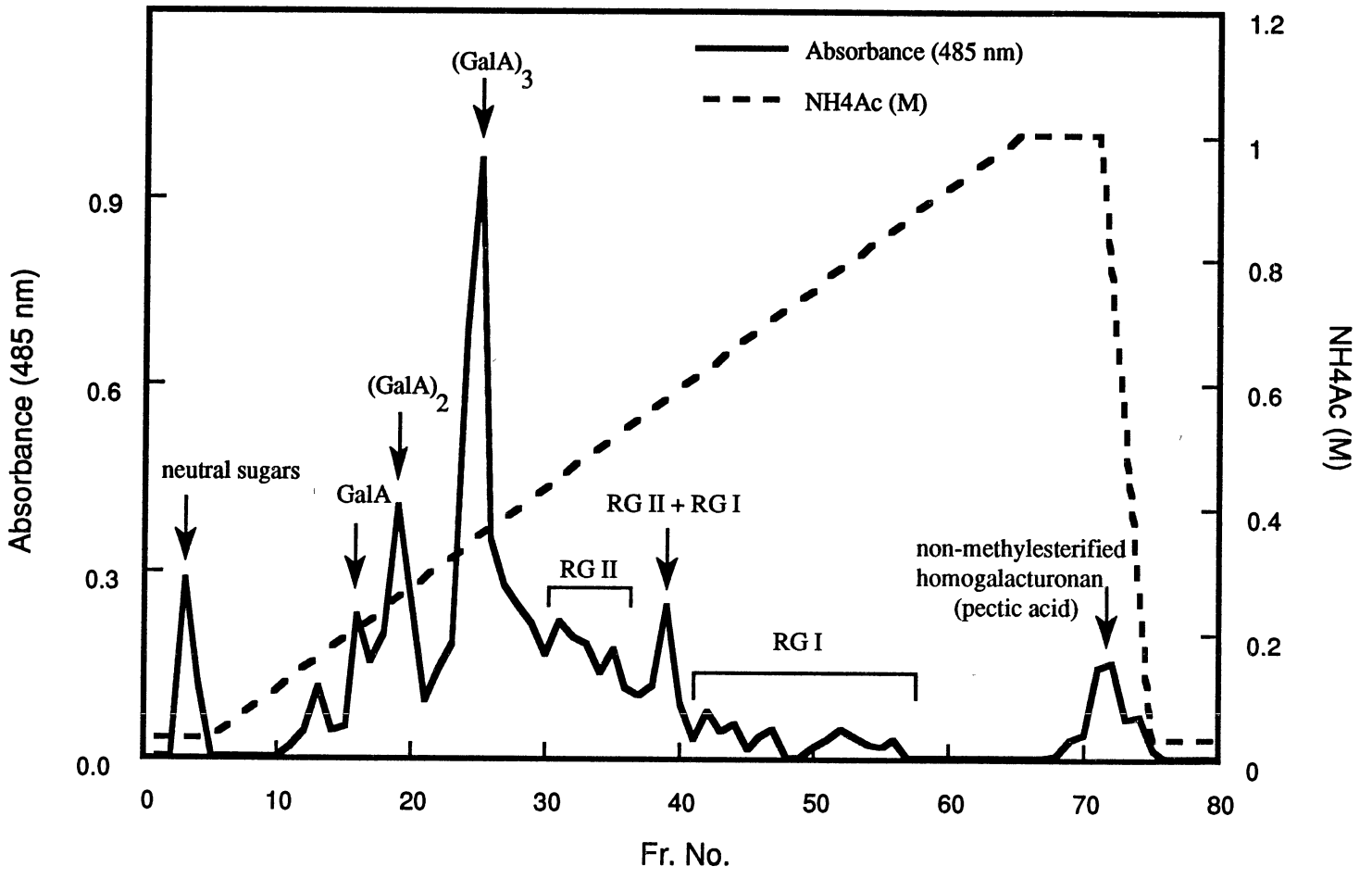
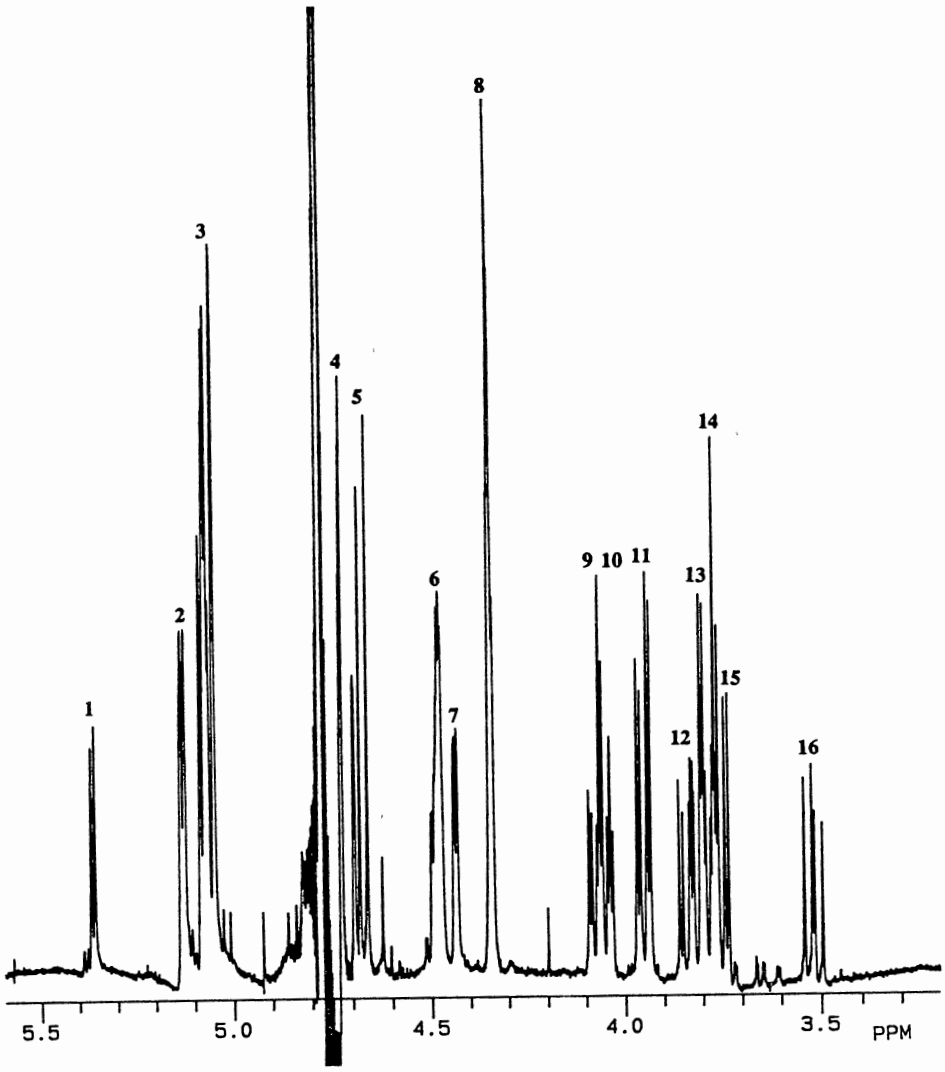
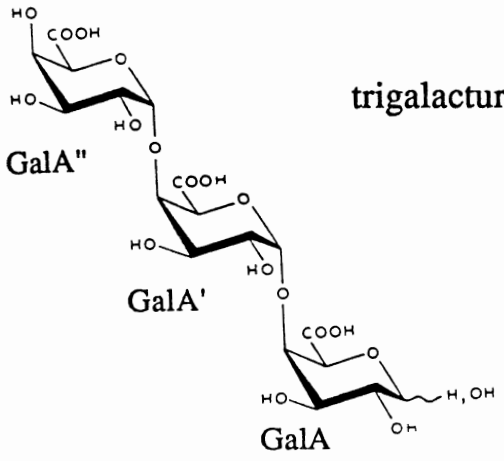

Absorbance (485 nm)
NH4Ac (M)
(GalA)3
(GalA)2
neutral sugars
GalA
RG II
RG II + RG I
RG I
non-methylesterified
homogalacturonan
(pectic acid)
Absorbance (485 nm)
NH4Ac (M)
Fr. No.
0.0
0.3
0.6
0.9
0
0.2
0.4
0.6
0.8
1
1.2
0
10
20
30
40
50
60
70
80

Figure 25. ^{1}H n.m.r. Spectrum of the Trigalacturonic Acid of Figure 24, and Structure of Trigalacturonic Acid.

The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25°C in D_2O. Peaks were identified as follows: 1) H-1α; 2) H'-1; 3) H"-1, H'-5, H"-5; 4) H-5α; 5) H-1β; 6) H-4α, H'-4; 7) H-4β; 8) H-5β, H"-4; 9) H-3α; 10) H'-3; 11) H"-3; 12) H-2α; 13) H-3β; 14) H'-2; 15) H"-2; 16) H-2β.

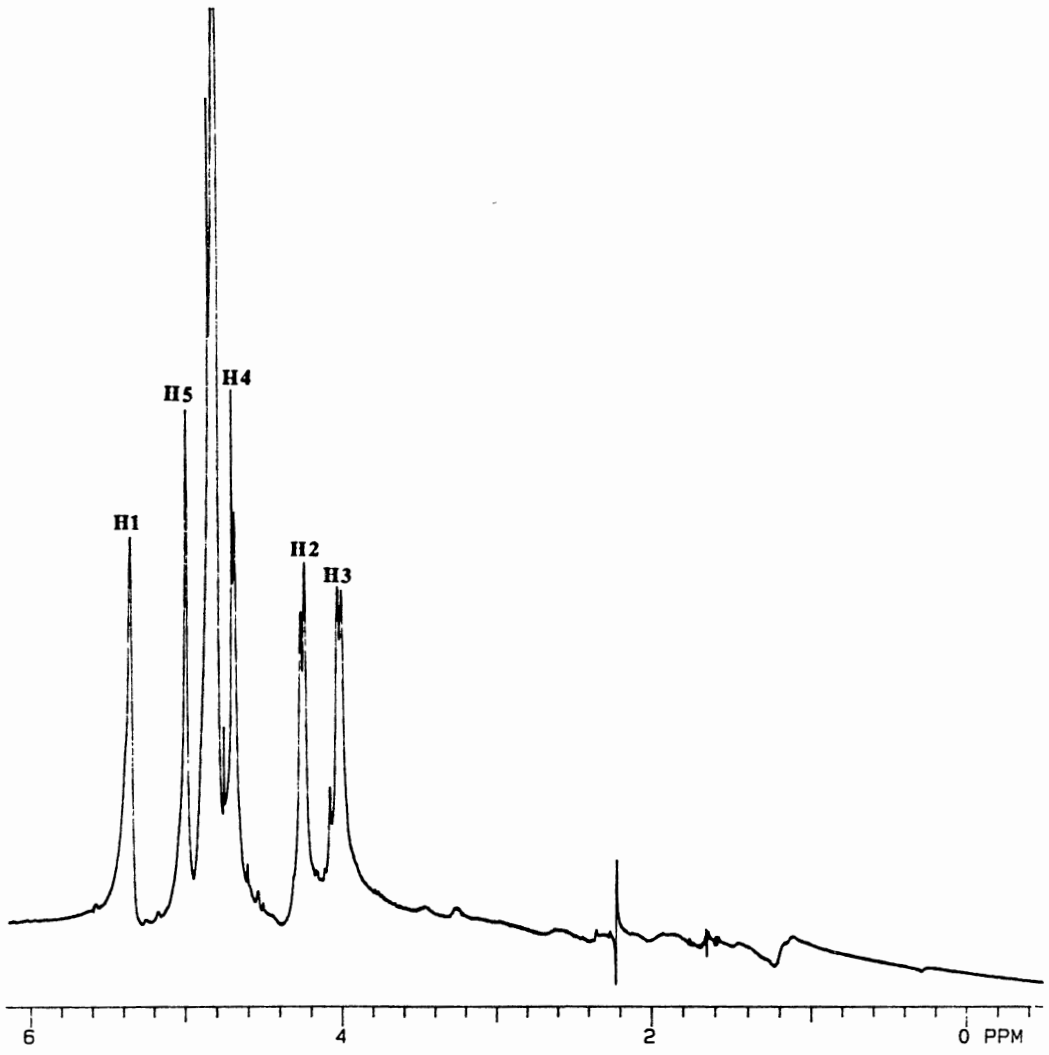


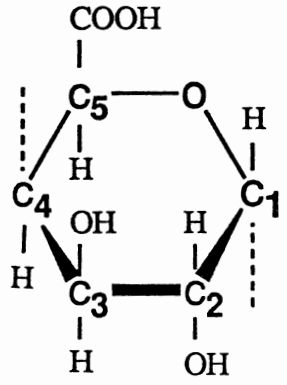


trigalacturonic acid

Figure 26. ^{1}H n.m.r. Spectrum of the Homogalacturonic Acid (Pectic Acid) of Figure 24, and Structure of Galacturonic Acid Residue.

The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25°C in D_2O. Peaks were identified as indicated in the spectrum.





α–D-galacturonic acid residue
in homogalacturonan

Worthington cellulase. Filtrate B contains xyloglucan oligosaccharides, cellulose in the form of cellobiose and glucose, as well as pectic polysaccharides. The mole percent and weight percent of the cellulase-treated filtrate B and residue B are shown in Table 11. In addition, less than two percent of the Hyp was liberated by the enzyme. As already discussed in the previous section, this may be due to proteases contained in the Worthington cellulase preparation.

The xyloglucan fragments in filtrate B after cellulase treatment were characterized using LSIMS mass spectrometry. The mass spectra are given in Figure 27. Two major xyloglucan fragments were found by direct LSIMS of the crude cellulase solubilized material.

In the control experiment, ten percent of the Hyp was liberated by trypsin after the wall was pretreated with both EGPase and cellulase. The amount of Hyp solubilized in this treatment is very close to the amount liberated with only cellulase prior to trypsin digestion. The result indicated that the release of Hyp by trypsin after pretreating cotton walls with EPGase or cellulase is not additive. This may be due to the presence of EPGase activity in the cellulase preparation. In fact, there were oligomers of galacturonic acid from homogalacturonan found in the soluble fraction of intact walls treated with cellulase (data are not presented).

