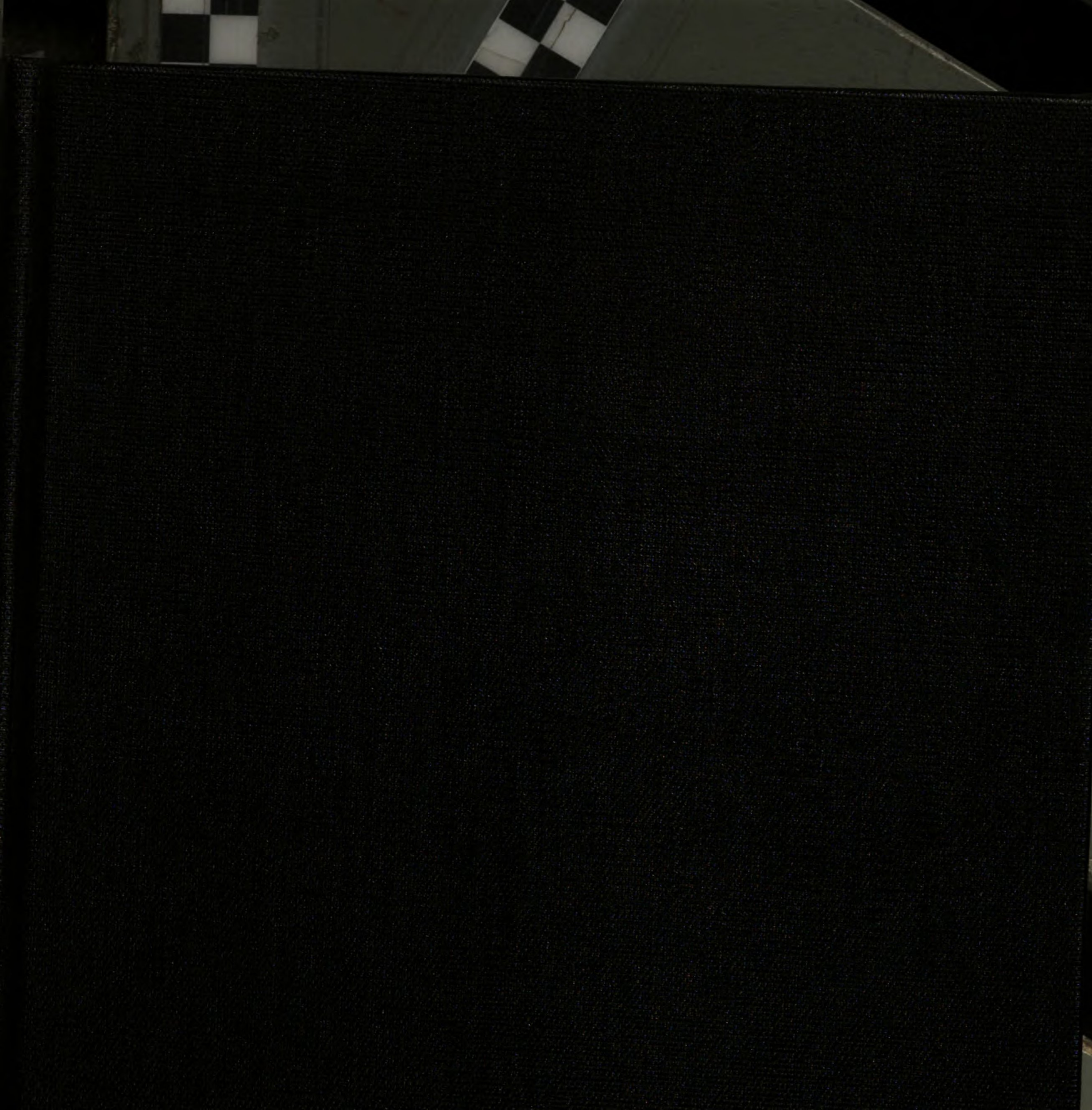

This is to certify that the

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ISOLATION AND CHARACTERIZATION OF EXTENSINS FROM THE NON-GRAMINACEOUS MONOCOT, ASPARAGUS

presented by

Lawrence L. Benbow III

has been accepted towards fulfillment
of the requirements for

Master's degree in Biochemistry

Major professor

Date May 7, 1991

ISOLATION AND CHARACTERIZATION OF EXTENSINS FROM THE NON-GRAMINACEOUS MONOCOT, ASPARAGUS

By

Lawrence L. Benbow III

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Submitted to
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ABSTRACT

