PARTIAL CHARACTERIZATION OF EXTENSIN STRUCTURES AND THEIR INTERACTION WITH POLYSACCHARIDES IN COTTON SUSPENSION CULTURE CELL WALLS

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AGPs	arabinogalactan proteins
Ala	alanine
Ara	arabinose
Asp	aspartic acid
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
cDNA	complementary DNA
ELISA	enzyme-linked immunosorbent assay
EPGase	endopolygalacturonase
ERC A	extensin rhamnogalacturonan I complexes A
ERC B1	extensin rhamnogalacturonan I complexes B1
ERC B2	extensin rhamnogalacturonan I complexes B2
ERC B3	extensin rhamnogalacturonan I complexes B3
ERC B	extensin rhamnogalacturonan I complexes B
Gal	galactose
GalA	galacturonic acid
Glc	glucose
Gly	glycine
GRPs	glycine-rich proteins
HF	hydrogen fluoride
HHRGP	histidine-hydroxyproline-rich glycoprotein
HPLC	high performance liquid chromatography
HRGPs	hydroxyproline-rich glycoproteins
Hyp	hydroxyproline

kD	kilodalton
IDT	isodityrosine
Lys	lysine
Man	mannose
MMNO	4-methyl-morpholine-N-oxide hydrate
M.W.	molecular weight
NBT	nitro blue tetrazolium
PITC	phenylisothiocyanate
Pro	proline
PRPs	proline-rich proteins
PTL	potato tuber lectin
RGI	rhamnogalacturonan I
RGII	rhamnogalacturonan II
Rha	rhamnose
Ser	serine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEM	transmission electron microscopy
Thr	threonine
Tyr	tyrosine
THRGP	threonine-hydroxy-proline-rich glycoprotein
Val	valine
Vi	included volume
Vo	void volume

CHAPTER I

INTRODUCTION

The cell walls of plants play an important role in the control of plant growth and morphogenesis as well as in resisting disease (Bell,1981). Plant cell walls consist primarily of polysaccharides, glycoproteins and phenolic compounds. In order to better understand the function of plant cell walls, one must have a clearer understanding of the exact nature of the structure of these cell walls than is currently available. Of particular interest is understanding the ways in which the various polymers are connected together.

Plant cell walls are classified as either primary or secondary cell walls. The primary cell wall is of the type which has a polysaccharide framework having been deposited over the entire surface area as the cell was expanding. The primary wall of most dicotyledonous plant cells is composed of cellulose, xyloglucan, pectin compounds, proteins and small amounts of lignin and suberin (Fry,1988). Cellulose, perhaps the best known polysaccharide, constitutes approximately 20% to 30% of the dry weight of a typical primary cell wall. Xyloglucan, another cell wall polysaccharide, makes up about 20% of the dry weight of the primary cell wall. Pectins, a class of complex cell wall polysaccharides, are mainly comprised of homogalacturonans, and the polymers rhamnogalacturonan I and II. Pectins constitute about 30% of the dry weight of the cell wall. Polysaccharides of cell walls have been investigated and reviewed extensively (Darvill et al., 1980; McNeil et al., 1984; Varner and Lin, 1989).

In contrast to the predominance of polysaccharides in the cell wall are proteins, important components which constitute only up to 10% by weight of dehydrated primary cell walls of dicotyledonous plants (Bacic et al., 1988). Among cell wall structural proteins, extensins are the most abundant in dicotyledons. Extensins are generally

characterized by the following: richness of hydroxyproline and serine and some combination of valine, tyrosine, lysine and histidine; extensins usually contain the repeating pentapeptide motif Ser-Hyp4, often within the context of other, larger repeating motifs; most of the hydroxyproline residues are glycosylated with one to four arabinosyl residues, while some of the serine residues are glycosylated with a single galactose unit; they are basic proteins due to their high lysine content; a polyproline II helical structure is generally assumed in solution while a rodlike appearance is observed when viewed by the electron microscope. These characteristics are exemplified by many dicot extensins (Lamport and Miller, 1971; Lamport et al., 1973; Cho and Chrispeels, 1976; Smith et al., 1984,1986; Masau et al., 1988; Heckman et al, 1988; Statstrom and Stalhelin, 1988; Qi, 1992) and extensin cDNA and genomic clones (Keller and Lamb, 1989; Zhou et al., 1992).

Extensins are known to be insolublized once they have been secreted and incorporated into the cell wall. The mechanisms that explain how this happens and what interactions occur on the molecular level are unknown. Although there are some clues, direct evidence is lacking about covalent linkages. It has been suggested that extensins may crosslink by intermolecular diphenylether linkages between tyrosines (Fry, 1982; Cooper and Varner, 1984) or link via intramolecular isodityrosine linkages (Epstein and Lamport, 1984). It has also been suggested that extensins may be covalently crosslinked to some wall polysaccharides (Keegstra et al., 1973; Mort, 1978; Selvendran, 1985).

Research on cell walls in our lab deals mainly with the study of the cotton plant. The polysaccharide structures of the cotton primary cell walls have been isolated and partially characterized (Komalavilas and Mort, 1989; Mort et al., 1993; El Rassi et al., 1991). Cotton cell wall extensins have also been partially characterized and three tryptic peptides have been sequenced (Qi, 1992). The research also showed some evidence that covalent cross-linkages exist between extensins and pectins (Qi, 1992). This is an important achievement , but we must know much more about the structure of extensins and

any cross links between extensins and polysaccharides in significantly greater detail in order to provide important insights to cell wall extensin function.

In this study, the structure of cotton suspension cell wall extensins and their interactions with wall polysaccharides have been investigated. The first part of this work describes the isolation, purification and partial characterization of extensin fragments from cotton suspension culture cell walls. The second part reports the solubilization and partial characterization of extensin- RG I fragments connected by putative covalent linkages.

CHAPTER II

LITERATURE REVIEW

Components of Plant Cell Walls

Polysaccharides:

Cellulose is one of three primary types of polysaccharides in the cell wall. It is a linear polymer of (1 → 4) linked β-D-glucopyranosyl residues. Within the microfibril, cellulose chains are arranged into an ordered crystalline lattice or near crystalline arrays that are nearly free of water (Roelofsen, 1965). The cellulose microfibrils in a typical primary cell wall are eliptical in cross section with axes 50-300 Å, constituting 20-30% of the dry weight of the wall. Although the enzymatic apparatus for cellulose synthesis is poorly understood, it is apparently contained in particles observed in the electron microscope as ordered groups of rosettes in the plasmalemma (Heath and Hausser, 1984). Microfibril orientation at deposition is determined by the microtubules and is usually transverse to the major axis of growth. As the cell increases in length, microfibrils in the outer layers of the wall may be pulled into an orientation nearly parallel to the axis of elongation.

Xyloglucan, a hemicellulose, was first isolated and characterized from tamarind (*Tamarindus indica*) seeds (Kooiman, 1961). Since then, xyloglucan has been isolated and studied from a variety of sources, making up about 20% of the dry weight of the primary wall of dicots. Members of the xyloglucan family have a linear extended backbone of (1 → 4)-linked β-D-glucopyranosyl residues with D-xylosyl side chains α-linked to O-6 of some of the glucosyl residues. However, some xylosyl side chains have D-galactose or L-fucosyl-2-α-D-galactose β-linked to the O-2 of the xylosyl residues (Darvill et al., 1980). Research on xyloglucan of cotton suspension cultures cell walls also showed the

similar structure as mentioned above (El Rassi et al., 1991). Xyloglucans in muro seem likely to form a layer on the surface of the crystalline regions of the microfibrils and penetrate into the amorphous regions.

Pectins, a complex class of cell wall polysaccharides, are mainly comprised of homogalacturonans, rhamnogalacturonans I and II in dicots. Homogalacturonans are mainly comprised of α-4-linked galacturonic acid residues. The study of the pectin structure of cotton suspension cell walls showed that GalA residues in homogalacturonans are methyl esterified in an non random manner (Mort et al., 1993). The rhamnogalacturonan I (RG-I) consists of a backbone of 4-linked D-galacturonic acid residues which are interspersed with 2-linked L-rhamnosyl residues. In some instances, the galacturonosyl and rhamnopyranosyl residues may be in a repeating sequence. For example, RG I from suspension cultured *Acer pseudoplatanus* walls has up to 300 alternating residues of each sugar and about half of the rhamnosyl residues glycosylated at O-4, with sidechains containing up to 15 glycosyl residues in length (McNeil et al., 1984; Lau et al., 1985). Research on the cell walls from carrot, cotton, tobacco, and tomato suggested that acetylation of pectin is confined to the RG I region (Komalavilas and Mort, 1989). Rhamnogalacturonan II (RG-II) is a very different molecule from RG I, consisting of about 30 to 60 glycosyl residues (Stevenson et al., 1988; Varner and Lin, 1989). It may be a substituent on either the $(1 \rightarrow 2)$-linked rhamnopyranosyl or $(1 \rightarrow 4)$ linked galacturonosyl residues of the rhamnogalacturonan backbone, and contains many unusual glycosyl residues, including 2-O-methylfucose, 2-O-methylxylose, apiose (a branched pentosyl residue), 3-C-carboxy-5-deoxy-L-xylose and 3-deoxy-manno-octulosonic acid.

Cell Wall Glycoproteins

There are five main classes of plant cell wall proteins which represent the most abundant , best studied and most widely documented plant cell wall proteins. These include the extensins, the glycine-rich proteins (GRPs), the proline-rich proteins (PRPs),

the solanaceous lectins, and the arabinogalactan proteins (AGPs). These proteins may be evolutionarily related to one another.

Arabinogalactan proteins (AGPs) are hydroxyproline-rich glycoproteins (HRGPs) that are usually quite soluble and highly glycosylated (Fincher et al., 1983; Showalter and Varner, 1989). Carbohydrates account for most of the weight of the AGPs and contain D-galactose and L-arabinose as the major carbohydrate constituents. AGPs are widely distributed in plants and are typically comprised of only 2-10% protein by weight. They are often found as constituents of the extracellular milieu. The protein moeity of AGPs is typically rich in hydroxyproline, serine, alanine, threonine and glycine. The N-terminal sequences of four different AGPs, three from carrot and one from ryegrass have been determined, and all four sequences were found to contain Ala-Hyp repeating units (Gleeson et al., 1985). More recently, sequences from five tryptic peptides from deglycosylated ryegrass AGP have also been determined, with some of these sequences likewise containing Ala-Hyp repeats (Gleeson et al., 1989). These repeats may become a diagnostic characteristic of AGPs since they are not known to occur in any of the other cell wall proteins, with the possible exception of the maize histidine-hydroxyproline-rich glycoprotein (HHRGP) (Kieliszewski et al., 1992).

Solanaceous lectins represent a unique class of plant lectins that can be distinquished from other lectins by their restricted occurrence in solanaceous plants, their ability to agglutinate oligomers of N-acetylglucosamine, their predominantly extracellular location, and their unusual amino acid and carbohydrate composition in which hydroxyproline and arabinose are the major constituents (Showalter and Varner, 1989). Potato tuber lectin (PTL) is perhaps the best studied member of the solanaceous lectins. It is a glycoprotein with a monomeric molecular weight of 50 kD and consists of 50% carbohydrate and 50% protein by weight (Allen et al., 1978). The protein moiety is especially rich in four amino acids; hydroxyproline, serine, glycine, and cysteine. The PTL is comprised of at least two distinct proteins domains, one of which is rich in serine

and hydroxyproline and contains the carbohydrate moiety consisting mainly of arabinose linked to hydroxyproline and galactose linked to serine. The ability of PTL to bind N-acetylglucosamine oligomers is known to be associated with the glycine-cysteine-rich domain (Allen et al., 1978) and it is likely that this chitin binding domain will be homologous to that found in other chitin binding proteins.

PRPs represent a relatively newly identified class of plant cell wall proteins of which most, if not all members contain hydroxyproline. There are at least two broad subclasses of PRPs, those that are components of normal plant cell walls (Sheng et al., 1991) and those that are plant nodulins (proteins produced in response to infection by nitrogen fixing bacteria) and constitute some of the nodule cell wall (Govers et al., 1991). The ability to distinguish between these two classes may not be clearcut, however (Govers et al. 1991). All of the PRPs are characterized by the repeating Pro-Pro units that are contained within a variety of other, larger repeating units (Govers et al., 1991). Recently, the cloning and characterization of the first PRP gene of the monocot maize was described (Jose-Estanyol et al., 1992). This maize PRP is comprised of an N-terminal proline rich domain having numerous Pro-Pro-Tyr-Val and Pro-Pro-Thr-Pro-Arg-Pro-Ser repeats and a C-terminal domain nearly void of proline that is hydrophobic containing several cysteine residues. This characteristic domain makeup is found in two different dicot PRP sequences, one from bean (Sheng et al., 1991) and another from tomato (Salts et al., 1991).

Another newly discovered class of proteins is represented by the Glycine-Rich Proteins (GRPs) characterized by their repetitive primary structure containing up to 70% glycine arranged in short amino acid repeating units. The first of the GRP genes was isolated by Condit and Meagher (1986). Keller et al. (1988) followed by isolating a bean genomic clone containing two linked GRP genes that coded for proteins containing 63% and 58% glycine in which the glycines are predominantly found in Gly-x repeating units. Several other research groups have also isolated and characterized GRP cDNAs or genes

from tomato (Showalter et al., 1991) and petunia (Linthorst et al., 1990). The idea that these GRPs are localized in the cell wall has been verified by immunolocalization studies with antibodies specific for GRP (Keller et al., 1988).

Extensins are a family of hydroxyproline-rich glycoproteins (HRGPs) found in the cell walls of higher plants. Research on these hydroxyproline-rich cell wall components was spurred by Lamport's discovery in 1960 that hydroxyproline is a major amino acid found in the hydrolyzates of cell walls. Since then, numerous researchers working with dicot extensins have shown that extendins are the most abundant structural proteins of dicot cell walls. These researches included the characterization of a number of hydroxyproline-rich glycopeptides and peptides obtained by the partial hydrolysis and enzymatic digestion (Lamport and Miller, 1971; Lamport et al., 1973; Cho and Chrispeels, 1976; Qi, 1992), characterization of extensin precursors obtained with salt extraction (Smith et al., 1984,1986; Mazau et al., 1988; Heckman et al., 1988), characterization of extensin by immunocytochemical location (Stafstrom and Stæhelin, 1988), and characterization of extensin genes by molecular biological techniques (Keller and Lamb, 1989; Zhou et al., 1992). The results of these researches also showed that extensins have the following characteristics in common: richness in hydroxyproline and serine as well as some combination of the the amino acids valine, tyrosine, lysine and histidine; the repeating pentapeptide Ser-Hyp4 is usually present often bounded by other, larger repeating motifs; most of the hydroxyproline residues are glycosylated with one to four arabinosyl residues, with some serine residues glycosylated with a single galactose unit; they are basic proteins with isoelectric points of ~10 due to their high lysine content; a polyproline II helical structure is assumed in solution; and electron microscopic examination yielding a rod-like appearance. A recently characterized extensin from sugar beet, a primitive dicot , provides a marked contrast to the many extensins characterized from more advanced herbaceous dicots (Li et al., 1990). This extensin lacks the repeating pentapeptide Ser-Hyp4, but does contain a repeating variation of this motif, specifically Ser-Hyp2-[X]-Hyp2-Thr-Hyp-Val-

Tyr-Lys, where [X] represents the insertion sequence Val-His-Glu/Lys-Tyr-Pro. In monocots, slightly differing versions of extensins have been shown to exist. For example, in the graminaceous monocot maize, both a threonine-hydroxy-proline-rich glycoprotein (THRGP) and a histidine-hydroxyproline-rich glycoprotein (HHRGP) have been observed with the THRGP being particularly well characterized (Kieliszewski and Lamport, 1987; Kieliszewski et al., 1990). Gymnosperms contain cell wall HRGPs as well. Analysis of the salt elutes of Douglas fir cell wall suspension cultures has revealed at least two distinct HRGPs, one of which having sequence characteristics very similar to the PRPs (Kieliszewski et al., 1992a) with the other containing both Ser-Hyp4 and Ala-Hyp repeating units (Fong et al., 1992).

The biosynthesis of extensin has been fairly thoroughly investigated by immunological techniques with antibodies developed against cell wall fractions. The extensin polypeptide backbone is synthesized on endoplasmic reticulum-bound polyribosomes before undergoing extensive post-translational modifications, including the hydroxylation of proline residues in ER lumen and glycosylation (Wienecke et al., 1982). Most of the proline residues in the polypeptide are hydroxylated by the action of peptidylproline hydroxylase (Chrispeels, 1970). The hydroxyproline containing protein is then transferred from the endoplasmic reticulum to the golgi apparatus, in which it is glycosylated and secreted (Wieneke et al., 1982). O-linked arabinosylation of the hydroxyproline residues of extensins occurs in cis-cisternae, and glycosylated molecules pass through all cisternae before they are packaged into secretory vesicles in the monensin-sensitive trans-golgi network (Moore et al., 1991). Following this packaging, the fully formed soluble glycoprotein is then secreted to the cell wall. Before it is insolubilized there, it may be conveniently extracted with salt solutions for analysis.

The direct functional evidence of extensins is lacking, although from their structural and regulatory properties extensins have been proposed to be structural proteins that may also function in development, wound healing, and plant defense. Masau and Esquerre-

Tugaye (1986) reported that HRGPs concentrations increase in some dicots by factors of 1.5 to 10 after infection by fungi, bacteria and viruses with this accumulation occurring earlier and reaching higher levels in resistant plants. The increased extensin deposition and suggested increased extensin cross-linking should lead to a more impenetrable cell wall barrier, thereby impeding pathogen infiltration and infection (Showalter, 1993). There are several other observations that provide evidence for the proposed role of extensin in disease resistance (Benhamou et al., 1990a; 1990b). Extensins have also been likened to a kind of "fly paper" for the cell wall capable of immobilizing certain plant pathogens (Showalter, 1993).

Other Polymers

In some specialized cells, lignin is found deposited in both primary and secondary walls after cell growth ceases. Lignin is a complex polymeric substance composed of di- and trihydric phenols derived from phenylalanine or tyrosine, and serves as a structural material to harden and stiffen the walls. From differing cells types of different species, lignins vary in monomeric composition, types of linkages between monomers, and organiztion of monomers within the macromolecule (Monties, 1985).

Cutin is essentially a three dimensional poly-esterified series of long chain fatty acids, ω-OH fatty acids, ω- and midchain -OH fatty acids, and midchain epoxide fatty acids. Suberin, another polyester, is recognized in walls by its characteristic staining with lipid specific stains (Sudan IV for example) and its appearance in the transmission electron microscopy (TEM). The outer walls of epidermal cells are coated with the waxes cutin and suberin that serve to protect the plant from water loss and as a mechanical barrier to bacteria, fungi, and insects.

Interactions Between Polymers in Plant Cell Walls

Cellulose-Xyloglucan Associations

It has been known that some xyloglucan is hydrogen bonded to cellulose in the cell wall. Studies on the solubilization of xyloglucan have shown that most chaotropic agents, e.g. 8M urea or guanidinium thiocyanate, solubilize only a small percentage of hemicellulose from walls, whereas the stronger chaotropic agent 4-methyl-morpholine-N-oxide hydrate (MMNO) at 120°C solubilized xyloglucan and cellulose completely (Joseleau, et al., 1981). This tends to support the existence of H-bonding. Furthermore, it has been shown that xyloglucan can self assemble via H-bonding and bind to cellulose in vitro (Bauer et al., 1973; Wada and Ray, 1978).

Associations Between Pectin / Pectin or Pectin/ Xyloglucan

There are several types of cross-links between pectins. Evidence for calcium bridges was obtained by treatment of walls with cold cyclohexane-1, 2-diamine tetra acetic acid (CDTA) at neutral pH, which removed essentially all of the calcium while also solubilizing a large portion of the pectin (Jarvis, 1982). Other proposed crosslinks include other ionic bonds (Smith et al., 1984), coupled phenols (Fry, 1985), glycosidic bonds (Mort and Lamport, 1977), and ester bonds (Fry,1984).

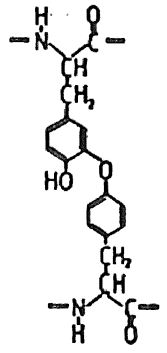
Fragments isolated from sycamore culture cell walls were reported to be the linkage point between xyloglucan and pectin (Keegstra, et al., 1973; Albersheim, 1978). Later work failed to isolate significant quantities of xyloglucan-pectin hybrid fragments (Monroe et al., 1976), but the possibility of a small proportion of glycosidic bonds between xyloglucan and pectin is not excluded (Jarvis, 1984; O'Neill and Selvendran, 1985).

Polysaccharide-Lignin Associations

There is a close association between lignin and polysaccharides in cell walls. It is reasonable that pectin molecules are cross-linked by oxidative coupling of phenolic substituents. Eriksson et al. (1980) presented the evidence for ether linkages between polysaccharides and lignin in black spruce. Pectins extracted with Driselase from spinach culture cell walls contained the cinnamate derivatives ferulate and p-coumarate (Fry, 1979), ester-linked through their carboxylic acid groups to pectins (Fry, 1983). There were about 10 feruloyl groups per 1000 pectic sugar residues (the estimated degree of polymerization of RGI). The goal of detecting a pair of pectin fragments, cross-linked by a diferuloyl (or similar) bridge is yet to be achieved.