Analysis of HF -73°C-Treated Filtrate C and Residue C

Liquid HF solvolysis at -73°C of residue B for 30 minutes was described in the previous section. About 5.7% total weight of the original walls became soluble and the residue C left behind was weighed approximately 16% of the original weight. Mole and weight percent of the sugars in the ether/HF and water extractions (filtrate C), and residue C are given in Table 12. Approximately 80% of the arabinose in the walls was extracted by ether after HF -73°C treatment. The ^{13}C-^{1}H n.m.r. spectrum of the ether/HF extraction is shown in Figure 28. By comparing ^{1}H n.m.r. spectrum of standard monoarabinoside, it

TABLE 11

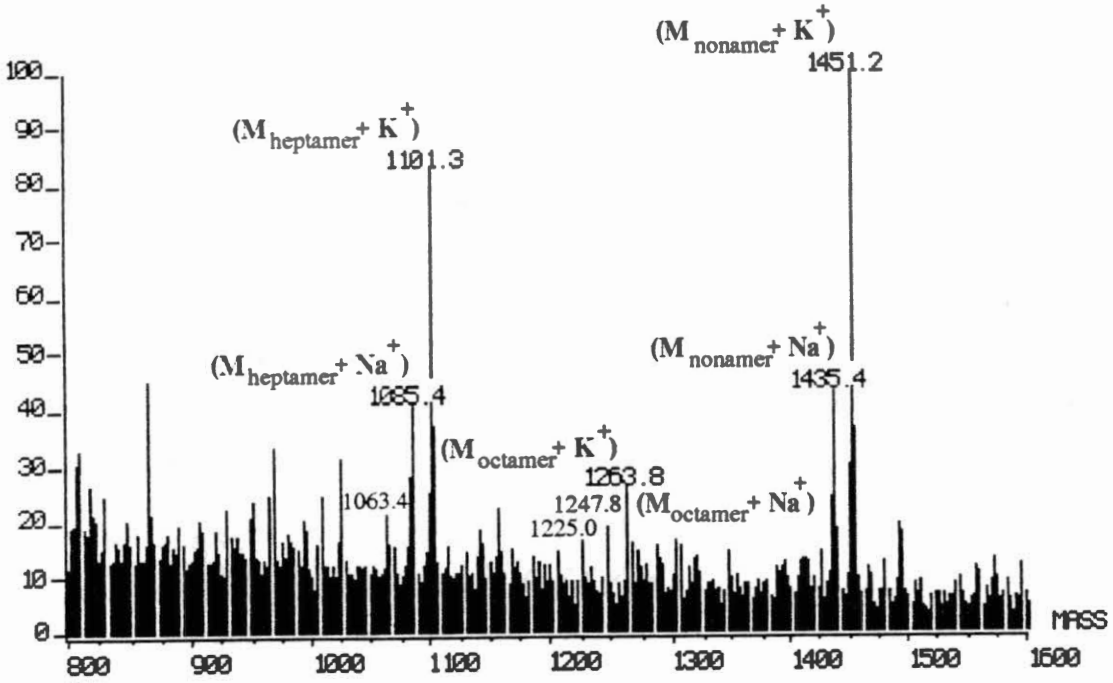
MOLE PERCENT OF SUGARS IN THE SOLUBLE AND THE INSOLUBLE PORTIONS OF ENDOPOLYGALACTURONASE TREATED-RESIDUE TREATED WITH CELLULASE FOLLOWED BY TRYPSIN DIGESTION

Material	Ara	Rha	Fuc	Xyl	GalA	Gal	Glc	wt%*	wt (mg)
Residue (EPGase)	24.0	11.4	—	21.3	12.6	13.3	17.3	33.5	700
Filtrate (Cellulase)	3.0	1.5	2.1	18.2	4.0	5.4	65.0	61.6	405
Residue (Cellulase)	32.1	15.7	—	8.2	17.9	11.2	14.7	44.5	250
Filtrate (Trypsin)	19.7	12.1	—	6.4	21.9	13.2	23.3	33.9	125
Residue (Trypsin)	22.8	3.8	—	4.9	5.5	5.9	56.5	52.3	120

* Same as indicated in Table 1.

Figure 27. Mass Spectra of the Filtrate B of Figure 23, and Structure of Xyloglucan Oligosaccharides Generated by the "Purified" Cellulase from Cotton Cell Walls.

The spectra were recorded on a ZAB-2SE spectrometer. Peaks at m/z of 1085.4 and 1101.3 correspond to the $[M+Na^+]$ and $[M+K^+]$ of heptamer, respectively; 1247.8: $[M+Na^+]$ of octamer; 1263.8: $[M+K^+]$ of octamer; 1435.4: $[M+Na^+]$ of acetylated nonamer; 1451.2: $[M+K^+]$ of acetylated nonamer.



β-D-Glc(1→4)β-D-Glc(1→4)β-D-Glc(1→4)D-Glc
6 6 6
↑ ↑ ↑
1 1 1
α-D-Xyl α-D-Xyl α-D-Xyl

heptamer

M.W. 1062

β-D-Glc(1→4)β-D-Glc(1→4)β-D-Glc(1→4)D-Glc
6 6 6
↑ ↑ ↑
1 1 1
α-D-Xyl α-D-Xyl α-D-Xyl
2
↑
1
β-D-Gal

octamer

M.W. 1224

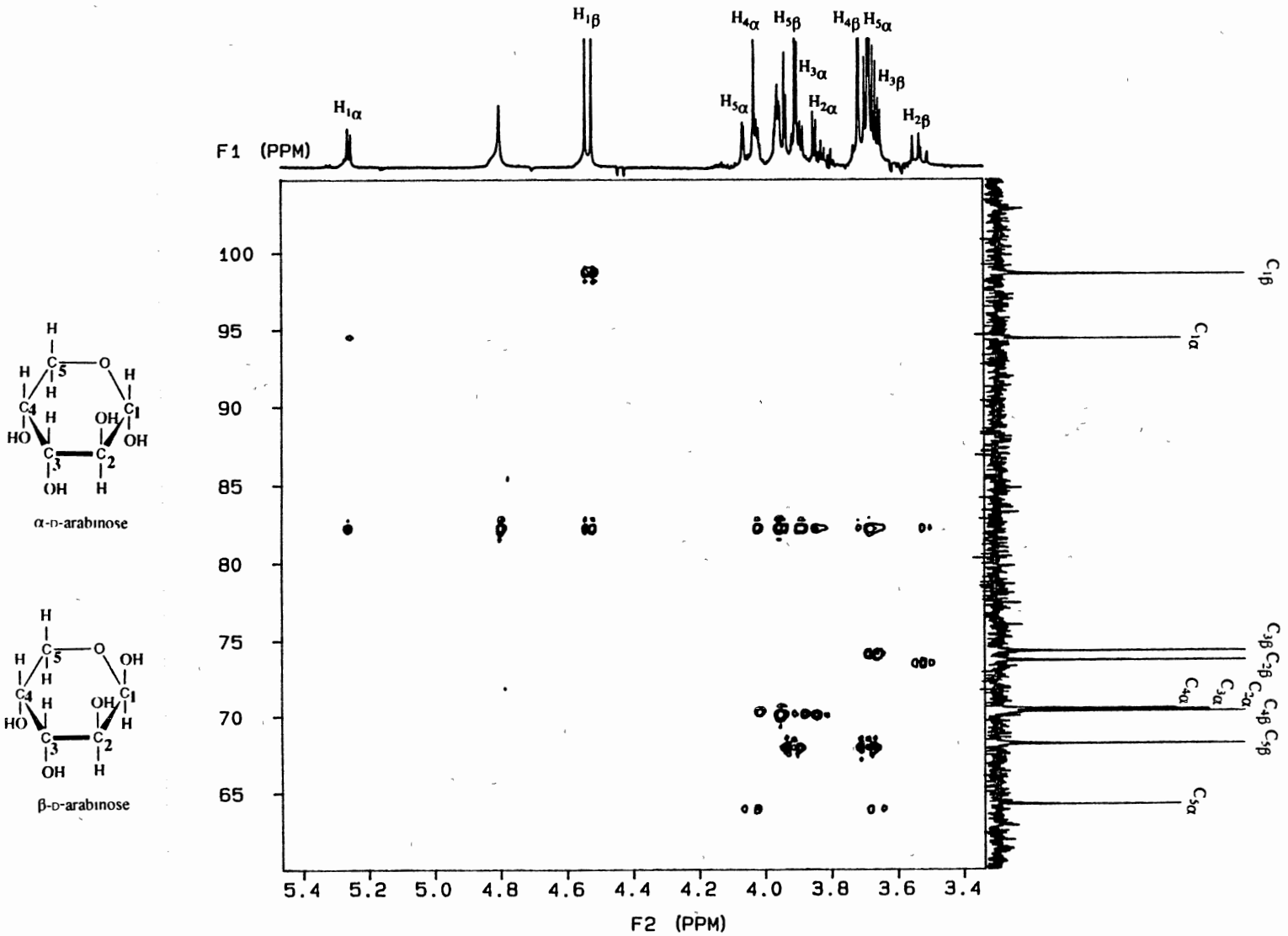
β-D-Glc(1→4)β-D-Glc(1→4)β-D-Glc(1→4)D-Glc
6 6 6
↑ ↑ ↑
1 1 1
α-D-Xyl α-D-Xyl α-D-Xyl
2
↑
1
β-D-Gal-Acetyl
2
↑
1
α-L-Fuc

acetylated nonamer

M.W. 1412

Figure 28. ^{13}C-^{1}H Heteronuclear Correlated n.m.r. Spectrum of Filtrate C (Ether/HF Extract) of Figure 23, and Structure of Arabinose Isomers.

The spectrum was recorded on a 400 MHz n.m.r. spectrometer at 25°C in D_2O. Peaks are identified as indicated in the spectrum.


H1β
H4α
H5β
H4β H5α
H3α
H3β
H1α
H5α
H2α
H2β
F1 (PPM)
100
95
90
85
80
75
70
65
5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4
F2 (PPM)
C1β
C1α
C3β C2β
C4α
C3α
C2α
C4β C3β
C5α
α-D-arabinose
β-D-arabinose

was determined that most of the sugar residues contained in the fraction are monomers of arabinoside. The monoarabinose isomers were determined using GC-MS analysis as shown in Figure 29 (DeJongh *et al.*, 1969).

Although less than 5% of the original walls from the ether/HF extracted residue was extracted with water, this fraction contains the sugars, namely Rha, GalA, Xyl, Gal, Glc, and Ara in the mole percentages of 6.4%, 12.8%, 10.9%, 13.9%, 27.0%, and 28.8% respectively. This suggests a combination of xyloglucan and rhamnogalacturonan I in the form of XG-RGI complexes.

Since these XG-RG I complexes were released by HF treatment at a very low temperature (-73°C), and were not solubilized by pretreatment with EPGase and cellulase, these complexes may be insolubilized into the wall with an unidentified arabinosidic linkage. Therefore, this experiment may provide a method to investigate crosslinks between XG and RG I wall polymers.

As in the sequential treatment (HF -23°C, cellulase, trypsin) described in Figure 19, fifty percent of the Hyp was liberated from residue C by trypsin digestion after pretreating the wall with EPGase, cellulase and HF at -73°C. However, it is safer to treat cell walls with HF at low temperature in order to leave as many unidentified crosslinks as possible between wall polymers. The composition of the sugars solubilized along with Hyp during this control experiment is shown in Table 12.

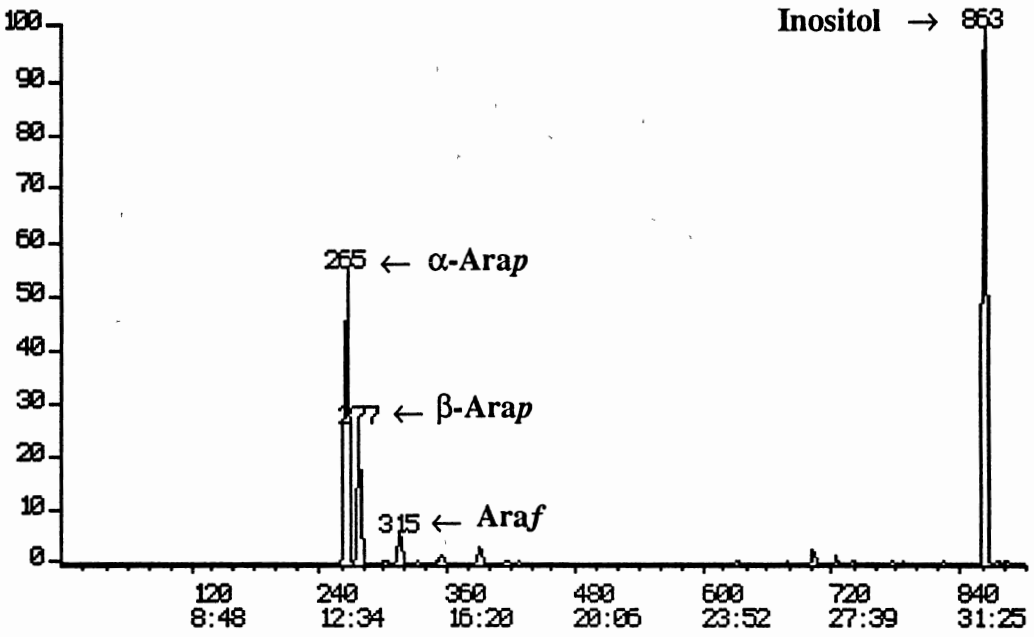
Analysis of Ammonium Bicarbonate Buffer-Extracted
Filtrate D and Residue D

As shown in Table 12, large amounts of galacturonic acid and rhamnose residues were found in the trypsin solubilized mixture. A question arises as to whether these RG I-like sugars were co-solubilized with Hyp-containing proteins because they occur in covalent crosslink with the wall proteins. Residue C was extracted with the same buffer used for trypsin digestion, 100 mM ammonium bicarbonate (pH 7.6). The extraction was carried

Figure 29. Gas Chromatogram of Trimethylsilyl Derivatives of Carbohydrates in Filtrate C (Ether/HF Extract) of Figure 23, and the Mass Spectrum of the Peaks of the Chromatogram.

(A) Gas chromatogram. Peaks were identified from mass spectra as follows: 265: α-arabinopyranose; 277: β–arabinopyranose; 315: arabinofuranose. (B) Mass spectra of α-arabinopyranose, β-arabinopyranose; and arabinofuranose.

A.


Inositol → 863
265 ← α-Arap
277 ← β-Arap
315 ← Araf
100
90
80
70
60
50
40
30
20
10
0
120
8:48
240
12:34
360
16:20
480
20:06
600
23:52
720
27:39
840
31:25

B.

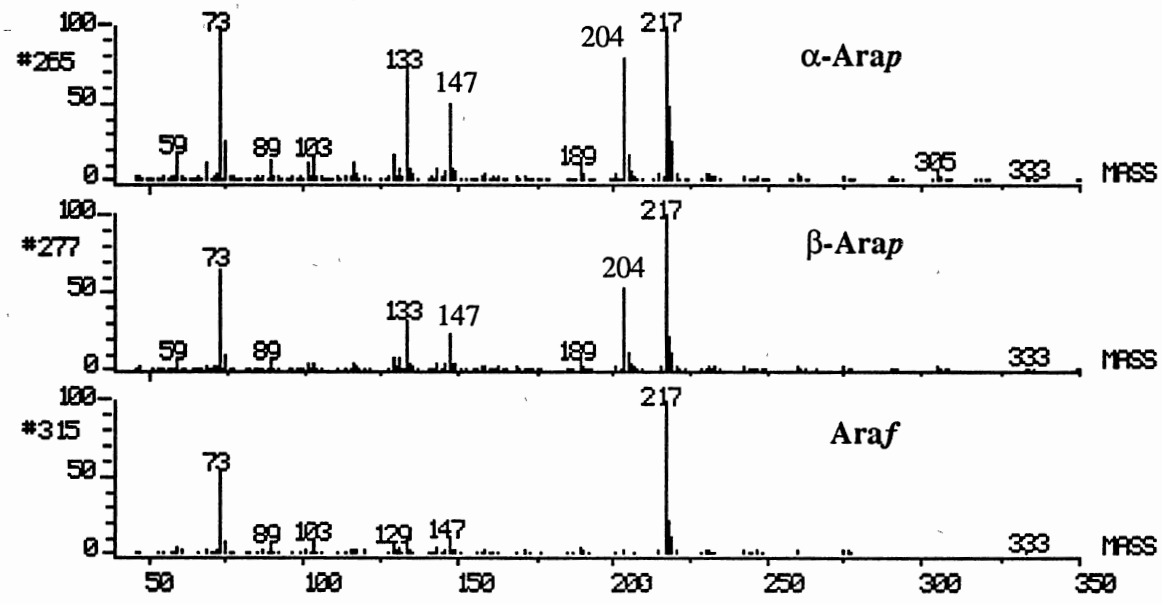

#265
α-Arap
#277
β-Arap
#315
Araf
MASS
73
59
89
103
133
147
189
204
217
305
333
129
50
100
150
200
250
300
350

TABLE 12

MOLE PERCENT OF SUGARS IN THE SOLUBLE AND THE INSOLUBLE PORTIONS OFCELLULASE TREATED-RESIDUE AFTER HF SOLVOLYSIS AT -73°C FOLLOWED BY TRYPSIN DIGESTION

Material	Ara	Rha	Fuc	Xyl	GalA	Gal	Glc	wt%*	wt (mg)
Residue (Cellulase)	32.1	15.7	—	8.2	17.9	11.2	14.7	44.5	250
Ether Extract (HF, -73°C)	100.0	—	—	—	—	—	—	—	—
Water Extract (HF, -73°C)	28.8	6.4	—	10.9	12.8	13.9	27.0	30.5	11
Residue (HF, -73°C)	10.2	26.2	—	5.6	35.8	15.4	6.4	41.3	160
Filtrate (Trypsin)	10.3	25.5	—	6.8	32.2	17.5	6.5	28.7	60
Residue (Trypsin)	9.1	27.7	—	5.5	35.7	15.7	6.4	54.6	95

* Same as indicated in Table 1.

out overnight at room temperature with gentle stirring. The extraction solution was then filtered and washed three times with distilled water. Residue D and combined filtrate D were assayed for sugar compositions which is given in Table 13. About 3.8% of the weight of the walls was extracted, and the filtrate contained mostly Rha (12.2%), Ara (20.8%), and GalA (39.4%). No Hyp was detected in the filtrate. At that point, only 11.5% of original weight of the walls remained insoluble in residue D. Residue D contains more than 98% of the Hyp content and some sugars characteristic of the RG I molecule. Indeed, this step removed the noncovalently-linked RG I like sugars from the residue C.