ISOLATION AND CHARACTERIZATION OF EXTENSINS FROM THE NON-GRAMINACEOUS MONOCOT, ASPARAGUS

By

Lawrence L. Benbow III

The structural glycoprotein component of plant primary cell walls, extensin, has been studied in several plant species including dicots (mostly advanced angiosperms) and the graminaceous monocot, Maize. These are highly repetitive proteins characterized by high content of hydroxyproline (Hyp), serine, valine, tyrosine, and lysine. These studies have shown both similarities in, and striking differences between the graminaceous monocot and dicot cell wall glycoproteins—Hyp content, protein sequence, glycosylation patterns, and occurrence of the crosslinked amino acid (IDT).

The object of this study was to examine the glycoprotein component of a non-graminaceous monocot primary cell wall. We chose Asparagus suspension cultures as a source of material. Examination included: amino acid compositions, hydroxyproline arabinoside profiles, sugar compositions, peptide generation and sequencing, and IDT detection. Results of this work support the view of the non-graminaceous monocot cell wall extensin as a "bridge" between the dicot and graminaceous monocot extensins.

ACKNOWLEDGEMENTS

I greatfully acknowledge my fellow lab members— Renate for tissue culture assistance, Marcia for technical and experimental advice, Brad for experimental advice and general discussion, Abdol for his stable character, and Derek for providing an interesting project and an interesting working environment. I would also like to thank Rawle Hollingsworth and Pam Green for being on my committee, their constructive criticism, and accepting this thesis.

Thanks, Sebastian, for the music and beer. And I especially want to thank Ursula for her patience and thoughtfulness, and for being the most beautiful person I know.

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HF

HF

HP

HR(

Hy

IDT

kD

M_r

OP/

P1

P2

PCV

Poly

Pro

LIST OF ABBREVIATIONS

Ara	Arabinose
AGP	Arabinogalactan protein
d w	dry weight
FPLC	Fast protein liquidchromatography
HF	Anhydrous hydrogen fluoride
HHRP	Histidine hydroxyproline-rich glycoprotein
HPL	High pressure liquid chromatography
HRGP	Hydroxyproline-rich glycoprotein
Hyp	Hydroxyproline
IDT	Isodityrosine
kD	Kilodalton
M_r	Relative molecular weight
OPA	Ortho-pthaldehyde
P1	Tomato extensin precursor P1
P2	Tomato extensin precursor P2
PCV	Packed cell volume
PolyLC	PolyHYDROXYETHYL Aspartamide
Pro3	Pronase peptide 3 from asparagus wall

Lis

PR

PT

SA

SA

SE

SD

TC

TP

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List of Abbreviations....continued

PRP	Polystyrene reverse-phase
PTH	Phenylthiohydantoin
SA1	SulfoETHYL Aspartamide fraction 1
SA2	SulfoETHYL Aspartamide fraction 2
SEC	Size exclusion chromatography
SDS-PAGE	Sodium dodecylsulfate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TPCK	L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone
Tris	Tris(hydroxymethyl)aminomethane

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INTRODUCTION

I. Composition and Function of the Primary Cell Wall

Why study the cell wall? The cell wall accounts for the bulk of all biomass and is the ultimate source of food for animals and man. Individual cell walls regulate the size, shape, growth rate, and morphogenesis of specific cells and collectively determine many characteristics of the entire plant. The wall provides a structural barrier to solutes and pathogens (Preston, 1974), and in some cases the wall confers disease resistance (by physically binding and imobilizing microbes or through enzymes which can harm the invading pathogen). Finally, studies have shown that wall composition is affected by various stresses such as cell culture and heat shock. Before we can understand the biology and chemistry of plant cell growth we must understand the underlying wall structure.