Extensin-Extensin Association

The insolubilization of extensins once they are secreted into the wall represents a major unsolved mystery in terms of how this insolubility occurs as well as what molecules interact with extensin. There are several clues but no direct evidence. Fry (1982) first found a new cross-linking amino acid, isodityrosine (structure 1) from plant cell wall , and



Structure 1

suggested that isodityrosine is an inter-polypeptide cross-link responsible for the insolubility of plant cell wall glycoprotein. Another study showed that the isolated cell walls from aerated root slices of *Daucus carata* have the ability to insolubilize extensin

through the formation of isodityrosine (Cooper and Varner, 1984). Epstein and Lamport (1984), isolated isodityrosine from cell wall hydrolysates and from two tryptic peptides of extensin. By determination of the molecular weights, net charges and composition of the peptides, they showed that isodityrosine (IDT) can form a short intramolecular linkage in sequences consisting of -1/2 IDT-(Tyr or Lys)-1/2 IDT. In another study, Stafstrom and Stæhelin (1986) using electron microscopic techniques found that "kinks" occured at several sites along the lengths of extensin monomers from carrot cell walls, the distribution of kinks is similar to that of Tyr-Lys-Tyr sequences, so they suggested that these kinks are visible manifestations of intramolecular IDTs. In addition, the electron micrographs also showed that there were extensin dimer, trimer, tetramer and polymer. They suggested that the oligomers likely result from IDT cross-links between monomers. From these studies, it seems possible that isodityrosine exists in both inter- and intramolecular forms, each of which having their own functions.

Extensin-Polysaccharide Associations

It was believed for a long time that extensin is insolubilized into the wall by crosslinking to wall polymers. It is likely that extensin interacts ionically with pectins. The positively charged lysine and protonated histidine residues of extensin are candidates for ionic interactions with the negatively charged uronic acids of pectins. Such interactions could be regulated by changes in cell wall pH and calcium ion concentration, thus altering the physiochemical properties of the wall. Lysine residues may also form Schiff base linkages with polysaccharides (Painter, 1983). These linkages could be reversibly altered by changes in the cell wall pH.

Extensin may also be covalently crosslinked to some wall carbohydrates, as was suggested by Keegstra et al. (1973). Covalent associations of wall protein and noncellulosic polysaccharides in the walls of a number of dicotyledons was proposed on the basis of coelution by Selvendran et al. (1985). There is also tentative evidence for

complexes between protein and pectic polysaccharides. In both cases, the hydroxyproline content of the protein eluted is low. Most recently, Qi (1992), using selective cleavage methods in combination with proteolysis, liberated extensin RG-I complexes, providing the biochemical evidence that supports the existence of an extensin-pectin crosslink.

Extensin-Lignin Associations

It has been proposed that lignin is complexed to hydroxyproline-rich glycoproteins in cell walls (Whitmore 1978a, 1982). This idea is based on a comparison of the amino acid composition of proteins associated with thioglycolic acid-lignin among different wall preparation from *Pinus elliotii* callus. On incubation of washed cells (containing bound peroxidase) with coniferyl alcohol and hydrogen peroxide, an incorporation of coniferyl alcohol into a Klason lignin-protein complex (obtained by sulfuric acid hydrolysis of cell walls) containing hydroxyproline was observed. It is possible that the solubilization of both lignin and protein by acid chlorite treatment of walls (O'Neill and Selvendran, 1980) could be due to the disruption of phenolic links between the wall protein and the lignin as well as to cleavage of intermolecular isodityrosine linkages in extensin.

Plant cell walls are an intriguing complex of carbohydrates, proteins, phenolic compounds and water that vary among plant species, cell types and even neighboring cells. Although much is currently known about the structure of the various cell wall components, relatively little is known about their intermolecular and intramolecular interactions. It will be of great utility to find out the exact nature of the crosslinking and between cell wall polymers.

CHAPTER III

MATERIALS AND METHODS

Preparation of Cell Walls

Cell walls were obtained from suspension-cultured cotton plant cells (*Gossypium hirsutum* L.). Explant tissue for callus initiation was obtained from plants 30 to 90 days old. Callus cultures from fully expanded leaves of cultivar 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 Ruyak et al. (1979). Cotton suspension cells were initiated from these cultures 3 to 4 weeks later by transferring 3 to 4 grams of callus (late log phase) from SH (Schenk and Hildebrandt) agar medium to 50 mL of liquid SH medium contained in 125 mL culture flasks. Suspension culture cells about two weeks old in late log phase were used in 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 followed by thorough washing with 100mM potassium phosphate (pH 7.0) 4 times to remove extracellular debris. Cells were collected using Nitex-Nylon Mesh HL3-15 (Tetko, Inc., Precision Woven Screening Media) after each washing. The cells were then resuspended in one volume of 500 mM phosphate buffer and homogenized with a polytron mixer (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, with samples kept in ice throughout the grinding procedure. Preparations were then microscopically examined to ensure 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,

followed by its suspension in five volumes of 500 mM potassium phosphate (pH 7.0) buffer, and subsequent washing 5 times with distilled water. The resulting solid was then suspended with vigorous stirring in 5 times its volume of 1:1 chloroform:methanol (v/v) and placed in a coarse scintered glass funnel. The organic solvent was removed by the application of gentle aspiration to the funnel. The cell walls were then 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 (10mg/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 then repeated until starch could not be observed bound to cell walls by microscopic examination.. The enzyme treated cell walls were then washed thoroughly with distilled water followed by acetone and air-dried. The prepared cell walls were stored in a desiccator until ready to use.

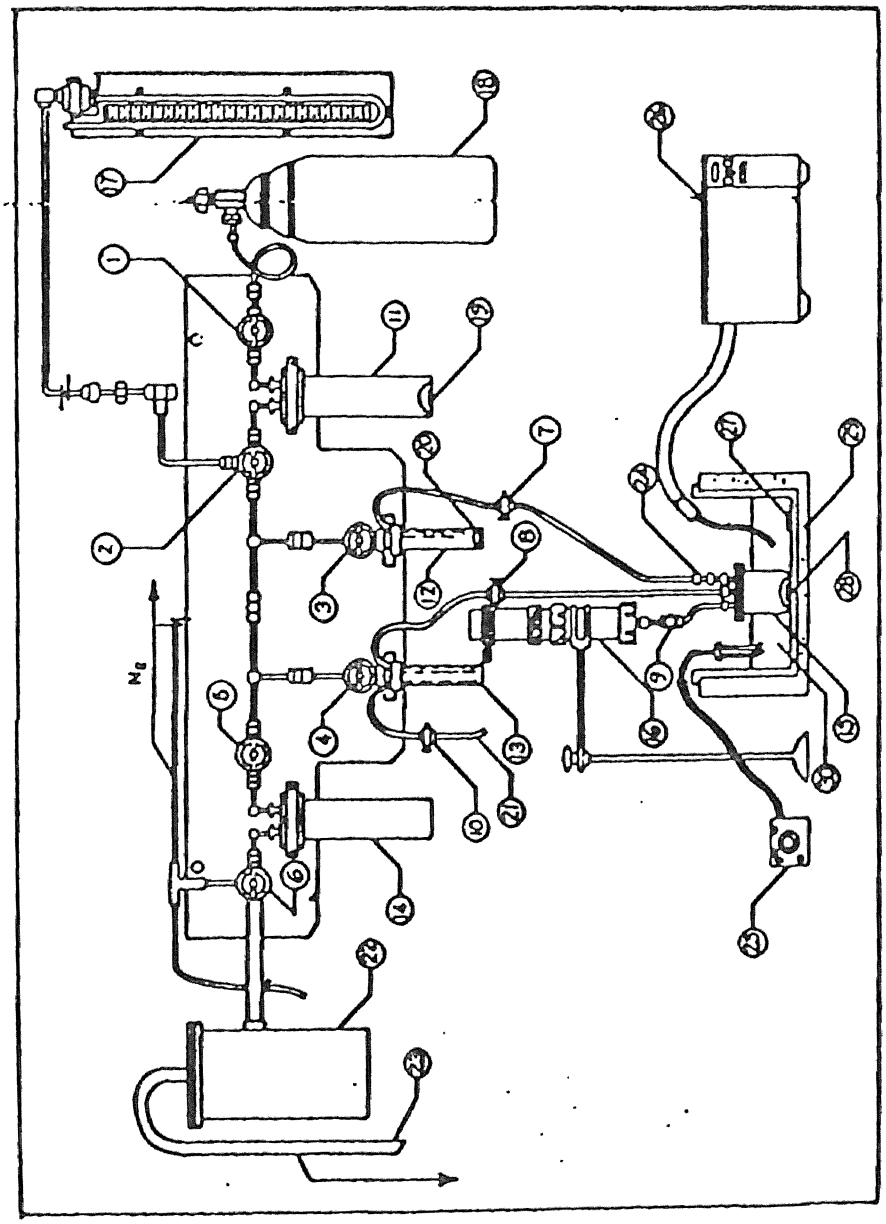
Selective Hydrogen Fluoride (HF) Solvolysis

HF Solvolysis and Apparatus

Hydrogen fluoride (HF) is a, volatile, fuming and corrosive liquid at room temperature. This substance is extremely reactive, toxic to living tissues and corrosive to glass and many metals. To safely handle HF, a special apparatus was assembled that is closed to the atmosphere and constructed of Teflon® and Kel-F®, which is inert to this solvent (Mort, 1983). The apparatus is shown in Figure 1 (Mort, et al., 1989). In a typical experiment, dry cotton cell walls and their residues (500mg) were placed in reaction vessel 15, along with a Teflon® coated stirring bar. The whole apparatus was evacuated, and leaks in any part of the system were checked and sealed. Approximately 20 mL of HF was transferred from resevoir 11 to the HF holding vessel 12. This was done by cooling the vessel 12 with dry ice in acetone (approximately -73°C) and allowing the HF to distill from resevoir 11. Both vessels 12 and 15 were next allowed to reach an appropriate

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.



temperature equilibrium through the use of a cooling bath and this temperature maintained during solvolysis. HF was transferred from vessel 12 to the reaction vessel 15 using positive nitrogen gas pressure. The reaction in vessel 15 was allowed to continue for 30 minutes with continuous stirring. The reaction was then stopped by freezing the reaction mixture in a liquid nitrogen bath. Approximately 300 mL of cold diethyl ether (cooled with dry ice) was added to the frozen samples from vessel 16. The liquid nitrogen bath was then removed, and the quenched mixture was allowed to warm. The mixture was then allowed to stir at room temperature for 3 hours and filtered using a Teflon® filter (50mm diameter, fine grade, Savillex Corporation). Sugars from the filtrate (HF/ether/sugar mixture) were recovered by evacuation under vacuum of the reaction vessel into a liquid nitrogen trap into which the HF:etherate was filtered, dissolved in water and freeze dried. Alternatively, the quenched reaction mixture (HF/ether solution) from the HF solvolysis of small quantities of sample (a few milligrams) was evacuated under vacuum to dryness without filtering. The remaining materials were dissolved in water, or in 50% acetic acid, then freeze dried.

HF Treatment of Different Samples at Different Temperatures

Acala 44 cotton suspension culture cell walls or enzyme-treated cell wall residues were treated with anhydrous hydrogen fluoride (HF) at two different selected temperatures. Enzyme-treated cell wall residues were treated with HF at a temperature of -73°C, obtained through the use of a dry ice/acetone bath or with 95% ethanol in an immersion cooling system (CC-100, NESLAB Instruments, Inc.) regulated by an immersion heater. HF treatment of Acala cell walls was done at a temperature of 0°C obtained by the use of an ice/water bath. 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 (EPGase)

Dry cell walls were suspended in 50mM ammonium acetate buffer, pH 5.2 (approximately 10 mg/mL) to which was added an EPGase solution (0.018 units/mg cell walls) and incubated at room tempereature with gentle stirring overnight. The addition of a few drops of toluene added to these solutions served to inhibit bacterial growth. After digestion, the reaction solution was filtered using a concentrator with NYLON 66 membrane (Alltech, 0.45 micron) under positive nitrogen gas pressure. The residue was washed with 3 volumes of water , and both the filtrate and residue were then lyophilized.

Digestion of Cell Walls or Their Residues with Cellulase

Dry cell walls or their residues (EPGase 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 to inhibit bacterial growth. After incubation, the digestion mixture was filtered and washed. Filtrates and residues were then lyophilized

Digestion of Wall Protein-Containing Samples With Trypsin

Dry samples (cell wall residues, deglycosylated Hyp-containing proteins or peptides) were treated with porcine pancreas trypsin (2% w/w, Sigma Chemical, type IX) in ammonium bicarbonate buffer (100 mM, pH 7.6, 10 mg sample/mL) at room temperature and gently stirred overnight. The product was subsequently filtered and the filtrate lyophilized.

Isolation of Extensin-RGI Complexes

Acala 44 cotton cell suspension culture 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 to obtain extensin-RG I complexes A and B. All filtration steps after each treatment were conducted with a NYLON 66 filter (Alltech, 0.45 micron, 47 mm) using a concentrator under positive nitrogen gas pressure.

Isolation of Extensin Peptides

Acala 44 cotton cell suspension culture cell walls were treated sequentially as described above. The final residue was treated with HF at 0°C followed by trypsin. The extensin-RG I complexes A and B were likewise treated with HF at 0°C followed by trypsin. The tryptic peptides were then separated on a reverse phase C18 column, and purified further by chromatography on a gel filtration HW 40 (S) column.

Production of Polyclonal Antibody to Extensin Precursor dp1a

Production of Polyclonal Antisera

A 7 pound young adult female New Zealand white rabbit was obtained from Laboratory Animal Resources, Veterinary Medicine, Oklahoma State University, OK. The antigen, an HF-deglycosylated tomato extensin precursor dp1a (Smith et al., 1986), was provided by Dr. Marcia Kieliszewski at Complex Carbohydrate Research Center, University of Georgia. The primary injection of antigen was a 500 µL aliquot of a water-in-oil emulsion. The emusion was prepared by vigorous mixing of 100µg of antigen contained in 250 µL PBS with 250 mL Freund's complete adjuvant (Sigma Chemical) and then injected subcutaneously into both hips of the rabbit one week after the preimmune bleeding. Booster injections of antigen containing 105 µg dp1a in 500 µL water-in-oil

emulsion of Freund's incomplete adjuvant (Sigma Chemical) were given 2 weeks after the primary immunization. Blood was obtained one week later by cardiac puncture under ketamine/rompun anesthesia and antisera was harvested by centrifuging the blood. The second bleeding was conducted the following week. All antisera obtained was stored at -70°C.

Determination of Polyclonal Antibody Titre by Enzyme Linked Immunosorbant Assays (ELISA)

The titre of polyclonal antisera was determined by the ELISA method. Each well of the 96 well microtest tissue culture plate was coated with 1 µg antigen dp1a contained in 100 µL coating buffer (30 mM sodium carbonate, 69 mM sodium bicarbonate, pH 9.5) for at least 2 hours at 37°C. The plate was washed twice with PBS/Tween (50 mM sodium potassium phosphate, pH 7.4; 150 mM sodium chloride; 0.02%, w/w potassium chloride; 0.05%, v/v, Tween-20). Remaining protein binding sites were blocked by adding 200 µL 1% bovine serum albumin (BSA) in PBS/Tween for at least 1 hour at 37°C, followed by washing twice with PBS/Tween. A series of antibody dilutions were made using diluting buffer (PBS/Tween). To each well was added 100 µL diluted antibody, and incubated for 30 minutes at 37°C. After incubation, the plate was washed four times with PBS/Tween. One hundred µL of goat anti-rabbit IgG-peroxidase conjugate (1:5000 in diluting buffer) was added to each well and allowed to incubate 30 minutes at 37°C. The plate was washed with PBS/Tween four more times. One hundred µL of fresh peroxidase substrate solution containing 0.02 mg 2,2'-azino-(3-ethylbenzthiazoline sulfonic acid), diammonium salt was added to each well and incubated at room temperature until a blue-green color appeared. The plate was then scanned by an automated microplate reader. The most diluted titer giving a positive color reaction (compared with preimmune sera control) gave the titer of the antibody.

Determination of Cross-reactivities Between Extensin RG-I Complexes and Anti dp1a

Cross-reactivities of extensin RG-I complexes as well as their deglycosylated forms with polyclonal antibody raised against deglycosylated tomato extensin precursor dp1a were also determined by ELISA in the same manner as described above. Micro-ELISA wells were coated with 2-10 μg of extensin RG-I complexes in 100 μL coating buffer and 100 μL of antisera and preimmune sera (1:40) dilution were added to each well. After comparison of the color reactions in antisera wells with preimmune sera control wells, the cross reactivity of dp1a antibody with extensin RG-I complexes was demonstrated.

Analytical Methods

Capillary Gas-Liquid Chromatography

The trimethylsilyl derivatives of methyl glycosides were separated on a fused silica capillary column (30m x 0.25 mm i.d., Durabond-1 liquid phase, J &W Scientific, Rancho Cordova, CA) installed in a Varian 3300 gas chromatograph equipped with an on-column injector and helium carrier gas. One or two μL aliquots of sample were injected at 105°C. After 1 minute the temperature was raised at the rate of 10°C/minute to 160°C for 4 minutes, and the raised at a rate of 1°C/minute until 200°C was reached, whereupon the temperature was immediately raised to 240°C and held there for 10 minutes in order to clean the column. Peaks were integrated using a Varian 4290 integrator.

Reverse Phase Chromatography

The mapping of tryptic peptides was accomplished using a procedure modified from that described by Aebersold et al. (1987). A Bakerbond wide pore (300 Å) octadecyl C18 (5 μm) reversed phase column (4.6 x 250 mm, J.T. Baker Inc.) was used in this experiment. Solvent A was 0.1% (v/v) of trifluoroacetic acid, 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 concetration: 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 UV absorption at 214 nm with those of interest collected. Peptide mappings were performed using the Beckman HPLC system consisting of two model 110B pumps, a model 421 controller and a model 163 variable wavelength detector.

Gel Filtration Liquid Chromatography

Tryptic peptides separated on reverse phase C18 column were further purified on a Fracto-gel HW 40 (S) gel filtration column (50 x 1 cm, Supelco Inc., Bellefonte, PA). The elution buffer was 0.1% (v/v) trifluoroacetic acid with a flow rate of 0.5mL/minute. Peptides were detected by a UV monitor set at 215 nm.

Soluble fractions containing extensin RG-I complex were fractionated on Fracto-gel HW 50 (S) gel filtration column (50 x 1 cm, Supelco Inc., Bellefonte, PA). Trifluoroacetic acid (0.1% v/v) was used as the elution solvent with a flow rate of 0.5 mL/minute. Peptides were detected by UV monitor set at 215 nm.

Determination of Carbohydrate Composition

The carbohydrate composition of samples was determined by gas chromatographic analysis of the trimethylsilyl glycoside derivatives. Methanolysis and derivatization were performed by a modification of the protocol of Chaplin (1982). About 50 µg each of dry samples were weighed on a CAHN 29 Electrobalance and placed in screw-cap glass vials with Teflon®-lined lids along with 100 nmoles of myo-inositol as an internal standard. Two hundred µL of 1.5M methanolic HCl and 50 µL of methyl acetate were added to the samples, after which the vials were tightly sealed and incubated in an 80°C heating block overnight. The vials were removed the following morning, and a few drops of t-butanol then added to each vial to quench residual HCl. The samples were then evaporated to

dryness under a stream of nitrogen gas. Trimethylsilylating reagent was prepared fresh in an exhaust hood by mixing 1 part Tri-Sil Concentrate (Pierce Chemical Company, Rockford, IL) with 3 parts of anhydrous pyridine. Twenty-five µL of the reagent was added to the dried samples and allowed react for a minimum of 15 minutes to effect derivatization. The derivatized samples were then evaporated under a gentle stream of nitrogen gas just to dryness, and then redissolved in 50 to 200 µL of 2,2,4-trimethylpentane. One µL aliquot was injected into a fused silica capillary column installed in a Varian 3300 gas-liquid chromatograph. Peak integration was performed using a Varian 4290 integrator.

Determination of Amino Acid Composition

Approximately 500 pmoles (on an amino acid basis) of dry protein samples were hydrolyzed in 200 µL of 6N HCl (sesquanal grade, Pierce Chemical) at 110°C for 18 to 24 hours in sealed, screw-cap glass vials with Teflon®-lined lids. The resulting amino acids were evaporated to complete dryness. All drying steps were carried out in a Speed Vac Concentrator (Savant). The hydrolyzed samples were then analyzed with a method of Seferiadis et al (1987). One hundred µL phenylisothiocynate (PITC) derivitization 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 those described by Heinriksona and Meredith (1984).

Five µL of PITC were added along with 100 µL of coupling buffer. The reaction time was five minutes for hydroxyproline and amino acid standards (Pierce-H), and 20 minutes for unknown protein or peptide hydrolyzates.

Prior to separation by HPLC, the PITC derivatized samples were dissolved in acetonitrile/water (1:1 v/v) and diluted in a like volume of solvent A. The eluent consisted of Sovent A, 0.05M ammonium acetate (pH 6.0) and Solvent B, 0.1M ammonium acetate in acetonitrile/methanol/water/ (44:10:46 v/v/v).

Analyses were performed using a Beckman HPLC system using a Lichrosorb C18 5 micron , 4.0 x 250 mm (Hibar) column, preceded by a 5 micron , 4.6 x 20 mm C18 guard column (Alltech). The column and guard column were covered by a glass jacket and held at 52°C by a model FS recirculating water bath (Haake).

Determination of Hydroxyproline Content

The hydroxyproline content was determined colorimetrically at 560 nm by the procedure of Drozda et al. in 1976. Between 150 and 500 µg of dry cell wall samples (or other samples) were hydrolyzed in 6N HCl at 110°C for 24 hours in screw-cap glass vials fitted with Teflon®-lined lids. Hydrolized samples were reacted with the oxidizing agent chloramine T, with the hydroxyproline in the samples being converted to pyrrole-like compounds. Formation of the chromophore is the result of the action of p-dimethylaminobenzaldehyde (Erlich's reagent) with pyrrole derivatives from hydroxyproline. The chromophore was then detected spectrophotometrically.