Analysis of Trypsin-Treated Filtrate E and Residue E

Fifty percent of the Hyp along with approximately 4% of the RG I-like sugars were liberated from residue D with trypsin digestion. The sugar compositions of the filtrate are given in Table 13. These RG I-like sugars were not solubilized by EPGase and cellulase, therefore the RG I fragments must be strongly attached to the wall polymers. Most likely, the RG I-like sugars are associated with the wall proteins by covalent crosslinks. Therefore, they would not become soluble unless the proteins were released from the walls by trypsin digestion. On the other hand, if any crosslinks exist in between extensin and the RG I polymers, they should remain in their original form in the wall under the sequential treatment conditions. Thus, the filtrate mixture is a reasonable candidate for investigating unidentified crosslinks which hold extensin and RG I macromolecules together.

In residue E, 48% of the Hyp and about 9% of sugars still remained insoluble. The amino acid and sugar compositions of residue E are presented in Table 13. Sugars contained in residue E are predominantly RG I-like sugars. The fact that the RGI-like sugars are insolubile in residue E indicates that they are crosslinked to the wall proteins. After the sequential treatment, the insoluble materials consisted of about ~40% proteins, ~35% the sugars, and ~25% other unidentified compounds. Further analysis of residue E will be described elsewhere.

TABLE 13

MOLE PERCENT OF SUGARS IN THE SOLUBLE AND THE INSOLUBLE PORTIONS OF -73°C HF TREATED-RESIDUE EXTRACTED WITH AMMONIUM BICARBONATE BUFFER FOLLOWED BY TRYPSIN DIGESTION

Material	Ara	Rha	Fuc	Xyl	GalA	Gal	Glc	Glc#	wt%*	wt (mg)
Residue (HF -73°C)	10.2	26.2	—	5.6	35.8	15.4	6.4	—	41.3	160
Filtrate (NH_4HCO_3 Buffer)	20.8	12.2	—	8.3	39.4	9.1	9.3	—	41.7	38
Residue (NH_4HCO_3 Buffer)	7.2	26.6	—	4.9	44.4	14.6	2.3	—	42.2	115
Filtrate (Trypsin)	21.4	17.5	—	4.8	35.3	15.7	2.9	—	33.5	32
Residue (Trypsin)	6.0	25.0	—	4.4	40.5	14.1		9.3	63.9	80

Glucose content was determined after HF solvolysis at 0°C.
* Same as indicated in Table 1.

Separation of Extensin-RG I Complexes by Gel Filtration Chromatography

The filtrate collected from the trypsin digestion of residue D was freeze-dried. The dried sample was dissolved in 0.1% trifluoroacetic acid (pH ~2.0), and applied to a Fracto-gel HW50(S) gel filtration column. The flow rate was 0.5 mL per minute, and the fractions were collected as 1 mL per tube. Extensin-RG I complexes were detected at 215 nm (see Figure 30). Three fractions were pooled as fraction A (from tubes 16 to 20) in void volume, B (from tubes 33 to 49) in the fractionation range, and C (from tubes 50 to 70) in the range near the included volume. Analyses of the amino acids and sugars are shown in Table 14. Fraction A contains 15% Hyp, fraction B contains 23% Hyp, and C contains 8% Hyp. Fraction A, called extensin-RG I complexes A, contains a high concentration of RG I-like polymers as determined using Dionex CarboPac PA-1 HPLC ion exchange chromatography (see Figure 31). Most of the Hyp-containing proteins were eluted in the range from fractions 40 to 60. Fraction B, called extensin-RG I complexes B, contains some arabinose residues and smaller amount of RG I-like sugars.

The previous study showed that the tryptic peptides of cotton extensin were fairly small molecules. These peptides were fractionated on a Fracto-gel HW40(S) column (fractionation range from MW 100 to 10,000 daltons). The extensin-RG I complexes from the sequential treatment behaved like very large molecules on Fracto-gel HW50(S) column (fractionation range from MW 800 to 90,000 daltons). There are at least two explanations for this experimental result. (1) since extensin peptides are heavily glycosylated with RG I-like sugars, the carbohydrate portion may contribute to the size of the complexes. (2) Extensin molecules may have cleaved at lower frequency since the RG I polysaccharides associated with the extensin and/or residual Hyp arabinosides may have prevented trypsin access to the proteins. Both of these explanations were verified by the following experiment.

Figure 30. Chromatography on an HW 50(S) Gel Filtration Column of the Filtrate E of Figure 23.

Sample was applied in and eluted with 0.1% trifluoroacetic acid. The flow rate was 0.5 mL/fraction/min. Detection was by UV monitor at 215 nm. Tubes 16-20 were pooled into fraction A (complexes A), tubes 33-49 were pooled into fraction B (complexes B), and tubes 50-70 were pooled into fraction C.

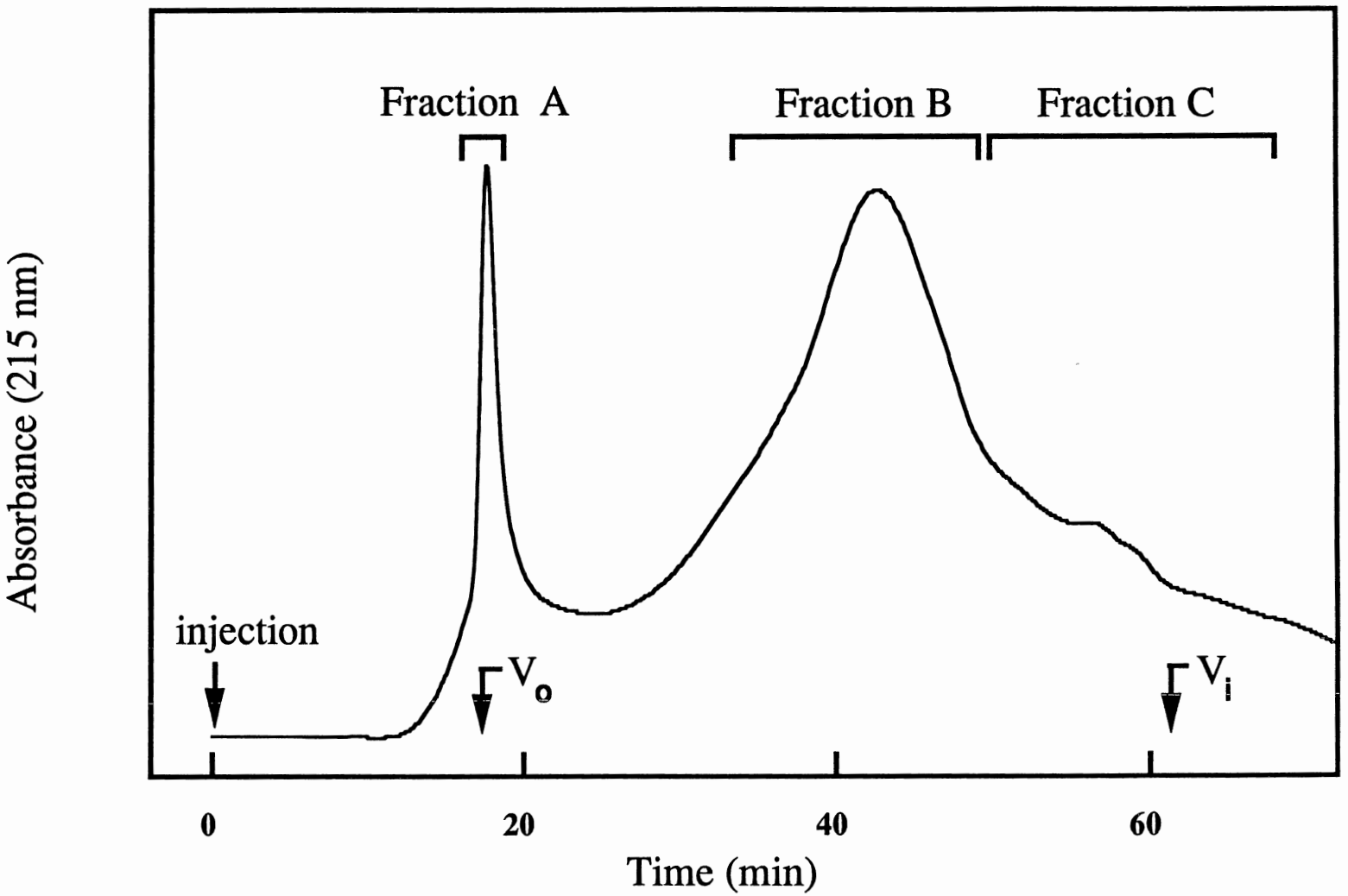

Fraction A
Fraction B
Fraction C
injection
V_o
V_i
Absorbance (215 nm)
0
20
40
60
Time (min)

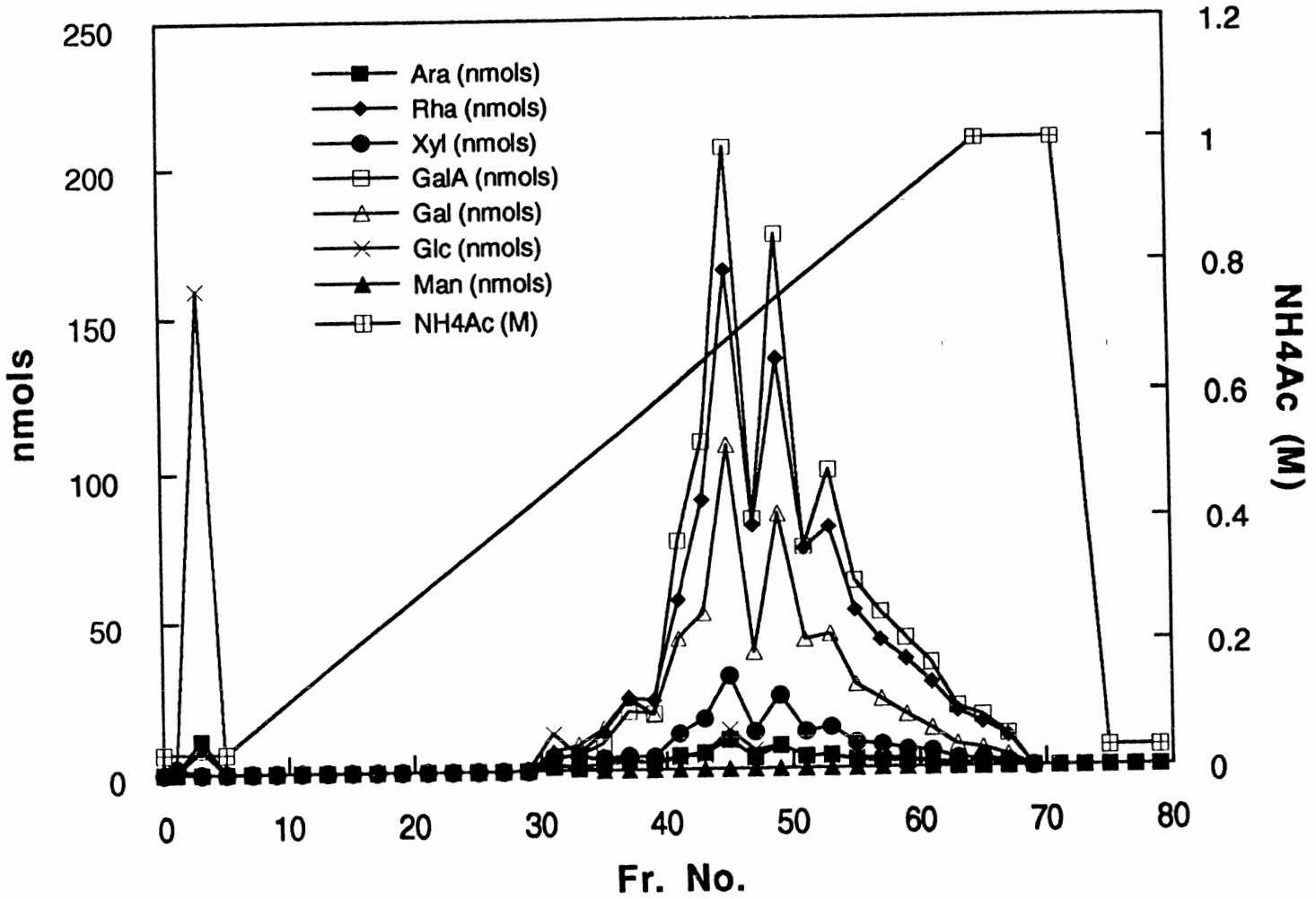
TABLE 14

AMINO ACID AND SUGAR COMPOSITIONS (NMOLES/MG) IN THE FRACTIONS OF HW50(S) GEL FILTRATION CHROMATOGRAPHY FROM THE FINAL FILTRATE AFTER TRYPSIN DIGESTION IN THE SEQUENCIAL TREATMENT OF ONE MILLIGRAM CELL WALLS

Amino Acid Residue	nmoles/mg Fraction A	Fraction B	Fraction C	Sugar Residue	nmoles/mg Fraction A	Fraction B	Fraction C
Hyp	624	825	389	Ara	390	546	74
Ser	149	527	343	Rha	1,246	95	6
Gly	48	425	441	Xyl	271	54	8
Thr	33	233	217	GalA	2,158	286	—
Arg	27	243	237	Gal	784	594	28
Ala	53	269	262	Glc	55	331	29
Pro	52	266	259				
Tyr	42	147	173				
Val	57	328	302				
Ile	22	175	181				
Leu	36	349	379				
Phe	66	93	175				
Lys	34	161	366				
wt (mg)	10	12	9	wt (mg)	10	12	9
protein (%)	13	42	39	sugar (%)	79	31	2
Hyp (μg)	705	1,119	396				

Figure 31. Chromatography on a Dionex CarboPac PA-1 HPLC Ion Exchange Column of Extensin-RG I Complexes A.

A linear gradient of ammonium acetate from 0.03 to 1.00 M over 60 minutes followed by a steady elution of 1.00 M for 10 minutes was applied to elute the sample. The sugar composition contained in each peak were analyzed using gas chromatography method. RG I polymer was used as "standard" to eluted on this column under the same conditions (chromatogram are not presented).