There are two major components of the plant cell wall: 1) the primary cell wall, and 2) the secondary cell wall. The primary cell wall is laid down by growing, undifferentiated cells beginning with cell division and continuing until cessation of growth and deposition of secondary wall material. It is responsible for the growth rate, size, and shape of the cell, and must resist bursting under high turgor pressures—requiring both plasticity and strength. The primary wall

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is ~ 0.1 μm thick and is laid down external to the protoplast. Approximately 90% of the dicot (also maize) primary cell wall is composed of polysaccharide (cellulose, hemicellulose, and pectin). The remaining 10% is made up predominantly of glycoprotein with up to ~ 1% lipid. These are approximate values and exceptions do exist. (A survey of 6 graminaceous monocots showed the protein content to vary from 7% to 17% dw; Burke *et al.*, 1974). Speculative models of the primary cell wall have been proposed (Lamport, 1965; Keegstra et al., 1973; Lamport, 1986); some have been refuted, but none confirmed. As my report deals with the primary cell wall, unless specified otherwise, cell wall refers to this wall component.

The secondary cell wall, on the other hand, is laid down by differentiating cells internal to the primary cell wall after growth has ceased. The secondary cell wall varies greatly depending on the tissue, but generally contains more cellulose than the primary cell wall and is lacking in pectin and glycoprotein. Functions associated with the secondary cell wall include: 1) defense, 2) support, and 3) storage.

Currently the main goal in this field is elaboration on the concept of the primary cell wall as cellulose fibers embedded in a matrix of polysaccharide and glycoprotein. Most work is directed toward isolation and identification of individual components, and elucidation of their primary and three-dimensional structures. Beyond this fundamental work, additional topics of study include the linkage, distribution, biosynthesis, and insertion of these wall components. The ultimate goal is a comprehensive wall model and

an understanding of wall synthesis and cell growth. (For review see Darvill *et al.*, 1980)

The focus of this report is on the structural glycoprotein found in the wall (extensin[1]). Extensins are HRGPs (hydroxyproline-rich glycoproteins). Three HRGPs are commonly associated with the cell wall: 1) extensin, 2) arabinogalactans, and 3) solanaceous lectins. Extensin is characterized by its insolubility (covalent attachment in the wall), repetitive nature, and sugar composition. These characteristics differentiate extensin from other HRGPs.

II. Extensin Structure

A major characteristic associated with all wall-bound extensins is insolubility. This has been a hindrance in the study of extensins. Complete insolubility has been shown in detergents (Fry, 1982), salts (Stuart & Varner, 1980), cold acids and alkalies (Blashek *et al.*, 1981), phenol/acetic acid/water (Fry, 1982), chelating agents, and anhydrous HF (Mort, 1978). Despite this stumbling block, important sequence data were gathered from enzymatically cleaved peptides from the covalently bound primary cell wall glycoprotein (Figure 1) (Lamport, 1973).

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[1] Extensin is the term coined by Lamport for the hydroxyproline-rich glycoprotein component of cell walls. This name emphasizes its postulated role in cell extension (Lamport, 1963).

A. Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-1/2IDT-Tyr-1/2IDT-Lys

B. Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Lys

C. Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys

D. Ser-Hyp-Hyp-Hyp-Hyp-Lys

E. Ser-Hyp-Hyp-Hyp-Hyp-Val-1/2IDT-Lys-1/2IDT-Lys

Figure 1. Amino acid sequences of five HRGP glycopeptides solublized from tomato cell walls (from Lamport, 1977).