Amino Acid Sequencing

Approximately 50 pmoles of Acala 44 cotton cell wall extensin peptides (freeze-dried samples) obtained from purification by reverse phase chromatography followed by gel filtration HW 40 (S) were sequenced commercially on a model 473A Amino Acid Sequencer (Applied Biosystems, Inc.) by Dr. Ken Jackson, Molecular Biology Resource Facility, William K. Warren Medical Research Institute, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma.

Peptide Sequence Searches

Peptide sequence homology searching was done by Dr. Ulrich K. Melcher, Biochemistry and Molecular Biology Department of Oklahoma State University, with

computations performed at the National Center for Biotechnology Information (NCBI) using the BLAST network service.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was carried out as described by Laemmli (1970). The acrylamide concentrations in the running gel was 15%. The samples (50μg to 110μg of Extensin RG-I Complexes and deglycosylated extensin fragments) were run at 195 volts for approximately 40 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 (Bio-Rad Laboratories, Richmond, CA). The broad range molecular weight standards, (Bio-Rad Laboratories) were as follows: Myosin, MW 200,000; β-Galactosidase, MW 116,250; Phosphorylase B, MW 97,400; Bovine Serum Albumin, MW 66,200; Ovalbumin, MW 45,000; Carbonic Anhydrase, MW 31,000; Soybean Trypsin Inhibitor, MW 21,500; Lysozyme, MW 14,400; Aprotinin, MW 6,500. The prestained broad range molecular weight standards , (Bio-Rad Laboratories) were as follows: Myosin, MW 205,000; β-Galactsidase, MW 116,500; Bovine Serum Albumin, MW 80,000; Ovalbumin , MW 49,500; Carbonic Anhydrase, MW 32,500; Soybean Trypsin Inhibitor, MW 27,500; Lysozyme, MW 18,500; Aprotinin, MW 6500.

Western Blot Analysis

Samples were separated by electrophoresis in SDS-PAGE (30:0.8% w/v acrylamide:bis). Analysis of the distribution of Extensin RG-I Complexes A and B were done using gels of 15% SDS-PAGE. Glycoproteins in the gels were electro-transferred at a constant current density of 2.5mA/cm^2 for 20 to 25 minutes using an ABN polyblot transfer system (Model SBD-1000) as described in the American Bionetics instruction manual. The PVDF membrane (0.2 micron, Bio-Rad Trans-Blot Transfer Medium) was

soaked in methanol and rinsed with distilled water before blotting. The blotted PVDF membrane was rinsed with Tris buffered saline (TBS) buffer containg 20mM Tris-HCl (pH 7.6) and 150mM NaCl for 5 minutes, then soaked for 1 hour at room temperature in 5% (w/v) non-fat dry milk in TBS buffer. After blocking, blots were incubated overnight at 4°C with 20mL TBS/1% (w/v) non-fat milk containg antibodies to deglycosylated tomato extensin precursor dp1a diluted 1:40. Blots were washed 5 minutes consecutively with TBS, then twice with TBS/0.5% Tween 20 (polyoxyethylene sorbitan mono laurate), then with TBS again. After reblocking for 10 minutes at room temperature with 5% (w/v) non-fat dry milk in TBS buffer, the blots were incubated for 2 hours at room temperature with 20mL TBS/0.125% non-fat milk containing goat anti-rabbit immunoglobulin G (H+C) alkaline phosphatase conjugate diluted 1:5000. Blots were again washed as described above. Bands were then visualized at 35°C using 5-bromo-4-chloro-3-indonyl -phosphate (BCIP). The BCIP substrate solution (30mL) consists of 10mg of nitro blue tetrazolium (NBT), 5mg of BCIP in AP buffer (pH 9.5) containing 100 mM Tris HCl, 100 mM sodium chloride and 100mM magnesium chloride, prepared just prior to use. The reaction was stopped immediately after bands appeared by soaking the blots in distilled water. Western blots were then dried with paper towels and photographed to provide a permanent record.

CHAPTER IV

RESULTS AND DISCUSSION

Characterization of Cotton Cell Wall Extensin

Isolation of Extensin Fragments from Cotton Cell Walls

Cell walls were prepared from cotton suspension cells Acala 44. There are several different combinations of treatments that can isolate different amount of extensin fragments from cell walls as judged by release of hydroxyproline-containing peptides. When the procedures shown in Figure 2 were used, most of the extensin peptides were solubilized. First, one gram of Acala 44 cotton suspension cultured cell walls was treated with 20 mL of liquid anhydrous hydrogen fluoride (HF) at 0°C for 30 minutes. This treatment completely removes the sugars from cell walls except for those linkages of amino sugars (which are in very low concentration in our samples) and some of the non-methyl esterified homogalacturonans without breaking any peptide bonds (Mort and Lamport, 1977). After HF solvolysis, the insoluble residue from Acala 44 cotton suspension cultured cell walls was extracted by ether and water to solubilize and remove most of the sugars. Furthermore, the residue was extracted by 50 mM ammonium acetate buffer (pH 5.2) at room temperature overnight to remove some homogalacturonan fragments. The resulting residue was then digested with trypsin (2% w/w) in 100 mM ammonium bicarbonate buffer (pH 7.6) at room temperature overnight. Extensin peptides were solubilized in the filtrate fraction.

The extensin peptide samples were then fractionated on a reverse phase HPLC, giving rise to two major fractions (I and II, see Figure 3) and many minor ones (Peaks

Figure 2. Flow Chart of Solubilization of Fragments Containing Hyp-Rich Extensin Peptides from Cotton Suspension Cell Walls.

Cotton cell walls were 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. Solubilized extensin peptides were contained in filtrate fraction. The value given in parentheses represents the weight of each fraction recovered after lypholization.

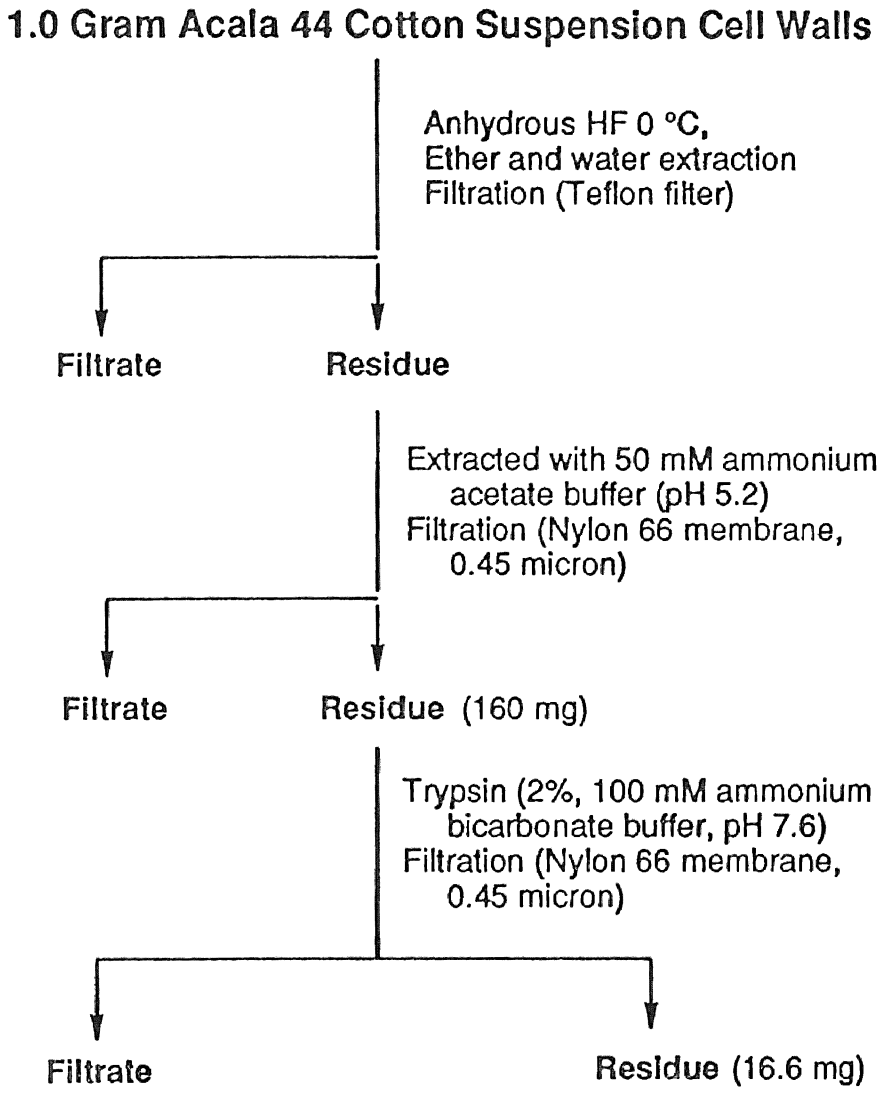
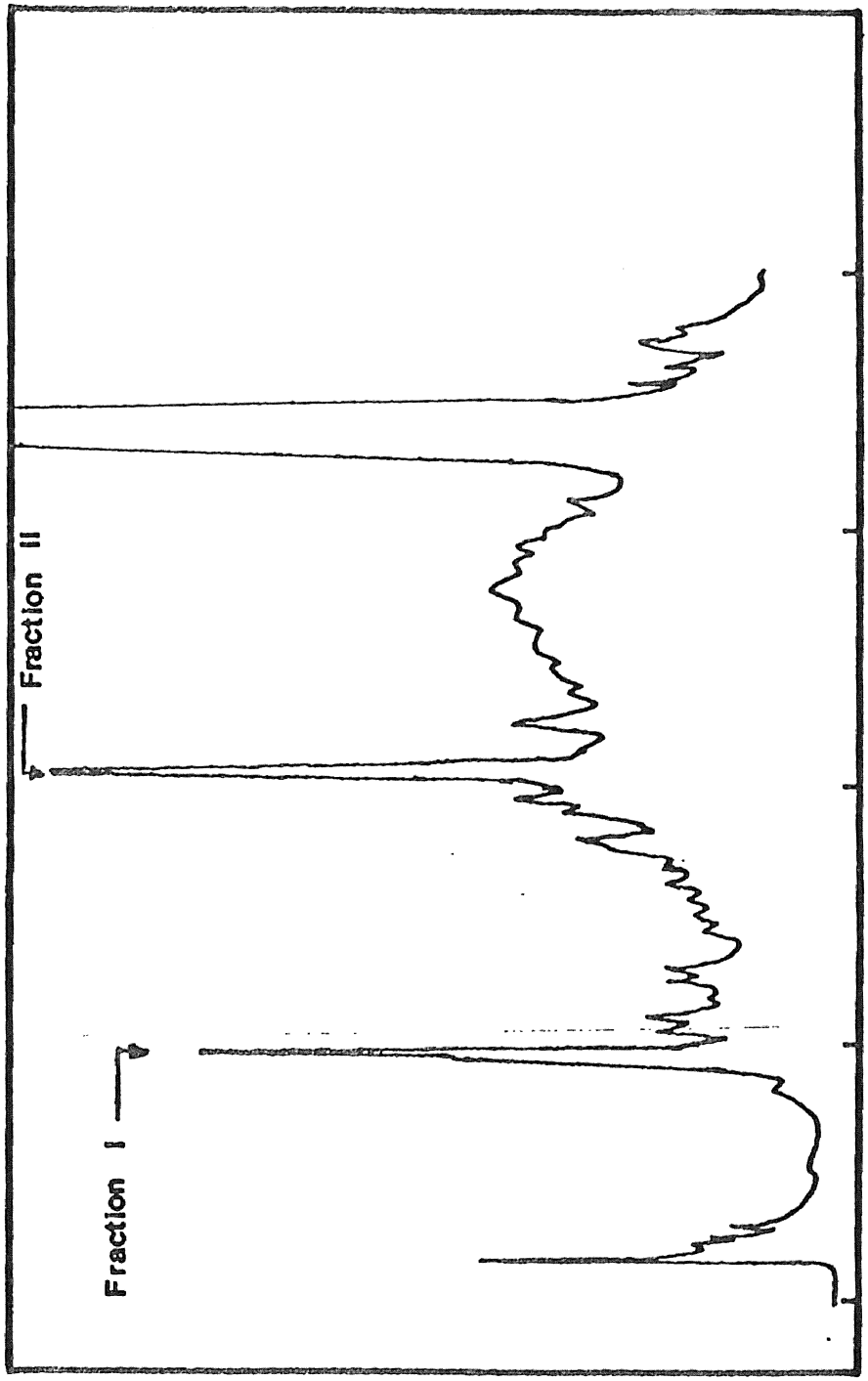

1.0 Gram Acala 44 Cotton Suspension Cell Walls
Anhydrous HF 0 °C,
Ether and water extraction
Filtration (Teflon filter)
Filtrate
Residue
Extracted with 50 mM ammonium acetate buffer (pH 5.2)
Filtration (Nylon 66 membrane, 0.45 micron)
Filtrate
Residue (160 mg)
Trypsin (2%, 100 mM ammonium bicarbonate buffer, pH 7.6)
Filtration (Nylon 66 membrane, 0.45 micron)
Filtrate
Residue (16.6 mg)

Figure 3. The HPLC Chromatogram of the Filtrate Solubilized by Trypsin Digestion in Figure 2 on Reverse Phase C18 Column.

The elution buffer consisted of solvent A: 0.1 % trfluoroacetic 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/minute. 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 41-42 in fraction II.


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

from reverse phase HPLC will be refered to as "fractions" while those from HW40 (S) chromatograms will be referred to as "peaks" in order to differentiate them from each other and facilitate the discussion). From amino acid composition analysis and hydroxyproline (Hyp) content determination, both major fractions (I and II) are rich in Hyp. Fraction I contains 23.2 % of the Hyp present in initial cell wall sample, and fraction II contains 19.2 % of the Hyp. This suggests that these two fractions contain repetitive Hyp-rich sequences of extensin. The Hyp content in different fractions is given in Table 1. Fractions I and II were further purified by gel filtration chromatography on a Fracto-Gel HW 40 (S) column. Peptides from fraction I of reverse phase HPLC followed by gel filtration have been sequenced (Qi, 1992). Attempts to sequence fraction II, however, indicated a broad range of N-termini (Qi, 1992). This may due to the impurity of samples, or as an artifact of HF 0°C treatment.

Therefore, a series of mild and selective treatments, shown in Figure 4, was used and showed that extensin peptides could be obtained from different fractions. One gram of Acala 44 cotton suspension cultured cell walls was treated with 18 units of endopolygalacturonase purified from *E. Coli* (Willis et al., 1987; Maness and Mort, 1989) in 50 mM ammonium acetate buffer (pH 5.2) to remove homogalacturonic acid. After filtration through a 0.45 micron NYLON 66 membrane, the residue was washed three times with distilled water and then freeze dried. The EPGase-treated residue A was digested with chromatographically purified Worthington cellulase (2% w/w) in 50 mM ammonuim acetate buffer (pH 5.2) overnight at room temperature to remove cellulose and xyloglucan. The residue B after cellulase digestion was treated with liquid HF at -73°C for 30 minutes to specifically cleave arabinose residues from Hyp residues in extensin since HF treatment at -73°C breaks only arabinofuranosyl glycosidic linkages (Qi et al., 1993). After extraction with ether, water and 100 mM (pH 7.6) ammonium bicarbonate buffer (to extract RG-I), the HF treated residue C was digested with trypsin (2% w/w) in 100 mM ammonium bicarbonate buffer (pH 7.6) at room temperature overnight to liberate extensin

TABLE 1

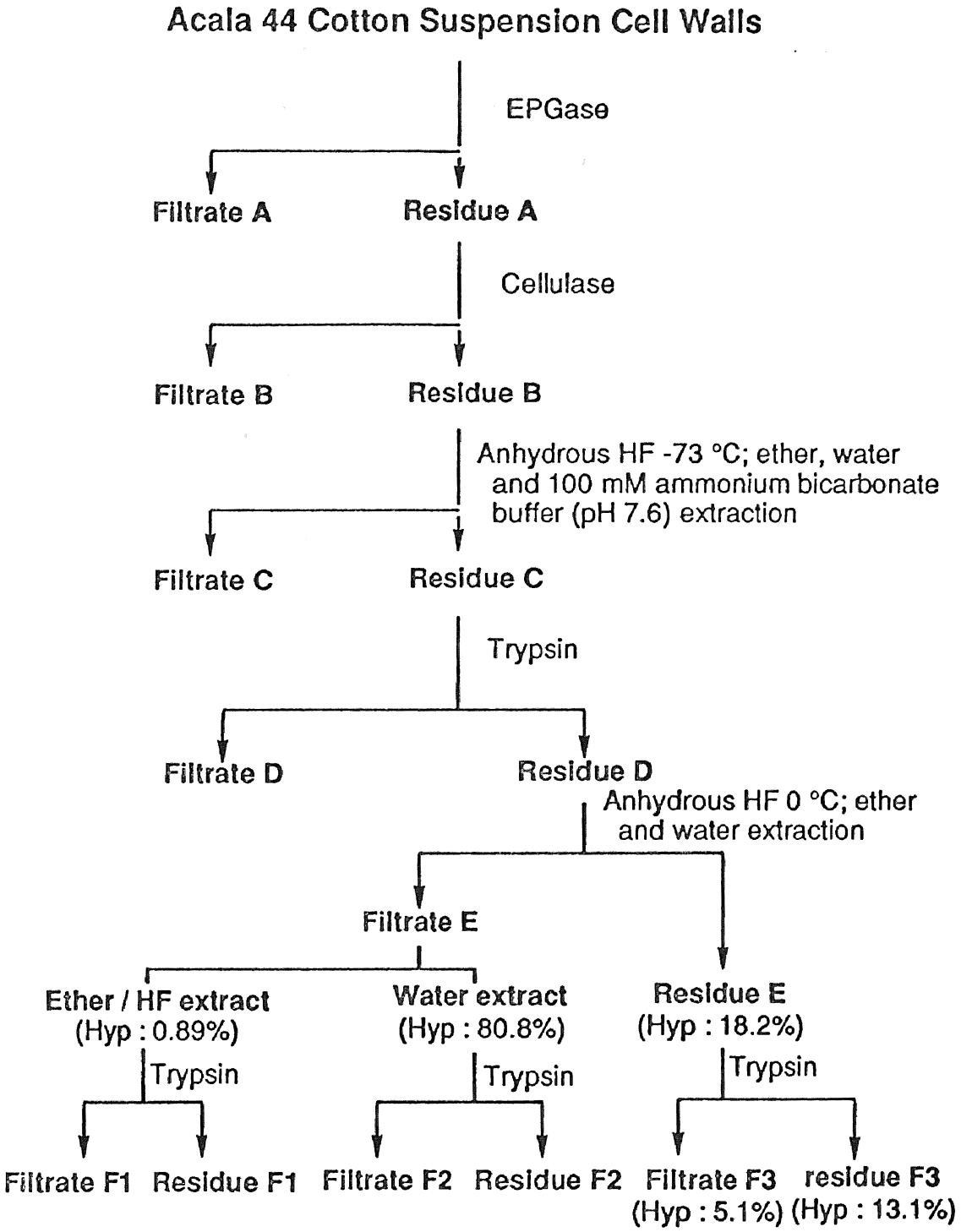
The Percentage of Hydroxyproline Content in Fractions with Different Retention Times on Reverse Phase HPLC Shown in Figure 3

Retention time (min.)	3 - 17	18 - 20	21 - 40	41 - 42	42 - 65	66 - 80
% Hyp*	2.4	23.2	12.8	19.2	18.4	4.4

* % Hydroxyproline of the original cotton suspension cell walls

Figure 4. Flow Chart of Isolation of Fragments Containing Hyp-Rich Extensin Peptides from Cotton Suspension Culture Cell Walls.

Cotton cell wall was treated with the combination of 1) EPGase digestion 2) cellulase digestion 3) HF solvolysis at -73°C following by ether, water and 100 mM ammonium bicarbonate buffer (pH 7.6) extraction 4) trypsin digestion 5) HF solvalysis at 0°C 6) trypsin digestion. The Hyp-rich extensin peptides were solubilized in filtrate F. The value given in parentheses represents the percentage of Residue D.


Acala 44 Cotton Suspension Cell Walls
EPGase
Filtrate A
Residue A
Cellulase
Filtrate B
Residue B
Anhydrous HF -73 °C; ether, water and 100 mM ammonium bicarbonate buffer (pH 7.6) extraction
Filtrate C
Residue C
Trypsin
Filtrate D
Residue D
Anhydrous HF 0 °C; ether and water extraction
Filtrate E
Ether / HF extract (Hyp : 0.89%)
Water extract (Hyp : 80.8%)
Residue E (Hyp : 18.2%)
Trypsin
Trypsin
Trypsin
Filtrate F1
Residue F1
Filtrate F2
Residue F2
Filtrate F3 (Hyp : 5.1%)
residue F3 (Hyp : 13.1%)

fragments into filtrate D (the characterization of these fragments will be discussed in next section). Samples of residue D were then treated with HF at 0°C followed by ether and water extraction. Hyp was contained in both filtrate E (ether/HF extract and water extract) and residue E fractions, with the water extract containing the most abundant Hyp (about 80.8% of the residue D or about 39% of the initial cell wall sample). All three fractions were then treated with trypsin to obtain the tryptic extensin peptides.

In residue D, about 48% of the Hyp and about 9% of sugars (which are mainly RG I like sugars) remained insoluble (Qi, 1992). Up to this step, the polysaccharides with noncovalent linkages should have been removed. However, further treatment of the residue D with HF at 0°C (cleaving glycosidic bonds), most of the Hyp-containing peptides which were not solubilized by the previous trypsin digestion were co-solubilized with polysaccharides (mainly RG I-like sugars). These results support the existence of covalent cross-links between extensin and pectin. In addition, after trypsin digestion of residue E, there was still about 13.1% Hyp of residue D or 6.3% Hyp of the original cell wall sample in residue F3. This indicates that there may be direct covalent cross-links between extensin molecules or extensin-phenolic compounds. This would help explain why the extensin remains in residue F3. Compared with the procedures shown in Figure 2 which left 20% Hyp unsolubilized (Qi, 1992), the milder series of procedures used in this work (see Figure 4) yields greater solubilization of extensin peptides. This may be due to the harsh treatment (HF at 0°C) leading to synthetic crosslinking between extensins or extensin-phenolic compounds by alkylation or Friedel-Crafts alkylation (Solomons, 1984) when all these potential crosslinking components exist together.