Ara (nmols)
Rha (nmols)
Xyl (nmols)
GalA (nmols)
Gal (nmols)
Glc (nmols)
Man (nmols)
NH4Ac (M)
nmols
NH4Ac (M)
Fr. No.
0
50
100
150
200
250
0.2
0.4
0.6
0.8
1
1.2
0
10
20
30
40
50
60
70
80

Arabinoside-Hydroxyproline Profile Analysis of Extensin-RG I Complexes

Fifty milligrams (~300 μg of Hyp) of Acala 44 cotton cell walls were hydrolyzed in excess 0.2 M $Ba(OH)_2$ at for 18 h at 105°C. Two milligrams (~50 μg of Hyp) each of extensin-RG I complexes A and B were hydrolyzed in the same way. Alkaline hydrolysis of the cell walls and isolated extensin-RG I complexes released racemic mixtures of free hydroxyproline and hydroxyproline arabinosides. The hydrolysates were neutralized with H_2SO_4, centrifuged, and then the supernatants were freeze dried (Lamport and Miller, 1971).

After redissolving the dry samples in 200 μL of water, the samples were applied to a Technicon Chromobeads C column at 65°C by Marcia Kieliszewski at Michigan State University. The free hydroxyproline and hydroxyproline arabinosides were separated in the following sequence: the *trans* and *cis* hydroxyproline tetraarabinosides, *trans* and *cis* hydroxyproline triarabinosides, *trans* and *cis* hydroxyproline diarabinosides, *trans* and *cis* hydroxyproline monoarabinosides, and *trans* and *cis* free hydroxyprolines.

The profiles of hydroxyproline arabinosides for cotton cell walls, complexes A and B are given in Table 15. As found in other dicotyledous plant species (Lamport and Miller, 1971), cotton cell walls showed Hyp-$(Ara)_4$ and Hyp-$(Ara)_3$ predominating, 48% (relative mole percent) and 31%, respectively. Only 12% of the Hyp was unsubstituted and very small amount of Hyp-$(Ara)_2$ (4%) and Hyp-Ara (5%) were found. Complexes A and B showed that the percentage of Hyp-Ara and free Hyp were dramatically increased and no Hyp-$(Ara)_4$, Hyp-$(Ara)_3$ and Hyp-$(Ara)_2$ were found in either sample. The results indicate that HF -73°C treatment cleaved most of the arabinose on the Hyp residues. On the other hand, the data showed that more than 50% of the Hyp contained in both complexes A and B are substituted with a single arabinose residue. It is suggested that the linkage between the arabinose residue and the hydroxyproline residue of the extensin molecule is

TABLE 15

HYDROXYPROLIN ARABINOSIDE PROFILES OF COTTON SUSPENSION CULTURE CELL WALLS AND EXTENSIN-RG I COMPLEXES A AND B

	Hydroxyproline Arabinoside				Unsubstituted
Sample	Hyp-$(Ara)_4$*(%)	Hyp-$(Ara)_3$ (%)	Hyp-$(Ara)_2$ (%)	Hyp-Ara (%)	Hyp (%)
Acala 44 Cotton Intact Cell Walls	48	31	4	5	12
Extensin-RG I Complexes A	0	0	0	61	39
Extensin-RG I Complexes B	0	0	0	55	45

* Hyp-$(Ara)_4$ is hydroxyproline tetraarabinoside.

somewhat resistant to the HF solvolysis at -73°C. Lamport has also noticed the significant acid stability of the carbohydrate-protein linkage which involves the hydroxyproline hydroxyl group in a glycosidic linkage with arabinose (personal communication).

Separation of HF-Deglycosylated Extensin-RG I Complexes

The extensin-RG I complexes A and B in fraction A and B of Figure 29 were treated separately with HF at 0°C for 30 minutes to remove all of the sugars from the proteins. The reaction mixtures were fractionated on a Fracto-gel HW50(S) column as shown in Figure 32 and 33. After HF solvolysis at 0°C, the deglycosylated form of the extensin-RG I complexes in fraction A eluted later on the HW50(S) column than the original complexes under exactly the same conditions (comparing Figure 32 to Figure 30). This indicated that the extensin fragments in fraction A are heavily glycosylated with RG I-like sugars, since 61% of the Hyp residues in extensin are only attached by a single arabinose residue (see Table 15). Comparing the deglycosylated form of extensin-RG I complexes in fraction B to the glycosylated form, there is a slight delay in the retention time on a Fracto-gel HW50(S) column (comparing Figure 33 and Figure 30). This indicates that the extensin fragments in fraction B are lightly glycosylated with RG I-like sugars. These results are consistent with the sugar composition analyses of fractions A and B which are listed in Table 14. In both cases, however, the deglycosylated forms of extensin-RG I complexes eluted mostly in the fractionation range of the Fracto-gel HW50(S) gel filtration column. The extensin fragments liberated by the sequential treatment are apparently large molecules.

The glycosylated and deglycosylated forms of extensin-RG I complexes in both fraction A and B were subjected to SDS-PAGE analysis. Fifty to one hundred μg of each sample was applied to the gel (12% of separating gel). The resulting gels were stained with coomassie blue to visualize the proteins, and with fuchsin sulfite dye to visualize the carbohydrates (see Figure 34). The glycosylated form of the complexes in fraction A

Figure 32. Chromatography on an HW 50(S) Gel Filtration Column of HF-Deglycosylated Extensin-RG I Complexes A.

Sample was applied in and eluted with 0.1% trifluoroacetic acid. The flow rate was 0.5 mL/fraction/min. Detection was by UV monitor at 215 nm. Tubes 16-24 were pooled into fraction A1, tubes 26-41 were pooled into fraction A2, and tubes 55-70 were pooled into fraction A3.

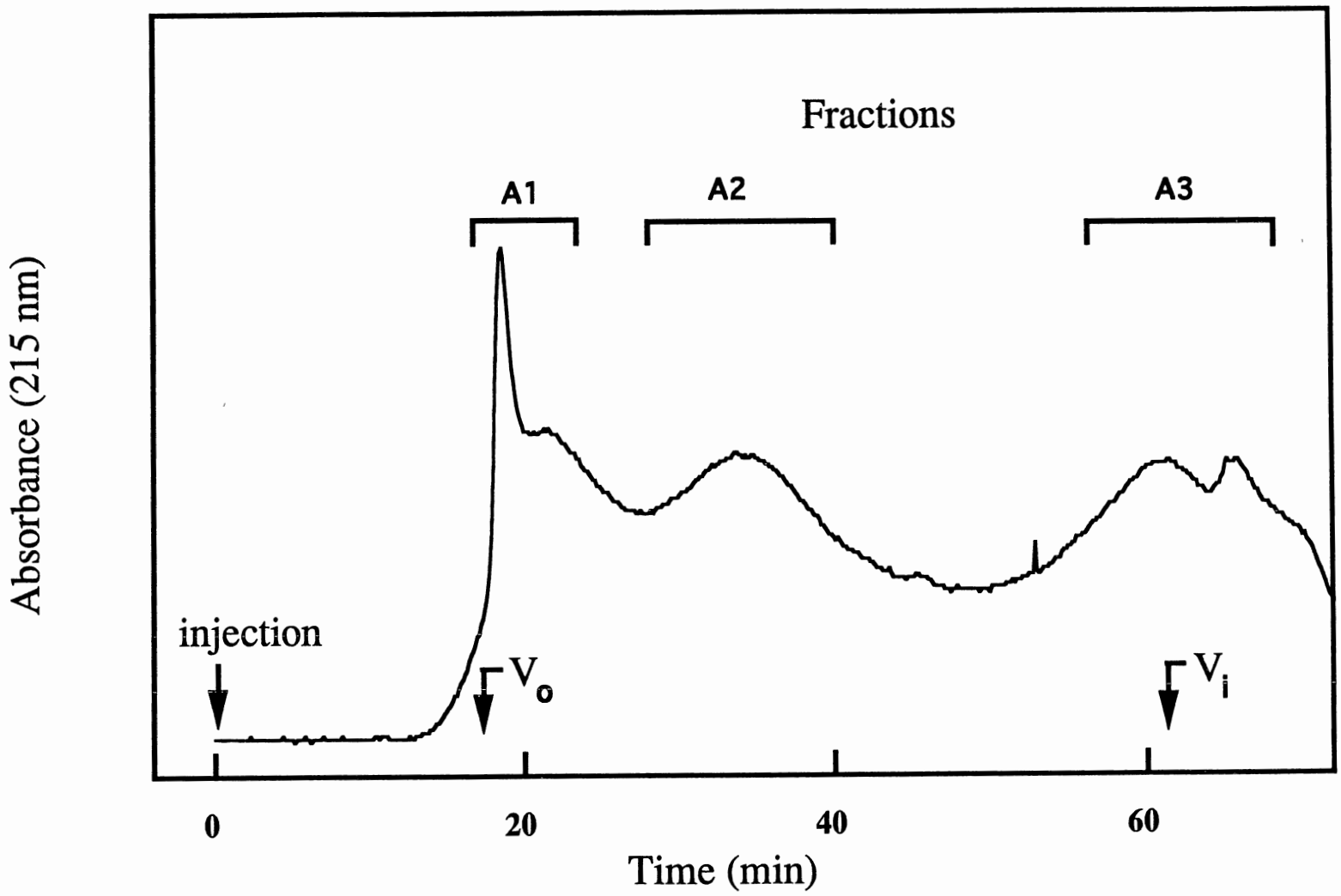

Fractions
A1
A2
A3
injection
V_o
V_i
Absorbance (215 nm)
0
20
40
60
Time (min)

Figure 33. Chromatography on an HW 50(S) Gel Filtration Column of HF-Deglycosylated Extensin-RG I Complexes B.

Sample was applied in and eluted with 0.1% trifluoroacetic acid. The flow rate was 0.5 mL/fraction/min. Detection was by UV monitor at 215 nm. Tubes 31-42 were pooled into fraction B1, tubes 43-48 were pooled into fraction B2, tubes 49-56 were pooled into fraction B3, and tubes 57-70 were pooled into fraction B4..

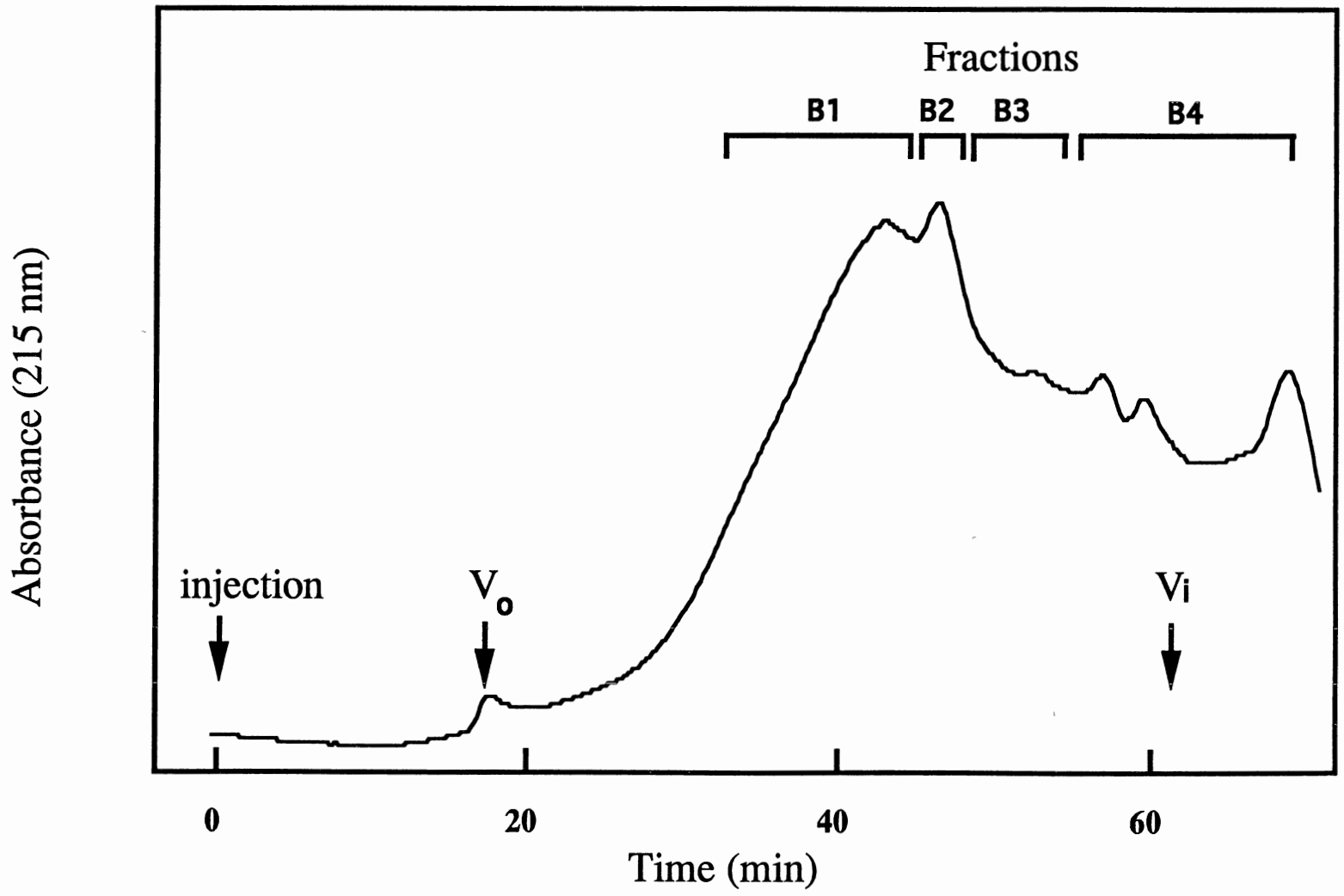
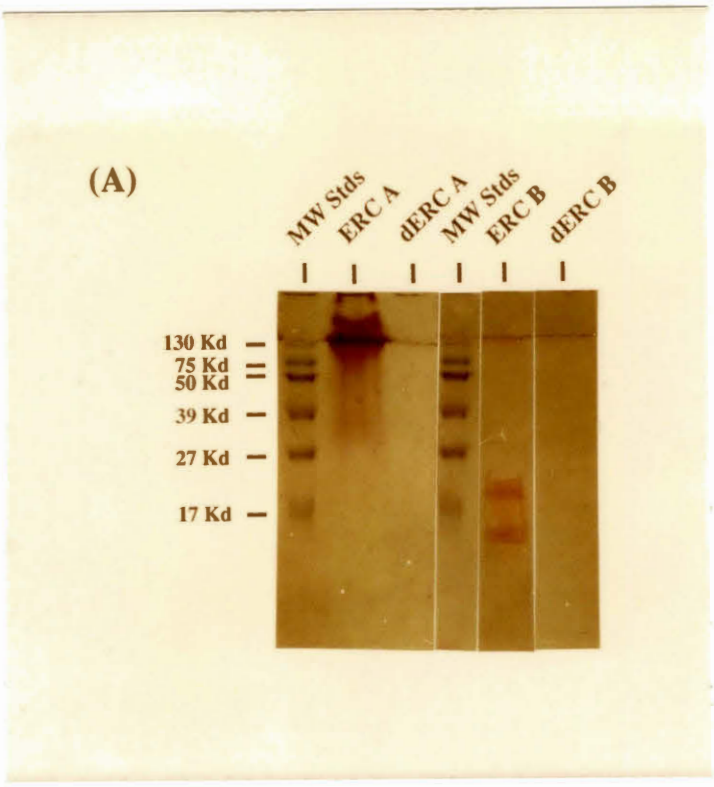
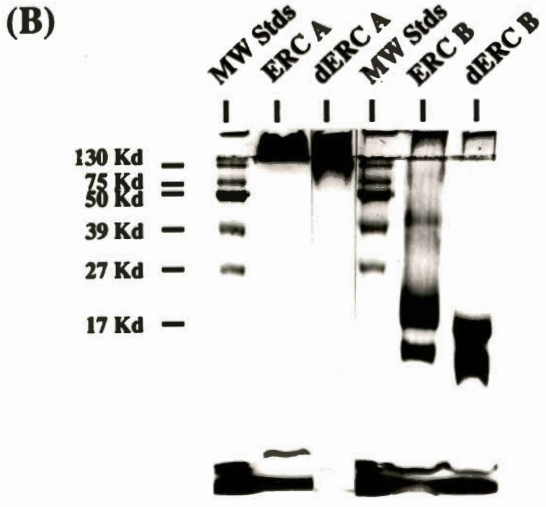

Fractions
B1
B2
B3
B4
injection
V_o
V_i
Absorbance (215 nm)
0
20
40
60
Time (min)

Figure 34. 12% SDS-PAGEs of Extensin-RG I Complexes A and B, and Their HF-Deglycosylated Forms.

