ISOLATION AND CHARACTERIZATION OF HYDROXYPROLINE-RICH GLYCOPROTEINS FROM CALLUS CULTURES OF SUSCEPTIBLE AND RESISTANT CULTIVARS OF COTTON PLANTS

By

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LIST OF ABBREVIATIONS

Ac 44	Acala 44
AG	arabinogalactan
AGP's	arabinogalactan proteins
A. pseudoplatanus	*Acer pseudoplatanus*
Ara	arabinose
cDNA	complementary DNA
CM	carboxymethyl
Fuc	fucose
Gal	galactose
Gal A	galacturonic acid
Glc	glucose
Glc A	glucuronic acid
Gly	glycine
HF	hydrogen fluoride
His	histidine
HRGP's	hydroxyproline-rich glycoproteins
hr	hour
Hyp	hydroxyproline
Im 216	Immune 216
Lys	lysine
Man	mannose
min	minute

Pro	proline
SbPRP1	soybean proline-rich protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
Thr	threonine
TMS	trimethyl silyl
Tyr	tyrosine
Val	valine
X. c. malvacearum	*Xanthomonas campestris* pv. *malvacearum*
Xyl	xylose

CHAPTER I

INTRODUCTION

Extensin is one member of a class of hydroxyproline-rich glycoproteins present in a wide variety of plants and algae [1]. Cell walls of higher plants usually contain small quantities of extensins. Their usually low level is strikingly increased in response to wounding [2,3,4], infection [5,6,7], elicitor treatment [8,9], and under tissue culture conditions [10]. A number of reports,cited in [1], have suggested that this accumulation of the glycoprotein may act as a defense mechanism of the plant to disease.

Isolation of extensins is a prerequisite to clearly understanding their roles. This step, however, is particularly difficult due to the high insolubility of the glycoprotein. Recent progress in the isolation has been accomplished either by direct elution of a precursor from carrot root and tomato cell walls with a salt solution [11,4] or by solubilizing intact extensins from a homogenate of potato tubers and tobacco callus at very acidic pH [12,13]. In addition to hydroxyproline-rich glycoprotein (HRGP)-extensin precursors, these procedures have led to the solubilization of hydroxyproline-rich arabinogalactan proteins.

The objectives of this research were as follows:
1) To isolate extensins from callus cultures of susceptible and resistant cultivars of *Gossypium hirsutum* (cotton). 2) To determine several biochemical parameters such as amino acid content, hydroxyproline content and carbohydrate content. 3) To relate the differences in the biochemical parameters such as amino acid content and hydroxyproline content to the

resistance of the plant to the bacterial pathogen, *Xanthomonas campestris pv. malvacearum.*

CHAPTER II

LITERATURE REVIEW

Plant Cell Wall Proteins

Plant cell walls are comprised of cellulose, hemicelluloses, pectic compounds, lignin, suberin, proteins and water [14]. Cell walls contain several types of structural proteins as well as various enzymes. Among these is the family of hydroxyproline-rich glycoproteins (HRGP's) which to date is the best characterized class. Plants contain at least three classes of HRGP's: 1) certain lectins found in the *Solanaceae* [15,16,17], 2) the arabinogalactan proteins (AGP's) [15,16,17,18,19 and 20], and 3) extensins [1,10,14,20,21 and 22]. They are distinguished from one another by their chemical composition. The first class is a group of hemaglutinating lectins whose activity is specifically inhibited by di- and tri-N-acetyl glucosamine [15]. The potato lectin is a cell wall glycoprotein whose synthesis is increased upon wounding [17]. The second class is the AGP's,widely distributed in the plant kingdom. They are primarily located in the extracellular matrix and are freely soluble although they are sometimes associated with the plasma membrane, and are major components of plant gums and exudates [16]. The third class is the extensins, major components of the primary cell wall where they may play a structural role. Extensins are insoluble HRGP's within the primary cell wall; however, soluble forms of this protein class have been purified [22].

With the use of molecular biological techniques (isolation and sequencing

of cDNA clones), some investigators have reported other structural cell wall proteins, like the glycine-rich proteins in petunia [23] , and bean [24], and the proline-rich proteins in carrot [25], and soybean [26,27,28]. Furthermore, a 28-kD glycoprotein that accumulated in the cell wall of growing stems of soybean at low water potentials has been reported [29]. Moreover, a 70-kD protein was extracted from mature cell walls of soybean stems [29]. Although graminaceous monocots generally contain low levels of HRGP's, a threonine-rich HRGP, homologous with dicot extensins has been isolated from maize cell cultures [30,31]. HRGP's from unicellular alga *Chlamydomonas reinhardtii*, similar to dicot HRGP's, were recently characterized [32].

Detailed information about structural cell wall proteins has only begun to develop. Future studies on the characterization, localization and assembly of other cell wall components will provide further information on how wall proteins affect the growth, development and function of plant cells.

Extensins

Extensins are largely confined to the primary cell walls that are undergoing extension, hence the name extensin accorded them by Lamport [10]. So far, the best characterized extensin is the one isolated by salt-elution from carrot root cell walls [4]. The amino acid composition of the soluble carrot extensin is mostly Hyp, Ser, His, Tyr, Lys and Val. The abundance of Lys and low content of Glu contribute to the high isoelectric point observed in this molecule. This glycoprotein consists of 35% protein and 65% carbohydrate. Arabinose represents 97% of the sugar present and galactose only 3%. The arabinose is attached via an O-glycosidic linkage to Hyp in short side chains of mainly four and three residues. Galactose is linked to serine in an O-glycosidic linkage.

This extensin has a highly repetitive pentapeptide sequence, Ser-$(Hyp)_4$ characteristic of the backbone of all extensins.

The secondary structure of soluble carrot, tomato and sycamore-maple extensins has been studied using electron microscopy [33,34,35] and circular dichroism [34]. Analysis by circular dichroism indicates that extensin is in the polyproline II conformation (an extended left-handed helix). Morever, it appears that the carbohydrate moiety of this glycoprotein stabilizes the helical conformation. The secondary structure of extensin is consistent with the information obtained from electron micrographs of the glycoprotein. The micrographs show thin rod-like molecules with an average length of 80-84 nm. True molecular weight of carrot and tobacco extensin was reported to be ~90 kD.

Extensins have also been isolated from different plants and tissues, such as potato tuber [12], tobacco callus [13], tomato cell suspension cultures [11], and soybean seed coats [36]. In all these glycoproteins, Hyp is the major amino acid representing 33-42 mole % of the total amino acids. Other abundant amino acids are Ser, His, Lys, Tyr, Val and Pro. Arabinose and galactose are the only carbohydrates present in the protein [36,12,13]. In tomato cell suspension cultures, Hyp-tetra-arabinosides and Hyp-tri-arabinosides predominate in both extensins isolated [11]. In soybean seed coat extensin, arabinose is the major sugar mainly bound to Hyp in side chains of three arabinosyl residues [36].

Amino acid sequences of two different extensin monomers, labelled P_1 and P_2 from tomato cell suspension cultures, have been reported [37]. The tryptic peptide maps show that both extensin precursors are highly periodic structures. P_1 contains primarily two different peptide blocks: Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys and Ser-Hyp-Hyp-Hyp-Hyp-Val-Lys-Pro-Tyr-His-

Pro-Thr-Hyp-Val-Tyr-Lys and P_2 consists entirely of a single repeating decapeptide, Ser-Hyp-Hyp-Hyp-Hyp-Val-Tyr-Lys-Tyr-Lys. These sequences of P_1 and P_2 show two different repeated domains, one of glycosylated Ser-$(Hyp)_4$ sequences and the other non-glycosylated. Lamport proposed that the glycosylated domain is relatively rigid and the non-glycosylated one flexible, a structure that might allow the weaving of the cellulose microfibrils of the primary cell wall with an extensin network of defined porosity, the so called "warp-weft" model.

It has been proposed that extensin is slowly insolubilized in the cell wall by covalent linkages [33,11]. One proposed covalent link is the isodityrosine formed between two tyrosine residues from different extensin molecules [39,40]. To date, no intermolecular cross-link has been characterized. Thus, the insolubilization of extensin may not be the consequence of covalent linkages.

Further analyses of the protein structure of extensins indicate that in sugar beet the Ser-$(Hyp)_4$ blocks appear to be split, indicating an unusually high interspecific variability for a putative structural molecule [41].

In summary, all the extensins that have been characterized are highly basic molecules with high Hyp and Ara contents. Most of Hyp is found in the Ser-$(Hyp)_4$ peptide sequences. The Hyp-Arabinosylation pattern varies from species to species, as does the content of non-Hyp amino acids.

Arabinogalactan Proteins

Arabinogalactan proteins (AGP's), as major components of plant exudates and secretions, have been studied extensively in terms of their chemistry [15,16], but they are known to occur in all plant tissues and in organ-specific forms [42].

AGP's contain a high proportion of carbohydrate and usually less than 10% by weight of protein. The major polysaccharides are D-galactopyranose and L-arabinofuranose with most samples containing more Gal than Ara. They may also contain smaller proportions of L-rhamnopyranose, D-mannopyranose, D-xylopyranose, D-glucopyranose, D-glucuronic acid and D-galacturonic acid. The polysaccharides are comprised of a backbone of (1 → 3) -linked β-D-galactopyranosyl residues branched through positions 6 with (1 → 6) -linked β-D-galactopyranosyl side-chains which are substituted with arabinopyranosyl residues, and the less abundant monosaccharides often in terminal positions [15]. Using Smith-degradation procedures, it has been shown for many AGP's, that the β-D-galactan framework contains blocks of galactopyranosyl residues which are interrupted at regular intervals by periodate-sensitive residues [43,44]. The blocks which remain resistant to Smith degradation consist largely of (1-->3) linked galactopyranosyl residues [15].

In contrast with the polysaccharide component, relatively little is known about the structure and organization of the protein core of the AGP's [20]. They are rich in hydroxyproline, serine, alanine and glycine. The nature of the carbohydrate-protein linkage has been demonstrated unequivocally only for the AG-peptide from wheat endosperm and involves galactopyranosyl residues linked *O*-glycosidically to hydroxyprolyl residues [45,46]. However, the hydroxyproline of rice-bran proteoglycan is substituted with oligo-arabinosides [47], and alkali-stable arabinosyl-hydroxyproline linkages have been detected in an AGP from Timothy grass pollen [48]. In *Cannabis sativa* leaf, both galactosyl-serine and alkali-resistant linkages have been detected [49], and carbohydrate is attached to serine, threonine and hydroxyproline in the AGP from *Phaseolus vulgaris* [50]. A large proportion of the polysaccharide in radish leaf AGP may be linked to the protein through 3-*O*-D galactosylserine [51].

It is not clear if one or more polypeptides are present in the AGP molecule. Three N-terminal amino acids, ser, gly and ala, in molar proportions 1:2:1 are found in *Acer pseudoplatanus* AGP [52], consistent with the presence of four peptide chains cross-linked in some way, each bearing several AG chains at Hyp sites. Attempts to sequence the peptides by the Edman procedure were thwarted by glycosylation of Hyp at residue 3 of the chain and by cross-linking of structures further into the polypeptide chain. The N-terminal amino acids in AGP's from *Ipomea batatas*, *Daucus carota*, *Pisum sativum*, *Acica elata*, *Pinus taeda*, and *Pseudosuga menziessi* are the same as in *A. pseudoplatanus* supporting the idea that the N-terminal amino acids of the AGP polypeptides are evolutionarily conserved [52].

The recent generation of monoclonal antibodies to the plasma membrane of plant cells has led to observations that glycoproteins associated with the plasma membrane contain carbohydrate components that also occur on soluble AGP proteoglycans [19,53]. Recently, it has been indicated that Ala-Hyp repeats are found in a ryegrass AGP [54].