Later, it was found that HRGPs could be eluted from walls prior to insolubilization (Brysk & Chrispeels, 1971; Stuart & Varner, 1980; Smith *et al.*, 1984). Brysk & Chrispeels (1971) demonstrated, unconvincingly, that extensin precursors could be extracted from carrot walls as a salt-elutable pool. Lamport initially dismissed these results as the kinetics data were inconclusive, the amino acid and carbohydrate compositions were unlike those of extensin wall peptides, and the experiments were not repeatable using sycamore suspension cultures (Pope, 1977). Smith *et al.* (1984) showed tomato HRGPs to be monomers of the extensin network. Examination of wall peptides and elutable monomers showed extensins to be rich in hydroxyproline, serine, valine, tyrosine, lysine, and occasionally histidine. As previously mentioned, extensins are highly repetitive molecules, most containing the pentapeptide, Ser-Hyp-Hyp-Hyp-Hyp. Until work was performed with sugar beet (a chenopod, primitive dicot) and maize, this pentapeptide motif (based on advanced

herbaceous dicot sequences) was considered to be the diagnostic feature of extensins. Work on these graminaceous and primitive dicot species, however, has revealed insertions and deletions within the decameric tomato P1-type extensin motif, Ser-Hyp$_4$-Thr-Hyp-Val-Tyr-Lys (Li *et al.*, 1990; Kieliszewski & Lamport, 1988) (Figure 2).

Beet:	Ser	Hyp	Hyp	[X]	Hyp	Hyp		Thr	Hyp	Val	Tyr	Lys
Tomato:	Ser	Hyp	Hyp		Hyp	Hyp	[Y]	Thr	Hyp	Val	Tyr	Lys
Tomato:	Ser	Hyp	Hyp		Hyp	Hyp		Thr	Hyp	Val	Tyr	Lys
Maize:	Ser	Hyp	Lys		Pro	Hyp		Thr	Pro	----------		Lys
Maize:	Ser	Hyp	Lys		Pro	Hyp	[Z]	Thr	Pro	----------		Lys

Insertion/Deletion sequences:

[X] = Val His Glu Tyr Pro

[Y] = Val Lys Pro Tyr His Pro

[Z] = Ala Thr Lys Pro Pro

Figure 2. Decameric motif of P1-Type extensins (from Kieliszewski & Lamport, 1988)

Carbohydrate comprises 40-65% of extensin's weight. The sugar portion of these glycoproteins is predominantly composed of arabinose and galactose in O-glycosyl linkage to hydroxyproline

(Lamport, 1967) and serine respectively. Galactose is found in the form of a single residue α-O-linked to serine (Allen *et al.*, 1988; Lamport *et al.* 1973). One to four arabinose residues are O-linked to hydroxyproline (Figure 3) (Lamport & Miller, 1971; Mazau & Esquerre-Tugaye, 1986) with the following configuration for the tetra-arabinoside:

α-L-Araf(1-3)-β-L-Araf(1-2)-β-L-Araf(1-2)-β-L-Araf(1-4)-Hyp

(Akiyama *et al.*, 1980). Configurations of other arabinosides have not yet been determined.