Certainly the questions should be raised as to why extensin peptides were solubilized in different steps of the series treatments. As shown in Figure 4, the Hyp-containing peptides in filtrate D were solubilized with the first trypsin digestion after sequential treatment; the extensin peptides in filtrate E were not liberated by trypsin but were co-solubilized with polysaccharides; peptides in filtrate F3 fraction were not

cosolubilized by HF 0° C treatment but were liberated following trypsin digestion. Are there differences in the composition and structure, therefore leading to associations with other cell wall polymers among these extensin peptides eluted in different fractions? Further characterization and comparision of these extensin peptide fragments as described below will help us to know what extensin peptide structure is involved and the nature of the interactions between extensin and other polymers in cell walls.

Partial Characterization of Tryptic Peptides

The soluble material containing extensin peptides from trypsin digestion of HF 0°C treated filtrate E (ether/HF extract, water extract) and residue E were fractionated on a reverse phase HPLC (see Figures 5, 6, and 7). All three of these different fractions had similar tryptic maps which contained two major fractions (I and II) and many minor ones. These tryptic maps are also similar to that of Hyp-rich fractions shown in Figure 3. Amino acid composition analysis showed that both fractions I and II are rich in Hyp. So, these two major fractions must contain the repeating sequences of Hyp rich extensin peptides. When the material in ether/HF extract before and after trypsin digestion was fractionated on reverse phase HPLC (see Figures 7 and 8), only the one after trypsin digestion had these similar fractions (I and II) which contained extensin peptide repetitive sequences as those shown in Figure 3. This could indicate that the peptides which co-solubilized with sugars from residue D by HF treatment were larger than the completely digested tryptic peptides and contained peptides involved in the cross-linkages with pectin.

The peptides contained in fractions I and II were further fractionated on a Fracto-Gel HW 40 (S) column (fractionation range from 100 to 10,000 Daltons). The gel filtration chromatography of Filtrate F2 fraction I (Figure 9) showed similar peaks as those that contained the repeated extensin peptides sequenced by previous work in our laboratary (Qi, 1992). The peptides contained in Filtrate F2 fraction II and from Filtrate F3 fraction II were purified by gel filtration chromatography as shown in Figures 10 and 11.

Figure 5. The HPLC Chromatogram of the Filtrate F2 Solubilized by Trypsin Digestion of Water Extract in Figure 4 on Reverse Phase C18 Column.

The elution buffer consisted of solvent A: 0.1% trifluoroacetic acid (pH~2), solvent B: 0.1% trifluorozcetic 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 1.0 mL/minute. Detection was by UV monitor at 214nm. The sample was dissolved in 0.1% trifluoroacetic acid and injected. Tubes 19-20 were pooled into fraction I, and tubes 38-39 into fraction II.

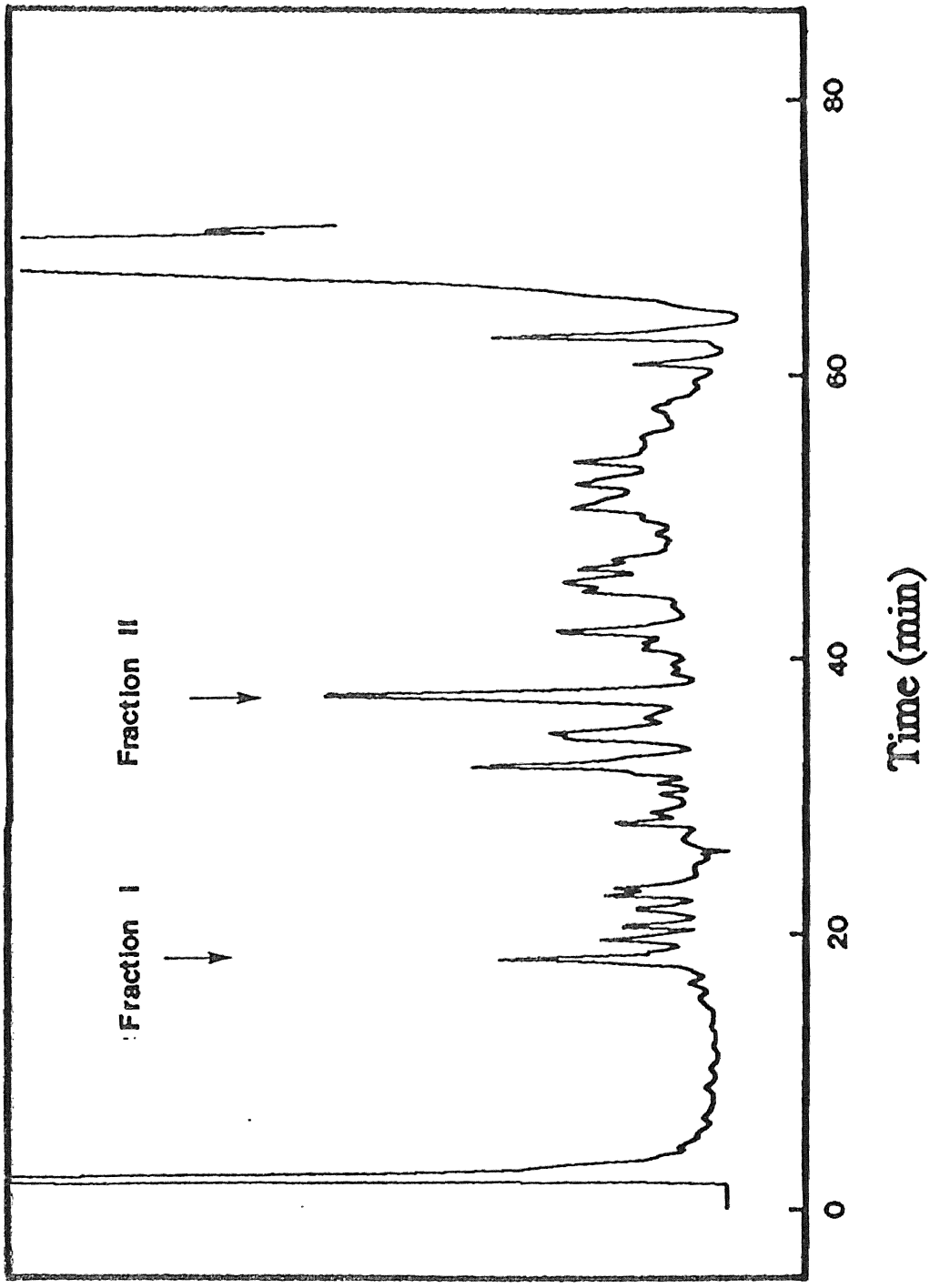

Absorbance (214 nm)
Fraction I
Fraction II
0
20
40
60
80
Time (min)

Figure 6. The HPLC Chromatogram of the Filtrate F3 Solubilized by Trypsin Digestion of Residue E in Figure 4 on Reverse Phase C18 Column.

The elution buffer consisted of solvent A: 0.1% trfluoroacetic 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 1.0 mL/minute. Detection was by UV *monitor at 214nm.* The sample was dissolved in 0.1% trifluoroacetic acid and injected. Tubes 19-20 were pooled into fraction I, and tubes 38-39 into fraction II.

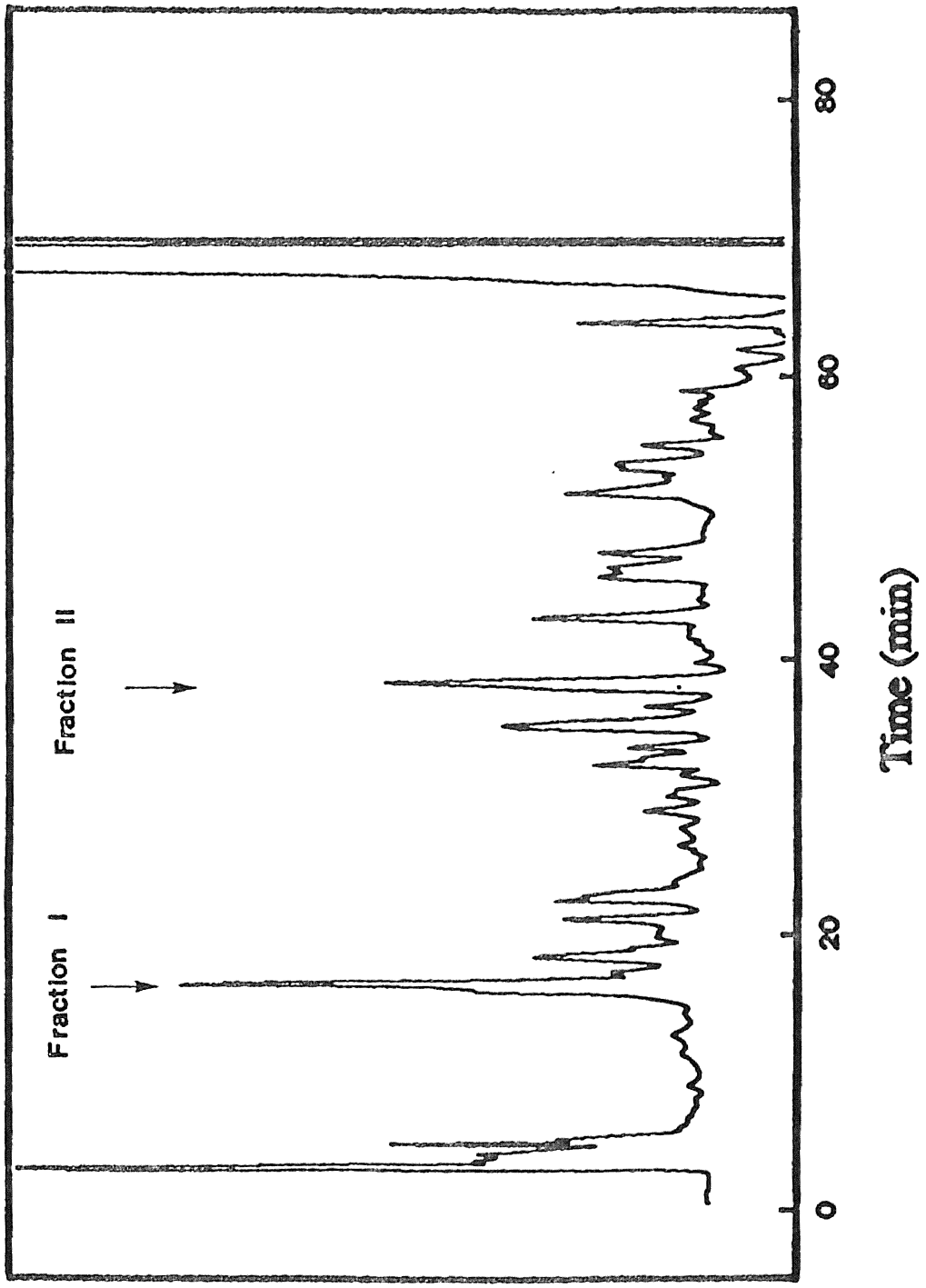

Absorbance (214 nm)
Fraction I
Fraction II
0
20
40
60
80
Time (min)

Figure 7. The HPLC Chromatogram of the Filtrate F1 Solubilized by Trypsin Digestion of Ether/HF Extract in Figure 4 on Reverse Phase C18 Column.

The elution buffer consisted of 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 1.0 mL/minute. Detection was by UV monitor at 214nm. The sample was dissolved in 0.1% trfluoroacetic acid and injected. Tubes 23-24 were pooled into fraction I, and tubes 48-49 into fraction II.

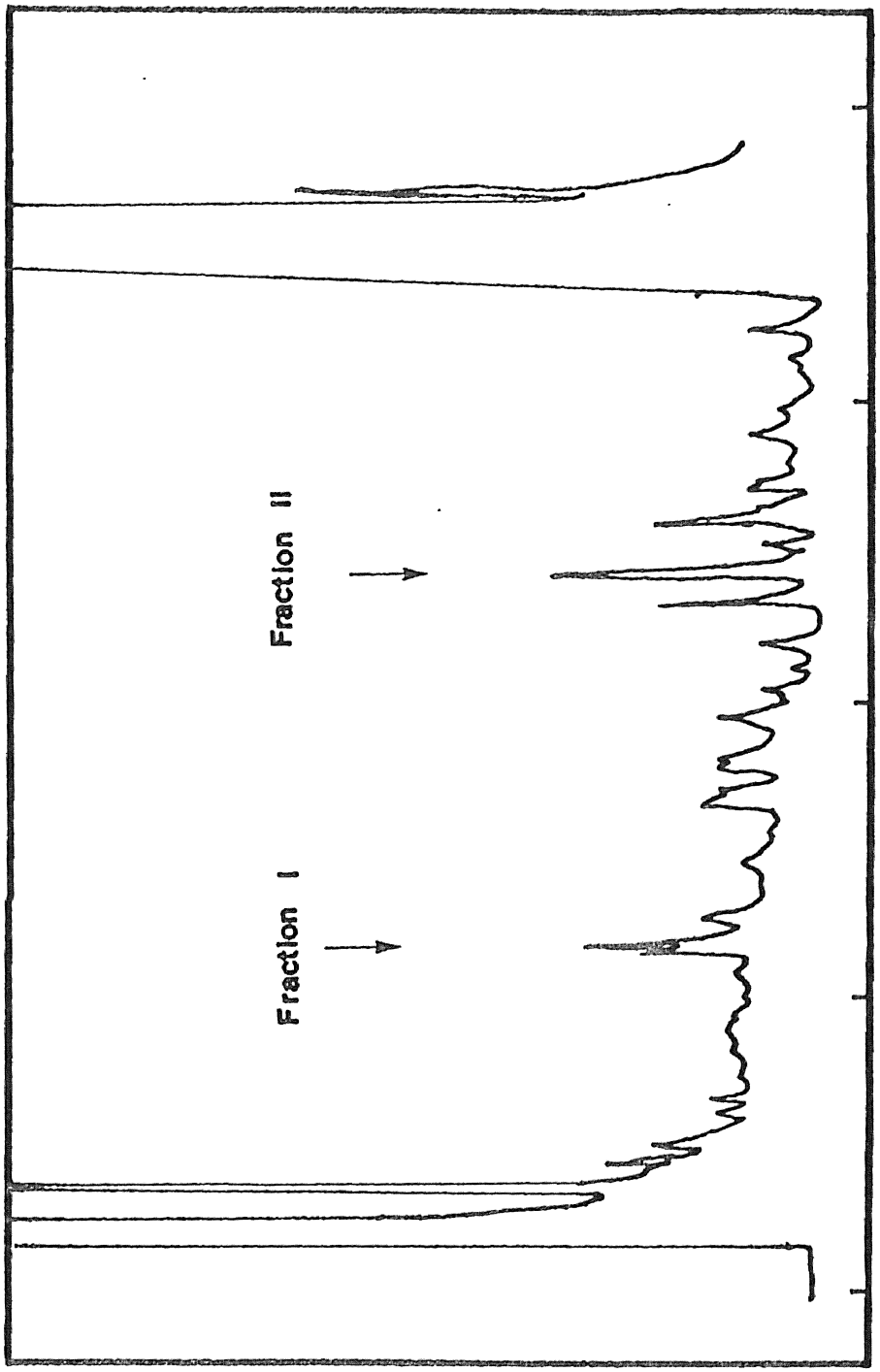

Absorbance (214 nm)
Fraction I
Fraction II
0
20
40
60
80
Time (min)

Figure 8. The HPLC Chromatogram of Ether/HF Extract in Figure 4 on Reverse Phase C18 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 1.0 mL/minute. Detection was by UV monitor at 214 nm.

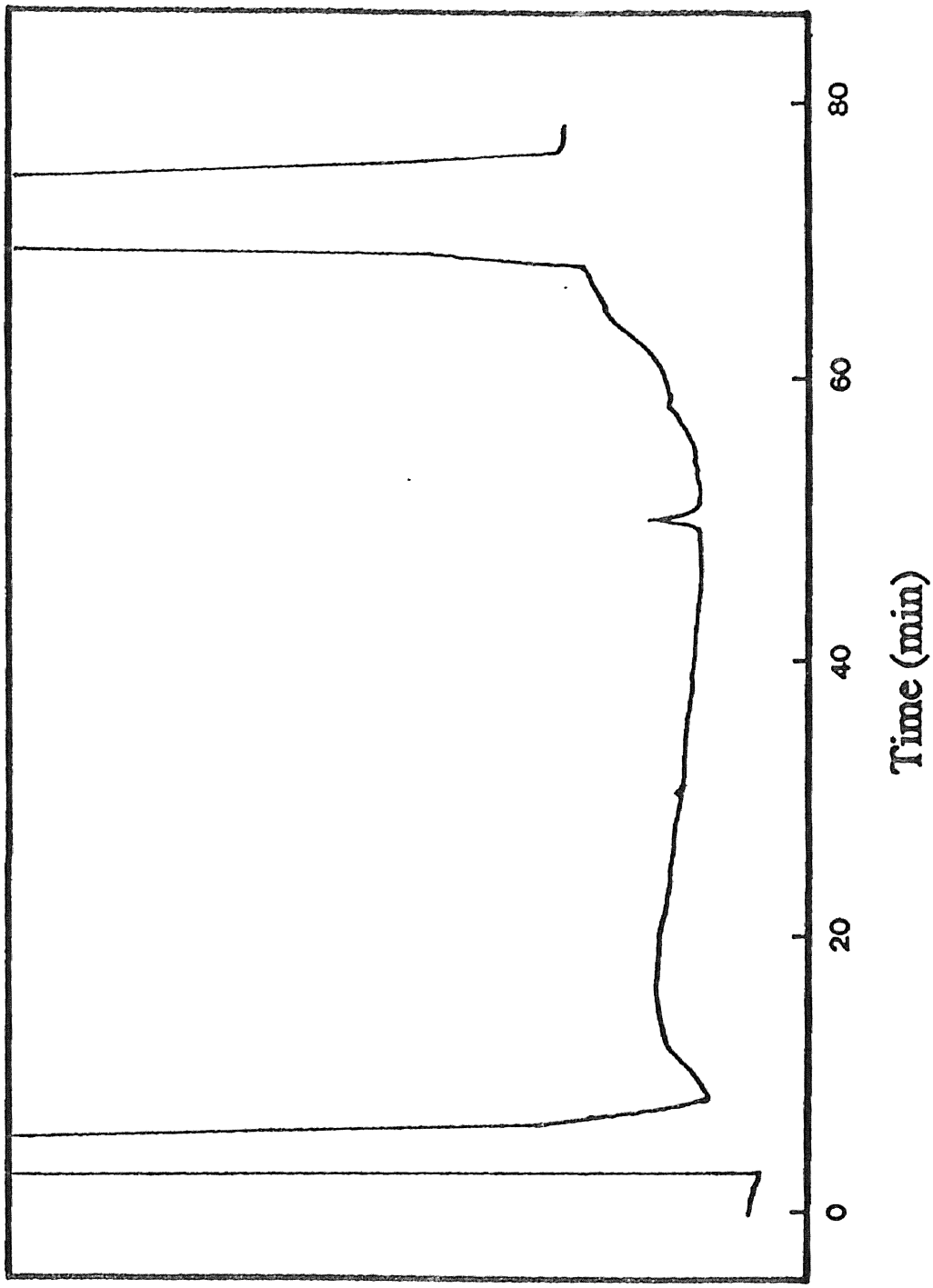

Absorbance (214 nm)
0
20
40
60
80
Time (min)

Figure 9. 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/minute. Detection was by UV monitor at 215 nm.