In summary, AGPs are acidic molecules rich in hydroxyproline, alanine, serine and threonine. Arabinose and galactose are the main sugars in addition to uronic acids. Although there is a wealth of information on the structure of AGPs, their function remains obscure. It has been proposed that they might be involved in cell-cell recognition and/or interaction, however no one has reported any attempts to establish their function by direct experimentation.

Molecular Biological Studies

Although the repetitive segments of the primary sequences of two different extensins from tomato cell suspension cultures have been determined [37],

completion of the sequence by protein chemical methods may prove difficult because of the presence of many imino acid residues and of many post-translational modifications. Nucleic acid studies offer the simplest means of determining the primary sequences of extensins.

Chen and Varner isolated and sequenced a partial cDNA clone for carrot root extensin, which provided a sequence of the carboxy-terminus [55]. This cDNA clone encodes a peptide containing Ser-$(Pro)_4$ repeats and Tyr-Lys-Tyr-Lys [55] sequences also found in tomato extensin [56,37]. Using cDNA clones as probes, six different clones from carrot genomic libraries were isolated [57]. One of the genomic clones was characterized and found to contain an open reading frame possibly encoding extensin, and a single intron in the 3'-noncoding region. The derived amino acid sequence contained a putative signal peptide, and 25 Ser-$(Pro)_4$ sequences. Two different extensin RNA transcripts were found corresponding to the genomic clone with different 5' start sites. Both transcripts increase markedly upon wounding, which correlates with the extensin accumulation seen in the cell wall after wounding in carrot roots [57,3,4].

A tomato extensin genomic clone has been isolated with the genomic clone for carrot extensin as a probe [58]. The sequence of this clone encodes a polypeptide with numerous Ser-$(Pro)_4$ repeats, which are usually followed by Val-His or Val-Ala.

Three extensin transcripts of *Phaseolus vulgaris* have been sequenced [59]. Two of these transcripts code for proteins rich in repeats of Tyr-Tyr-Tyr-Lys-Ser-Pro-Pro-Pro-Pro-Ser-Pro-Ser-Pro-Pro-Pro-Pro, and the third for repeats of Tyr-Tyr-Tyr-His-Ser-Pro-Pro-Pro-Pro-Lys-His-Ser-Pro-Pro-Pro-Pro.

One of the tobacco extensin transcripts has been sequenced [60]. It codes for 17 repeats of Ser-Pro-Pro-Pro-Pro, seven repeats of Ser-Pro-Pro-Pro-Pro-

Thr-Pro-Val, and eight repeats of Ser-Pro-Pro-Pro-Pro-Lys-Lys-Pro-Tyr-Tyr-Pro-Pro-His-Thr-Pro-Val-Tyr-Lys.

Rape extensin gene, *extB,* has been isolated [61] from an oilseed rape (*Brassica napus* L). It represents a sub-family of extensin genes different from the sub-family containing *extA* [62].

A cDNA clone from carrot root has been isolated [55] that encodes a proline-rich 33 kD protein, p33, containing three repeats of Pro-Pro-Val-Tyr-Thr-Pro-Pro-Val-His-Lys. Subsequent studies have shown that this proline-rich protein is present in the cell wall of carrot root [22]. A petunia gene encoding a protein that is 67% glycine has been characterized [23]; the bulk of the sequence consists of $(Gly\text{-}X)_n$ sequences in which X is frequently glycine. It has been proposed that the protein encoded by this gene is likely to function as a cell wall structural protein [23].

Another gene that encodes a proline-(Hyp)-rich protein has been isolated from soybean [26]. The amino acid sequence deduced from the gene for the protein, designated $SbPrP_1$, is composed primarily of 43 repeat units of Pro-Pro-Val-Tyr-Lys and a putative signal sequence of 26 amino acids. Recently a new proline-rich protein has been isolated from soybean seedlings [28]. This protein shows similarities in composition and sequence to that predicted for the carrot p33 protein and $SbPrP_1$.

Although the most-studied HRGP's, so far, have been those from dicot species, a maize HRGP has been characterized at the protein and genomic levels [30,31,63]. This maize HRGP has the main features of dicot extensins, although its main repeated motif is different from the dicot ones and it is very rich in threonine [63]. The sequence of a HRGP gene from sorghum [64] resembles maize HRGP gene (60% overall homology).

As more genes that encode cell wall proteins are isolated and

characterized, it will be possible to gain further information on their structure and function.

Roles of Extensins

Structural-Functional Role

Casab and Varner [21] demonstrated that extensin is most abundantly localized in cells that belong to the sclerenchymal tissue. The sclerenchymal cells act as the skeletal elements of the plant body. These cells enable the plant body to withstand various strains, such as stretching, bending, compression and tension [66]. Thus the presence of extensin in the sclerenchymal cell walls together with other wall components, may determine the unique characteristics of these cells.

An extremely hydroxyproline-rich sulfated glycoprotein is expressed under strict developmental control in inverting Volvox colonies, which supports the idea of a functional role of HRGP in inversion [67].

Perhaps, the best example of a structural role for extensin in the wall comes from studies of Chlamydomonas cell walls in which several hydroxyproline-rich glycoproteins constitute the major structural components [68,69]. The structure and assembly of cell walls in *Chlamydomonas reinhardtii* have been analyzed *in vitro*. Each wall consists of chaotrope-insoluble (W_1, W_2) and chaotrope-soluble (W_4, W_6) layers. There are four major glycoproteins in the extracellular matrix of *Chlamydomonas reinhardtii*. Three of four are the hydroxyproline-rich glycoproteins that co-polymerize to form the W6 layer.The fourth is a glycine-rich glycoprotein apparently found within the W4 layer.

Role of Extensin in Plant Defense

Although the numerous reports on the biosynthesis and structure of extensin has been published, we still know very little about its role inside the cell wall.

Several studies suggest a role for extensins in plant defense. Extensins accumulate in the plant cell wall in response to infection, mechanical wounding, elicitor treatment, and under tissue culture conditions [65].

It has been demonstrated that hydroxyproline and thus HRGP's increased markedly in the cell walls of melon seedlings during infection by *Colletotrichum lagenarium* [70,5,6]. Although the accumulation of HRGP was first reported in melon in a susceptible reaction, it was later demonstrated that it accumulated earlier in melons in which resistance had been induced against the fungal pathogen [6]. In cucumber, cell wall Hyp levels increased more rapidly in resistant than in susceptible cultivars of cucumber infected by the fungus *Cladosporium cucumerinum* [71]. Since then, it has been shown that Hyp increases in the cell walls of other hosts infected by different parasites [71,72].

There is an accumulation of cell wall HRGP's in bean hypocotyls [8] following slicing (mechanical wounding). Similarly, wounded tomato stems have been shown to accumulate cell wall HRGP's and HRGP mRNA [73]. Chrispeels *et al.* [3] have shown that slicing and aeration enhance the synthesis and secretion of HRGP in carrot discs and suggested that this process may be involved in structural reformation of the wall or, perhaps, in disease resistance following wounding [3].

The earliest detectable event during plant-pathogen interaction is a rapid increase in ethylene biosynthesis [74]. It has been proposed that ethylene produced in response to biological stress is a signal for plants to activate

defense mechanisms against invading pathogens. It has been reported that extensin mRNA's accumulated in response to ethylene. In carrot, two HRGP mRNA's, 1.8 and 1.4 kb have been induced by ethylene [74]. Additionally, it has been found that treatment of melon plants with ethylene leads to induction of 1.4 and 1.65 kb mRNA's which hybridize to a genomic clone of HRGP from carrot. Treatment of melon and soybean hypocotyls with elicitors of fungal origin resulted in the stimulation of HRGP synthesis [8]. Also, elicitor treatment of bean cell cultures resulted in the accumulation of cell wall hydroxyproline [9], a direct sign of HRGP accumulation.

All these works indicate that enrichment of the cell wall HRGP is a common response of plants to infection, and this response may be involved in the general defense reactions of plants.

In summary, a precise role of extensins in the cell wall is not clear and may not be necessary, but they are assumed to play a role in the structure of plant cell walls and may therefore be important in controlling growth, development, and disease resistance. Further research is necessary to more clearly define their precise role.

CHAPTER III

MATERIALS AND METHODS

Plant Materials

Cotton Lines and Callus Cultures

Two different tissue cultured cell lines were used in this research. They are cell cultures from cotton lines Acala 44 (Ac 44) and Immune 216 (Im 216).

The cotton line Ac 44 (blight-susceptible) possesses no genes for resistance to *Xanthomonas campestris* pv. *malvacearum* (*X.c. malvacearum)* [75] and is completely susceptible to all North American races of the bacterial pathogen. Resistant line Im 216 (blight-immune) possesses homozygous resistance to *X.c. malvacearum*, having three major resistance genes B_2, B_3, b_7 and the polygenes [75,76,77]. It is resistant to all North American races of *X.c.malvacearum*. Ac 44 and Im 216 callus cultures were established in 1985 (Janet Rogers, Dept. of Biochemistry, Oklahoma State University, OK) using the method of Ruyack et al. [78]. The callus cultures which were used in this research represent end of the log phase.

HRGP Extraction

HRGP's were extracted from cotton callus tissue according to a modification of the acid-ethanol procedure of Marinkovich [79] (Figure 1). The callus tissues (100 g fresh weight) were placed in 100 ml of a mixture of absolute ethanol:1.25 N HCl (3:1 v/v) to which 4 mM sodium metabisulfite had

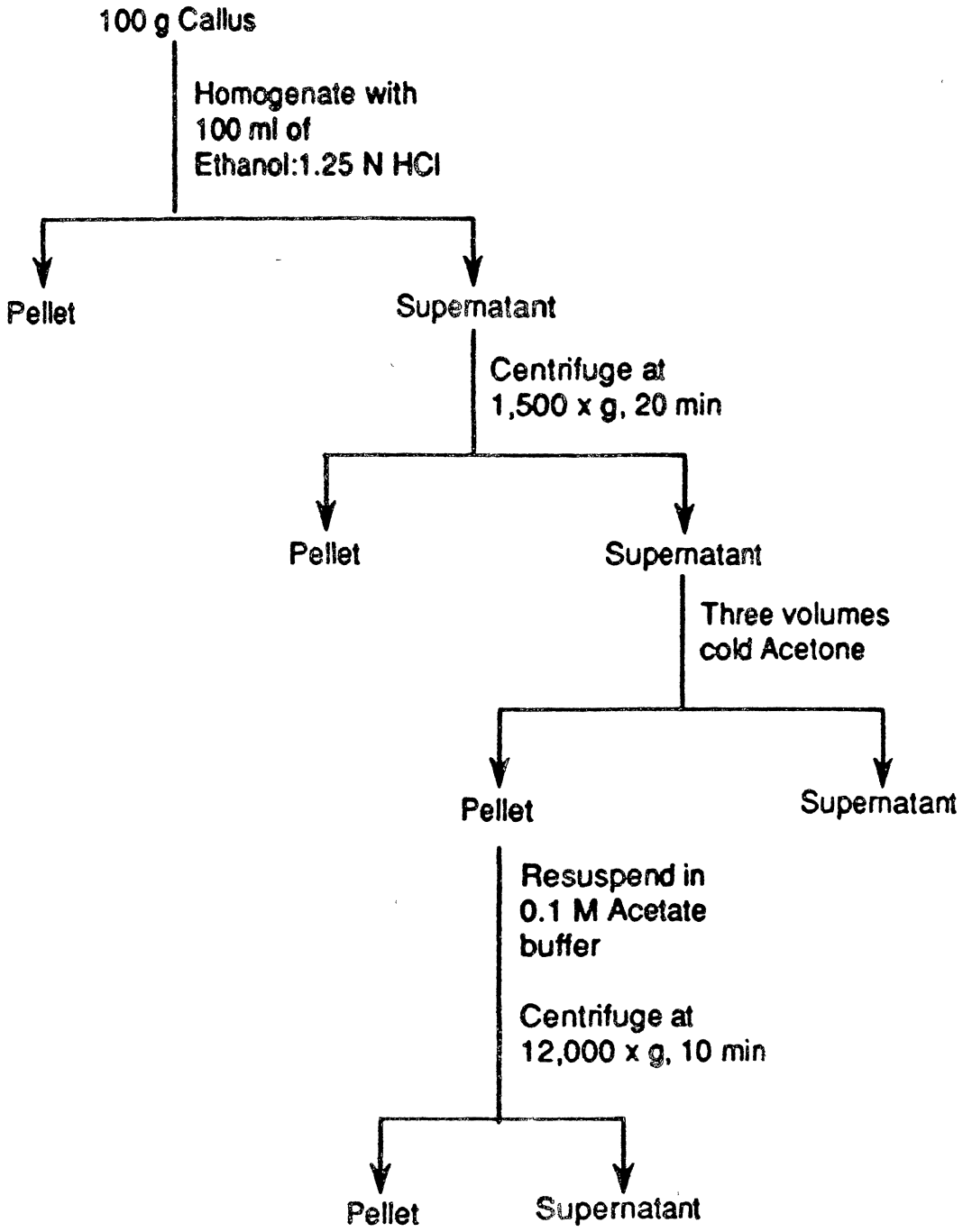


Figure 1. Scheme for the Extraction of Cotton HRGPs by Acidic-Ethanol Method.