(A) Staining of carbohydrates in the SDS-PAGE. (B) Staining of proteins in the SDS-PAGE. Lanes 1 and 4 contain MW markers as indicated on the far left (Phosphorylase b, 130 kD; Bovine Serum Albumin, 75 kD; Ovalbumin, 50 kD; Carbonic Anhydrase, 39 kD; Soybean Trypsin Inhibitor, 27 kD; Lysozyme, 17 kD). Lane 2 and 3 in both (A) and (B) contain 100 μg of extensin-RG I complexes A (ERC A) and deglycosylated extensin-RG I complexes A (dERC A), respectively. Lane 5 and 6 in (A) contain 100 μg of extensin-RG I complexes B (ERC B) and deglycosylated extensin-RG I complexes B (dERC B), respectively. Lane 5 and 6 in (B) contain 50 μg of ERC B and dERC B, respectively.


(A)
MW Stds
ERC A
dERC A
MW Stds
ERC B
dERC B
130 Kd
75 Kd
50 Kd
39 Kd
27 Kd
17 Kd


(B)
MW Stds
ERC A
dERC A
MW Stds
ERC B
dERC B
130 Kd
75 Kd
50 Kd
39 Kd
27 Kd
17 Kd

remained in the stacking gel because of its heavy glycosylation. Although the glycosylated form of the complexes in fraction B moved into the separating gel, the bands were diffuse. However, both of the deglycosylated forms of the complexes in fraction A and B resolved well in the gels. Migration was greatly increased on the gels. Once again, the results are consistent with the results obtained from the gel filtration chromatography.

Tryptic Peptide Mapping of HF-Deglycosylated Extensin-RG I Complexes

Tryptic peptide mapping of HF-deglycosylated extensin-RG I complexes A and B (after separation by an HW50(S) gel filtration chromatography) was done using exactly the same procedure as described in the section 'characterization of cotton cell wall extensin'. Fractions A1, A2 and A3 of the HF-deglycosylated complexes A collected from the HW50(S) gel filtration column were mapped with tryptic peptides. Only fraction A2 contains peptides in fraction I' (see Figure 35) which has exactly same retention time as cotton extensin peptides characterized in fraction I of Figure 5. Fractions A1 and A3 have other unidentified peptides. Likewise, the fractions B1, B2, B3 and B4 of HF-deglycosylated complexes B collected from HW50(S) gel filtration column were mapped for tryptic peptides. Fractions B1, B2 and B3 were mapped as cotton extensin peptides in fraction I of Figure 5. Among them, fraction B2 contains large amount of these extensin peptides which eluted in fraction I" of Figure 36. These results together with previous experiments shown that both complexes A and B contain cotton extensin peptide-containing proteins, and those proteins were glycosylated with RG I fragments.

Cross-Reactivities of dP1 and dP2 Antibodies with Extensin-RG I Complexes

Enzyme-linked immunosorbent assay (ELISA) has been used to determine the cross reactivities of polyclonal antibodies obtained from Marcia Kieliszewski at Michigan State

Figure 35. The HPLC Chromatogram of the Trypsin Treated Fraction A2 of Figure 32 on Reverse Phase C_{18} Column.

The elution buffer consisted of solvent A; 0.1% trifluoroacetic acid (pH ~2), solvent B; 0.1% trifluoroacetic acid/acetonitrile (37.5:62.5). The gradient composition was 0-30% solvent B in 60 minutes, 30-100% B in 5 minutes and 100% B for 10 minutes. The flow rate was 1 mL/min. Detection was by UV monitor at 214 nm. The sample was dissolved in 0.1% trifluoroacetic acid and injected. Fraction I' was eluted exactly at the same time as fraction I of figure 5 under the same conditions.

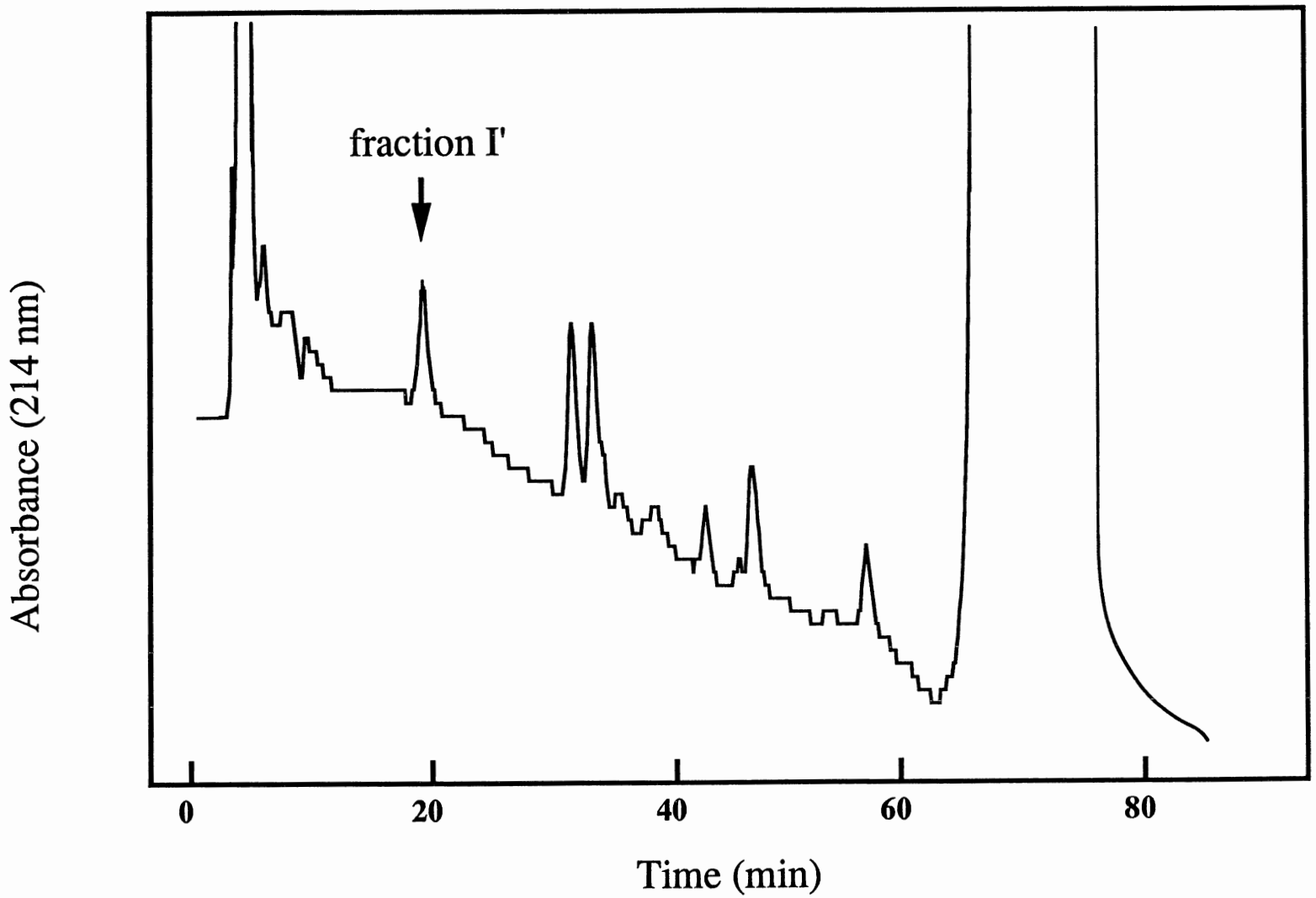
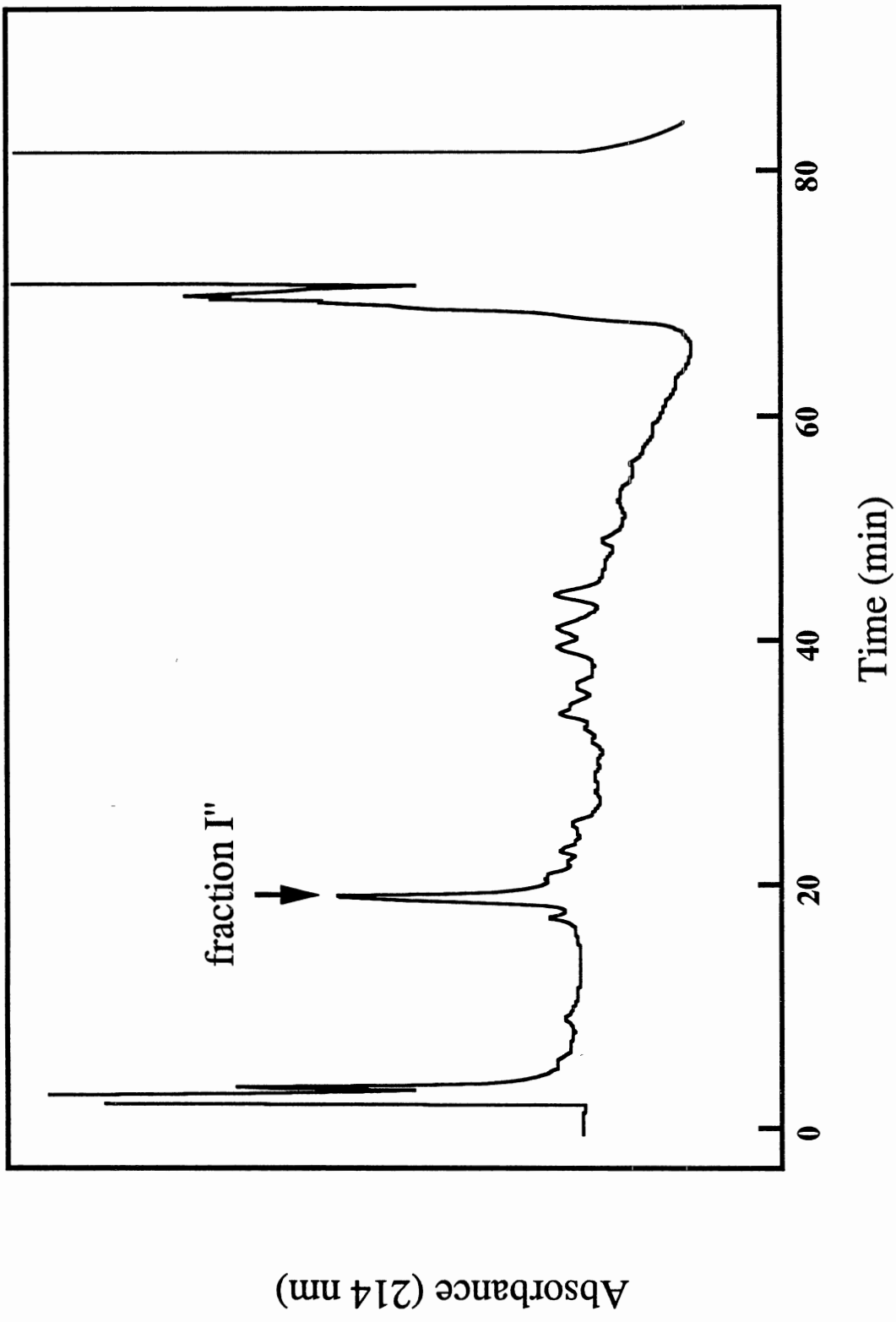

fraction I'
Absorbance (214 nm)
0
20
40
60
80
Time (min)

Figure 36. The HPLC Chromatogram of the Trypsin Treated Fraction B2 of Figure 33 on Reverse Phase C_{18} Column.

The elution buffer consisted of solvent A; 0.1% trifluoroacetic acid (pH ~2), solvent B; 0.1% trifluoroacetic acid/acetonitrile (37.5:62.5). The gradient composition was 0-30% solvent B in 60 minutes, 30-100% B in 5 minutes and 100% B for 10 minutes. The flow rate was 1 mL/min. Detection was by UV monitor at 214 nm. The sample was dissolved in 0.1% trifluoroacetic acid and injected. Fraction I" was eluted exactly at the same time as fraction I of figure 5 under the same conditions.


Absorbance (214 nm)
fraction I"
0
20
40
60
80
Time (min)

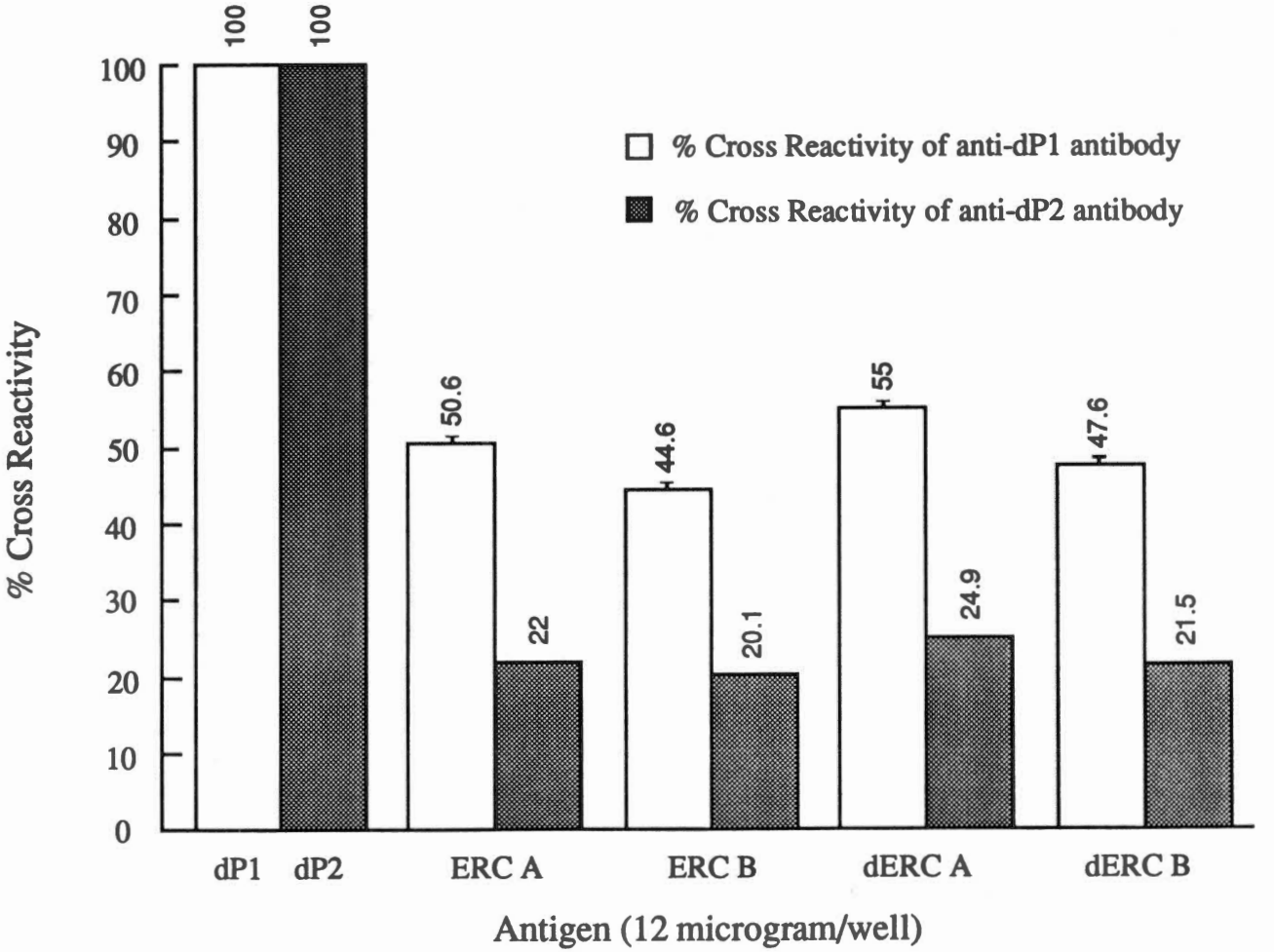
University raised against the HF-deglycosylated tomato extensins dP1 and dP2 precursors with extensin-RG I complexes A and B in fractions A and B of figure 30.