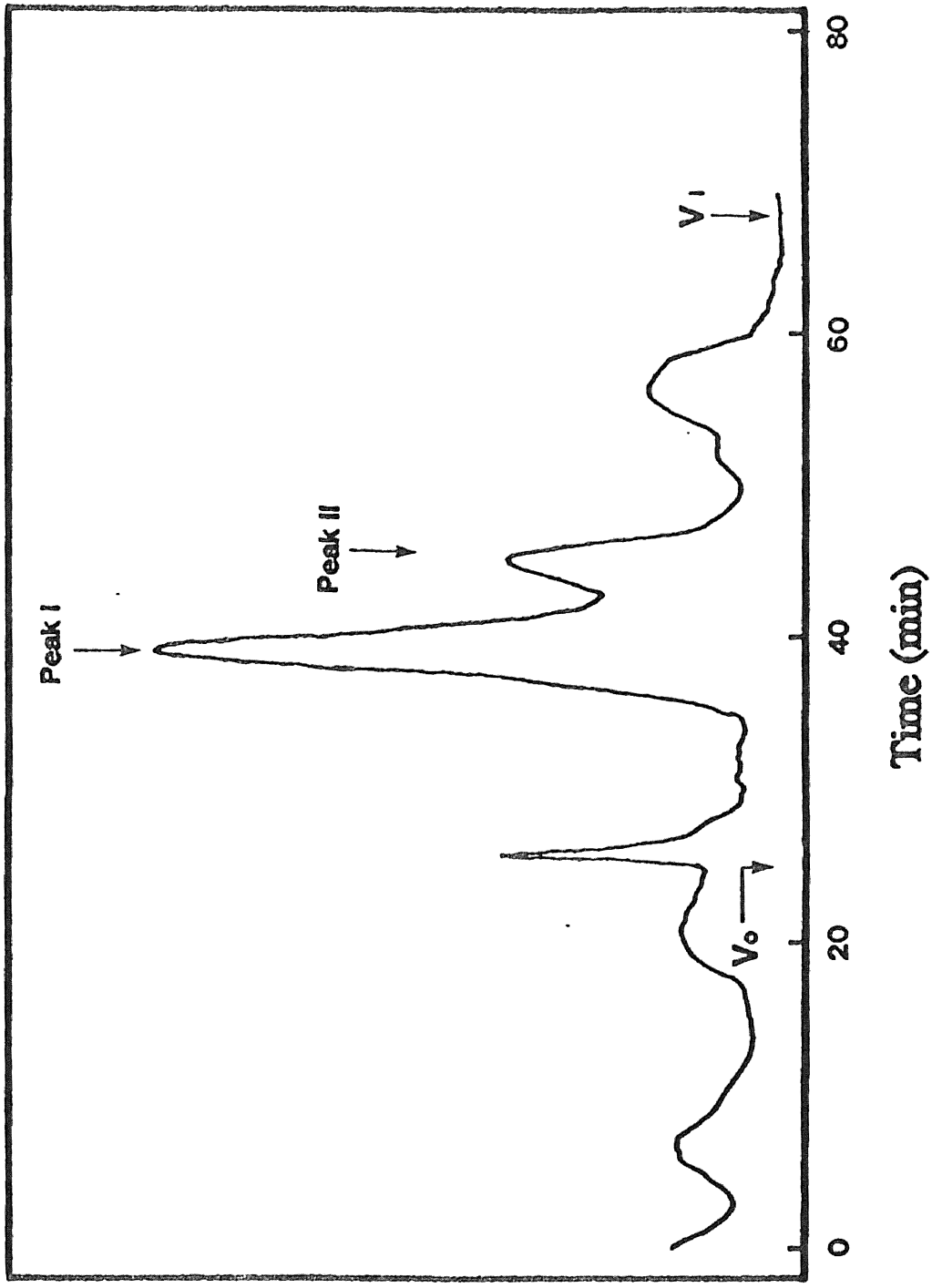

Peak I
Peak II
V_o
V_t
0
20
40
60
80
Time (min)
Absorbance (215 nm)

Figure 10. HW 40 (S) Gel Filtration Chromatogram of Fraction II from Reverse Phase HPLC (Figure 5) of HF 0° C Water Extract of Residue D in Figure 4.

Sample was applied in and eluted with 0.1% trifluoroacetic acid. The flow rate was 0.5 mL/fraction/minute. Detection was by UV monitor at 215 nm. Fractions 35-39 were pooled into peak I, fractions 40-45 were pooled into peak II, fractions 46-49 were pooled into peak III, fractions 54-58 were pooled into peak IV, and fractions 67-71 were pooled into peak V.

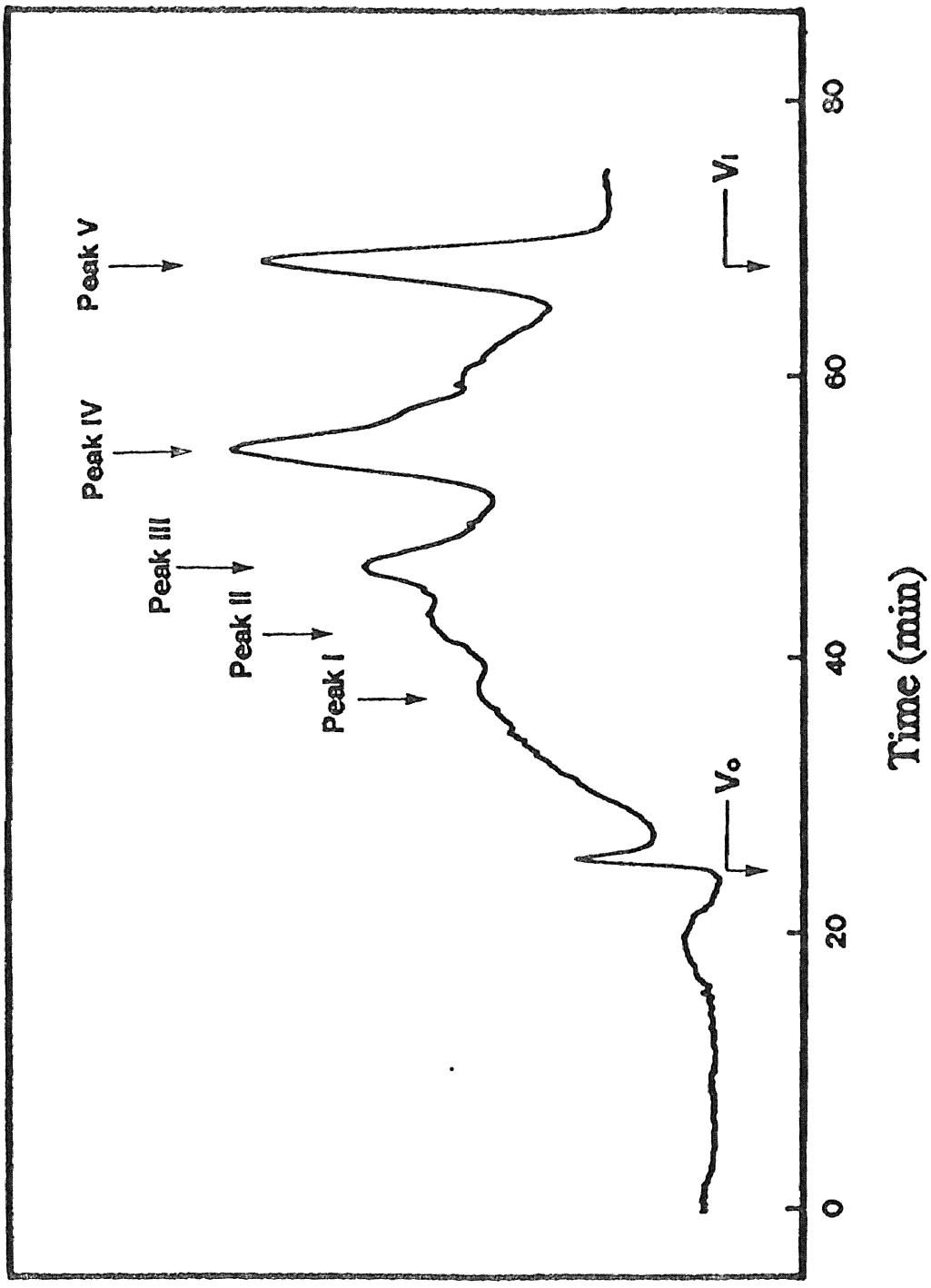
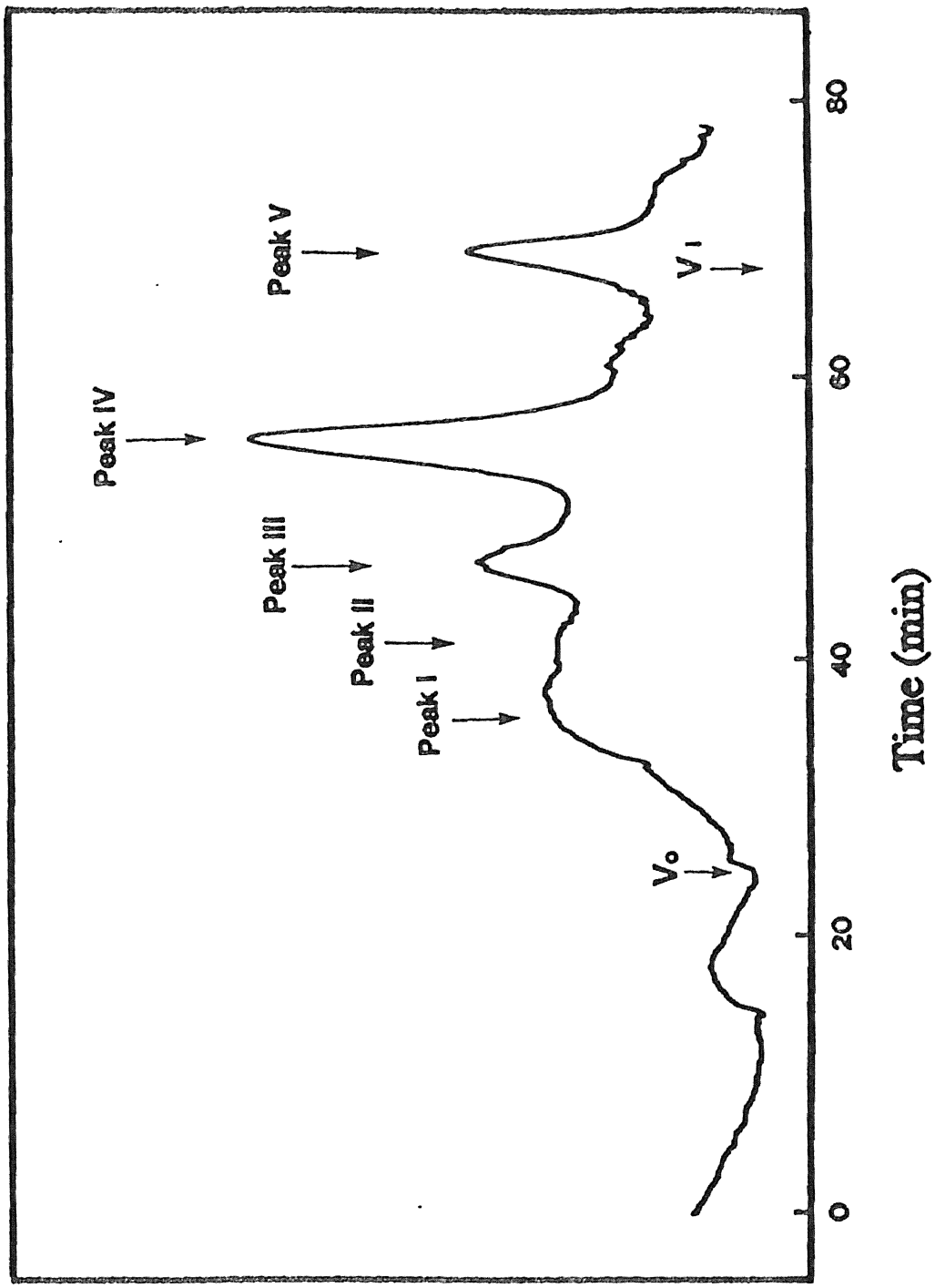

Peak V
Peak IV
Peak III
Peak II
Peak I
Vo
Vt
0
20
40
60
80
Time (min)
Absorbance (215 nm)

Figure 11. HW 40 (S) Gel Filtration Chromatogram of Fraction II from Reverse Phase HPLC (Figure 6) of HF 0°C Unsoluble Residue E in Figure 4.

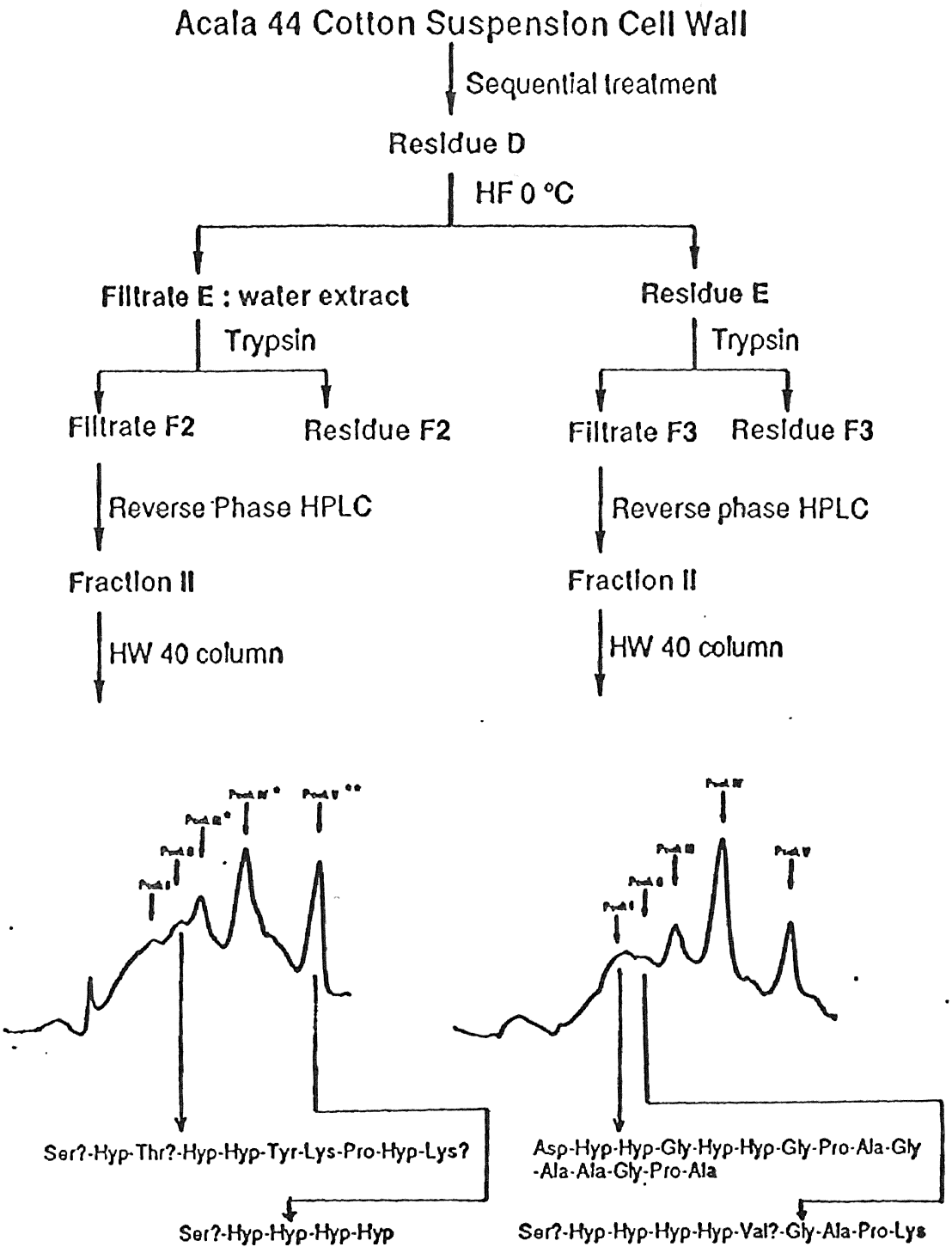
Sample was applied in and eluted with 0.1% trifluoroacetic acid. The flow rate was 0.5 mL/fraction/minute. Detection was by UV monitor at 215 nm. Fractions 35-39 were pooled into peak I, fractions 40-45 were pooled into peak II, fractions 46-49 were pooled into peak III, fractions 54-58 were pooled into peak IV, and fractions 67-71 were pooled into peak V.


Peak IV
Peak V
Peak III
Peak II
Peak I
V₀
V t
0
20
40
60
80
Time (min)
Absorbance (215 nm)

These peptides, after purification with gel filtration chromatography, were sequenced and peaks I, II and V were found to contain Hyp-rich peptides characteristic of extensins. In Figure 10, peak II from Filtrate F2 (water extract of HF 0°C treatment) contains one peptide with sequence of Ser?-Hyp-Thr?-Hyp-Hyp-Tyr-Lys-Pro-Hyp-Lys?. Sequence comparision by searching the GenBank (R) database at the National Center for Biotechnology Information (NCBI) showed that this sequence has similarity with almond extensin which has a sequence (deduced from cDNA) of Pro-Thr-Pro-Val-Tyr-Lys-Pro-Pro (the score of confidence is 43) and a proline rich protein with a sequence (deduced from cDNA) of Pro-Thr-Pro-Pro-Tyr-Val-Pro-Pro-Thr found in maize (score of confidence is 50). Other similar proteins include laminin B1 chain variant from chicken, enhancer-binding protein C/EBP delta from mouse and calmodulin-binding protein from fruit fly. Sequencing results from peak V showed that it contains a short peptide which is Ser?-Hyp-Hyp-Hyp-Hyp. In Figure 11, peak I contains a major sequence of Asp-Hyp-Hyp-Gly-Hyp-Hyp-Gly-Pro-Ala-Gly-Ala-Ala-Gly-Pro-Ala and some minor or trace amount of other amino acids in some cycles. Peak II was sequenced as: Ser?-Hyp-Hyp-Hyp-Hyp-Val?-Gly-Ala-Pro-Lys. The sequences of these four repeated peptides and their origin were shown in Figure 12. As shown above, two of the sequencing peptides did not show Lys as the C-terminal amino acid as one would expect since these are tryptic peptides. A possible explanation may be that the acid in the samples from HF treatment could remove Lys by partial hydrolysis (Epstein and Lamport, 1984), or the sequencing was simply not complete. Furthermore, some amino acids (indicated with question marks) of the peptides sequenced are not certain because there were multiple amino acids present in those sequencing cycles. The amino acids selected for those uncertain sequences were based on chromatograms from the sequencing lab showing the relative abundance of the amino acids present in that cycle as well as previous cycles. The most abundant amino acid present in one cycle and decreasing in the following cycle was often chosen. Of course judgement calls had to be made in these decisions, and the exact amino acid sequence remains unclear

Figure 12. Flow Chart Showing the Origin of Extensin Peptides Sequenced.

Extensin peptide-containing filtrate F2 and F3 were obtained by the series treatments shown in Figure 4. Filtrate F2 and F3 were fractionated on reverse phase HPLC. The fraction II then was separated by HW 40 (S) gel filtration chromatography column. Peaks II and V from filtrate F2 and peaks I and II from filtrate F3 were sequenced as indicated.



* From trypsin
**Also contains peptide from trypsin

as indicated by the question marks. We chose to include these questionable amino acids since they were the more logical choices. Although the sequencing results were not completely satisfactory, these possible peptide sequences gave us preliminary data, and provided more information for extensin structure of cotton suspension cell walls. The reasons for this uncertainty are due to the following: 1) the samples were not of sufficient purity and 2) the existence of post translationally modified, unusual amino acids in the peptide sample. Thus, further purification of samples from peaks I, II, and V (by rechromatography, for example) may improve the sequencing results. But if there are unusual, post translational amino acids present, the best approach is to find out the extensin gene (cDNA) sequences to know more about the extensin structure.

As shown above, the tryptic peptides of cotton suspension cell wall extensin gave only two major fractions and some minor fractions on reverse phase HPLC. And those four sequenced Hyp-rich extensin peptides comprise almost all of one of the two major fractions (fraction II), therefore they must be repeated several times within the cotton extensins. Comparing these peptide sequences of fraction II with those of fraction I (Qi, 1992), more hydrophobic amino acids were contained in peptides of fraction II as it was expected. Interestingly, the peptide sequences of peak II from filtrate F2 fraction were different from those of peak II from filtrate F3 fraction (see Figure 12) although these two peptides have the same character on reverse phase HPLC and HW40(S) gel filtration chromatography. Further investigation is necessary, however, since the sequencing results were preliminary.

Sequencing and homology comparision results also show that there are two types of peptides from trypsin in peak III, IV and V of Figure 10. Peak III was sequenced as Ala-Pro-Val-Leu-Ser-Asp-Ser-Ser-Cys-Lys. Peaks IV and V contained the same peptides and were sequenced as Ile-Val-Gly-Gly-Tyr-Thr-Cys-Ala-Ala-Asn. These are peptides from trypsin itself. It has been known for a long time that trypsin can undergo autolysis. It was suggested that an enzyme to protein (w/w) ratio of 1/25 (4%) is sufficient to

adequately digest the protein while keeping the amount of trypsin required to a minimum (Stone et al., 1989). At this weight/weight ratio, peptides resulting from trypsin autolysis are usually not a problem. Even so, this possibility can be evaluated by searching all of the resulting peptide sequences against the protein identification resource database to alert oneself to the possibility of a sequence arising from trypsin. In our case, although only 2 % (w/w) trypsin was used, the actual amount of protein in our glycoproteins was considerably less, so the amount of trypsin used may have been excessive.