been added as antioxidant and homogenized with a Polytron Homogenizer at full speed. Homogenization was done at room temperature for two-minute bursts until the cells were completely broken (examined under the microscope). The homogenate was maintained under constant stirring for 48hr at 4°C before being filtered through cheese cloth. After centrifuging at 1,500xg for 20 min, the supernatant was recovered and the proteins were precipitated by addition of three volumes of cold acetone. The protein pellet was recovered by centrifugation (1,500xg for 20 min) after sitting for 24hr at 4°C and resuspended in 25 ml of a 0.1 M sodium acetate buffer (pH =3.8). The undissolved material was discarded after centrifugation (12,000xg for 10 min) and the resulting soluble crude extract was first dialyzed for 12hr against 2.5L of 0.05 M sodium acetate buffer (pH = 3.8) at 4°C. Second, the extract was dialyzed against 2.5L of water at 4°C. Dialysis solutions were changed every six hours. Following dialysis, the crude extract was lyophilized. The lyophilized samples were resuspended in half ml of 0.05 M sodium acetate buffer.

Analytical Methods

CM-Sepharose Chromatography

The resuspended extract was applied to a column of CM-Sepharose (1.1 x 10 cm) equilibrated with 0.05 M sodium acetate buffer at pH =3.8. The column was eluted at 20°C with 40 ml of the same buffer followed by 100 ml of a 0 to 2 M NaCl linear gradient in the same buffer. The elution profile was determined by measuring the absorbance at 280 nm of each fraction (2 ml) and the fractions corresponding to eluted material were pooled, dialyzed against water for 12hr, and lyophilized.

Gas-Liquid Chromatography

Sugar composition was determined by the method which allows for complete analysis of most sugars in a single procedure [80]. A weighted dry sample (~50 μg) was put into a small screw cap vial containing 100 nanomoles of Inositol as an internal standard. A dry aliquot of sugar standards containing 100 nanomoles of each sugar and 100 nanomoles of Inositol in a screw cap vial was prepared. Approximately 200 μl of methanol in 1.5 M HCl was added to each vial; they were capped tightly and placed into a heating block at 80°C overnight. In this methanolysis step, methyl glycosides are formed. After 16hr, vials were removed from the heating block and cooled. A few drops of t-butanol were added followed by evaporation under a stream of nitrogen at room temperature. The butanol co-evaporates with the HCl, helping to remove the HCl without degrading the sugars. Using a syringe, 25 μl of a 1: 3 mixture of "Tri-Sil " concentrate:pyridine was added to each vial. The vials were capped and let stand for 15 min (this step adds trimethylsilyl groups to all free OH groups, making them more volatile). After 15 min, the samples were evaporated just barely to dryness under the stream of nitrogen at room temperature. With a syringe, 100 μl of isooctane was added and swirled gently to dissolve all of the sample. One microliter (1μl) of sample was injected into the gas chromatograph for analysis. The column was a DB-1 bonded-phase capillary column of 0.27-mm inside diameter and 30 m long. The carrier gas was helium flowing about 30 cm/min. Initial column temperature was 105°C and immediately increased to 160°C following sample injection and remained at that temperature for 4 min. Then, the temperature was programmed to increase at 1°C/min. Analysis of peaks was done on a Macintosh Microcomputer using Microsoft Excel. The sugar analysis program was written by Dr. Andrew Mort. The program provided

us nanomole, mole percent, relative mole, micrograms and weight percent of each sugar present in the sample. By knowing the weight (micrograms) of the sample methanolyzed, carbohydrate content (weight percent) of each sample was calculated from total weight of sugar present in the sample divided by weight of the sample methanolyzed. The carbohydrate content of each peak was calculated in this way and expressed in the thesis. Only the second peak for glucose was used to calculate its abundance because a contaminant comigrated with the first peak.

Amino Acid Analysis

Amino acid analyses were done commercially by Dr.Kenneth Jackson, the Molecular Biology Resource Facility, Saint Francis Hospital of Tulsa Medical Research Institute. Approximately 1 mg dry samples were sent for analysis. They hydrolized the samples in 400-600 µl of 6 N HCl for 24 hr at 110°C. Then resulting amino acids were evaporated to dryness and resuspended in 200 µl 0.01 N HCl. Two separate amino acid analyses were performed by that laboratory. One amino acid analysis was used to quantitate all amino acids except hydroxyproline. The second amino acid analysis was used to quantitate hydroxyproline. The two analyses were correlated by equating the glycine from each analysis. The second analysis was needed to separate hydroxyproline and aspartic acid by dropping the analysis temperature from 50°C to 30°C for separation. In all analyses proline, hydroxyproline and glutamic acid were quantitated by absorbance at 400 nm, while all other amino acids were detected at 570 nm. Data analyses and peak integrations were performed using the Dionex D-500 data system and later the Beckman System Gold Chromatography software.

Gel Electrophoresis

SDS-PAGE was performed by using the Laemnli buffer system [81]. An acrylamide to bisacrylamide ratio of 30/0.8 was used and the acrylamide concentrations in the running and stacking gels were 12 and 4 % respectively. The samples (from each peak of both cultivars) were carefully weighed (on a Cann electrobalance) and they were directly suspended in SDS sample buffer and applied to the SDS-PAGE gels. The samples were run at 200 volts for 40-45 min using Mini-Protean II Dual Slab Cell System. The gels were stained using the Bio-Rad silver staining method (Bio-Rad Laboratories, Richmond, CA).

Western Blot Analysis

The protein samples were run at 200 volts for 40-45 min using a Mini-Protean Dual Slab Cell System. After electrophoresis, the proteins were transferred to nitrocellulose filters at a current density of 2.5 mA / cm for 30 min using ABN Polyblot apparatus as described in the American Bionetics Instruction manual. The nitrocellulose papers (blots) were rinsed with phosphate buffered saline (PBS) once. The filters were placed in blocking solution (10 % Inactivated Fetal Calf Serum) for 45 min to block non-specific antibody binding sites on the nitrocellulose papers The blots were incubated with the antibody to deglycosylated tomato extensin precursor,dP_1, for 1hr at room temperature and overnight at 4°C (dP_1 antibodies and deglycosylated extensin precursors were kindly provided by Marcia Kielisiewski). Next day, the nitrocellulose filters were washed for 5 min with PBS once, PBS/ 0.5 % Tween 20 twice and PBS once.more. The filters were placed in the blocking solution for 5 min, followed by incubation for 2 hr with secondary antibody. After

2 hr, the filters were washed for 5 min with PBS once, PBS/ 0.5 % Tween 20 twice and PBS twice. Into 30 ml of Tris-Cl buffer (pH=9.5), 200µl Nitro Blue Tetrazolium and 100µl 5-Bromo-4-Chloro-3-Indoyl Phosphate were added to prepare developing solution. Blots were developed in this solution (~3-4 min) and dried.

Chemical Analysis

Protein Determination

The protein content of HRGP was measured according to Lowry *et al.* [82].

Carbohydrate Determination

The carbohydrate content was determined by the Gas-liquid chromatography and double checked by phenol-sulfuric acid method [83]. The carbohydrate and protein determinations do not account for all of the weight in the HRGP fractions and the rest of the material is not identified yet.

HF Solvolysis at 0ºC

HRGP's were deglycosylated with HF solvolysis at 0ºC following the procedure of Mort and Lamport [84]. Anhydrous HF is an effective and facile method for the chemical deglycosylation of glycoproteins [84]. The basic components of the apparatus are shown in Figure 2.

In a typical experiment, the dried sample (~50 mg) and a stirring bar were placed in a Teflon reaction vessel. The whole apparatus was vacuum evacuated for 15 min and checked for leaks. Then, HF was transferred from an HF reservoir to the HF holding vessel. This was done by cooling the HF holding vessel in a dry-ice/acetone bath and allowing the HF to distill from the HF

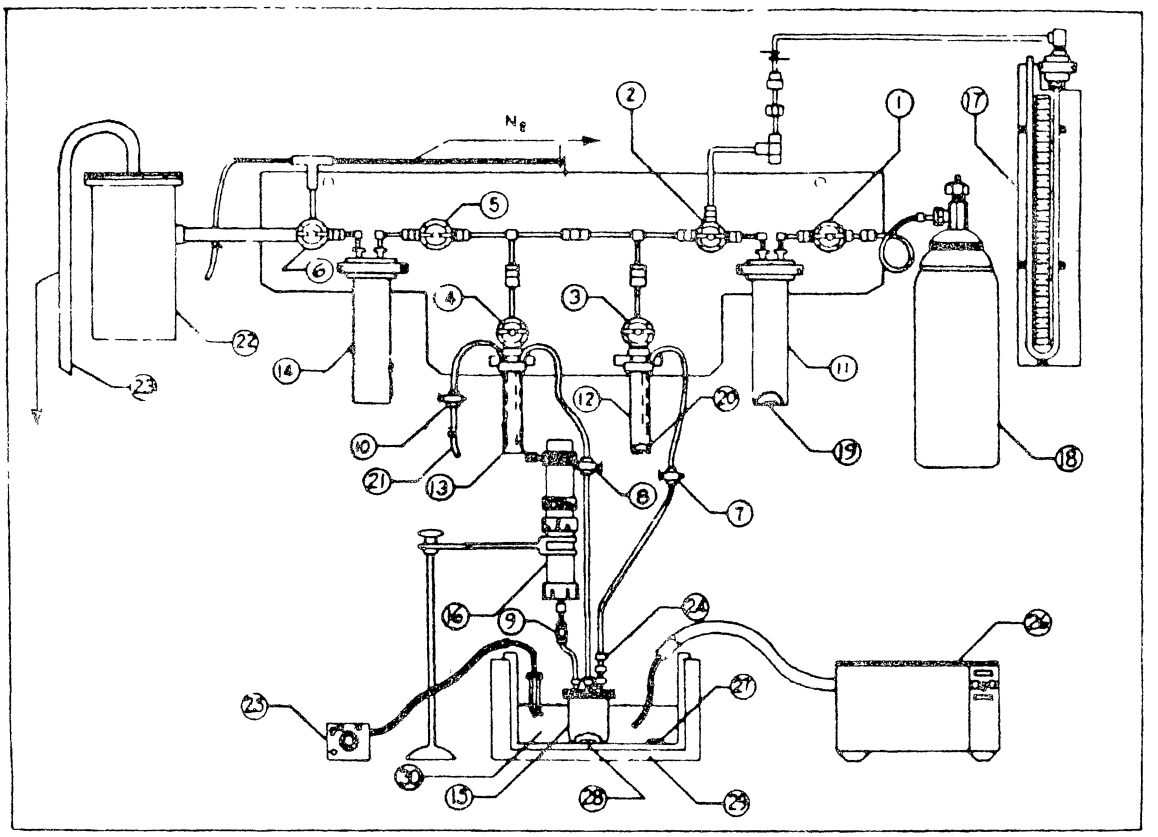


Figure 2. Schematic Representation of the Hydrogen Fluoride Solvolysis Apparatus. 1-8, 10, stopcocks; 9 teflon needle valve; 11-16 Teflon and Kel-F vessel; 17, manometer; 18, hydrogen fluoride tank; 19-20, stirrer bars; 21, exit to the sink for pressure release, if necessary; 22, calcium oxide trap; 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 (Mort, A.J., P.Komalavilas., G.L.Rorrer and D.T.A.Lamport. (1989) In Modern Methods of Plant Analysis, page 40).