ELISA is an especially sensitive, indirect, non-competitive immunological technique which is used in detecting common antigenic determinants on molecules. Therefore, a definitive identification of cotton extensins in the complexes A and B can be made by this assay. The extent of antibody cross-reactivity depends upon the number of common epitopes that exist between the deglycosylated tomato precursors, dP1 and dP2, the complexes A and B, and their HF-deglycosylated molecules. There are two types of antigenic determinants (epitopes) in each deglycosylated antigenic precursor of concern: nonglycosylated epitopes of the intact glycoprotein and epitopes exposed only after deglycosylation. From the analysis of the tryptic peptide map, dP1 contains two different peptide blocks: Ser-(Hyp)$_4$-Thr-Hyp-Val-Tyr-Lys and Ser-(Hyp)$_4$-Val-Lys-Pro-Tyr-His-Pro-Thr-Hyp-Val-Tyr-Lys. dP2 consists of a single-repeating decapeptide, Ser-(Hyp)$_4$-Val-Tyr-Lys-Tyr-Lys (Smith *et al.*, 1986). It has been suggested that the sequence Val-Lys-Pro-Tyr-His-Pro is the major nonglycosylated epitope of tomato extensin precursor P1, and Val-Tyr-Lys-Tyr-Lys is the major nonglycosylated epitope of tomato extensin precursor P2 (Kieliszemski and Lamport, 1986).

Figure 37 shows the cross-reactivities of dP1 and dP2 antibodies with extensin RG I complexes A and B, including their HF-deglycosylated forms as well. Results indicated that the polyclonal antibodies raised against the HF-deglycosylated antigens dP1 and dP2 cross-react with complexes A and B, and their deglycosylated forms. The cross-reactivities of dP1 antibody with complexes A and B, and deglycosylated complexes A and B are 50.6±1.0%, 44.6±1.0%, 55.0±2.2% and 47.6±0.6%, respectively. The cross-reactivities of dP2 antibody with the complexes A and B, and deglycosylated complexes A and B are 22.0±0.0%, 20.1±0.1%, 24.9±0.6% and 21.5±0.2%, respectively. Therefore, the cross-reactivities of dP1 antibody with the cotton extensins are more than two times higher than dP2 antibody. Slightly stronger cross-reactivities with either dP1 or dP2 antibody, 1.9-

Figure 37. Cross-Reactivity of dP1 and dP2 Antibodies with Extensin-RG I Complexes A (ERC A), Extensin-RG I Complexes B (ERC B), Deglycosylated ERC A (dERC A) and Deglycosylated ERC B (dERC B).

After coating micro-ELISA wells with 12 μg of ERC A, ERC B, dERC A and dERC B, 200 μL of diluted serum (x 800 for dP1 and dP2) was added expressing cross-reactivity of a given antibody with each antigen, as a percentage of the appropriate homologous antibody/antigen reaction (e.g. dP1/dP1 or dP2/dP2).


100
100
100
90
80
70
60
50
40
30
20
10
0
% Cross Reactivity
% Cross Reactivity of anti-dP1 antibody
% Cross Reactivity of anti-dP2 antibody
50.6
22
44.6
20.1
55
24.9
47.6
21.5
dP1
dP2
ERC A
ERC B
dERC A
dERC B
Antigen (12 microgram/well)

7.4%, is shown for complexes A and their deglycosylated form than complexes B and their deglycosylated form. Interestingly, the cross-reactivities of antibody dP1 (or dP2) with the glycosylated forms of complexes A and B are very close to the cross-reactivities with their deglycosylated forms. Taken together, the results indicated a great similarity in the nonglycosylated domains (not those exposed after deglycosylation) of intact glycoprotein between cotton extensins in the isolated complexes and tomato extensin precursor P1 as compared to precursor P2.

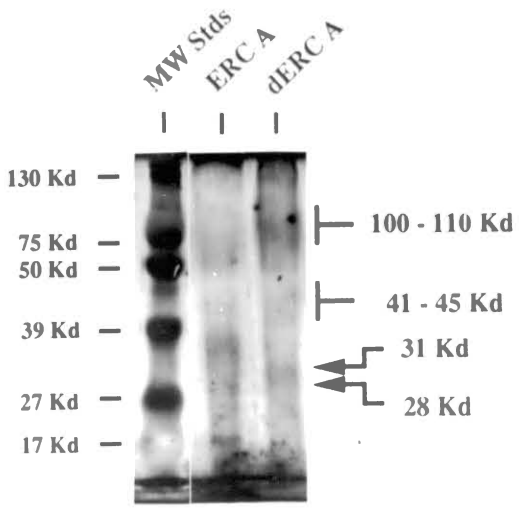
Western Blot Analyses of Extensin-RG I Complexes

Western blot analyses of the reactivities of complexes A and B with polyclonal antibodies raised against the deglycosylated tomato extensin precursor dP1 (selected because of its high cross-reactivities with cotton extensin molecules) were performed in order to specifically determine whether the isolated cotton cell wall proteins in complexes A and B after SDS-PAGE (see Figure 38) are "extensin-like molecules". The results are shown in Figure 39. In Figure 39 (A), extensin-RG I complexes A (ERC A) recognized by the dP1 antibody did not migrate into the separating gel, probably because of its heavy glycosylation. The deglycosylated extensin-RG I complexes A (dERC A) were separated in the separating gel as shown in Figure 39 (B). Two resolved bands (molecular weight relative to standards 28 kD and 31 kd) stained with the dP1 antibody, and several bands were not well resolved in between 41 to 45 kD, and others between 100 to 110 kD. These bands strongly stained with the dP1 antibody were also found in an SDS-PAGE gel (Figure 38, A) after blotting, as major protein bands stained with silver reagent. This result indicated that the majority of proteins of complexes A are cotton extensin-like molecules. In figure 39 (B), although extensin-RG I complexes B (ERC B) migrated into the separating gel, the bands stained with the dP1 antibody were diffuse and not resolved at all. After HF 0°C treatment, the deglycosylated extensin-RG I complexes B (dERC B) recognized by the dP1 antibody were resolved very well in the gel. Two bands (molecular weight relative to

Figure 38. SDS-PAGE of Extensin-RG I Complexes A and B, and Their HF-Deglycosylated Forms.

(A) 10% SDS-PAGE of extensin-RG I complexes A. (B) 15% SDS-PAGE of extensin-RG I complexes B. Lanes 1 in both (A) and (B) contain MW markers as indicated on the far left (Phosphorylase b, 130 kD; Bovine Serum Albumin, 75 kD; Ovalbumin, 50 kD; Carbonic Anhydrase, 39 kD; Soybean Trypsin Inhibitor, 27 kD; Lysozyme, 17 kD). Lane 2 and 3 in (A) contain 20 μg of extensin-RG I complexes A (ERC A) and deglycosylated extensin-RG I complexes A (dERC A), respectively. Lane 2 and 3 in (B) contain 50 μg of extensin-RG I complexes B (ERC B) and deglycosylated extensin-RG I complexes B (dERC B), respectively.

(A)


MW Stds
ERC A
dERC A
130 Kd
75 Kd
50 Kd
39 Kd
27 Kd
17 Kd
100 - 110 Kd
41 - 45 Kd
31 Kd
28 Kd

(B)

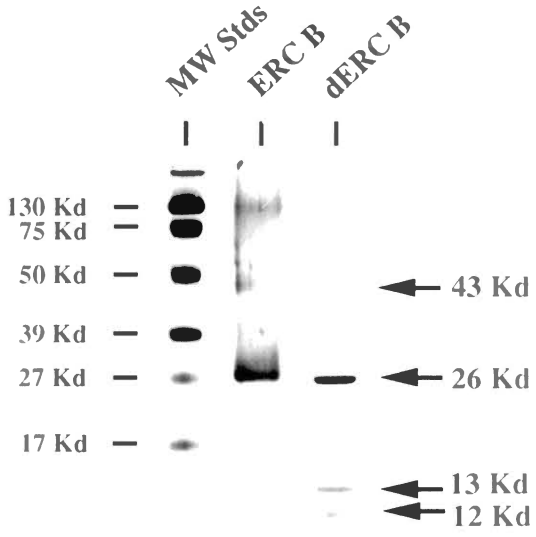
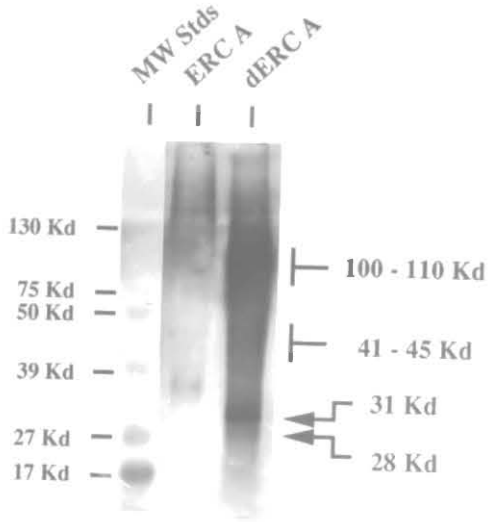

MW Stds
ERC B
dERC B
130 Kd
75 Kd
50 Kd
39 Kd
27 Kd
17 Kd
43 Kd
26 Kd
13 Kd
12 Kd

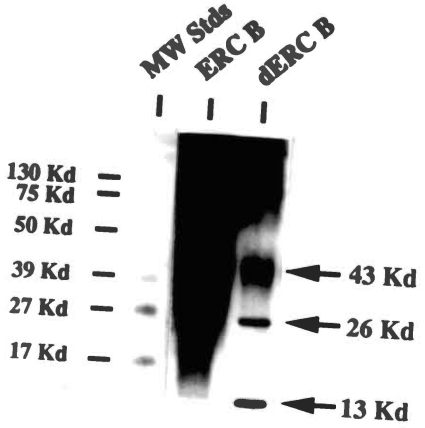
Figure 39. Western Blot Analyses of Extensin-RG I Complexes A and B, and Their HF-Deglycosylated Forms.

(A) Western blot analysis of extensin-RG I complexes A with a 10% SDS-PAGE. (B) Western blot analysis of extensin-RG I complexes B with a 15% SDS-PAGE. Lanes 1 in both (A) and (B) contain MW markers as indicated on the far left (Phosphorylase b, 130 kD; Bovine Serum Albumin, 75 kD; Ovalbumin, 50 kD; Carbonic Anhydrase, 39 kD; Soybean Trypsin Inhibitor, 27 kD; Lysozyme, 17 kD). Lane 2 and 3 in (A) contain 20 μg of extensin-RG I complexes A (ERC A) and deglycosylated extensin-RG I complexes A (dERC A), respectively. Lane 2 and 3 in (B) contain 50 μg of extensin-RG I complexes B (ERC B) and deglycosylated extensin-RG I complexes B (dERC B), respectively.

(A)


MW Stds
ERC A
dERC A
130 Kd
75 Kd
50 Kd
39 Kd
27 Kd
17 Kd
100 - 110 Kd
41 - 45 Kd
31 Kd
28 Kd

(B)


MW Stds
ERC B
dERC B
130 Kd
75 Kd
50 Kd
39 Kd
27 Kd
17 Kd
43 Kd
26 Kd
13 Kd

standards 13 and 26 kD) are very sharp. Comparing the bands stained with silver reagent on the SDS-PAGE (Figure 38, B) after blotting, only one major band (12 kD) is not stained with dP1 antibody. It is not known yet whether this protein should be an extensin-like molecules which may not cross-react with the dP1 antibody, or if it is from a wall protein other than cotton extensin. This question still remains for further investigation. This result, however, also demonstrated that most of the proteins contained in complexes B are cotton extensin-like molecules.

Selective Liberation of *O*-Linked Polysaccharides from Extensin-RG I Complexes A

Extensin molecules contain many modification sites for *O*-glycosylation, since they are rich in hydroxyproline and serine, and sometimes threonine. *O*-linked oligosaccharides from *N*, *O*-glycoproteins were selectively release by treatment with alkaline sodium borohydride in the presence of cadmium salt (Likhosherstov *et al.*, 1990). This reported procedure was used to determine whether or not RG I fragments are *O*-linked to cotton extensin proteins.

Extensin-RG I complexes A was treated with following reagent mixture: 27 mM NaOH, 2 M $NaBH_4$, 6 mM cadmium acetate, 6 mM EDTA·Na_2, and 5% (v/v) butanol. The reaction was carried out for 16 h at 50°C. The reaction mixture was then cooled, diluted with water, and acidified to pH 6. After freeze drying, the sample was redissolved in 50 mM ammonium acetate buffer (pH 5.2) and applied to an HW50(S) gel filtration column. Three fractions (Aa, Ab, and Ac) were collected (see Figure 40). The amino acid and sugar compositions are presented in table 16. Fraction Aa eluted in the void volume which contains predominantly RG I like sugars and a small amount of Hyp-containing proteins. While fraction Ab and Ac contained high concentrations of Hyp residue, very little of the sugar residues were eluted in the fractionation range. This experiment demonstrated the selective liberation of RG I fragments in fraction Aa from extensin molecules in fraction Ab

Figure 40. Chromatography on an HW 50(S) Gel Filtration Column of Mild Alkali Treated Extensin-RG I Complexes A.

Sample was applied in and eluted with 50 mM ammonium acetate buffer (pH 5.2). The flow rate was 0.5 mL/fraction/min. Detection was by UV monitor at 280 nm. Tubes 16-20 were pooled into fraction Aa, tubes 30-41 were pooled into fraction Ab, and tubes 42-53 were pooled into fraction Ac.