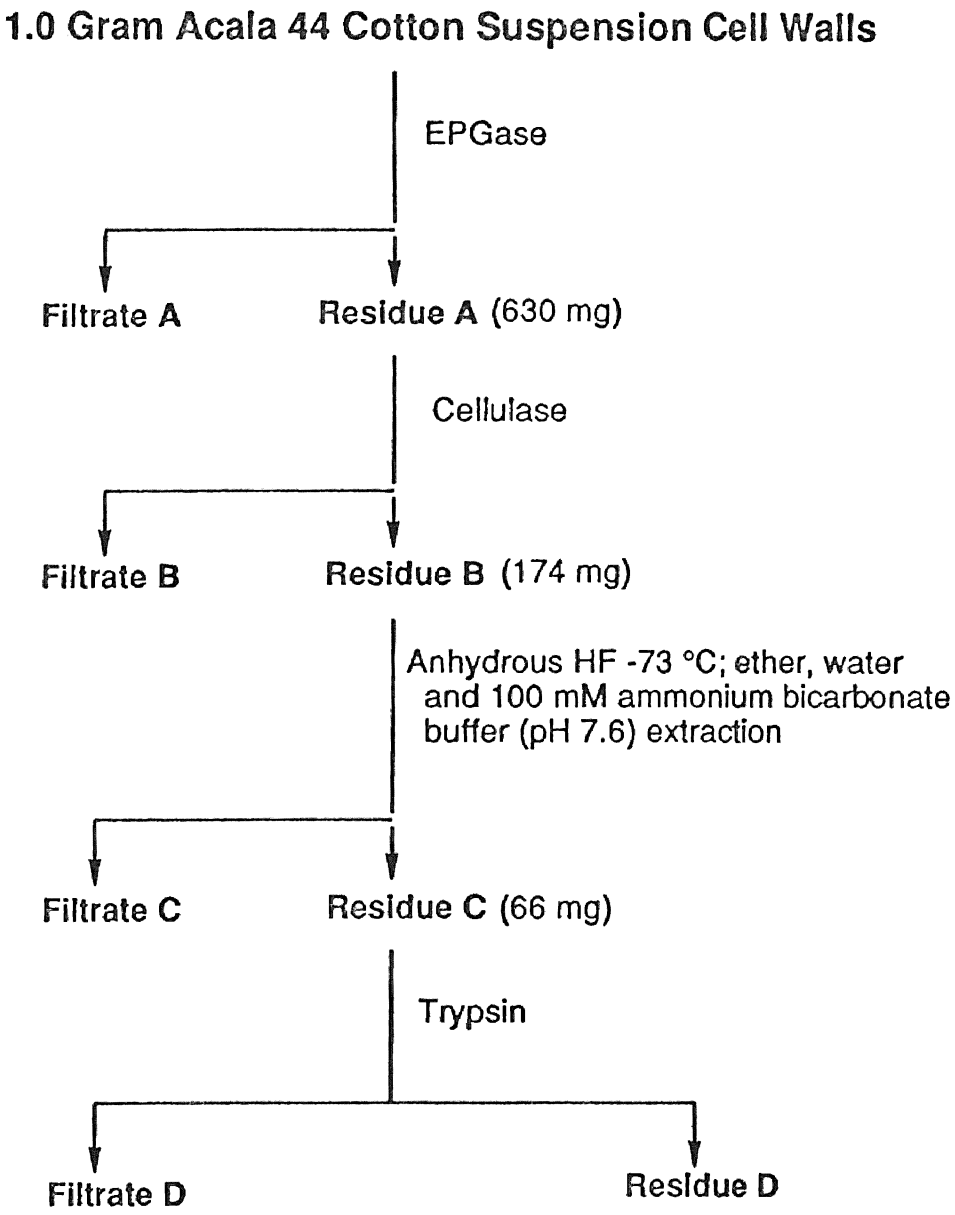
Investigation of Interaction between Extensin and Polysaccharides by Characterization of Extensin-RG I Complexes of Cotton Cell Walls

Solubilization of Extensin-RG I Complexes from Cotton Cell Walls

In order to ascertain whether there are covalent cross-links between extensin and polysaccharides in cotton cell walls, many highly specific methods for cleavage of polysaccharides and proteins were used to isolate possible cross-linked fragments which contained both amino acids and sugars. These cross-linking fragments were then characterized. Previous research in our laboratory (Qi, 1992) has shown that arabinose sidechains on the Hyp residues are important for preventing trypsinization of the protein and that the presence of cellulose inhibits the action of trypsin on extensin, and that a combination of specific sequential treatments can solubilize 50% of Hyp in the original cell wall sample. Therefore, the procedures shown in Figure 13 adopted from Qi (1992) were used to isolate the extensin-RG I complexes from cotton suspension cell walls. First, EPGase was used to remove the homogalacturonic acid followed by cellulase digestion of EPGase-residue to remove cellulose and xyloglucan. HF solvolysis at -73°C was then used to cleave arabinose from Hyp residues in extensin followed by ammonium bicarbonate buffer extraction to remove noncovalently linked RG I-like sugars. Finally, trypsin was used to liberate the Hyp-containing extensin proteins or peptides by cleaving the peptide bond at C-termini of lysine and arginine residues.

Figure 13. Flow Chart of the Isolation of Cotton Extensin RG-I 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. Extensin RG-I complexes were solubilized in filtrate D. The value given in parentheses represents the weight of each fraction recovered after lypholization.


1.0 Gram Acala 44 Cotton Suspension Cell Walls
EPGase
Filtrate A
Residue A (630 mg)
Cellulase
Filtrate B
Residue B (174 mg)
Anhydrous HF -73 °C; ether, water and 100 mM ammonium bicarbonate buffer (pH 7.6) extraction
Filtrate C
Residue C (66 mg)
Trypsin
Filtrate D
Residue D

Procedures shown in Figure 14 in which EPGase treatment was omitted from the sequential treatment were also tried. That is, digesting cell wall samples with cellulase (which contains some EPGase activity), followed by HF -73°C treatment, then trypsin digestion. However, only 36% of Hyp was solubilized by these procedures. This may due to incomplete digestion of homogalacturonic acid without EPGase application. These results imply that homogalacturonic acid could also inhibit the action of trypsin. Thus, the complete sequential treatment solubilizes more extensin-RG I complexes than the other procedures do.

After sequential treatment, the soluble portion contains 50% of the Hyp and 4% of the RG I-like sugars (Qi, 1992). These RG I-like sugars were not solubilized by EPGase and cellulase but cosolubilized with proteins. So it is possible that the RG I fragments covalently crosslink the extensin protein. Thus the filtrate of sequential treatment contains extensin-RG I complexes and is a reasonable candidate for study of the unidentified crosslinks.

Separation of Extensin-RG I Complexes by Gel Filtration Chromatography

The lyophilized sample from the filtrate portion of the complete sequential treatment was dissolved in 0.1 % trifluoroacetic acid (pH ~2.0), and applied to a Fracto gel HW 50 (S) gel filtration column with a flow rate of 0.5 mL/min., and 0.5mL/tube fractions were collected. The extensin RG I complexes were detected at 215 nm (Figure 15). Fractions were pooled as fraction A (from tubes 17 to 25), B1 (from tubes 37 to 44), B2 (from tubes 45 to 48), B3 (from tubes 49 to 52), and C (from tubes 53 to 63). Fraction A was eluted in the void volume, fraction B1, B2, and B3 in the fractionation range, and C in the range near the included volume. These results show that the extensin-RG I complexes are quite large molecules compared with the extensin tryptic peptides shown in Figure 9, 10 and 11 of the previous section since the fractionation range of this Fracto-gel HW 50 column is from MW 800 to 90,000 Daltons. The following reasons may explain this result.

Figure 14. Flow Chart of the Isolation of Extensin RG-I Complexes from Cotton Suspension Cell Walls.

Cotton cell walls were treated with the combination of 1) cellulase digestion 2) HF solvolysis at -73°C 3) 100 mM ammonium bicarbonate buffer (pH 7.6) extraction 4) trypsin digestion. Thirty six percent of Hyp content was solubilized.

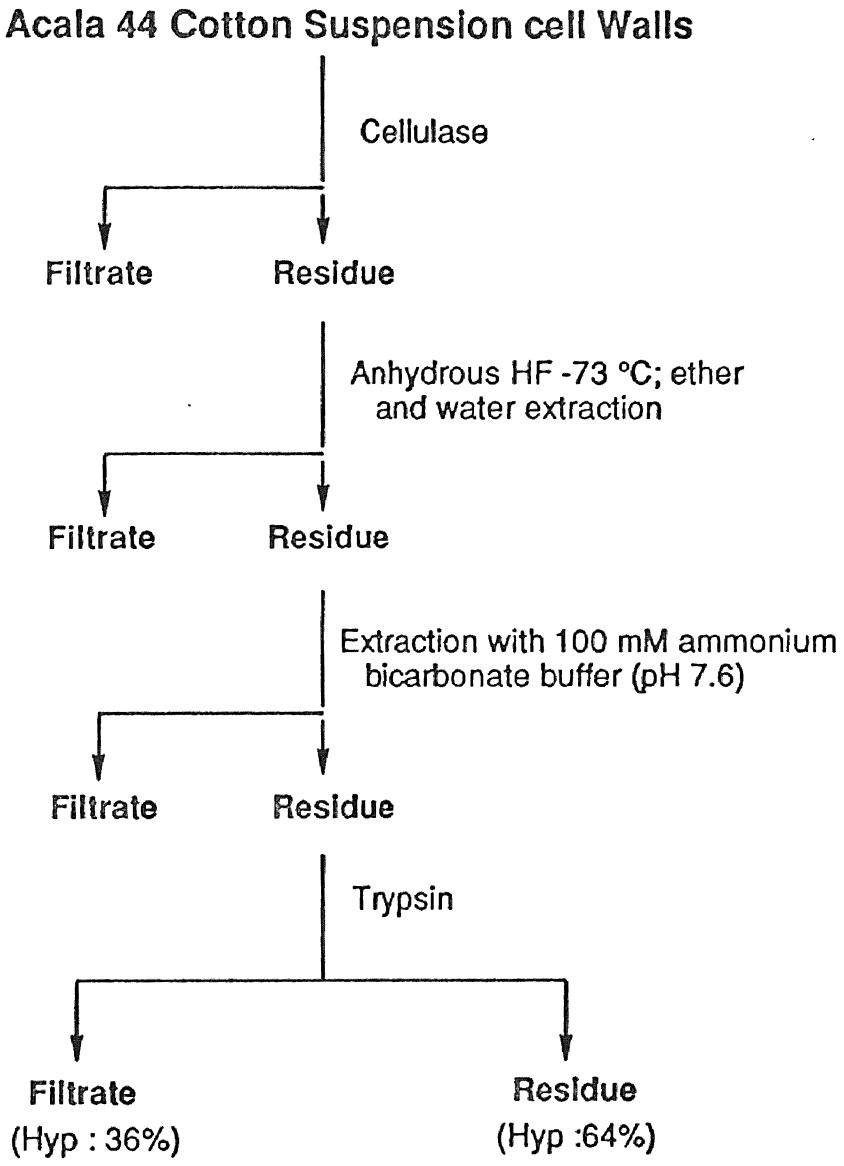
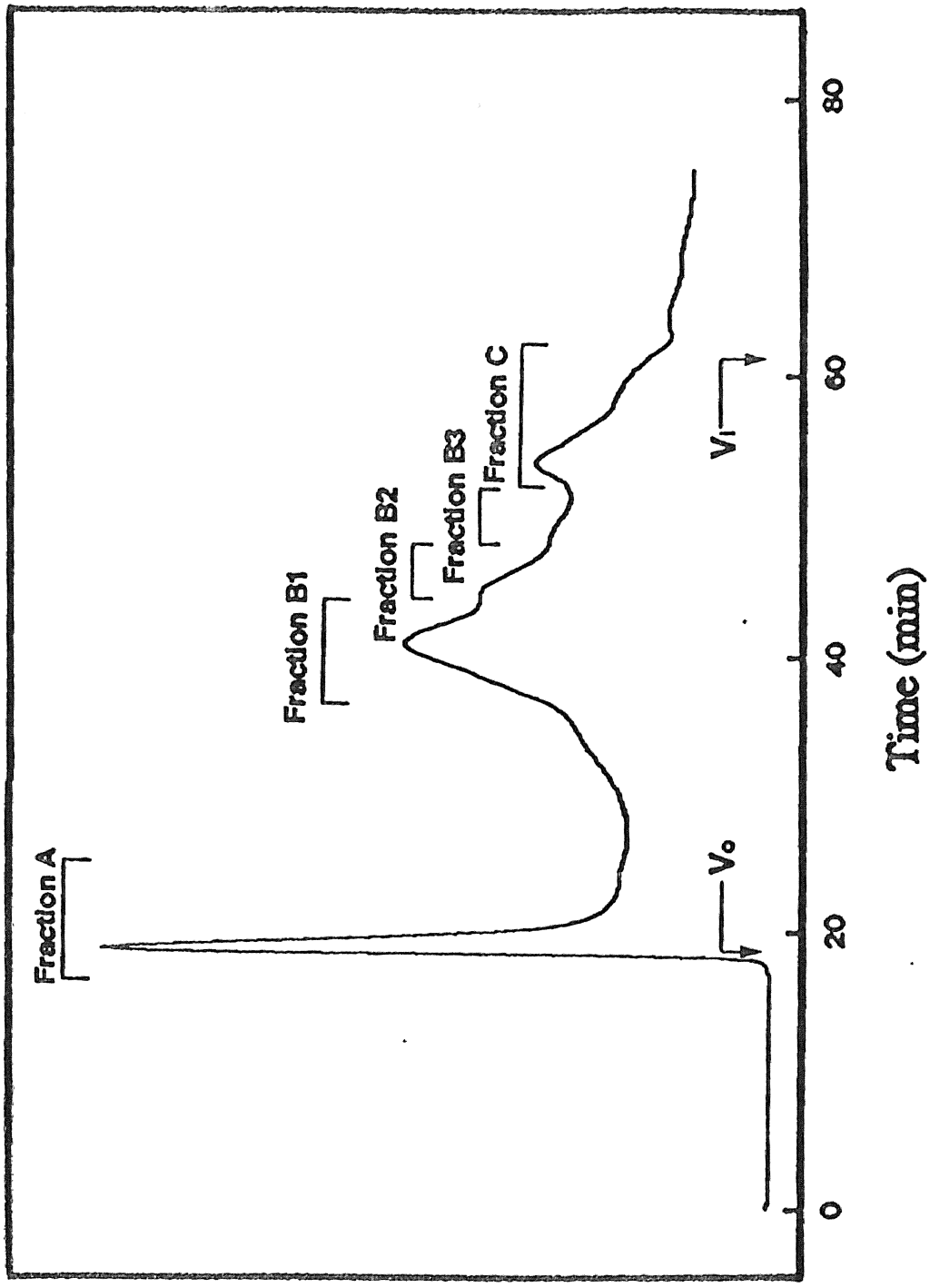

Acala 44 Cotton Suspension cell Walls
Cellulase
Filtrate
Residue
Anhydrous HF -73 °C; ether and water extraction
Filtrate
Residue
Extraction with 100 mM ammonium bicarbonate buffer (pH 7.6)
Filtrate
Residue
Trypsin
Filtrate
(Hyp : 36%)
Residue
(Hyp :64%)

Figure 15. Chromatography on an HW 50(S) Gel Filtration Column of the Filtrate D of Figure 13.

Sample was applied in and eluted with 0.1% trifluoroacetic acid. The flow rate was 0.5 mL/fraction/minute. Detection was by UV monitor at 215 nm. Fractions 17-25 were pooled into fraction A , fractions 37-44 were pooled into fraction B1, fractions 45-48 were pooled into fraction B2, fractions 49-52 were pooled into fraction B3, and fractions 53-63 were pooled into fraction C.


Absorbance (215 nm)
Time (min)
0
20
40
60
80
Fraction A
Fraction B1
Fraction B2
Fraction B3
Fraction C
Vo
Vi

First, extensin molecules may have been 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. Secondly, since extensin peptides are heavily glycosylated with RG I like sugars, the carbohydrate may contribute to the size of the complexes.

Comparatively, these gel filtration chromatography results are better than those of previous investigations (Qi, 1992), since some of these extensin-RG I complexes with smaller molecular weight are better resolved. For example, instead of only one fraction of B, the more specific B1, B2, and B3 fractions were obtained. Although these fractions were not separated completely, it is an improvement over previous attempts. It is possible that the cleavage of arabinose residues in this work was more complete, so extensin was more exposed to trypsin digestion giving smaller extensin RG I fragments. There were two results which could indirectly support this suggestion. First, as shown in Figure 13, after HF -73°C treatment followed by ether, water and ammonium bicarbonate buffer extraction, only 37.9% by weight of residue B remained in residue C. In previous results (Qi, 1992), there was 46% by weight of residue B remaining in residue C. This could mean that more arabinose residues were cleaved in this work although it also could be more complete removal of other sugars in previous steps. Another result, as shown in Table 2, after HF -73°C solvolysis of cellulase treated residue for 30 minutes, there was still about 7.6 mole percent arabinose in residue. But the mole percentage of arabinose after HF -73°C for one hour was only 6.5. This preliminary result showed that HF -73°C solvolysis for one hour may remove more arabinose from cell wall residues than HF -73°C treatment for 30 minutes could. So, the reaction time variation between different experiments may be a cause for differing extent of cleaving arabinose residues.

TABLE 2

MOLE PERCENT OF SUGARS IN THE INSOLUBLE PORTION OF HF-73 °C TREATMENT FOR 30 MINUTES OR 60 MINUTES AFTER CELLULASE DIGESTION

Material	Ara	Rha	Xyl	GalA	Gal	Glc	wt%*
Residue (30 min)	7.6	20.9	7.0	42.5	18.0	4.0	40.2
Residue (60 min)	6.5	22.1	6.9	42.4	17.4	4.7	39.1

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

SDS-PAGE Analysis of the Glycosylated and Deglycosylated Extensin-RG I Complexes

Half of the amount of the extensin-RG I complexes A (ERC A), extensin-RG I complexes B1(ERC B1), extensin RG I complexes B2(ERC B2), extensin RG I complexes B3 (ERC B3) and extensin RG I complexes B (ERC B) in fractions A, B1, B2, B3, and B (from B1 to B3) of Figure 15 were treated separately with HF at 0°C for 30 minutes to remove sugars from the proteins. These deglycosylated extensin-RG-I complexes were named dERC A, dERC B1, dERC B2, dERC B3, and dERC B respectively. The glycosylated and deglycosylated forms of extensin-RG I complexes in fraction A, B1, B2, B3 or B were all subjected to SDS-PAGE analysis. Eighty to 110 μg of each sample was applied to the gel (15% of separating gel). The resulting gels were stained with silver reagent.

The SDS-PAGE result of sample ERC B1 and dERC B1 is shown in Figure 16. Both glycosylated and deglycosylated form of extensin-RG I complexes B1 migrated into the separating gel and were resolved quite well. There are four bands (molecular weight of about 24 kD; 11.1 kD; 8.3 kD; and 4.1 kD) in ERC B1. After deglycosylation, dERC B1 contains three bands which are nearly the same as in B1. This result indicates that the B1 fraction of extensin RG I complexes is not heavily glycosylated. SDS-PAGE results of ERC B2, dERC B2, ERC B3, dERC B3 are shown in Figure 16 and Figure 17. Both ERC B2 and ERC B3 of glycosylated and deglycosylated forms predominantly contain the smallest proteins which are nearly the same size as those of ERC B1 and dERC B1 (MW about 4.1 kD). ERC B2 and dERC B2 also have some amount of protein bands of medium sizes (MW about 11.1 kD; 8.3 kD) and a smaller amount of a large protein band (approximate MW 24 kD) as those observed in ERC B1 and dERC B1. But in ERC B3 and dERC B3, there are almost no high molecular weight bands and there are minimal amounts of protein bands of medium weight. These results also show that ERC B2 and ERC B3 are not heavily glycosylated. It can also be seen that there are overlaps of different

Figure 16. 15% SDS-PAGE of Extensin RG-I Complexes B1, B2, and their HF-Deglycosylated Forms.

MW markers are indicated on the far left (Myosin, 200 kD; Serum albumin, 66.2 kD; Ovalbumin, 45 kD; Carbonic anhydrase, 31 kD; Trypsin inhibitor, 21.5 kD; Lysozyme, 14.4 kD; Aprotinin, 6.5 kD). Lanes 1, 2, 3, and 4 contain extensin RG-I complexes B1 (ERC B1), deglycosylated extensin-RG-I complexes B1 (dERC B1), extensin RG-I complexes B2 (ERC B2), and deglycosylated extensin RG-I complexed B2 (dERC B2), respectively.

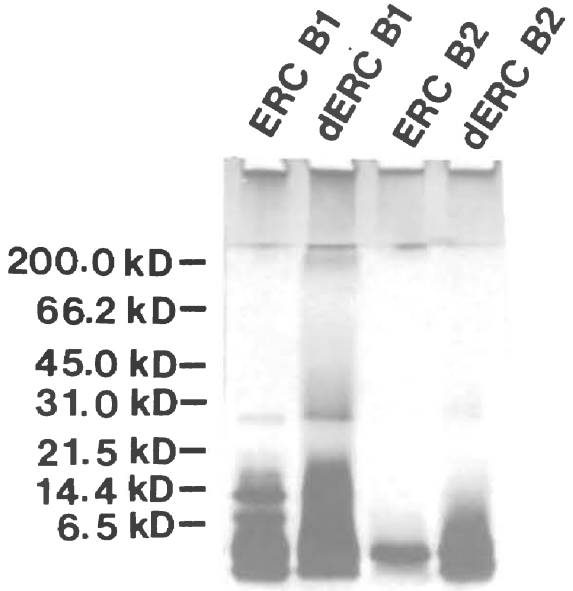
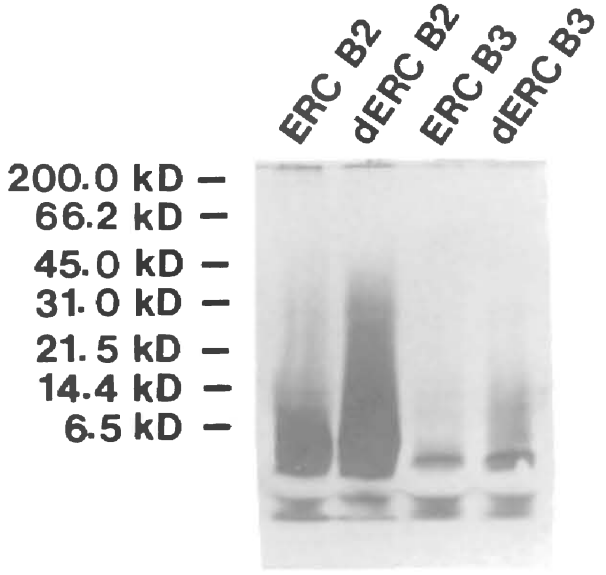

ERC B1
dERC B1
ERC B2
dERC B2
200.0 kD
66.2 kD
45.0 kD
31.0 kD
21.5 kD
14.4 kD
6.5 kD

Figure 17. 15% SDS-PAGE of Extensin-RG I Complexes B2, B3, and Their HF-Deglycosylated Forms.

MW markers are indicated on the far left (Myosin, 200 kD; Serum albumin, 66.2 kD; Ovalbumin, 45 kD; Carbonic anhydrase, 31 kD; Trypsin inhibitor, 21.5 kD; Lysozyme, 14.4 kD, Aprotinin, 6.5 kD). Lanes 1, 2, 3, and 4 contain extinsin RG-I complexes B2 (ERC B2), deglycosylated extensin RG-I complexed B2 (dERC B2), extensin RG-I complexes B3 (ERC B3), and deglycosylated extensin RG-I complexes B3 (dERC B3), respectively.