reservoir to the HF holding vessel. After ~20 ml HF was transferred, HF holding vessel and the reaction vessel containing the sample were cooled to 0°C and maintained at that temperature during solvolysis using an ice bath. Next HF was transferred from the HF holding vessel to the reaction vessel by using slight nitrogen pressure. Then the solution in the reaction vessel was stirred for 30 min to allow the reaction to continue. The reaction was stopped by adding cold (cooled by adding dry ice) ether (~300 ml) from the adjacent ether holding vessel. The cooling bath was removed and the quenched reaction mixture was allowed to stir for 30 min. After 30 min, the reaction mixture was filtered using a Teflon filter. To filter the reaction mixture, the reaction vessel was removed and an inline Teflon filter unit (containing preweighed filter paper) was attached to it. Upon filtering, the ether-insoluble residue (containing mainly protein) was collected on the filter paper whereas HF/Ether/sugar mixture (filtrate, which contains mainly monosaccharides and disaccharides as sugar) was collected in a Teflon container. The filter paper containing ether-insoluble residue was dried in vacuum oven over night. At the same time the HF/Ether-soluble extract (filtrate) was put into the reaction vessel and the reaction vessel was put into a warm water bath and stirred. The HF/Ether was evaporated from the reaction vessel and condensed in a cooled collection vessel. Sugars (mainly monosaccharides and some disaccharides) in the reaction vessel were dissolved in water (~1 ml) and freeze-dried. The freeze-dried sample (~20 mg) was saved, but no analysis was done to this sample. The HF/Ether complex in the collection vessel was allowed to come to room temperature and poored into solid calcium carbonate to neutralize it before disposal. The next day the dried filter paper was weighed and the dried sample (~500μg) was saved. Given the very low amount of sample, no water extraction was done, and portions of the sample were directly used for SDS-PAGE and Western blot analysis.

CHAPTER IV

PURIFICATION OF COTTON HRGP'S

Callus Culture of Cotton Line Ac 44

Acetone-insoluble material in the acidic-ethanol soluble extract was fractionated by column chromatography on CM-Sepharose. CM-Sepharose is a weak-cation exchanger which separates proteins on the basis of their charge. Two main peaks were obtained from CM-Sepharose chromatography of five extracts using Ac 44 Cotton callus. The first peak corresponds to acidic proteins and it came always as a single peak upon five extractions. The second peak corresponds to basic proteins and it came with a shoulder upon three extractions and as a single peak upon two extractions. Existence of a shoulder might be characteristic of Acala cotton. Figure 3 shows the elution profile of the third extract of Acala 44 callus. Also total A_{280}'s pooled for each peak for the five extracts of Ac 44 were determined and shown in Table I.

Callus Culture of Cotton Line Im 216

The elution profile for the acidic-ethanol extract of Im 216 callus gave two major peaks. The second peak in the profile did not show any extra peak or shoulder upon three extractions. Figure 4 shows the elution profile of the first extract of Im 216 callus. Total A_{280}'s pooled for each peak for the three extracts of Im 216 were shown in Table I.

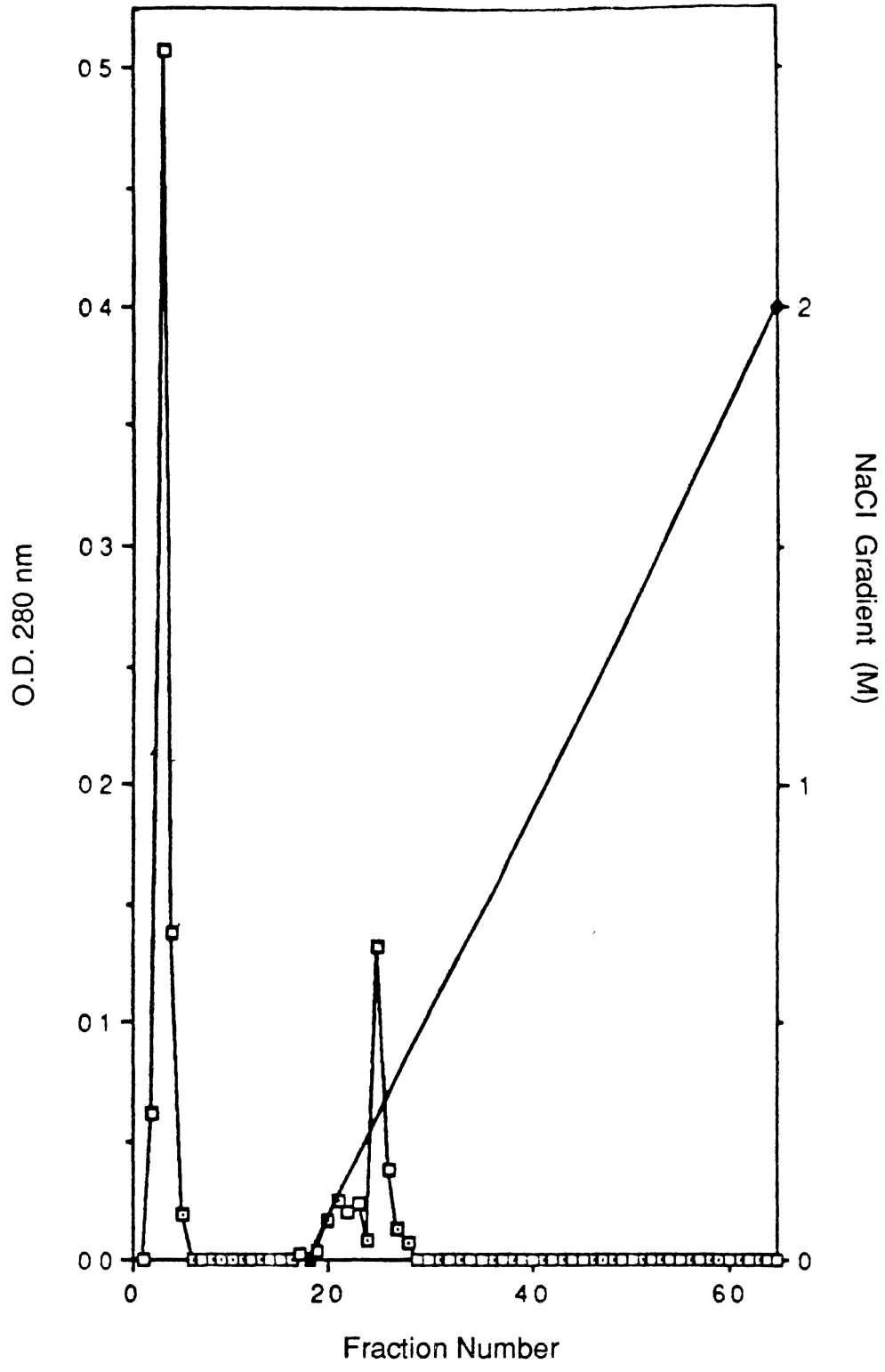


Figure 3. Elution profile of Acidic Extract from Ac 44 callus from a CM-Sepharose Column eluted with 50 mM acetate buffer pH =3.8 followed by a 0 to 2 M NaCl gradient in the same buffer. The pooled fractions for peak-1 are 1 → 6 and the pooled fractions for peak-2 are 18 → 29.

TABLE I

TOTAL ABSORBANCE 280 VALUES FOR EACH PEAK FOR ALL EXTRACTS OF AC 44 AND IM 216 CALLUS

Total A_{280} for Ac 44 peak-1	Total A_{280} for Ac 44 peak-2	Total A_{280} for Im 216 peak-1	Total A_{280} for Im 216 peak-2
0.756	0.290	2.121	0.877
0.734	0.231	2.004	0.864
0.738	0.233	1.241	0.862
1.018	0.344		
1.352	0.347		

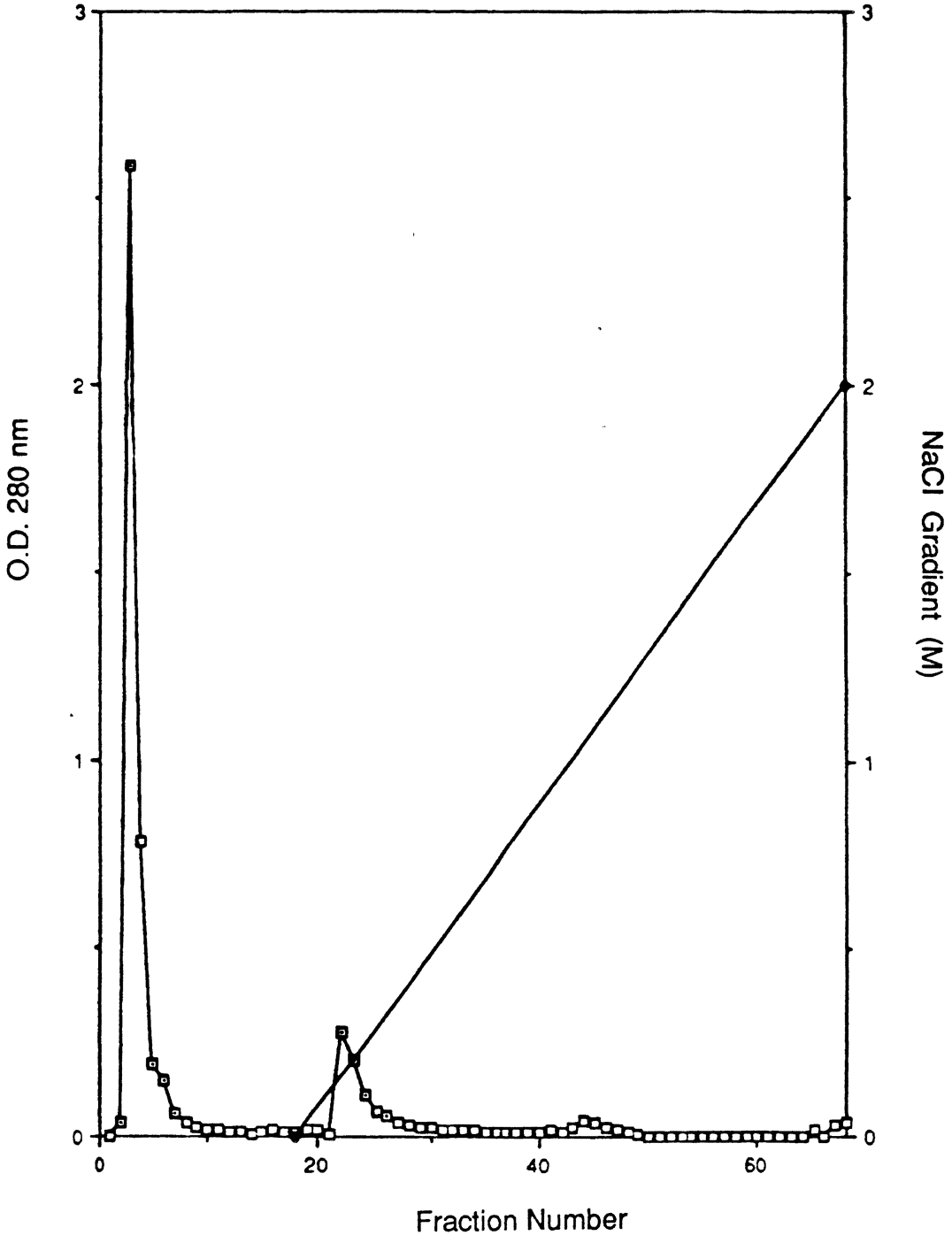


Figure 4. Elution profile of Acidic Extract from Im 216 callus from a CM-Sepharose Column eluted with 50 mM acetate buffer pH =3.8 followed by a 0 to 2 M NaCl gradient in the same buffer. The pooled fractions for peak-1 are 1 → 10 and the pooled fractions for peak-2 are 21 → 31.