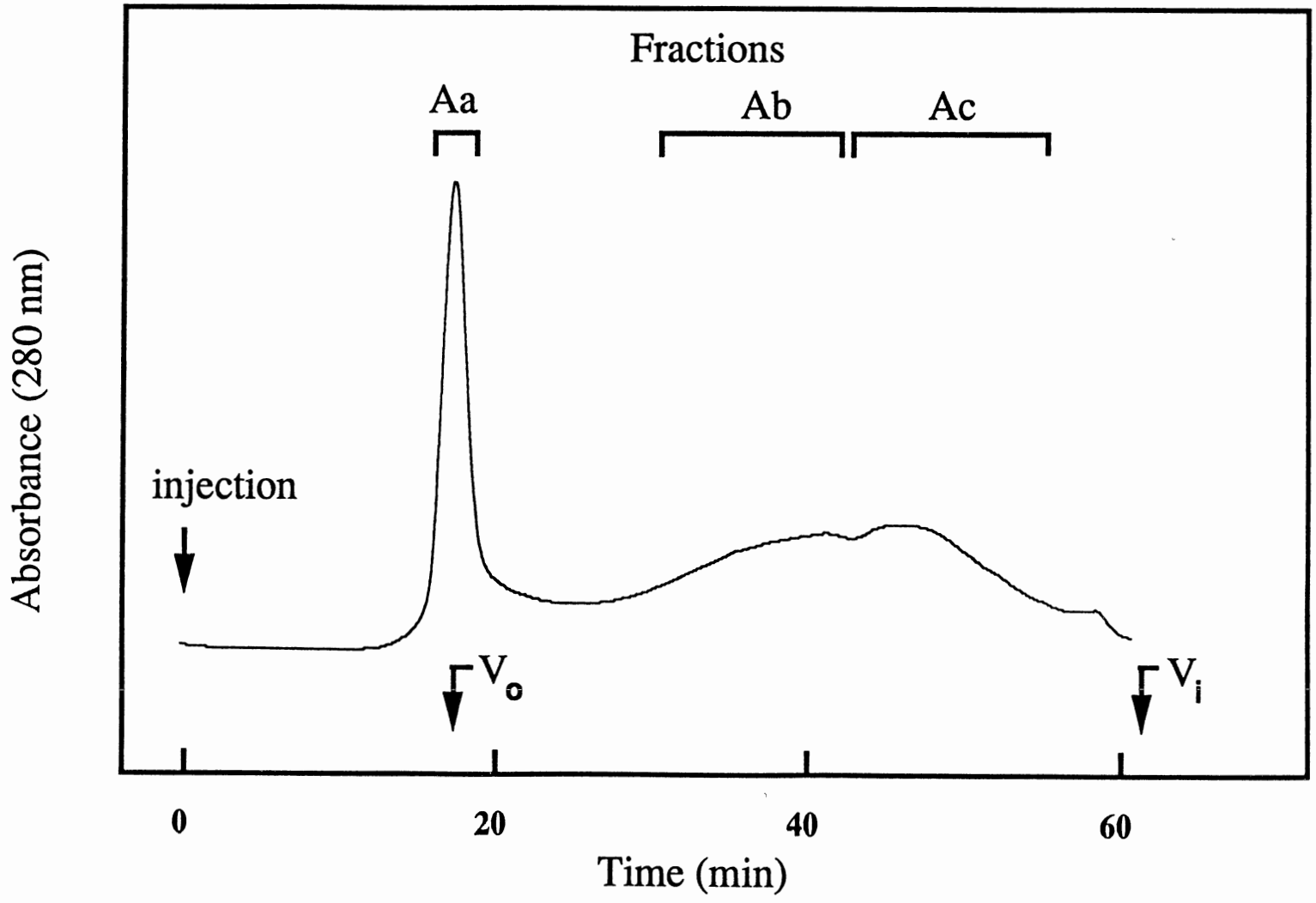

Fractions
Aa
Ab
Ac
injection
V_o
V_i
Absorbance (280 nm)
0
20
40
60
Time (min)

TABLE 16

AMINO ACID AND SUGAR COMPOSITIONS (NMOLES/MG) IN THE FRACTIONS OF HW50(S) GEL FILTRATION CHROMATOGRAPHY FROM THE MILD ALKALINE TREATED EXTENSIN-RG I COMPLEXES A

Amino Acid Residue	nmoles/mg			Sugar Residue	nmoles/mg		
	Fraction Aa	Fraction Ab	Fraction Ac		Fraction Aa	Fraction Ab	Fraction Ac
Hyp	46	47	104	Ara	86	182	155
Ser	128	110	134	Rha	1,224	239	—
Gly	—	108	—	Xyl	321	53	16
Ala	93	—	—	GalA	1,442	57	9
Pro	70	—	—	Gal	735	26	8
Tyr	27	—	—	Glc	193	63	11
Val	98	40	53				
Ile	47	28	32				
Leu	109	36	44				
Phe	33	24	29				
Lys	58	12	20				

and Ac. The result indicate that RG I polysaccharides are attached to cotton extensin proteins through *O*-glycosylation. This is also an indication that most extensin molecules contained in complexes A were eluted in the void volume (see figure 30) because of the size of RG I fragment, not because the size of extensin proteins themselves. Since this is a partial hydrolysis, some of the extensin peptides may still have been linked to RG I fragments and therefore eluted into fraction Aa.

Glycosyl Linkage Composition Analyses of
Extensin-RG I Complexes A

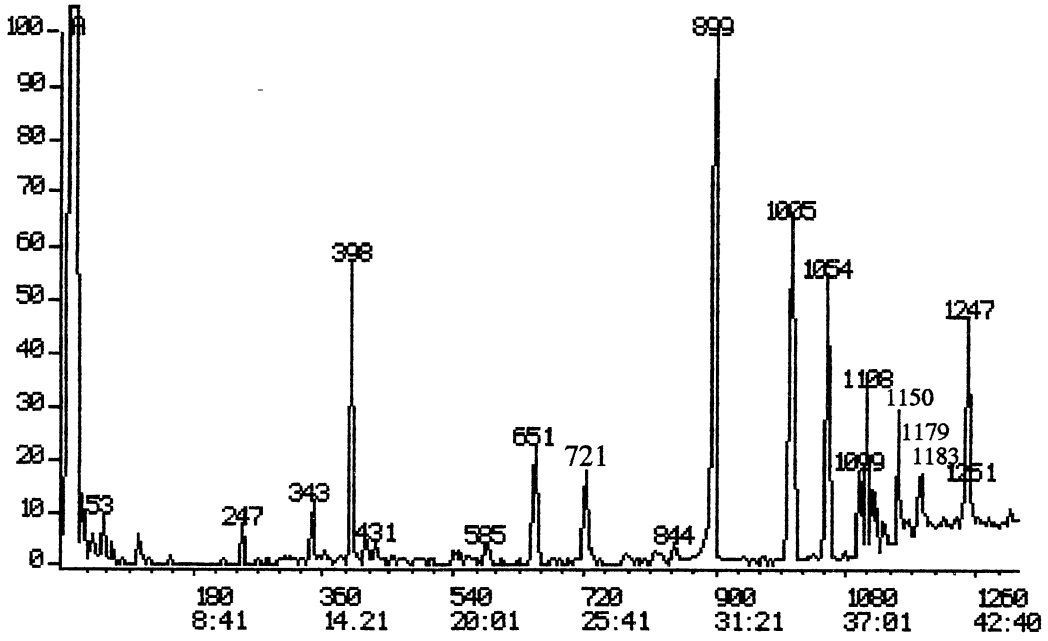
Glycosyl linkage compositions of extensin-RG I complexes A were determined by GC-MS of the partial alditol acetates as described in materials and methods. The gas chromatograms of the derivatized complexes A is given in Figure 41. The different linkage components of the sugar portions were identified according to their fragmentation patterns by mass spectrometry.

Complexes A contains large proportions of 2-linked and 2,4-linked rhamnosyl residues which are characteristic of the repeating disaccharides unit of RG I backbone in the cell walls. The relative amounts of 2-linked and 2,4-linked rhamnosyl residues of RG I fragments in complexes A is about 2 to 1, suggesting that ~33% of the rhamnosyl residues of RG I fragment in complexes A are branched, and that the attachment sites of these branched rhamnoses are at position 4.

In addition, complexes A contains a relatively high content of terminal-galactose residues. There are at least two types of galactose sidechains in the cell wall structures. One is a branch on the RG I backbone and the other is attached to the serine residues of the extensin protein. The majority of these galactose residues belong to the former, since complexes A contain a large quantity of RG I-like sugars. Moreover, there were no 2-linked or 3-linked arabinose residues found in complexes A, indicating that most of the arabinose sidechains were removed by HF -73°C solvolysis.

Figure 41. Gas Chromatogram of Partially Methylated Alditol Acetate Derivatives of Extensin-RG I Complexes A.

Gas Chromatogram of extensin-RG I complexes A. Major peaks were identified from mass spectra as follows: 651: terminal-Ara; 721: terminal-Xyl; 899: 2-Rha; 1005: terminal-Gal; 1054: 2,4-Rha; 1108: 3,5-Gal or 3,5-Glc; 1150: 6-Gal; 1179: 2,3,5-Gal; 1183: 2,3,5-Ara; 1247: inositol.


100
90
80
70
60
50
40
30
20
10
0
153
247
343
398
431
585
651
721
844
899
1005
1054
1099
1108
1150
1179
1183
1247
1251
180
8:41
360
14.21
540
20:01
720
25:41
900
31:21
1080
37:01
1260
42:40

Solid State n.m.r. Study of Cotton Cell Wall Residue E

Besides polysaccharides and glycoproteins, some plant cell walls contain insoluble complexes of hydroxy components such as lignin, suberin, cutin and carotenoids (Espelie *et al.*, 1982; Kadouri *et al.*, 1988). Most likely, plants make such resistant materials to facilitate resistance to pathogens. In fact, a dramatic increase in the levels of these types of compounds has been found after a challenge with pathogens. Also found was an increase in the levels of enzymes (and their mRNAs) which could take part in forming these complexes (Dixon and Lamb, 1990). Residue E (~8% of the initial walls weight) of the sequential treatment contains about 40% proteins, ~35% sugars, and ~25% unidentified materials. These unidentified compounds are more concentrated in residue E than they were in the original walls. In order to determine the nature of these resistant materials in cell walls, a preliminary 25 MHz cross-polarization/magic angle spinning (CP/MAS) ^{13}C n.m.r. spectrum of residue E was obtained by Dr. F. McEnroe of Conoco on an IBM WP-100 (see Figure 42).

The CP/MAS ^{13}C spectrum of extracted cell walls (residue E) showed a great enrichment over intact cell walls for signals in the region where one would expect aliphatic carbons, e.g. amino acid side chains and/or fatty acids, and in the aromatic region where one would expect signals from phenylalanine, tyrosine, lignin and other phenolic compounds. Unfortunately, we were not able to distinguish between the contributions of proteins and those of lignin-like or suberin-like materials.

Light Microscopy of Cotton Cell Walls and Extracted Walls
from the Sequential Treatment

The structures of intact cotton cell walls and of walls after each of the extraction shown in Figure 23 were examined using light microscopy. Figure 43 shows the light micrographs of intact cotton cell walls and their residues after these extractions.

Figure 42. CP/MAS ^{13}C n.m.r. Spectrum of Intact Cotton Cell Walls and Residue E (Extracted Cell Walls) of Figure 23.

The spectrum was recorded on a CP/MAS n.m.r. spectrometer. Peaks were identified as indicated in the spectrum.

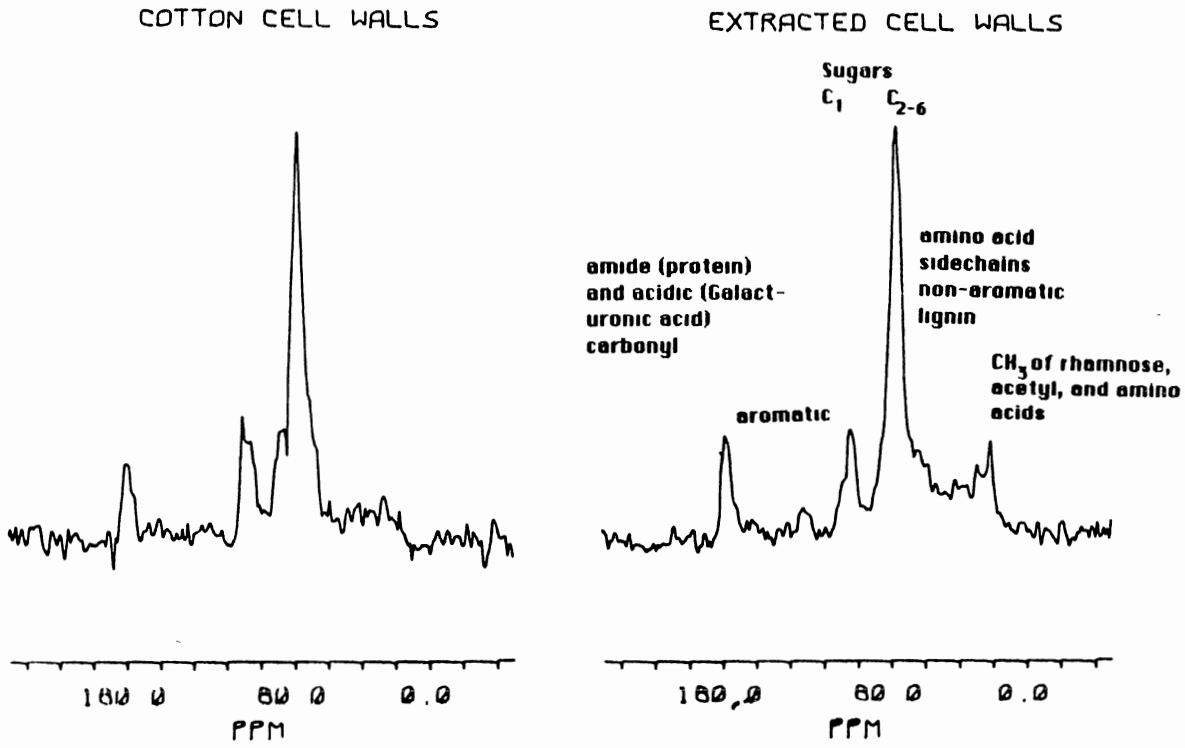
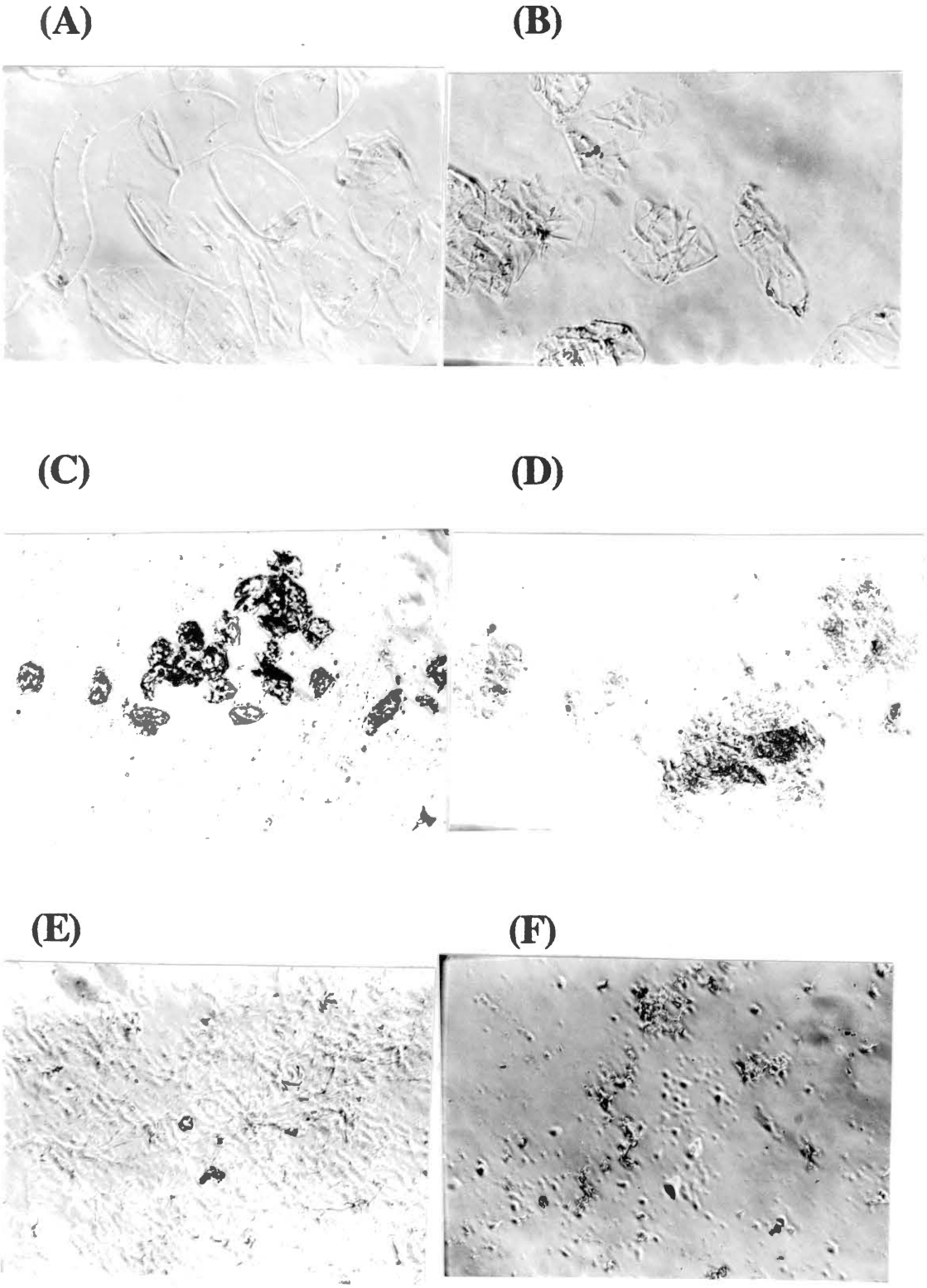

COTTON CELL WALLS
EXTRACTED CELL WALLS
Sugars
C1
C2-6
amino acid
sidechains
non-aromatic
lignin
amide (protein)
and acidic (Galact-
uronic acid)
carbonyl
aromatic
CH3 of rhamnose,
acetyl, and amino
acids
180 0
80 0
0.0
PPM
180.0
80 0
0.0
PPM

Figure 43. Light Micrographs of Acala 44 Cotton Cell Walls and Their Residues from the Sequential Treatment.