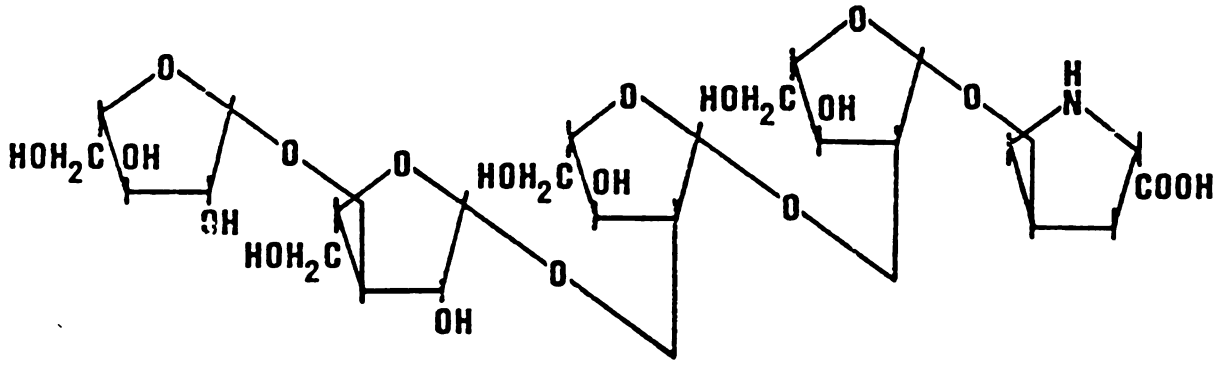

Figure 3. Hydroxyproline tetra-arabinoside

A third characteristic of some extensins is the presence of the unique amino acid derivative, isodityrosine (IDT) (Figure 4). This component was first described as "an unusual modified tyrosine residue" in two tryptic peptides from tomato cell walls (Figure 1, A & E). Later, Stephen Fry characterized IDT from wall hydrolysates as

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two tyrosine residues bridged by a diphenyl ether linkage (Fry, 1982). This led to speculation that IDT functions as a crosslink within the extensin network. Further investigation (Epstein & Lamport, 1984) has demonstrated that IDT is the unknown tyrosine derivative first described by Lamport, and that contrary to earlier expectations and conventional thought it forms an <u>intramolecular</u> crosslink with no direct evidence of an <u>intermolecular</u> IDT crosslink. Indirect evidence suggesting the existence of intermolecular IDT crosslinks includes: 1) tomato cell walls contain a lower Hyp:IDT ratio (i.e. more IDT) than tomato dP2 extensin (15:1 vs 20:1) suggesting that more crosslinks occur after insertion of the monomer into the wall (Smith *et al.*, 1984; Smith *et al.*, 1985) and 2) insolubilization of carrot extensin into the wall occurred with an increase of IDT at the expense of tyrosine (Cooper & Varner, 1983).

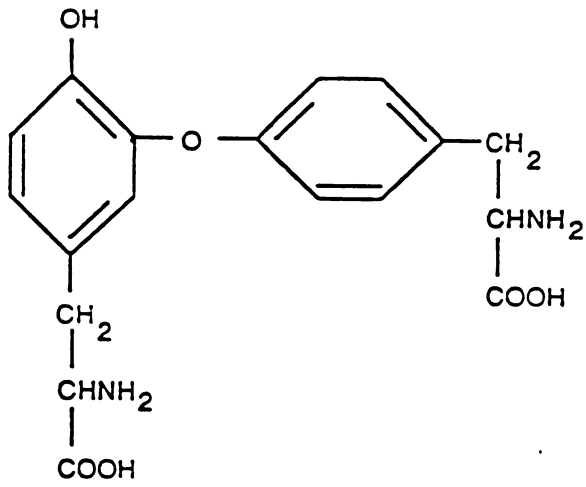

Figure 4. Isodityrosine (IDT)

III. Extensin Function

The characteristics of extensin (periodicity, high hydroxyproline content, extracellular location, and apparent lack of enzymatic activity) suggest a structural role in the cell wall (Lamport, 1970). These characteristics are shared with collagen, the major extracellular matrix protein of the animal kingdom. An inverse correlation between HRGP levels and cell elongation (Cleland & Karlsnes, 1967; Winter *et al.*, 1971; Bailey & Kauss, 1974; Sadava & Chrispeels, 1973; Klis & Eeltink, 1979), implies a role in cell growth. Additional evidence indicates that expression of extensins may be altered by forms of stress including: infection (Esquerre-Tugaye & Lamport, 1979; Esquerre-Tugaye & Mazau, 1974; Showalter *et al.*, 1985), elicitors (Roby *et al.*, 1985; Tierny *et al.*, 1988), and wounding (Showalter & Rumeau, 1990).

As a structural protein, extensin's function is intimately associated with its structure as both a monomer and a network. Cell wall models must take into consideration interaction of extensin monomers with each other and with other wall components such as pectins and carbohydrates. Several models have been proposed. Albersheim proposed a model of the wall as one huge macromolecule (Keegstra *et al.*, 1973). This model assumed that glycosidic bonds were responsible for holding the entire wall together (including the extensin). However, it has been shown that extensin is not released even after treatment with anhydrous HF (Mort & Lamport, 1977) which completely solublizes the carbohydrate. The "warp-weft" model proposed by Lamport (Lamport, 1986) postulates that two

independent, interpenetrating networks make up the bulk of the wall. Cellulose microfibrils compose the periclinal "warp" which is interpenetrated by the transmural extensin "weft". This model proposed that IDT crosslinking of the extensin network couples the cellulose microfibrils into a rigid, defined structure.