Chemical Characterization of Cotton HRGPs

Callus Culture of Cotton Line Ac 44

The carbohydrate composition of peak-1 from cotton line Ac 44 was determined. Gas chromatogram of the trimethyl silyl (TMS) derivatives of sugars from this peak is shown in Figure 5. Peak-1 contained 38% carbohydrate, mainly galactose (68%) and arabinose (27%) (see Table II). The gas chromatogram of peak-1 also showed some glucose (2.8%).

The protein content of peak-1 was about 13%. Hydroxyproline was the major amino acid (22%). Peak-1 was also rich in serine (19%), alanine (16%) and threonine (11%) (Table III). As we see, the glycoprotein portion of peak-1 was 75% carbohydrate and 25% protein. Having galactose and arabinose as main sugars and being rich in hydroxyproline, serine, alanine and threonine, peak-1 showed the characteristics of an AGP. It is not known yet whether the proteins in peak-1 are AGP's or not. Another possibility, some other materials which were in the acetone precipitate, and did not bind to the column, also eluted in peak-1. For the better identification of proteins in peak-1, electrophoretic and immunochemical analyses were done and will be discussed later.

Carbohydrate analysis of peak-2 was completed. A gas chromatogram of the TMS derivatives of sugars is shown in Figure 6. The carbohydrate content (9 .1%) of peak-2 was less than that of peak-1. Being rich in galactose (49%) and arabinose (28%), peak-2 does not show much difference from that of peak-1 (Table IV). Peak-2 also contained considerable amounts of glucose (18%). Such a high amount of glucose had not been detected in the chromatograms done early in this research. The glucose looks like an artifact resulting from vial caps or dialysis tubing. Experiments to test the reason for high amounts of

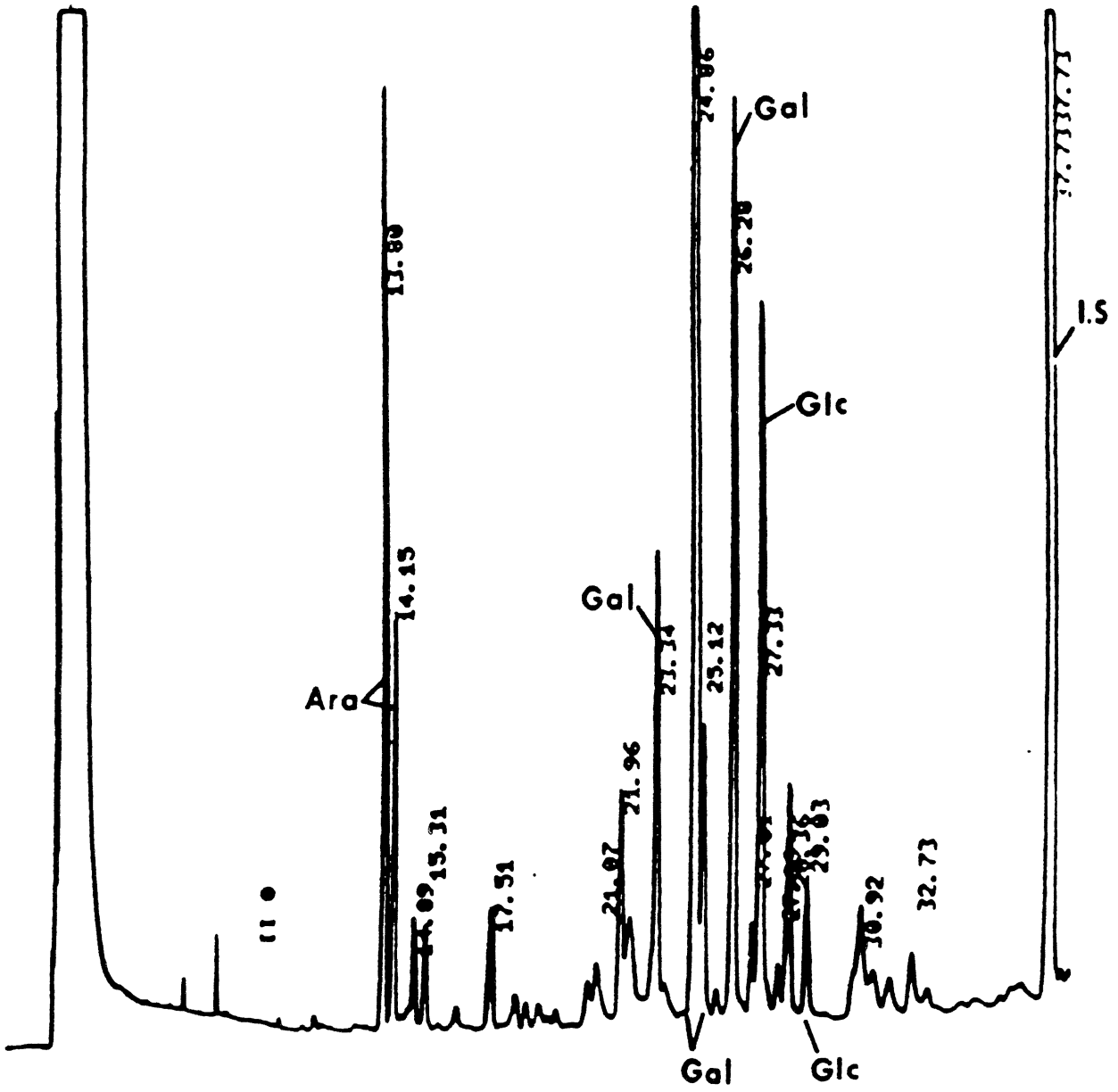


Figure 5. Gas Chromatogram of the Trimethyl Silyl Derivatives of the Methyl Glycosides of Peak-1 from Ac 44 Callus.Peaks are identified as follows: Ara, arabinose; Gal, galactose; Glc,glucose;I.S, myo-inositol, internal standard.

TABLE II

CARBOHYDRATE COMPOSITION OF PEAK -1
FROM AC 44 CALLUS

Carbohydrate Moiety[a]	38
Sugar Residues[b]	
Ara	27
Gal	68
Glc	2

[a]Weight %
[b]Mole %

TABLE III

AMINO ACID COMPOSITION OF PEAK-1 FROM AC 44 CALLUS

Protein Moiety[a]	13
Amino Acid[b]	
Hyp	22.1
Ser	18.9
Ala	16.4
Thr	11.3
Glx	5.5
Gly	5.0
Asx	4.7
His	0.7
Val	3.1
Lys	3.6
Pro	2.6
Leu	2.3
Ile	1.7
Phe	0.5
Tyr	0.4
Met	0.6
Arg	0.5

[a]Weight %
[b]Mole %

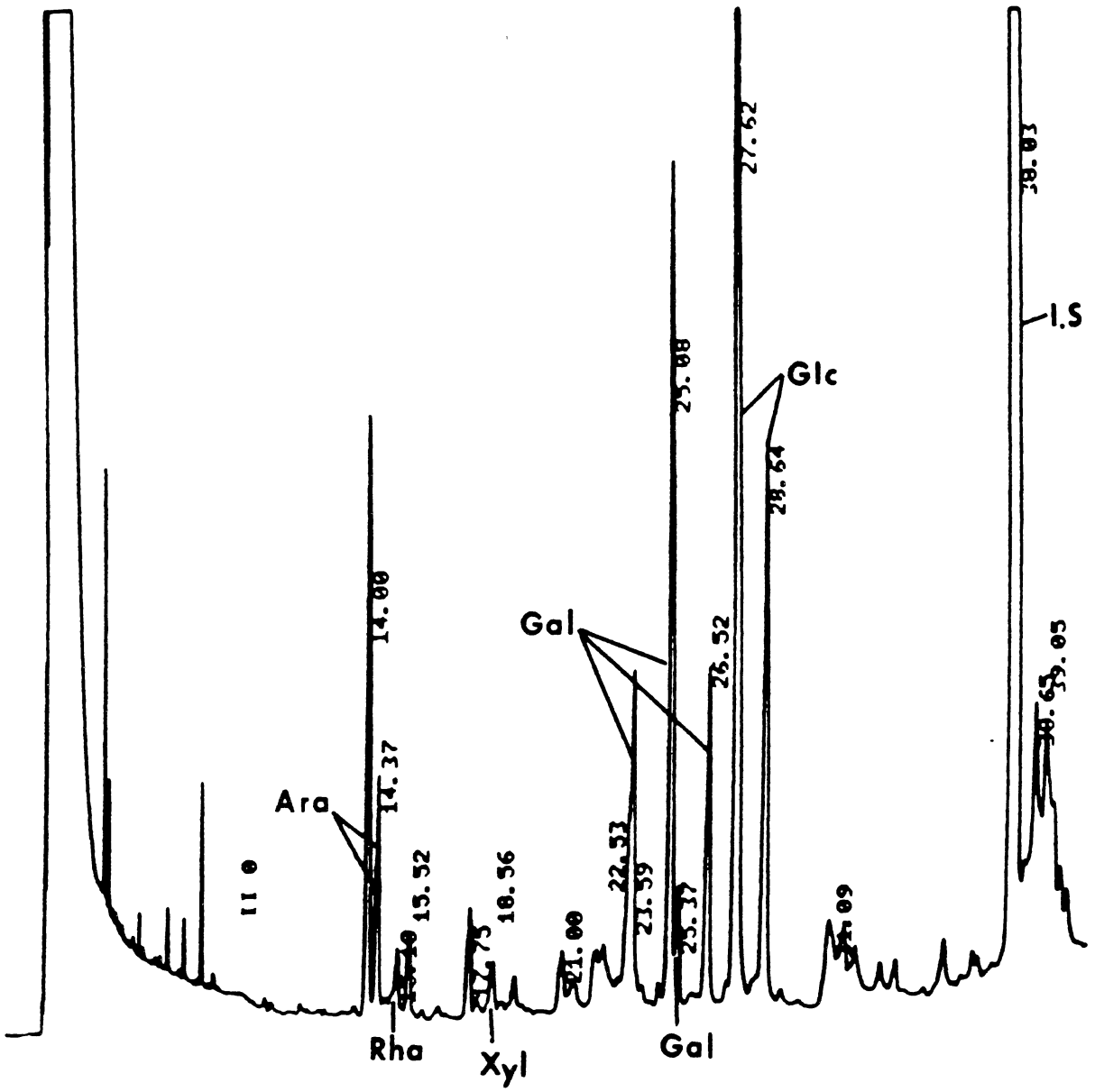


Figure 6. Gas Chromatogram of the Trimethyl Silyl Derivatives of the Methyl Glycosides of Peak-2 from Ac 44 Callus. Peaks are identified as follows: Ara, arabinose; Rha, rhamnose; Xyl, xylose; Gal, galactose; Glc, glucose; I.S, myo-inositol, the internal standard.