ERC B2
dERC B2
ERC B3
dERC B3
200.0 kD
66.2 kD
45.0 kD
31.0 kD
21.5 kD
14.4 kD
6.5 kD

sizes of proteins in ERC B1, ERC B2, and ERC B3. These results are consistent with the gel filtration chromatography results.

Because ERC B1, ERC B2, and ERC B3 contain similar proteins and these fractions were not separated completely in gel filtration column, fractions B1, B2, and B3 were pooled together as ERC B.

Figure 18 shows the SDS-PAGE result of both glycosylated and deglycosylated forms of ERC A and ERC B. From Figure 18, we can see that most of the complexes in the glycosylated form of extensin-RG I A fraction remained in the stacking gel because of heavy glycosylation. These are the greater abundant components in ERC A and they are consistent in size with the result from HW 50 gel filtration chromatography. Only a small amount of the complexes migrated into separating gel and gave five different sizes of protein bands of different density (approximate MW : 26.3 kD; 14.4kD; 11.5 kD; 7.2 kD; 4.7 kD). After deglycosylation, most of dERC A which remained in stacking gel migrated into separating gel (around MW 100 kD), and the protein bands which had already entered the separating gel had an increased migration. The MW of these increasingly migrating bands are about 25.7 kD, 14.4 kD, 11.1 kD, 6.9 kD and 4.6 kD, respectively. This result indicates that there are covalent cross linkages between extensin and pectins. For ERC B and dERC B, the results are similar to those in ERC B1 and dERC B1 of figure 16. Both glycosylated and deglycosylated extensin-RG I complexes B were resolved quite well in the gel without an increase in migration after deglycosylation.

Analysis of sugars in ERC A and ERC B is shown in Table 3. Fraction A (ERC A) contains a high concentration of RG I-like sugars such as GalA (34.7 mole %) and Rha (14.0 mole %). This implies that the extensin peptides in A fraction may crosslink with RG I polymer. After HF 0°C treatment and removal of this RG I polymer, the extensin peptides (dERC A) migrated into the separating gel and had an increased migration in the SDS-PAGE. Fraction B (ERC B) contains mainly Ara residues (47.5 mole %) and Gal residues (15.7 mole %). Only small amount of RG I-like sugars are in ERC B fraction

Figure 18. 15% SDS-PAGE of Extensin RG I Complexes A, B, and Their HF-Deglycosylated Forms.

MW markers are indicated on the far left (Myosin, 200 kD; Serum albumin, 66.2 kD; Ovalbumin, 45 kD; Carbonic anhydrase, 31 kD; Trypsin inhibitor, 21.5 kD; Lysozyme, 14.4 kD; Aprotinin, 6.5 kD). Lanes 1, 2, 3, and 4 contain extensin RG-I complexes A (ERC A), deglycosylated extensin RG-I complexes A (dERC A), extensin RG-I complexes B (ERC B), and deglycosylated extensin RG-I complexes B (dERC B), respectively.

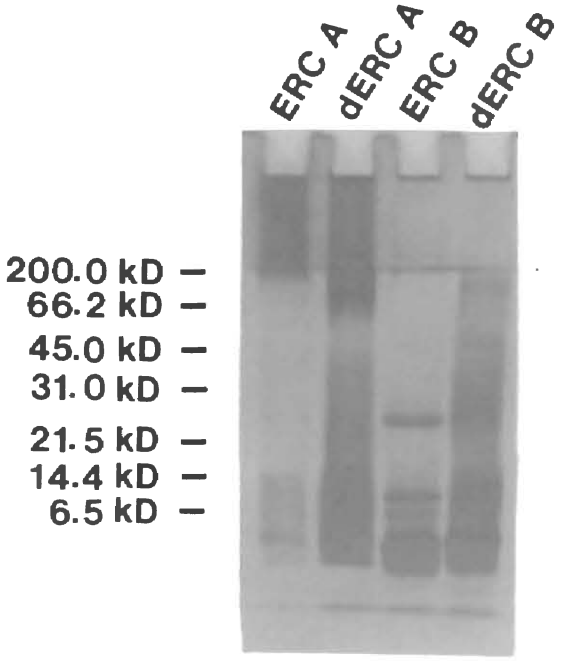

ERC A
dERC A
ERC B
dERC B
200.0 kD
66.2 kD
45.0 kD
31.0 kD
21.5 kD
14.4 kD
6.5 kD

TABLE 3

SUGAR COMPOSITIONS (MOLE %) IN THE FRACTIONS A AND B OF HW 50 (S) GEL FILTRATION CHROMATOGRAPHY FROM THE FILTRATE OF SEQUENTIAL TREATMENT OF CELL WALLS

Sugar Residue	Mole%	
	Fraction A	Fraction B
Ara	6.5	47.5
Rha	14.0	3.5
Xyl	6.6	4.1
GalA	34.7	7.1
Man	1.1	9.6
Gal	17.9	15.7
Glc	19.2	12.5

(GalA: 7.1 mole %; Rha: 3.5 mole %). It has been known that most hydroxyproline residues of extensin are glycosylated with one to four arabinosyl residues (Lamport and Miller, 1971), and a single galactose residue is attached O-glycosidically to some serine residues of extensin (Lamport et al., 1973; Cho and Chrispeels, 1976). In our samples (extensin-RG I complexes), HF-73°C treatment has removed most of the arabinose residues attached to Hyp of extensin, leaving some of the single arabinosyl residues. So, in ERC B, the extensin peptides link to mainly single Ara and Gal. Therefore, even removal of these sugars from peptides by HF at 0°C, dERC B migration did not increase on SDS-PAGE.

As shown on SDS-PAGE (see Figure 18), in addition to those bands which were separated well in separating gel, there were diffuse silver reagent stainable bands around the high MW areas in dERC A and dERC B lanes but not in ERC A and ERC B lanes. These could possibly be crosslinked proteins caused by HF 0°C treatment. It is also possible that the high MW materials in dERC A lane were from those proteins which did not enter the separating gel before deglycosylation. Besides, from Figure 18, we could see that protein bands in dERC A and dERC B lanes were not as sharp as those in ERC A and ERC B lanes. These could also be the artifact caused by HF 0°C treatment.

Comparing these results with those of a previous study in our laboratory (Qi, 1992), better resolution was obtained and the molecular weights of most of these extensin-RG I complexes were smaller in these latest attempts. This may also due to more complete cleavage of arabinose from HF -73°C treatment so that extensin was more exposed to trypsin digestion as mentioned in previous section.

Western Blot Analysis of Extensin-RG I Complexes

The cross reactivities of polyclonal antibodies raised against the HF-deglycosylated tomato extensin precursor (dp1a) with extensin-RG I complexes A and B were determined by enzyme-linked immunosorbent assay (ELISA). As we know, the extent of antibody

cross-reactivity depends upon the number of common epitopes that exist between dp1a and complexes A and B and their HF-deglycosylated forms. There are two different peptide blocks (decapeptide and hexadecapeptide) in dp1a : Ser- (Hyp)4-Thr-Hyp-Val-Tyr-Lys and Ser- (Hyp)4-Val-Lys-Pro-Tyr-His-Pro-Thr-Hyp-Val-Tyr-Lys (Smith et al., 1986). The major nonglycosylated epitope of tomato extensin precursor p1 may be the sequence Val-Lys-Pro-Tyr-His-Pro which is in the hexadecapeptide (Kieliszemski and Lamport, 1986).

The ELISA results of this study showed that the polyclonal antibody raised against dp1a cross-reacts with complexes A and B. Western blot analyses of the reactivities of complex A and B with Anti-dp1a were performed in order to specifically determine whether the isolated cotton cell wall proteins in complexes A and B after SDS-PAGE are extensin-like molecules. The control was performed by reacting complex A and B with pre-immune sera (see Figure 19). In Figure 20, the extensin-RG I complex A (ERC A) was recognized by Anti-dp1a, mainly near the stacking gel area, but only very slightly observed in the separating gel area. This may be because the amount of ERC A that migrated into the separating gel is not sufficient. In the lane of deglycosylated extensin-RG I complex A (dERC A), two bands (with MW of 14.4 kD and 4.6 kD) were reacted with the dp1a antibody. The other three bands of dERC A shown in SDS-PAGE (Figure 18) were not stained clearly in the western blot. This may also be due to a small amount of protein in those bands or no cross reaction of these protein bands with Anti-dp1a. In Figure 20, all the bands shown in SDS-PAGE (figure 18) of ERC B and dERC B except one band of ERC B (MW of about 8.3 kD) were reacted with Anti-dp1a. It is not known whether this one protein band is an extensin-like molecule which may not cross react with Anti-dp1a or is from other cell wall proteins. This question needs further investigation. However, the results from Western blots indicated that many of the proteins contained in complex A and B are cotton extensin-like molecules. The protein bands with or without specific reactivities shown by Western blot analysis are listed in Table 4.

Figure 19. Control of Western Blot Analyses of Extensin RG-I Complexes A, B, and Their HF-Deglycosylated Forms with a 15% SDS-PAGE.

MW markers are indicated on the far left (Myosin, 205 kD; Ovalbumin, 49.5 kD; Carbonic anhydrase, 32.5 kD; Soybean trypsin inhibitor, 27.5 kD; Lysozyme, 18.5 kD; Aprotinin, 6.5 kD). Lanes 1, 2, 3, and 4 contain deglycosylated extensin RG-I complexes B (dERC B), extensin RG-I complexes B (ERC B), deglycosylated extensin RG-I complexes A (dERC A), and extensin RG-I complexes A (ERC A), respectively.


dERC B
ERC B
dERC A
ERC A
205.0 kD
49.5 kD
32.5 kD
27.5 kD
18.5 kD
6.5 kD

Figure 20. Western Blot Analyses of Extensin RG-I Complexes A, B, and Their HF-Deglycosylated Forms with a 15% SDS-PAGE.

MW markers are indicated on the far left (Myosin, 205 kD; Ovalbumin, 49.5 kD; Carbonic anhydrase, 32.5 kD; Soybean trypsin inhibitor, 27.5 kD; Lysozyme, 18.5 kD; Aprotinin, 6.5 kD). Lanes 1, 2, 3, and 4 contain extensin RG-I complexes A (ERC A), deglycosylated extensin RG-I complexes A (dERC A), extensin RG-I complexes B (ERC B), and deglycosylated extensin RG-I complexes B (dERC B), respectively.

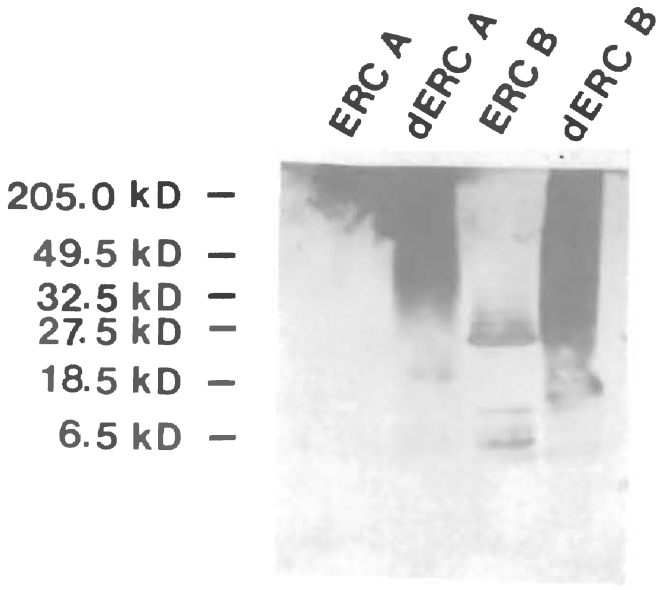

ERC A
dERC A
ERC B
dERC B
205.0 kD
49.5 kD
32.5 kD
27.5 kD
18.5 kD
6.5 kD

TABLE 4

THE MOLECULAR WEIGHT (KD) OF PROTEIN BANDS AND THEIR SPECIFIC REACTIVITIES WITH ANTI-dp1a* IN EXTENSIN-RG I COMPLEXES

Band	ERC A		dERC A		ERC B		dERC B	
Number	M W	S R**	M W	S R**	M W	S R**	M W	S R**
1	26.3	no	25.7	no	24	yes	24	yes
2	14.4	no	14.4	yes	--		--	
3	11.5	no	11.1	no	11.1	yes	11.1	yes
4	7.2	no	6.9	no	8.3	no	--	
5	4.7	no	4.6	yes	4.1	yes	4.1	yes

*polyclonal antibody raised against the deglycosylated tomato extensin precursor 1 a (dp1a)
**specific reactivities

Similarly, as in the SDS-PAGE gels mentioned previously, there were strongly stained proteins by dp1a antibody in the high MW areas of dERC A and dERC B lanes. Some of these proteins may be due to nonspecific binding because the similar results were shown in the control of Western blot analysis (Figure 19). In addition, there was one very slightly stained protein band with MW of about 24 kD in ERC B lane of the Western blot analysis control. This could also due to the nonspecific binding because of the abundance of protein amount in that band.

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

Tryptic peptides were obtained by digestion of HF-deglycosylated extensin RG I complexes A (dERC A) and B (dERC B) with trypsin, and were mapped on a reverse phase HPLC column. As shown in Figures 21 and 22, both dERC A and dERC B contain peptides in fractions I and II which have similar retention times as cotton extensin peptides characterized in fractions I and II of figures 3, 5, and 6. There is insufficient data as to whether the fraction I from dERC A and fraction I from dERC B contain similar peptides, and further analysis will be needed to justify this contention.

Furthermore, fraction I of tryptic peptides from dERC B was fractionated on a gel filtration HW 40 (S) column (see Figure 23), and showed nearly the same chromatogram as that in Figure 9. Also, fraction II after reverse phase HPLC of tryptic peptides from both dERC A and dERC B were fractionated in gel filtration HW 40 (S) column (see Figures 24 and 25). However, the HW 40 gel filtration chromatogram of fraction II of tryptic peptides from dERC A showed only similar peaks as the peaks III, IV and V that contained mainly peptides from trypsin in fractions described in Figure 12. The gel filtration chromatogram of fraction II from dERC B showed that there were similar peaks as those of both extensin peptide-containing peaks (but only peak II and V) and trypsin peptide-containing peaks (III, IV, and V). Although the chromatograms from different samples show peaks with similar retention times, this does not necessarily mean that they

Figure 21. The HPLC Chromatogram of the Tryptic Peptides from HF-Deglycosylated Fraction A of Figure 15 on Reverse Phase C18 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 1.0 mL/minute. Detection was by UV monitor at 214 nm. The sample was dissolved in 0.1% trifluoroacetic acid and injected.

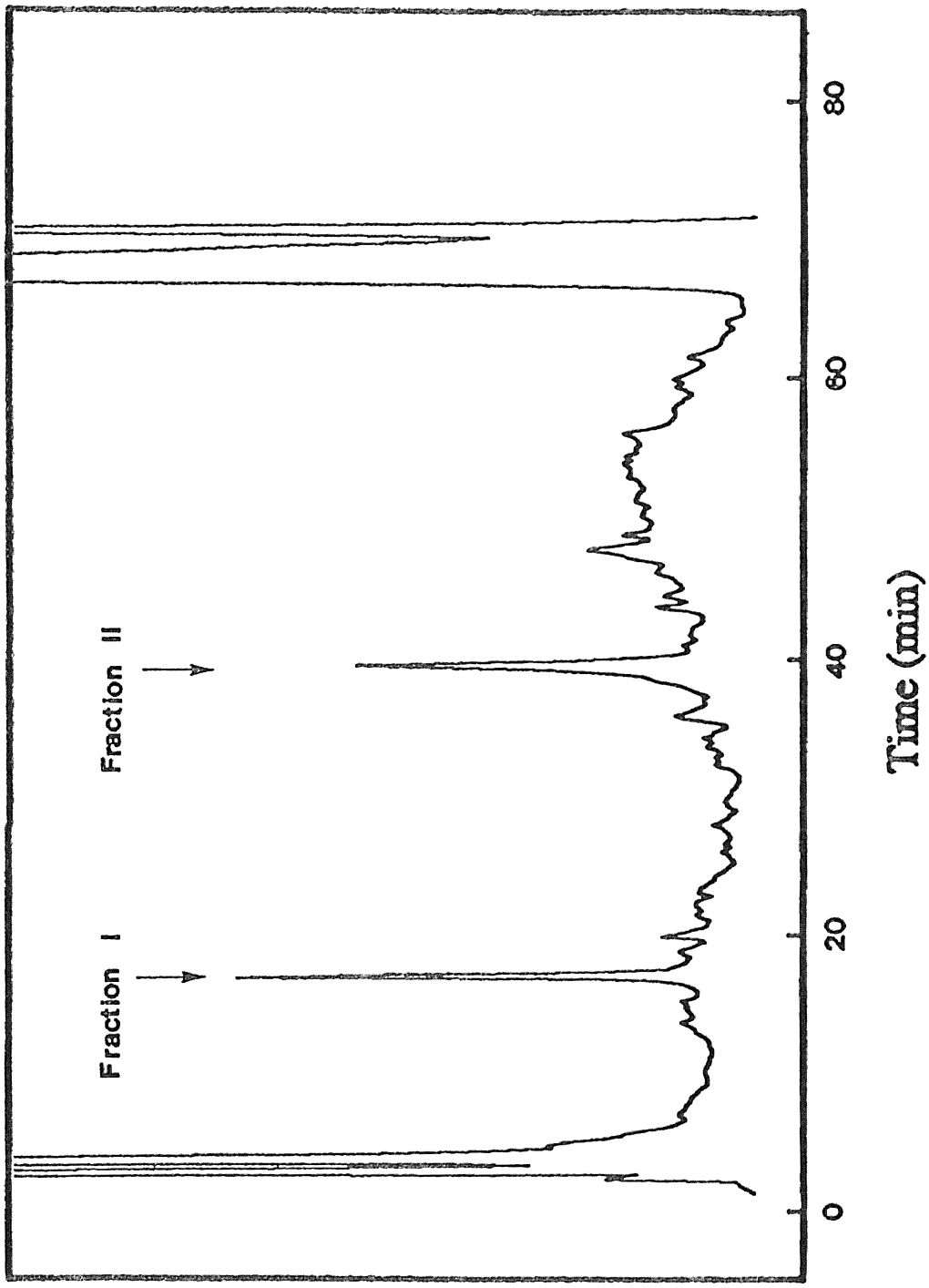

Absorbance (214 nm)
Fraction I
Fraction II
0
20
40
60
80
Time (min)

Figure 22. The HPLC Chromatogram of the Tryptic Peptides from HF-Deglycosylated Fraction B of Figure 15 on Reverse Phase C18 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 1.0 mL/minute. Detection was by UV monitor at 214 nm. The sample was dissolved in 0.1% trifluoroacetic acid and injected.

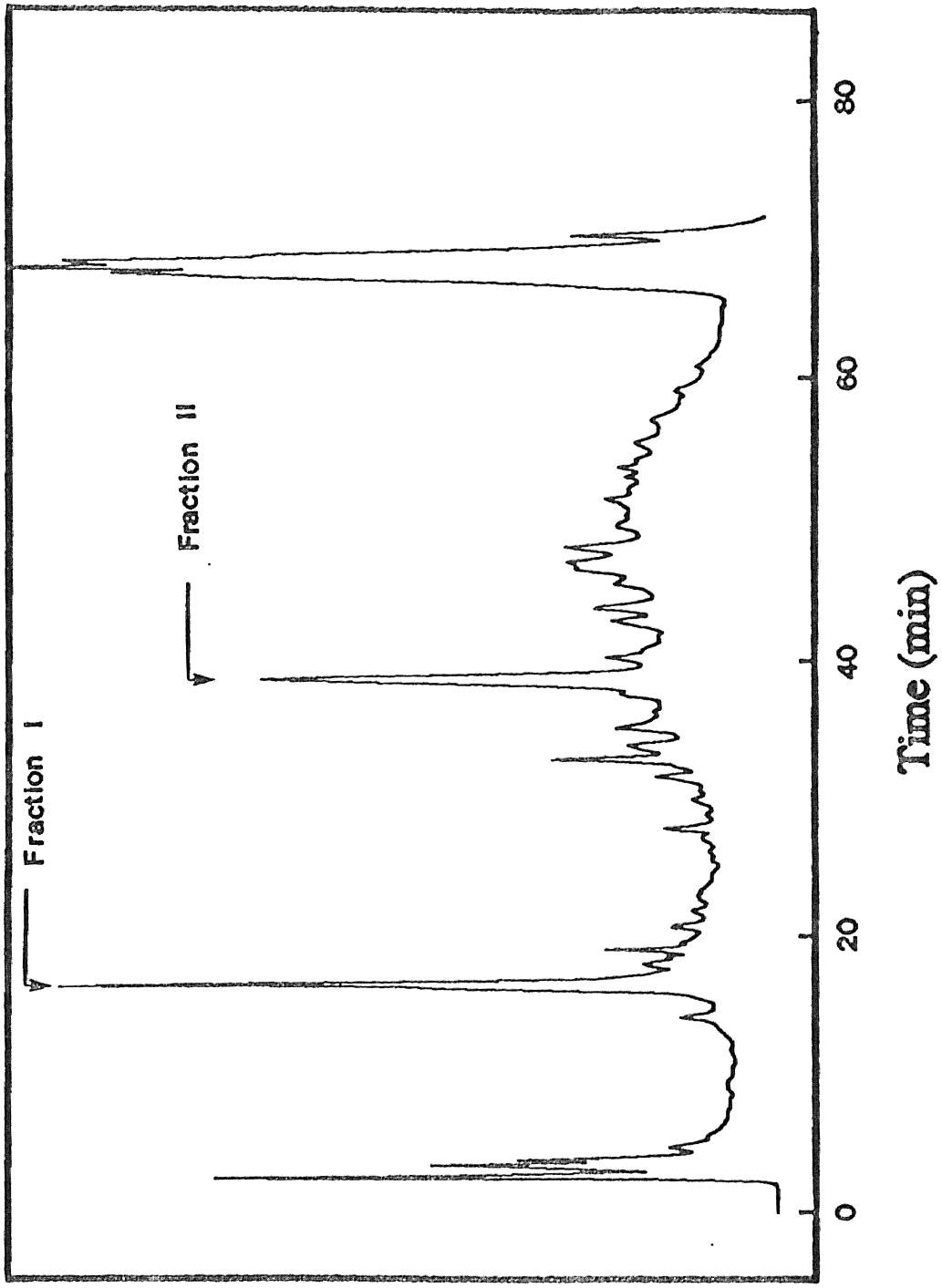

Absorbance (214 nm)
Fraction I
Fraction II
0
20
40
60
80
Time (min)

Figure 23. Chromatography on an HW 40(S) Gel Filtration Column of Fraction I of Figure 22.