Samples were completely hydrated with water, and the picture were taken by a Nikon light microscope BIOPHOT (130 X, phase contrast 2 DL). (A) Intact cell walls. (B) Residue A of Figure 23. (C) Residue B of Figure 23. (D) Residue C of Figure 23. (E) Residue D of Figure 23. (F) Residue E of Figure 23.


(A)
(B)
(C)
(D)
(E)
(F)

After removal of polygalacturonans with EPGase, the residue A has a similar structural appearance to the unextracted walls (see Figure 43, A and B). This is smooth in appearance, indicating that the integrity of the cell walls is maintained (Figure 43, B). Figure 43, C presents the structure of residue B from subsequent cellulase treatment. This residue shows a shrunken and broken wall structure resulting from the degradation of xyloglucan coated-cellulose microfibrils. Thus, the picture shows that cellulase digestion destroyed the rigidity of the cotton cell walls. Interestingly, the structure of residue B still appears as an extended and elongated shape, despite most of sugars being removed, leaving the heavily arabinosylated cell wall proteins and ~60% of the RG I. This may imply that the glycoprotein (extensin) is important to the network construction of the cell walls. The protein can be made susceptible to the protease trypsin by removing arabinose residues from it by a very mild HF treatment at -73°C. The resulting residue C shows a very condensed and shortened structure (see Figure 43, D). This is circumstantial evidence that the arabinose sidechains on the protein reinforce the polyproline II conformation of extensin, causing the molecules to be more extended and elongated (van Holst and Varner, 1984; Stafstrom and Staehelin, 1986a). Extraction of associated RG I fragments with buffer, does not alter appearance of residue D beyond that of residue C. The last step in this sequential treatment solubilizes approximately 50% of the protein, leaving a residue E which contains about 40 % protein, some RG I fragments and other minor constituents. Residue E appears as a mass of small pieces and fragments (see Figure 43, E). Apparently, the shape of the cotton cell walls was completely destroyed. The ultimate conclusion of the experiment is that the cellulose microfibrils and extensin glycoproteins are two major skeleton components of cell walls.

CHAPTER V

SUMMARY AND CONCLUSION

It is believed that the interactions between wall polymers are very important to maintain the functional cell wall. Extensins, a type of hydroxyproline-rich glycoproteins, are major structural proteins in plant cell walls. What is the structure of extensin in cotton cell wall and how does it interact with other polymers in the walls? These two questions led this research to be divided into two projects. In general, one project was isolation and characterization of the peptides of cotton cell wall extensins; the other project was undertaken to investigate the interactions of cotton extensins and other cell wall polymers, and determine the covalent cross-links between extensin molecules and rhamnogalacturonan I polysaccharides.

Cell walls were prepared from cotton suspension cells Acala 44. Sugars accounted for about 80% of dry weight of Acala 44 cotton suspension walls. The protein content of Acala 44 cotton suspension wall was about 7%. Cotton cell walls contain 0.5% hydroxyproline.

For some years the insolubility of extensin prevented its structure elucidation. In order to isolate and characterize the cotton extensin structure, hydrogen fluoride solvolysis of Acala 44 cell walls at 0°C was used to removes all of the sugars from the wall except those of amino sugars (which are in very low concentration) and some of those of nonmethylesterified homogalacturonans. After ether and water extraction the insoluble residue of cotton suspension cultures contained 98% of the Hyp present in the initial sample. The homogalacturonan left behind in the residue was removed by extraction with ammonium acetate buffer (50 mM, pH 5.2). No peptide bonds in cotton extensin should be

broken by HF 0°C treatment. Digestion of this residue with trypsin solubilized 80% of the Hyp, showing that the wall polysaccharides removed by HF 0°C treatment may interact with extensin to contribute to the insolubility of the proteins. On the other hand, the remaining residue (approximately 2% of the original dry cell walls) contained 20% of the Hyp. This result may imply that the interactions (such as isodityrosine cross-links) of extensin molecules are keeping these proteins insolubilized into the cell walls.

The cotton extensin peptides solubilized by trypsin digestion were separated on reverse phase HPLC, and give rised to two major fraction. Both of these fractions I and II were rich in Hyp, accounting for about 23% and 19% of the total cell wall Hyp, respectively. Three peptides contained in fraction I were purified by gel filtration chromatography on an HW 40(S) column. These were sequenced and found to be quite similar to peptides already isolated from extensins of other plants. They are:

(a) Ser-Hyp-Hyp-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Hyp-Lys

(b) Ser-Hyp-Hyp-Hyp-Hyp-Val-Lys

(c) Ser-Hyp-Hyp-Ser-Ala-Hyp-Lys

The exact amino acid stoichiometry of these peptides were verified by LSIMS. Pseudomolecular ions were seen at 1225.8, 786.0, 731.8, respectively. Interestingly, the most abundant peptide (a) contained the sequence Ser-Hyp-Hyp-Hyp-Hyp-Hyp-Hyp, which is two hydroxyproline residues longer than the most commonly encounted Ser-Hyp-Hyp-Hyp-Hyp. These three peptides accounted for 23% of the Hyp in the cell walls and must , therefore, be repeated several times within the extensins of cotton. In addition to two major fractions I and II fractionated by reverse phase HPLC, there were many minor peaks which account for about 33% Hyp of cell walls. This is an indication that the cotton cell wall contains only one or a few extensins rich in repetitive Hyp rich sequence (such as peptides a, b, and c), and an array of more minor Hyp poor or nonhydroxyproline-containing proteins.

Treatment of cotton cell walls in liquid HF at 0°C failed to solubilize the extensin, implying its existence *in muro* as a highly crosslinked network. In order to study the nature of cotton cell wall extensin and its interactions with other polymers, various permutations of highly specific methods have been used. These methods were selected to interrupt the interconnections of extensin and polysaccharides to liberate the extensin (as assessed by solubilization of Hyp), and included, (1) endopolygalacturonase digestion of homogalacturonans; (2) cellulase digestion of cellulose and xyloglucan; (3) hydrogen fluoride (HF) treatment at -73°C to selectively remove most arabinose residues from the Hyp residues of extensin; (4) HF treatment at 0°C to completely remove sugars from the cell walls; (5) HF treatment at -23°C to remove pectic and hemicellulosic sugars; (6) imidazole and EDTA buffer extractions to remove homogalacturonan fragments; (7) ammonium bicarbonate buffer extraction to remove unassociated RG I fragments; (8) trypsin digestion to degrade proteins; (9) α-chymotrypsin digestion to degrade proteins; and (10) esterase digestion to cleave ester bonds. The results of different combinations of these treatments were summarized in Table 17.

None of the treatments alone liberated significant quantities of extensin. Considerations of the extent of release by combination of treatments (3) and (8) showed that the arabinose side chains on the Hyp residues are important for preventing trypsinization of the extensin, since more Hyp (about four times more) was liberated by trypsin digestion after HF -73°C pretreatment than by trypsin treatment alone. Relatively small quantities of Hyp (5-10%) were liberated by trypsin after endopolygalacturonase, or cellulase, or endopolygalacturonase and cellulase pretreatment(s). If multi-pretreatments (HF at -23°C solvolysis and extractions with imidazole and EDTA buffer) were applied before trypsin digestion to remove pectic and hemicellulosic sugars, 19% of Hyp was liberated by proteolysis. This experiment gives the same indication as that of HF -73°C pretreatment, however, addition of cellulase pretreatment after EDTA buffer extraction

TABLE 17

SUMMARY OF HYP AND SUGAR CONTENTS SOLUBILIZED WITH VARIOUS TREATMENTS

Sample	wt (mg)	Hyp* (mg)	Hyp (%)	Sugar (mg)	Sugar (%)
Acala 44 Cotton Cell Walls	1,000	4.80	100	387	100
Filtrate					
Cellulase-α-Chymotrypsin	136	Trace	Trace	59	15
Cellulase-Esterase	140	0.08	2	59	15
Trypsin	76	0.24	5	7	2
EPGase-Trypsin	64	0.24	5	5	1
EPGase-Cellulase-Trypsin	125	0.50	10	42	11
Cellulase-Trypsin	149	0.42	9	57	15
HF -23°C-Imidazole-EDTA-Trypsin	68	0.90	19	Trace	Trace
HF -73°C-Trypsin	100	0.95	20	39	10
HF -23°C-Imidazole-EDTA-Cellulase-Trypsin	35	2.38	50	Trace	Trace
EPGase-Cellulase-HF -73°C-Trypsin	60	2.40	50	17	4
EPGase-Cellulase-HF -73°C-NH_4HCO_3-Trypsin	32	2.40	50	11	3
HF 0°C-NH_4Ac-Trypsin	65	3.84	80	Trace	Trace

* Hyp content was determined by colorimetric method.

allowed trypsin to liberate 2.5 times more Hyp (50%) from cell walls. This result showed that cellulose somehow inhibits proteolysis of wall extensin proteins.

By realizing that arabinose oligosaccharides and cellulose polysaccharides are two major factors in the protection of cotton extensin from trypsin digestion, the following sequential treatment was designed: (1) endopolygalacturonase digestion; (2) cellulase digestion; (3) HF-73˚C solvolysis; (4) ammonium bicarbonate buffer extraction; and (5) trypsin digestion. This sequential treatment liberated 50% of the Hyp along with the sugars characteristic of pectin from cotton cell walls. What is solubilized is a mixture of slightly glycosylated peptides and some very heavily glycosylated peptides. Both the lightly and heavily glycosylated peptides were shown by Marcia Kieliszewski in Derek Lamport's laboratory to contain Hyp residues glycosylated with single arabinose residues. In comparison to the intact cell wall, one can estimate that most of the hydroxyproline arabinosides were cleaved, except for some of the arabinose directly linked to the Hyp residues. Lamport has suspected for many years that in aqueous acid the Hyp-ara linkage is slower to hydrolyze than ara-ara linkage (personal communication). The resulting residue of the sequential treatment (about 8% of total cell walls) contains approximately 40% of proteins and some pectic sugars. Compared to the combination treatment (HF 0˚C, ammonium acetate buffer, and trypsin), less than 30% of the Hyp was liberated from by this sequential treatment. This is a indication that a single arabinose remained on the extensin and that pectin fragments blocked the trypsinization of the wall proteins.

The heavily glycosylated peptides, called extensin-RG I complexes A, contain a high proportion of pectic sugars. After complete deglycosylation by a HF 0˚C treatment, most of the peptides became much smaller as indicated by gel filtration chromatography and polyacrylamide electrophoresis. On an SDS PAGE gel, two bands (molecular weights relative to standards 28 kD and 31 kD) were resolved, and two other groups of bands (41-45 kD and 100-110 kD) were not resolved very well. All these bands were stained on western blots with an antibody obtained from Derek Lamport's laboratory to the

deglycosylated peptides of tomato extensin. In addition, the deglycosylated peptides of extensin-RG I complexes A were shown to contain the repeated sequences in fraction I by reverse phase chromatography.

The slightly glycosylated peptides, called extensin-RG I complexes B, fractionated by HW 50(S) gel filtration column (fractionating range from 800 to 90,000 daltons). These peptides only shifted their mobility slightly on the column and polyacrylamide electrophoresis after HF 0°C deglycosylation. There were four sharp bands (molecular weights relative to standards 12 kD, 13 kD, 26 kD and 43 kD) on an SDS PAGE gel. Three of these (13 kD, 26 kD and 43 kD) were stained on western blots with an antibody raised against the deglycosylated peptides of tomato extensin. The deglycosylated peptides of extensin-RG I complexes B were characterized as cotton extensin peptides by mapping on a reverse phase HPLC column. after digestion with trypsin.

Arabinosyl-hydroxyproline profiles of extensin-RG I complexes A and B were analyzed using alkaline hydrolysis and chromatographic separation methods described by Lamport and Miller (1971). No $Hyp(Ara)_4$, $Hyp(Ara)_3$ and $Hyp(Ara)_2$ have been found in either of the complexes, A or B. More than half of the Hyp residues contained in complexes A and B were not glycosylated. This is an indication that HF -73°C solvolysis cleaved most of arabinose side chains from the hydroxyproline residues, but that some of the glycosyl bonds between arabinose residue and Hyp were resistant to this treatment, or that there was insufficient time for all of these bonds be cleaved.

Mild alkaline treatment (17 mM $NaBH_4$ and 50 mM NaOH in the presence of cadmium salt) has been reported for selectively cleaving the O-linked oligosaccharide chains from glycoproteins. Most likely, cross-linkages between extensin and RG I fragments are in the O-linked form in complexes A and B, because cotton extensins contain high contents of serine and hydroxyproline residues which provide the O-glycosylation sites. In fact, extensins contain single β-D-Gal*p* residues glycosidically linked to some of the serine residues (Lamport, 1980a). In order to investigate this idea, the extensin-RG I complexes A

was treated with alkaline sodium borohydride in the presence of cadmium salt to selectively release a putative O-linked RG I fragments from the extensin peptides. Before the alkaline reaction, complexes A was eluted in the void volume of an HW 50(S) gel filtration column. After the selective liberation of O-linked sugars under the mild alkaline condition, most of the extensin peptides were eluted within the fractionation range on the same column. The released RG I fragments were still eluted at void volume. The result indicates that the RG I fragments are covalently attached to the extensin peptides in O-link forms.

Methylation of sugars contained in extensin-RG I complexes A provides some evidence about which sugar residues are likely to be in the complexes. 2-linked and 2,4-linked rhamnose residues were found in high concentration in complexes A. These two sugars are known to exist in the backbone of RG I. Most of the Hyp residues in extensin bear short oligosacchride side chains which contain 2-linked and 3-linked arabinose furanose residues (Fry, 1988). No 2-linked or 3-linked arabinose furanose residues were found in complexes A, indicating that most of the arabinose sidechains on extensins were removed by HF -73°C solvolysis.

In summary, isolation, purification and partial characterization of extensin from primary cell walls of cotton suspension cultures has been accomplished. A Ser-Hyp-Hyp-Hyp-Hyp-Hyp-Hyp repeat found in cotton extensin is different from other plant extensins which have been characterized. Although the majority of extensin peptides have been liberated with HF 0°C treatment followed by trypsin digestion, twenty percent of the Hyp still remained insoluble in the wall residue. This implies that there are unidentified cross-links between extensin molecules or extensin and polymers other than polysaccharides. Carbohydrate components of the cell walls, such as arabinose side chains on extensin, cellulose, and pectin, protect the extensin protein from proteolysis. Removal of polysaccharides sequentially with selective cleavage methods in combination with proteolysis allowed liberation of extensin-RG I complexes. Western blot analysis showed an increase in electrophoretic mobility of the antibody reactive material after it was

deglycosylated by a 0°C HF treatment. The results strongly indicate the existence of covalent cross-links between the cell wall extensin and pectin.

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