TABLE IV

CARBOHYDRATE COMPOSITION OF PEAK-2
FROM AC 44 CALLUS

Carbohydrate Moiety[a]	9
Sugar Residues[b]	
Ara	28
Gal	50
Glc	19
Xyl	2
Rha	1

[a]Weight %
[b]Mole %

glucose will be done. Peak-2 also contained small amounts of xylose (2.2%), and rhamnose (0.9%).

The protein content of peak-2 was about 9%. The abundant amino acids were serine (12%), alanine (11%), glycine (9.9%), hydroxyproline (8.2%), glutamine/ glutamic acid (8.5%), lysine (7.5%) and proline (6.9%) (Table V). The glycoprotein portion of peak-2 was 50% carbohydrate and 50% protein. The amino acid composition of peak-2 differed from that of peak-1. Low levels of hydroxyproline in the peak-2 was one of the differences between two peaks. Peak-1 differed from peak-2 with low levels of arginine, tyrosine, histidine, proline, phenylalanine and leucine. Also, carbohydrate and protein portions of peak-2 were equal, whereas peak-1 contained three times as much carbohydrate as protein. As a result, Peak-2 does not show characteristics of an AGP having high amounts of lysine, proline, and glycine, rather it looked more like an extensin-like protein. But it differed from other extensins from melon (65), tomato (11), carrot (4), and potato (12), having lower amounts of hydroxyproline and tyrosine and having galactose as the major sugar. Low levels of hydroxyproline in peak-2 might be characteristic of cotton extensin. So, if peak-2 consists of cotton extensins, they definitely have very unique, unusual features. Another possibility is the existence of other basic glycoproteins together with extensins in peak-2. Electrophoretic and immunochemical analyses would give an answer to this question.

The ratio of mole percents of Gal/Ara in peak-1's of Ac 44 upon five extractions changed from 2.5 to2.7 and mole percents of Hyp (as one of the most abundant amino acids) varied no more than 9%. The ratio of mole percents of Gal/Ara peak-2's of Ac 44 upon five extractions changed from 1.7 to 1.8 and mole percents of Hyp varied no more than 2%.

TABLE V

AMINO ACID COMPOSITION OF
PEAK-2 FROM AC 44
CALLUS

Protein Moiety[a]	9
Amino Acid[b]	
Hyp	8.2
Ser	12.1
Ala	10.6
Thr	7.3
Glx	8.5
Gly	9.9
Asx	7.5
His	2.1
Val	4.4
Lys	7.5
Pro	6.9
Leu	5.0
Ile	2.3
Phe	1.1
Tyr	2.3
Met	0.7
Arg	3.7

[a]Weight %
[b]Mole %

Callus Culture of Cotton Line Im 216

The carbohydrate composition of peak-1 was determined. A gas chromatogram of the TMS derivatives of sugars is shown in Figure 7. The carbohydrate content (63%) of peak-1 from Im 216 was more than that of peak-1 from Ac 44. Like peak-1 of Ac 44, this peak contained galactose (53%) and arabinose (22%) as main sugars (Table VI). It also contained small amounts of glucuronic acid (9.5%), fucose (6.0%), glucose (4.6%), rhamnose (1.8%), and xylose (1.0%). The protein content was about 10%. The amino acid composition was rich in serine (19%), hydroxyproline (16%), alanine (15%), and threonine (9.5%) (Table VII). The amino acid composition of peak-1 from Ac 44 callus differed from that of peak-1 from Im 216 callus by having lower amounts of hydroxyproline and threonine, and higher amounts of arginine. With galactose and arabinose as main sugars and hydroxyproline, serine, alanine, and threonine as main amino acids, peak-1 looked like an AGP. As we see, the glycoprotein portion of peak-1 was 86% carbohydrate and 14% protein. At this point, the glycoprotein of peak-1 of Im 216 showed similarity to that of peak-1 of Ac 44 (75% carbohydrate and 25% protein). But we do not know yet peak-1 contained AGP or not, or some other proteins. Electrophoresis and western blot analysis were done to find this.

Carbohydrate analysis of peak-2 from Im 216 callus is shown in Figure 8. It contained less carbohydrate (12%) than peak-1 of Im 216. Carbohydrate content of peak-2 of Im 216 differs from that of peak-1 of Im 216 having more arabinose (60%) than galactose (19%) (Table VIII). Peak-2 contained small amounts of glucose (9.8%), fucose (7.7%) xylose (2.3%), and rhamnose (1.3%). Protein content of peak-2 was about 6%, it contained hydroxyproline (18%), lysine (11 5), serine (9.9%), and proline (8.3%) as major amino acids (Table IX).

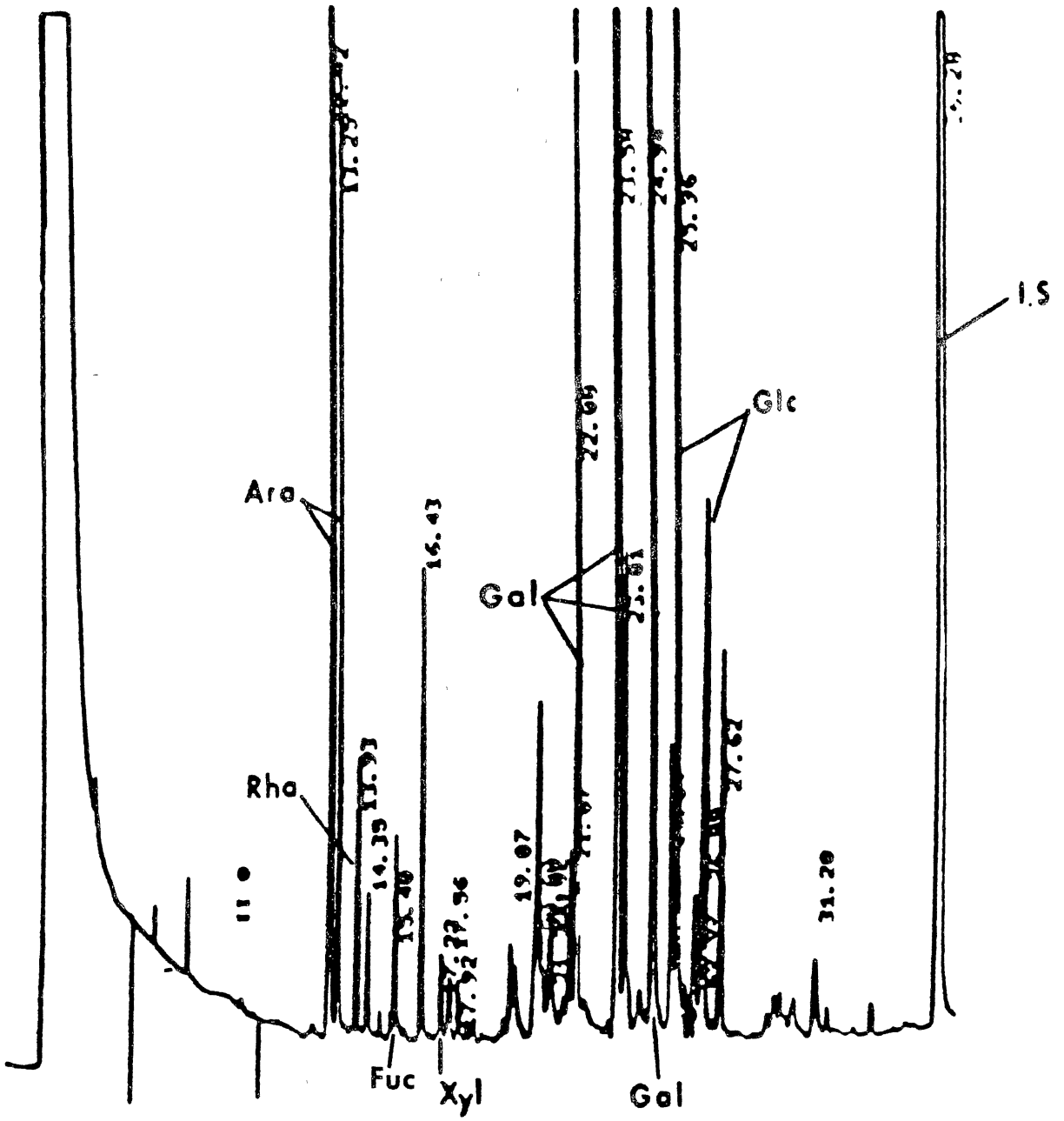


Figure 7. Gas Chromatogram of the Trimethyl Silyl Derivatives of the Methyl Glycosides of Peak-1 from Im 216 Callus.Peaks are identified as follows: Ara, arabinose; Rha, rhamnose; Fuc, fucose; Xyl, xylose; Gal, galactose; Glc A, glucuronic acid; Glc, glucose; I.S, myo-inositol, internal standard.

TABLE VI

CARBOHYDRATE COMPOSITION OF PEAK-1
FROM IM 216 CALLUS

Carbohydrate Moiety[a]	63
Sugar Residues[b]	
Gal	53
Ara	22
Glc	5
Glc A	10
Fuc	6
Rha	2
Xyl	1

[a]Weight %
[b]Mole %

TABLE VII

AMINO ACID COMPOSITION OF
PEAK-1 FROM IM 216
CALLUS

Protein Moiety[a]	10
Amino Acid[b]	
Hyp	15.9
Ser	18.5
Ala	15.2
Thr	9.5
Glx	6.1
Gly	6.6
Asx	5.2
His	1.0
Val	3.5
Lys	3.8
Pro	2.7
Leu	2.7
Ile	2.3
Phe	0.6
Tyr	0.6
Met	0.7
Arg	5.4