Sample was applied in and eluted with 0.1% trifluoroacetic acid. The flow rate was 0.5 mL/fraction/minute. Detection was by UV monitor at 215 nm.

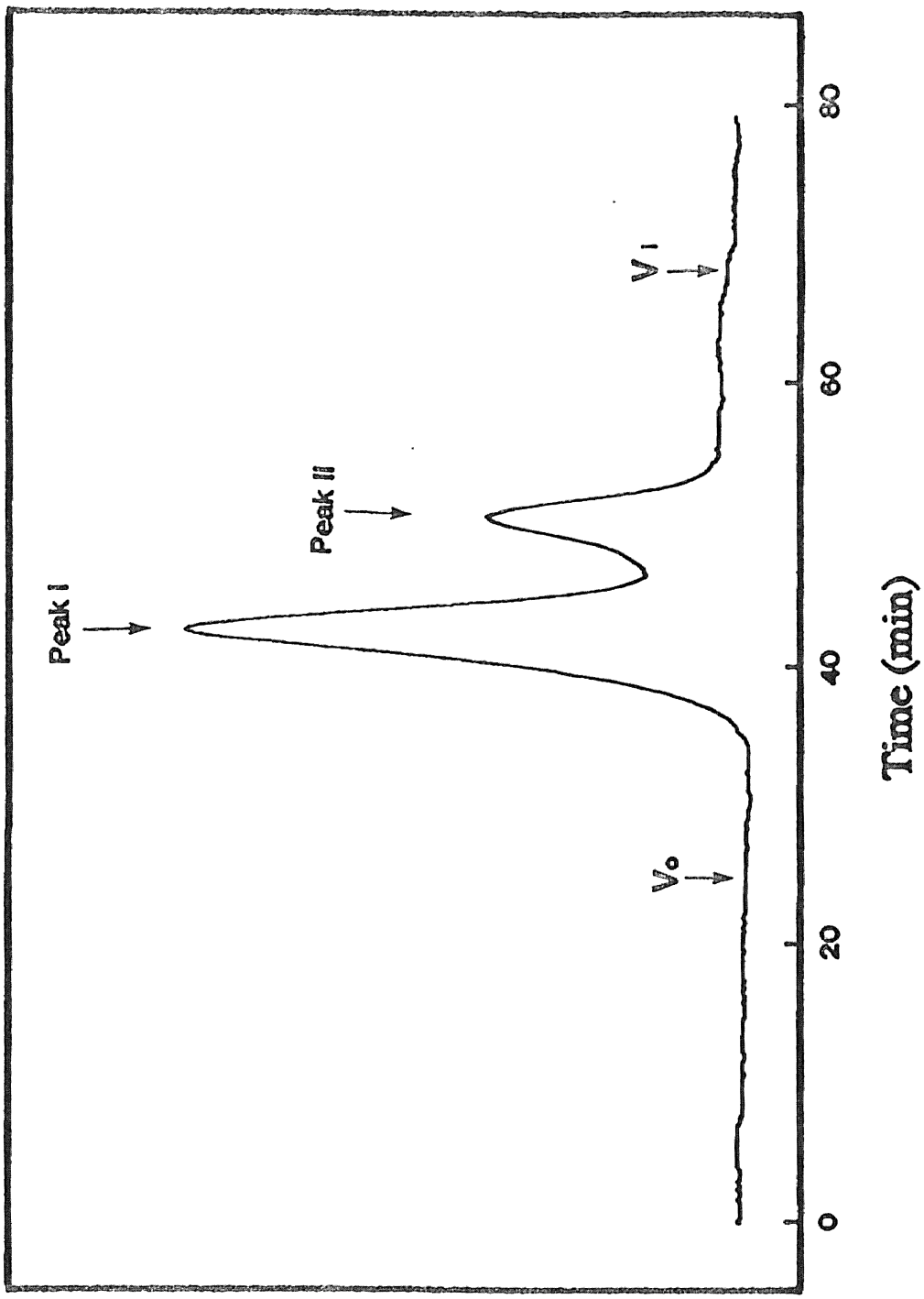

Absorbance (215 nm)
Peak I
Peak II
V_o
V_i
0
20
40
60
80
Time (min)

Figure 24. Chromatography on an HW 40(S) Gel Filtration Column of Fraction II of Figure 21.

Sample was applied in and eluted with 0.1% trifluoroacetic acid. The flow rate was 0.5 mL/fraction/minute. Detection was by UV monitor at 215 nm.

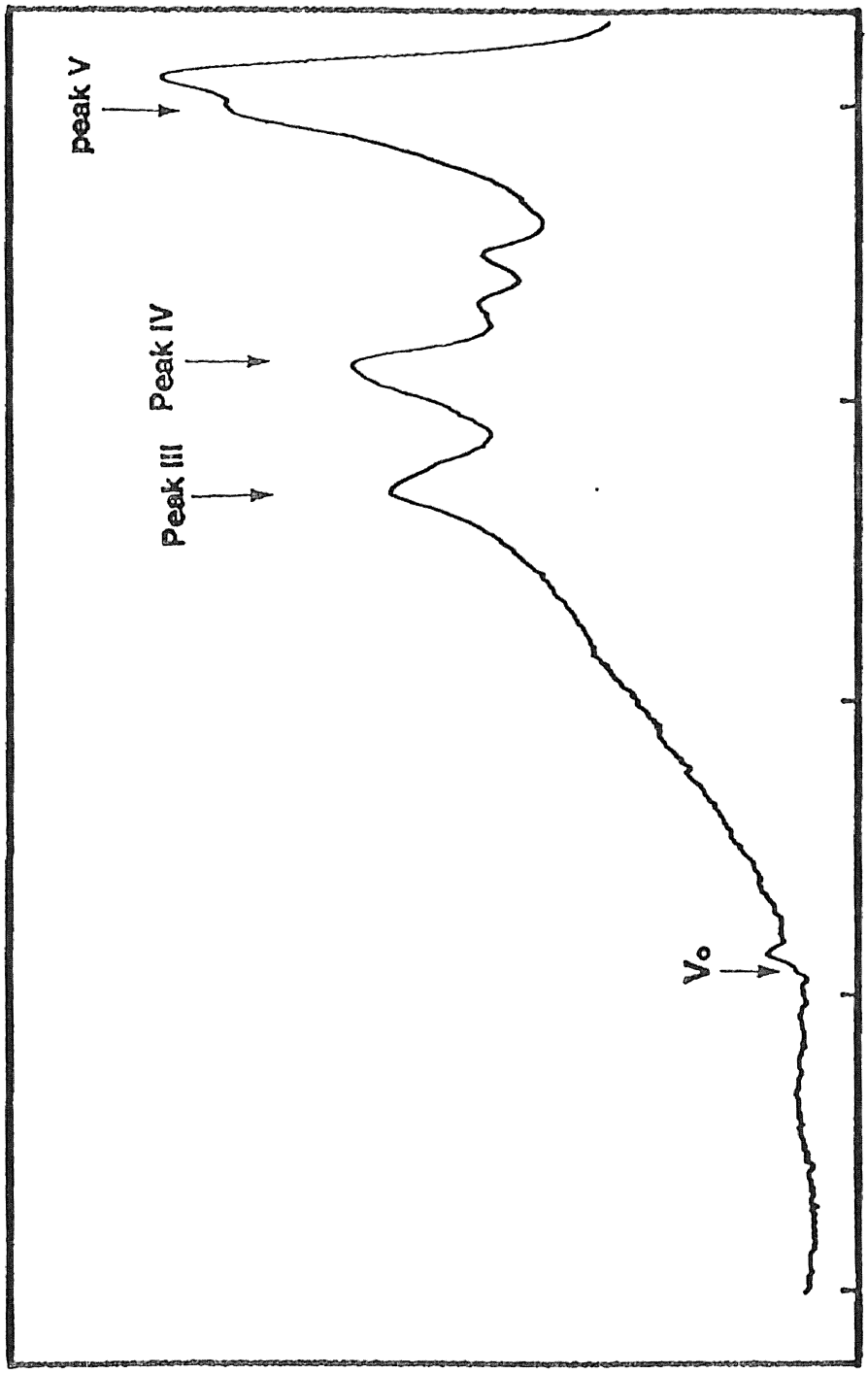
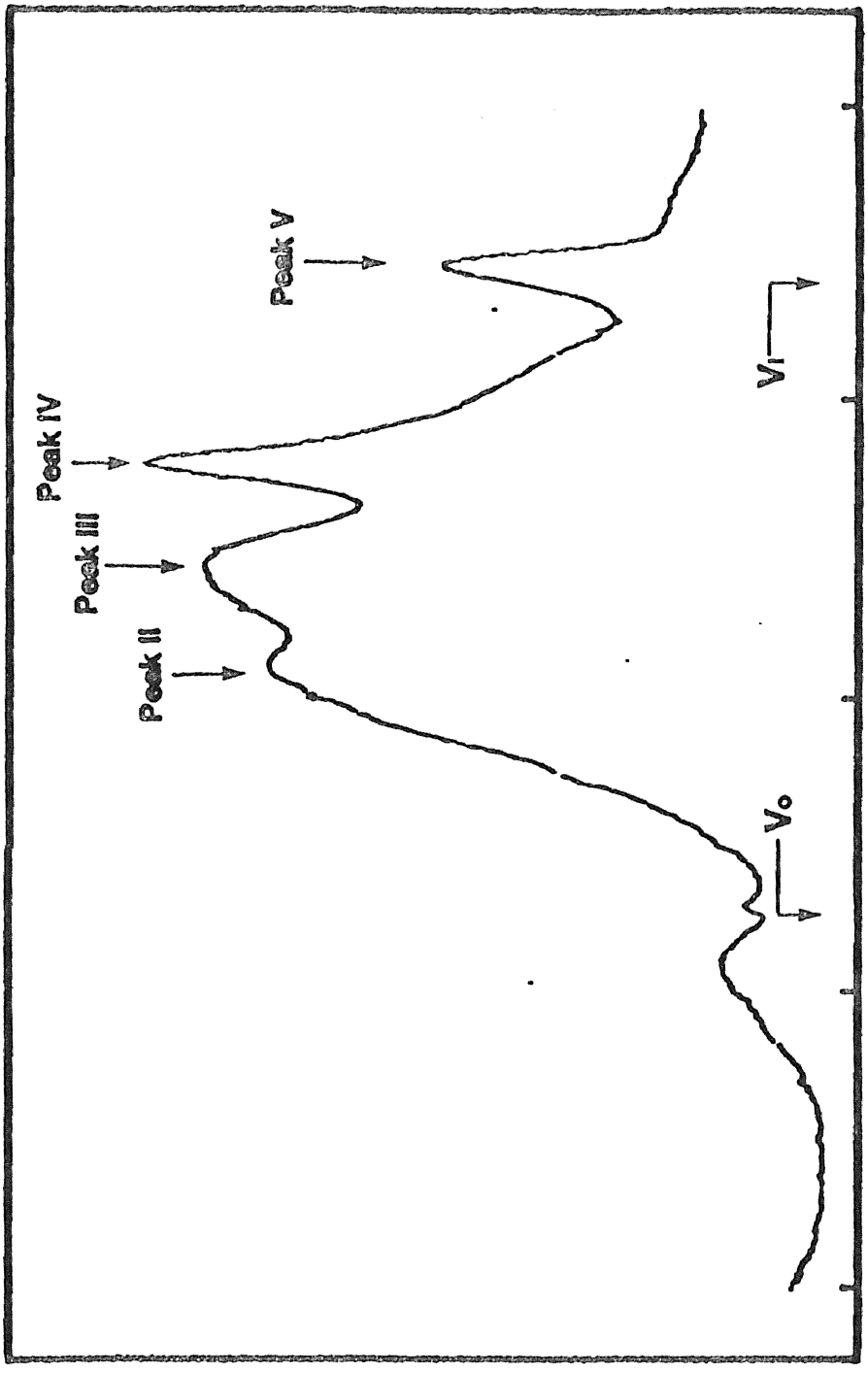

Peak V
Peak IV
Peak III
V₀
0
20
40
60
80
Time (min)
Absorbance (215 nm)

Figure 25. Chromatography on an HW 40(S) Gel Filtration Column of Fraction II of Figure 22.

Sample was applied in and eluted with 0.1% trifluoroacetic acid. The flow rate was 0.5 mL/fraction/minute. Detection was by UV monitor at 215 nm.


Peak II
Peak III
Peak IV
Peak V
V_o
V_t
0
20
40
60
80
Time (min)
Absorbance (215 nm)

have the same peptides. However, the results of tryptic peptide analysis provided more evidence suggesting that both extensin-RG I complexes A and B contain cotton extensin peptide-containing protein (mainly the extensin peptides in fraction I of reverse phase HPLC), and these proteins were glycosylated with RG I fragments (mainly in ERC A). Again, these results showed that the extensin peptides liberated from different steps of the series treatment have different character.

CHAPTER V

SUMMARY AND CONCLUSION

It is well known that the plant cell wall plays an important role in the control of plant growth, morphogenesis and pathogen defense. The interactions between cell wall polymers are very important to maintain these functions of cell walls. What these interactions are or how they work is either unclear or unknown. There are a number of well studied starting points, however, and we build upon them, reworking and reevaluating our conceptions of the cell wall and its functions as we learn more. This project has focused on extensins and their interaction with polysaccharides in the cotton cell wall. Extensin, a hydroxyproline-rich glycoprotein, is a major structural protein in plant cell walls. In order to know the structure of extensin and the interaction between extensin and polysaccharide, a two part work has been undertaken. The first part was isolation and partial characterization of extensin peptides of cotton suspension cell walls. The second part involved investigation of interactions between extensin and polysaccharides by characterization of extensin-RG I complexes of cotton cell walls.

Cell walls were prepared from cotton suspension cells Acala 44. For years, the insolubility of extensin prevented its structural elucidation. To isolate extensin peptides from cotton suspension cell walls, a mild, selected series of treatments was used in this study. First, cotton suspension cell walls were treated with EPGase to remove the homogalacturonic acid followed by cellulase digestion of EPGase-residue to remove cellulose and xyloglucan. The HF solvolysis at -73°C was used to cleave arabinoses from Hyp residues in extensin followed by ammonium bicarbonate buffer extraction to remove the noncovalently linked RG I-like sugars. Trypsin was used to liberate extensin fragments

which will be discussed later. A sample of this residue was further treated with HF at 0°C followed by ether and water extraction. Extensin peptides were solubilized in all of these fractions (i.e., ether/HF extract, water extract and residue), although the water extract contained the most abundant Hyp (about 39% Hyp of the original cell wall). Interestingly, this HF 0°C treatment, which cleaves the glycosylic bonds, co-solubilized most extensin peptides which could not be solubilized by the first trypsin treatment. This is an indication that there may be covalent cross-links between these extensin peptides and polysaccharides (pectin). After trypsin digestion of this HF 0°C treated residue, there was about 6.3% Hyp of the original cell wall sample remaining in the residue. This indicated that there may be direct covalent cross-links between extensin-extensin molecules or extensin-phenolic compounds that kept the extensins insoluble. However, the procedures used above were shown to be an efficient way for solubilizing extensin peptides since only about 6.3% Hyp of the original cell wall sample remained insoluble.

After digestion with trypsin, the cotton extensin peptides from ether/HF extract, water extract and residue were separated on reverse phase HPLC, each giving two major fractions, I and II, both of which were rich in Hyp. After purification of fractions II by gel filtration chromatography on an HW 40 (S) column, these peptides were sequenced. Four of the peptides sequenced are Hyp-rich peptides characteristic of extensin. They are: (a) Ser?-Hyp-Thr?-Hyp-Hyp-Tyr-Lys-Pro-Hyp-Lys?, (b) Ser?-Hyp-Hyp-Hyp-Hyp, (c) Asp-Hyp-Hyp-Gly-Hyp-Hyp-Gly-Pro-Ala-Gly-Ala-Ala-Gly-Pro-Ala, (d) Ser?-Hyp-Hyp-Hyp-Hyp-Val?-Gly-Ala-Pro-Lys. These four peptides comprise the majority of fractions II, so they must be repeated many times within cotton suspension extensins. Interestingly, peptide (a) from water extract and peptide (d) from residue of HF 0°C solvolysis of the residue after sequential treatment had similar characteristics on reverse phase HPLC and HW 40(S) gel filtration chromatography but did not have the same sequences. Is it possible that these peptide structural differences led to the differences of the interaction between these peptides and other cell wall polymers, which consequently led to these

peptides being solubilized in different steps of the treatment? This question needs further investigation. Sequencing and similarity comparision also showed that there were two trypsin derived peptides in fraction II. Although the sequencing results were not completely satisfactory, these results provided needed preliminary information about the structure of cotton cell wall extensin. Further purification as well as knowing extensin cDNA sequences will aid in this understanding.

In order to study the nature of cotton cell wall extensin and its interactions with polysaccharides, a sequential combination of highly specific methods which interrupt the interaction of extensin and polysaccharides were used. They were: EPGase digestion to remove homogalacturonans; cellulase digestion to remove cellulose and xyloglucan; HF -73° C solvolysis to remove arabinofuranosyl residues from the Hyp residues of extensin followed by ammonium bicarbonate buffer extraction; and trypsin digestion. This sequential treatment co-liberated 50% of the Hyp of the original cell wall and some pectin sugars. This is further evidence showing covalent linkage between extensin and pectin. The solubilized material called extensin-RG I complexes contains heavily glycosylated peptides and slightly glycosylated peptides.

After separation by gel filtration chromatography on a HW 50(S) column (fractionating range from 800 to 90,000 Daltons), the extensin-RG I complexes were separated into fraction A called extensin-RG I complexes A, fraction B called extensin-RG I complexes B, and fraction C. The extensin-RG I complexes A, eluted in the void volume, are heavily glycosylated peptides. The sugars in this fraction (A) are mainly RG I-like sugars such as GalA and Rha. After complete deglycosylation by HF 0°C treatment, most of the peptides became much smaller as indicated by SDS-PAGE. On the SDS-PAGE, five bands with molecular weight of about 26.3 kD, 14.4 kD, 11.5 kD, 7.2 kD and 4.7 kD were resolved before deglycosylation although these were minor components as the majority did not enter the separating gel. After deglycosylation, most of the peptides which remained in stacking gel migrated into the separating gel (around MW 100 kD), and the

protein bands which had already entered the separating gel had a faster migration. The molecular weights of these protein bands with faster migration are about 25.7 kD, 14.4 kD, 11.1 kD, 6.9 kD and 4.6 kD, respectively. Two of these bands, with molecular weights of 14.4 kD and 4.6 kD, were stained on a western blot with antibody raised against deglycosylated peptides of tomato extensin precusor (dp1a). The deglycosylated extensin-RG I complexes A contain again two fractions (I and II) on reverse phase HPLC of tryptic peptide mapping. The fraction II of ERC A only has the peaks suspected to be those of trypsin derived peptides, but not extensin peptide repeats as shown by HW 40 gel filtration chromatography.

Extensin-RG I complexes B, eluted in the fractionation range after HW 50 gel filtration chromatography column, are slightly glycosylated peptides. The major sugars in these complexes are arabinose, galactose, and a smaller amount of RG I-like sugars. Both glycosylated and deglycosylated extensin-RG I complexes ERC B and dERC B migrated into the separating gel in gel electrophoresis. There were four bands observed in ERC-B with molecular weights of about 24 kD, 11.1 kD, 8.3 kD and 4.1 kD. After deglycosylation, there were only three bands observed with mobilities similar to the glycosylated forms (the molecular weights of them are : 24 kD, 11.1 kD and 4.1 kD). This may be because the sugars of ERC B are mainly single galactose and arabinose which do not contribute very much to the MW of ERC B. All of the bands except one in ERC B (8.3 kD) were stained on western blot with anti-dp1a. The deglycosylated tryptic peptides of extensin-RG I complex B also have the characteristics of cotton extensin peptides on reverse phase HPLC peptide mapping and HW 40 gel filtration chromatography.

In conclusion, the results of this work indicated that the combination of specific selective treatments is an efficient way to isolate extensin peptides from cotton suspension primary cell walls. After purification, four repeated peptides were sequenced from extensin. Although these procedures solubilized most of extensin peptides, about 6.3% of the Hyp still remained insoluble. This implies that there may be crosslinks between

extensins or extensin-phenolic compounds. In addition, the sequential treatment, in which the last step was trypsin digestion cleaving peptide bonds, allowed the liberation of extensin-RG I complexes. SDS-PAGE and western blot analysis showed that after deglycosylation, the mobility of extensin peptides (mainly extensin-RG I complexes A) increased. The HF 0°C solvolysis of glycosidic bonds from the residue of sequential treatment co-solubilized the extensin peptides. These results strongly support the suggestion that the cell wall extensin may link to pectin covalently, and provide a background for further investigation. Finally, some of the extensin peptides solubilized from different steps of the series treatments seem to have different structure. Further characterization and comparision of these extensin peptide fragments may help to understand the nature of the interaction between extensin and other cell wall polymers.

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