IV. Extensin in the Graminaceous Monocot, Maize

Graminaceous monocot walls are notably hydroxyproline-poor (Lamport, 1965; Burke *et al.*, 1973). For this reason there has been little study of monocot extensin. Recently, structural studies of extensin isolated from maize confirmed that there is 10 to 20 fold less hydroxyproline in the graminaceous wall than in the dicot wall (Kieliszewski & Lamport 1987); however, two Hyp-rich fractions were obtained from salt-eluted cell walls. One of these fractions contained a threonine-rich (25.3 mole%) HRGP (THRGP). A second histidine-rich (13.3 mole%), HHRGP, fraction was also isolated (Kieliszewski & Lamport, 1986). While unique, the THRGP is homologous with tomato P1 extensin through both sequence analysis and antibody studies (Kieliszewski & Lamport, 1986). The HHRGP is currently under investigation.

Another difference between the graminaceous monocot salt-elutable HRGPs and the dicot extensins is the sugar composition. Tomato P1 and P2 extensins contain ~ 60% (w/w) sugar (Smith, 1985), whereas the maize THRGP contains 27% to 33% (w/w) sugar and the HHRGP contains ~ 60% (w/w) sugar (Kieliszewski & Lamport,

1987). Both the tomato extensins and maize HRGPs contain predominantly arabinose and galactose. However, the tomato extensins contain ~ 90% arabinose and ~ 7% galactose (total sugar = 100%), whereas the THRGP contains 100% arabinose and the HHRGP contains ~ 63% arabinose and ~ 37% galactose. In addition, the hydroxyproline-arabinoside profiles of both salt-elutable maize extensins and the covalently bound wall are different from tomato profiles. Advanced dicot profiles show predominantly tetra- and tri-arabinosides while there is a greater degree of free Hyp in the graminaceous extensins (Lamport, 1965; Kieliszewski, 1989). The lesser extent of substituted Hyp residues in the graminaceous monocot HRGPs is paralleled by a similar pattern in the more primitive dicot, sugar beet (Li *et al.*, 1990), and a gymnosperm, Douglas Fir (Kieliszewski, to be submitted). Monocots are widely believed to have diverged early from the dicot lineage. Similarity between the graminaceous monocot and primitive dicot cell walls (amino acid composition of the covalently bound wall protein and Hyp-arab profiles of elutable HRGPs) supports this hypothesis.

Investigations (Lamport, 1965; Burke *et al.*, 1974; Kieliszewski & Lamport, 1988) show that there is another (glyco)protein in Hyp-poor walls from several graminaceous monocots, sugar beet (Li *et al.*, 1990), and Douglas Fir (Kieliszewski, to be submitted). Although hydroxyproline levels in these species are low, these walls contain as much as 20% protein (dicot walls generally contain ~ 10% protein). There has been no extensive study of this protein component, but amino acid analyses of walls from these species show high amounts

of the hydrophobic amino acids (glycine, alanine, valine, leucine, and isoleucine), and asparagine/aspartic acid and glutamine/glutamic acid (amino acid analysis of acid hydrolysates does not differentiate these two sets of related amino acids) (Kieliszewski, 1989) (Table 18, Appendix). In fact, the only amino acids found in similar amounts in these other species' wall fractions and dicot wall fractions are proline, threonine, valine, and methionine (Table 2). Since extensin is clearly not the major protein component of these other cell walls (Kieliszewski & Lamport, 1988), a model of these walls must take into account this Hyp-poor component.

A fourth major difference between maize and the advanced dicot walls is the lack of IDT which leads to the question of a proposed crosslink in this Hyp-poor (glyco)protein network. In the acid hydrolysate of maize HF-insoluble cell wall, Kieliszewski (1989) found an unknown UV-absorbing peak which eluted between tyrosine and IDT. She suggested the possibility of another "tyrosine derivative" which may serve to crosslink the Hyp-poor (glyco)protein of the graminaceous wall.