[a]Weight %
[b]Mole %

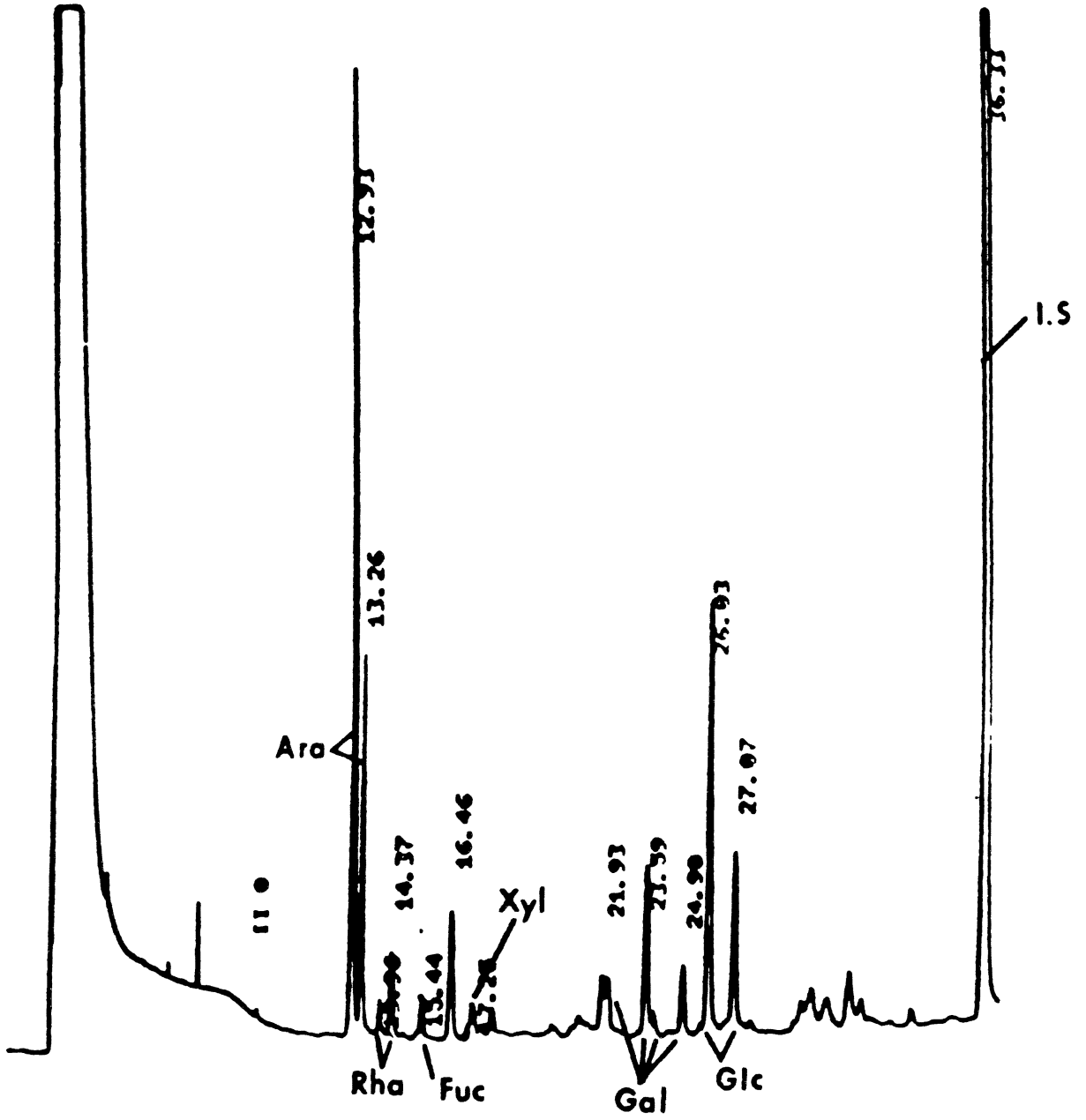


Figure 8. Gas Chromatogram of the Trimethyl Silyl Derivatives of the Methyl Glycosides of Peak-2 from Im 216 Callus. Peaks are identified as follows: Ara, arabinose, Gal, galactose; Glc, glucose;I.S, myo-inositol, internal standard.

TABLE VIII

CARBOHYDRATE COMPOSITION OF PEAK-2
FROM IM 216 CALLUS

Carbohydrate Moiety[a]	12
Sugar Residues[b]	
Gal	19
Ara	60
Glc	9
Fuc	8
Xyl	2
Rha	1

[a]Weight %
[b]Mole %

TABLE IX

AMINO ACID COMPOSITION OF
PEAK-2 FROM IM 216
CALLUS

Protein Moiety[a]	6
Amino Acid[b]	
Hyp	18.3
Ser	9.9
Ala	5.8
Thr	4.7
Glx	9.9
Gly	5.1
Asx	4.4
His	3.1
Val	4.3
Lys	10.9
Pro	8.3
Leu	5.3
Ile	3.0
Phe	0.9
Tyr	3.3
Met	0.4
Arg	2.2

[a]Weight %
[b]Mole %

The amino acid content of peak-2 of Im 216 callus showed differences from that of peak-1 of Im 216 callus. Peak-2 of Im 216 contained considerably more lysine, proline, histidine, and tyrosine. Peak-2 contained considerable less alanine, arginine, threonine, and serine. The amino acid composition of this peak differed from that of peak-2 of Ac 44 which was higher in alanine, serine, glysine, asparagine/ aspartic acid, and threonine and lower in hydroxyproline, lysine and proline. The glycoprotein portion of peak-2 from Im 216 was 67% carbohydrate and 33% protein. The carbohydrate and protein portions of peak-2 from Im 216 differed from that of peak-2 of Ac 44. The amino acid and carbohydrate composition of peak-2 of Im 216 showed characteristics of an extensin. But electrophoretic and immunochemical analysis were done for better identification.

The ratio of the mole percents of Gal/Ara in peak-1's of Im 216 upon three extractions changed from 2.3 to 2.5 and mole percents of Hyp varied by no more than 5%.The ratio of the mole percents of Gal/Ara in peak-2's of Im216 upon three extractions changed from 3.2 to 3.3 and mole percents of Hyp varied by no more than 1.6%.

Electrophoretic Analysis of Cotton HRGP's

Callus Cultures of Cotton Line Ac 44 and Im 216

For better characterization of the proteins found in peak-1 and peak-2 of the Ac 44 extract, electrophoretic analyses were performed by using 10% SDS-PAGE. One electrophoretic analysis was done using glycosylated and deglycosylated proteins from peak-1 of Ac 44. Because of the very low recovery of the glycoproteins in peak-1 after each extraction, all peak-1's obtained from five extractions were combined and used for HF 0^{o} C solvolysis. Results of the

SDS-PAGE of the glycosylated and deglycosylated proteins are shown in Figure 9.

Deglycosylated peak-1 proteins (Figure 9, lane 2) showed a major band with apparent molecular weight of 57.5 kD and some minor bands. The major band does not follow molecular weight range of deglycosylated AGP's (~100 kD). This led us to presume that AGP's in peak-1 failed to run on SDS-PAGE, as reported by other researchers. But no evidence of AGP's on the top of the gel is surprising and unexplained. The molecular weight of the major band was comparable to those obtained after SDS-PAGE by other researchers for deglycosylated extensins in melon (65), tomato (11) and potato (12), ~55 kD. SDS-PAGE electrophoresis of glycosylated peak-1 proteins (lane 1, containing ~1.2µg protein) did not show much difference from that of deglycosylated peak-1 proteins. The only difference was a band in lane 2 at 42.8 kD not detected in lane 1. So this may represent a glycoprotein in peak-1 that was deglycosylated. But this band does not follow molecular weight range of deglycosylated AGP's or extensins. No big difference in mobility of proteins in peak-1 upon deglycosylation is very unusual and it led us to assume that deglycosylation did not work. Also existence of some minor bands in both lanes raises the possibility of proteins which are not heavily glycosylated and therefore neither AGP's nor extensins. Western blot analysis was performed to obtain more information and is discusssed later. Because of the very low recovery of the proteins in the other peak's from both cultivars, no further HF analysis could be done. Also no further analysis of deglycosylated protein in peak-1 was done.

Figure 10 shows SDS-PAGE electrophoresis of the glycosylated proteins in peak-1 and peak-2 from extract 3 of Ac 44 callus. Lane 2 contained ~ 0.9µg protein from peak-1. Glycosylated peak-1 proteins showed a major band with apparent molecular weight of 57.5 kD. There were also some minor bands in

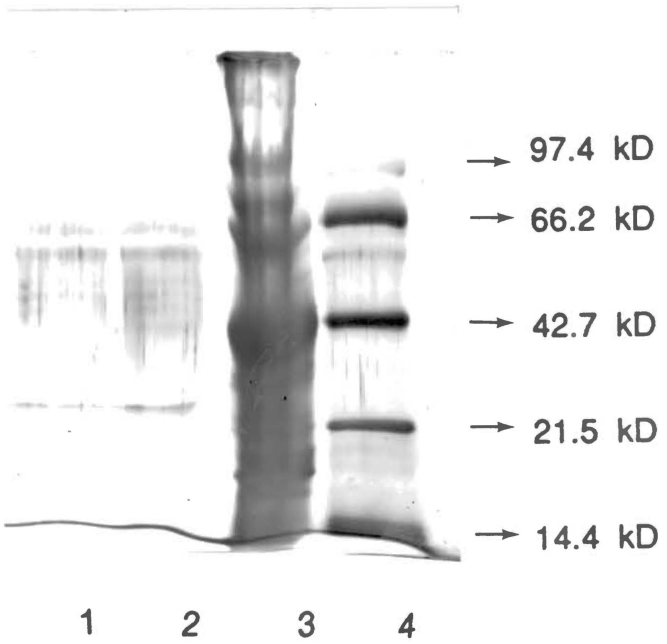


Figure 9. 10 % SDS-PAGE of the glycosylated and deglycosylated proteins from peak-1 of Ac 44 callus. Lane 1 contained 7µg glycosylated peak-1 and lane 2 contained 7µg deglycosylated peak-1. Lane 3 contained 10µg high mw markers and lane 4 contained 10µg low mw markers. Only one of the lanes of mw markers,lane 4, was identified (Phosphorylase B, 97.4 kD; BSA, 66.2 kD; ovalbumin, 42.7 kD; soybean trypsin inhibitor, 21.5 kD; lysozyme, 14.4 kD) and used for all calculations.

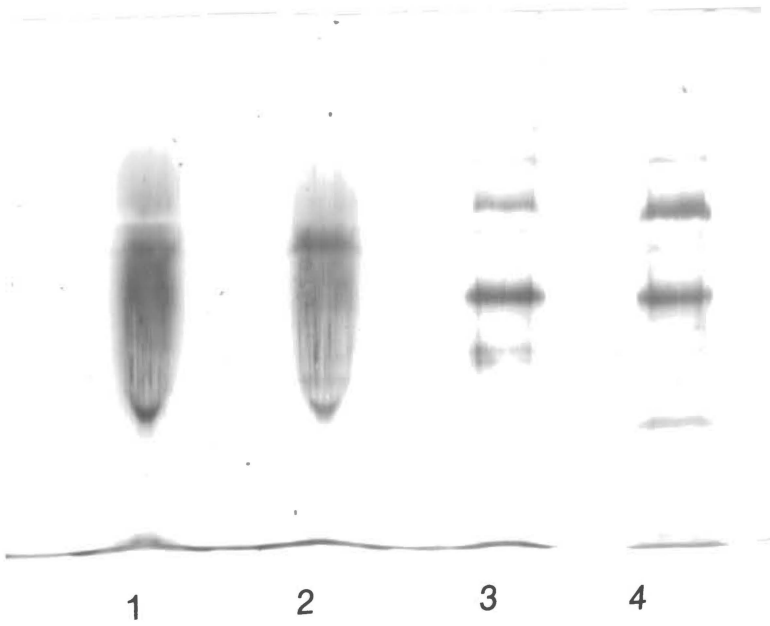


Figure 10. 10 % SDS-PAGE of the glycosylated proteins from peak-1 and peak-2 of Ac 44 callus. Lane 1 contained 7µg peak-2 and lane 2 contained 7µg from peak-1 of Ac 44 callus. Lane 3 contained 10µg high mw markers and lane 4 contained 10µg low mw markers (as described in in figure 9).

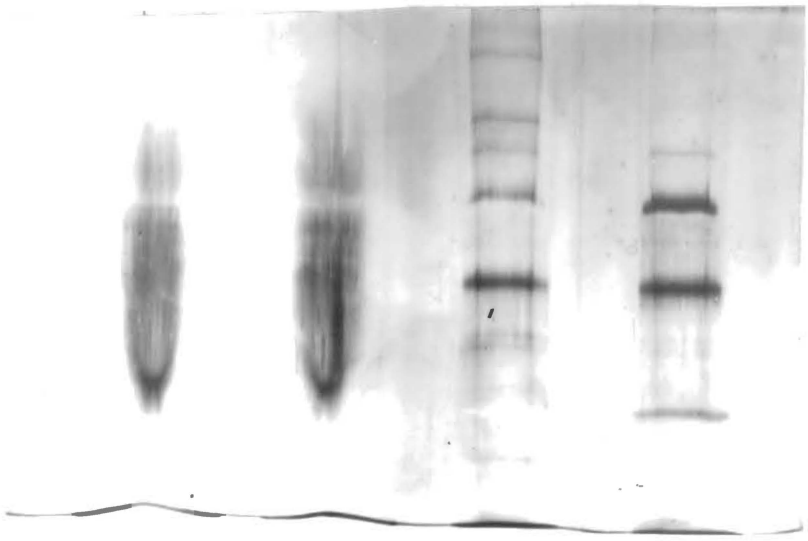
addition to this major band. Glycosylated peak-2 proteins (lane 1, containing ~0.6 μg protein) showed only one major band with apparent molecular weight of 57.5 kD. The molecular weight of the major band in both peaks does not follow the molecular weight range of either glycosylated AGP's or extensins. Besides this, as observed by other researchers, usually AGP's or extensins do not enter SDS-PAGE gels, staining on top of the gel. But, unaccountably no proteins were observed on top of the gels in our case. For better identification of the proteins in both peaks, Western blot analysis was performed and is discussed later.

Figure 11 shows SDS-PAGE electrophoresis of glycosylated proteins from peak-1 and peak-2 of extract 1 of Im 216 callus. Lane 2 contains ~0.7μg glycosylated proteins from peak-1. Glycosylated peak-1 proteins showed a major band with apparent molecular weight of 57.7 kD and some minor bands. This protein band corresponds closely in molecular weight to the major band in peak-1 of Ac 44. Lane 1 containing ~0.5μg glycosylated peak-2 proteins showed only a major band with apparent molecular weight of 57.7 kD, corresponding closely to that of the major band in peak-2 of Ac 44. As in Ac 44, the major band does not show characteristics of glycosylated AGP's or extensins. Therefore, for better identification of the proteins in both peaks, Western blot analysis was performed and is discussed later.

Western Blot Analysis

Callus Cultures of Cotton Line Ac 44 and Im 216

Western blot analysis of the reactivities of the deglycosylated and glycosylated proteins from peak-1 of Ac 44 with polyclonal antibody against the deglycosylated tomato extensin precursor, dP_1, was shown in Figure 12.



1 2 3 4

Figure 11. 10 % SDS-PAGE of the glycosylated proteins from peak-1 and peak-2 of Im 216 callus. Lane 1 contained 7µg peak-2 and lane 2 contained 7µg peak-1 of Im 216 callus. Lane 3 contained 10µg high mw markers and lane 4 contained 10µg low mw markers (as described in figure 9).

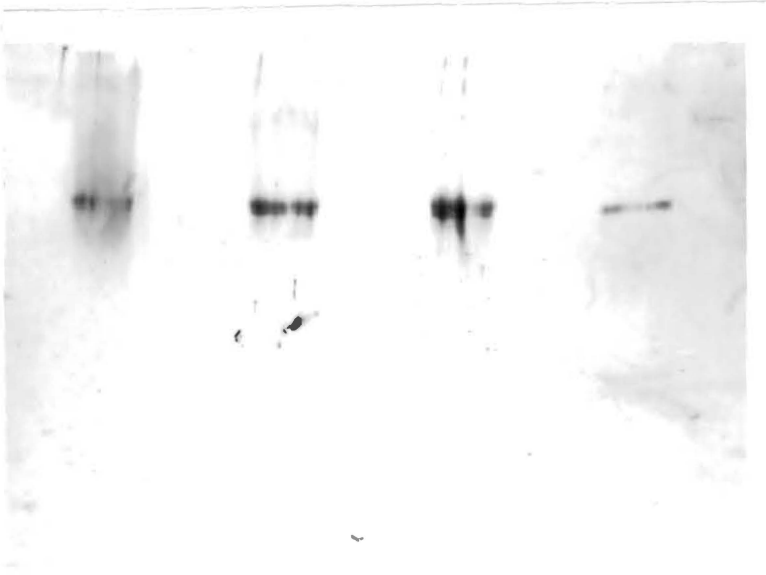
1 2 3 4 5

Figure 12. Western Blot Analysis of the reactivities of the glycosylated and deglycosylated proteins from peak-1 of Ac 44 callus. Lane 1 contained 15µg glycosylated peak-1, lane 2 contained 15µg deglycosylated peak-1, lane 3 contained 0.2µg of the dP_1 and lane 4 contained 0.05µg of the dP1, lane 5 contained 10µg low mw markers (as described in figure 9).

Deglycosylated peak-1 proteins (lane 2) showed reactivity with dP_1 antibody. Glycosylated peak-1 proteins (lane 1, containing ~2.5μg protein) also showed cross-reactivity with dP_1 antibody. The cross-reactivity of the dP_1 antibody against other HRGP's such as AGP's has been studied in melon (65), and tomato (11). In both cases dP_1 antibody did not cross react with AGP's. But in our case, there is some cross-reactivity in the low molecular weight standard lane. This brings the possibility of non-specific binding of other proteins to tomato extensin antibody, dP_1. Under this circumtance, a test with pre-immune serum is the best thing to see if the binding in the sample lanes (lane 1 and lane 2) is specific for the extensin antibody, dP_1.

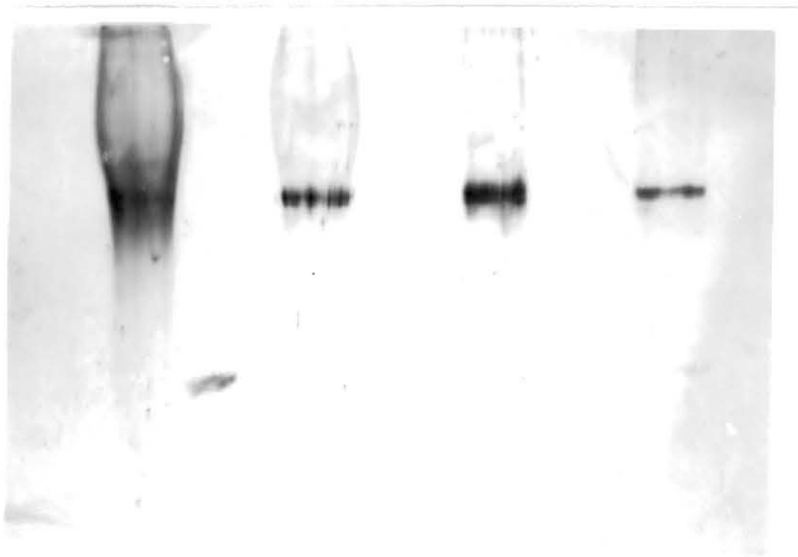
Western blot analysis of the reactivities of the glycosylated proteins from peak-1 and peak-2 of Ac 44 with polyclonal antibody against the deglycosylated tomato extensin precursor, dP_1 is shown in Figure 13. Lane 2 contained ~2μg glycosylated proteins from peak-1 and lane 1 contained ~1.4μg glycosylated proteins from peak-2. Proteins from both peak-1 and peak-2 reacted with dP_1 antibody. But also the low molecular weight standard lane showed reactivity with dP_1 antibody, indicating non-specific binding (heavier binding than figure 12). Again a pre-immune test is needed to decide if the binding in the sample lanes is specific for dP_1 or not.

Western blot analysis of the reactivities of the glycosylated proteins from peak-1 and peak-2 of Im 216 against dP_1 is shown in Figure 14. Both glycosylated proteins from peak-1 (lane 2 containing ~1.5μg protein) and peak-2 (lane 1 containing ~0.9μg protein) reacted with dP_1 antibody as did a low molecular weight standard.



1 2 3 4

Figure 13. Western Blot Analysis of the reactivities of the glycosylated proteins from peak-1 and peak-2 of Ac 44 callus. Lane 1 contained 15µg glycosylated peak-2, lane 2 contained 15µg glycosylated peak-1. Lane 3 contained 0.05µg of the dP_1,lane 4 contained 10µg low mw markers (as described in figure 9).



1 2 3 4

Figure 14. Western Blot Analysis of the reactivities of the glycosylated proteins from peak-1 and peak-2 of Im 216 callus. Lane 1 contained 15µg glycosylated peak-2, lane-2 contained 15µg glycosylated peak-1. Lane 3 contained 0.05µg of the dP_1, lane 4 contained 10µg low mw markers (as described in figure 9).

CHAPTER V

SUMMARY AND CONCLUSIONS

A carbohydrate/protein fraction has been extracted from cotton callus from susceptible and resistant cultivars of cotton. These acidic-ethanol extractions have led us to solubilize HRGP's from both cultivars, and we have accumulated evidence for the presence of both arabinogalactan proteins and extensin-like proteins.

The protein/carbohydrate moieties in the crude extract of Ac 44 and Im 216 cultivars were separated by ion-exchange chromatography on CM-Sepharose. The glycoprotein portion of peak-1 from Ac 44 callus was 75% carbohydrate, 25% protein and the glycoprotein portion of peak-1 from Im 216 callus was 86% carbohydrate and 14% protein. Comparison of carbohydrate portions of peak-1 from both Ac 44 and Im 216 callus showed that the extract from Im 216 callus was more heavily glycosylated. Both cultivars contained galactose and arabinose as major sugars. The extracts from both Ac 44 and Im 216 callus were rich in hydroxyproline, serine, alanine, and threonine. This led us to think of the existence of AGP's in peak-1 from both cultivars. But SDS-PAGE and Western blot analyses indicated the presence of some discrete bands and this is not characteristics of AGP's. SDS-PAGE analyses of peak-1's from both Ac 44 and Im 216 gave a major band with apparent molecular weights of 57.5 and 57.7 kD respectively. That weight is comparable to those obtained after SDS-PAGE by other researchers for deglycosylated extensins in other plants. However, deglycosylated and glycosylated peak-1 proteins did

not differ in their mobility in SDS-PAGE. This led us to conclude that the deglycosylation attempt was unsuccessful or that these proteins of peak-1 were not heavily glycosylated ones. Deglycosylated peak-1 of Ac 44 did show an additional band at 42.8 kD.

Western blot analysis showed reactivity of the major bands on SDS-PAGE gels with the antibody against deglycosylated tomato extensin precursor in the case of peak-1 from both Ac 44 and Im 216. This result raised the possibility that peak-1 contained extensin-like proteins. But the existence of smaller-sized minor bands in both peak-1's from the two cultivars showed us that peak-1 contained some other proteins which were precipitated in acetone and did not bind to the Sepharose column and eluted in peak-1. Existence of extensin in peak-1 would be surprising as that would show that some extensin did not bind to the column and eluted in peak-1 with other unbound material. Presumably the majority of peak-1 was AGP that failed in SDS-PAGE.

The glycoprotein portion of peak-2 from Ac 44 callus was 50% carbohydrate, 50% protein and the glycoprotein portion of peak-2 from Im 216 callus was 67% carbohydrate, 33% protein. Both peak-2's contained smaller amounts of carbohydrate than peak-1's in two cultivars. Both peak-2's contained arabinose and galactose as main sugars, but peak-2 from Im 216 was the only fraction that contained more arabinose than galactose. The amino acid composition of peak-2 from Ac 44 and Im 216 showed some differences. One of the major difference was the amount of the hydroxyproline. Peak-2 of Im 216 callus contained considerably higher amounts of hydroxyproline than peak-2 of Ac 44. Peak-2 of Im 216 also contained higher amounts of lysine, glutamine/glutamic acid and proline. Peak-2 of Ac 44 was higher in alanine, serine, glycine, asparagine/aspartic acid and threonine. SDS-PAGE of peak-2 from both cultivars gave a major band with apparent molecular weights of 57.5

and 57.7 kD. Reactivity of the proteins from peak-2's with the specific antibody suggested that peak-2 contained extensin-like proteins in both cultivars. Although peak-2 from both Ac 44 and Im 216 appeared to contain extensins, differences in the amino acid composition of the two peaks were very clear, especially higher levels of hydroxyproline in peak-2 of the resistant cultivar, Im 216, with respect to the susceptible cultivar, Ac 44. This higher level of hydroxyproline in the resistant cultivar might be related to the defense of the plant to pathogenic attack.

Total A_{280}'s for peak-1's were higher than those for peak-2's.Total A_{280}'s from peak-1 of Im 216 were usually higher than those of Ac 44, and total A_{280}'s from peak-2 of Im 216 were consistently higher than those from peak-2 of Ac 44.

In summary, even though the precise role of extensin in the plants was not clear, this research showed some differences in amino acid composition of HRGP's from Im 216 and Ac 44 callus cultures which suggest that they might be related to disease resistance. Further experiments are neeeded to be more definitive.

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