Table 1. Amino Acid Compositions of Asparagus, Maize[b], and Tomato[c] Cell Walls

Amino Acid [a]	Maize Wall	Tomato Wall
Hyp	1.1	28.5
Asp	10.4	4.0
Thr	5.1	4.6
Ser	6.9	14.2
Glu	9.3	2.8
Pro	3.7	3.9
Gly	10.7	3.3
Ala	10.6	3.2
Val	6.4	7.0
Met	1.7	0.3
Ile	4.2	1.8
Leu	10.3	2.5
Tyr	1.9	6.3
Phe	4.0	1.3
Lys	6.2	10.5
His	2.1	2.7
Arg	4.7	1.2

[a] Represented as Mole %
[b] from Kielisewski, 1989
[c] from Smith *et al.*, 1984

V. Speculation on the Monocot-Dicot Divergence

Monocots and dicots are generally accepted to have a common angiosperm origin. The divergence of monocots and dicots is obscure due to a lack of fossil data from progenitor angiosperms. Two theories exist for this lack of fossil data (Wolfe *et al.*, 1989): 1) the original habitats were refractory to fossilization, and/or 2) angiosperms first appeared in the early Cretaceous period (~ 140 million years ago) and radiated explosively. Table 2 shows the major

criterion for distinguishing monocot vs dicot. Monocot characteristics vary greatly and some characteristics carry over the monocot-dicot division. Wolfe *et al.* (1989) studied the mutation rate of chloroplast DNA and determined the time of the monocot-dicot split to be ~ 200 million years ago. Martin *et al.* (1989), studied cDNA sequences from glyceraldehyde-3-phosphate dehydrogenase (GADPH) genes from plants, animals, and yeast, and suggested the monocot-dicot split to have occurred more than 300 million years ago. These studies focused solely on graminaceous monocots (maize, rice, and wheat); however, the Graminae are a distinct, very specialized group of monocots, and we suggest that monocots as a whole should not be judged solely on graminaceous data.

Evidence from the primary cell wall of the graminaceous monocots supports this view. The primary cell walls of graminaceous monocots are known to have low amounts of pectin (~ 3% compared with ~ 35% in dicots) (McNeil *et al.*, 1984), but how widespread this trend is among monocots was unknown. Jarvis *et al.* (1988) showed that low levels of pectin were common in four (the Graminae being one) of thirty-three monocot species surveyed. Some species related to these four had intermediate pectin contents, while other species had high pectin contents comparable with dicots. I asked the question of whether the non-graminaceous monocot cell wall HRGP(s) is more closely related to HRGPs from the Graminae or from dicots. For comparison, a non-graminaceous monocot with a relatively high amount of pectin in its wall was the obvious choice, hence our

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selection of asparagus. (A crude estimation of pectin in the asparagus primary cell wall indicated a content of roughly 20% (dw).

Table 2. Main Differences Between Monocots and Dicots

Characteristic	Dicots	Monocots
Flower Parts	In fours or fives (usually)	In threes (usually)
Pollen	Basically tricolpate (having three furrows or pores)	Basically monocolpate (having one furrow or pore)
Cotyledons	Two	One
Leaf venation	Usually netlike	Usually parallel
Primary vascular bundles in stem	In a ring	Scattered
True secondary growth, with vascular cambium	Commonly present	Absent

from Biology of Plants, 3rd Ed. (1981) Raven, Evert, and Curtis

VI. Goals and Approach

Work on maize and speculation on the monocot-dicot divergence presented us with two obvious questions: "What are the characteristics of the non-graminaceous monocot cell wall HRGP(s)?", and "How are the cell wall HRGPs of the two monocot groups

(graminaceous and non-graminaceous) and the dicots related?" In particular I wanted to determine whether the non-graminaceous monocot (asparagus) cell wall HRGPs more closely resemble the graminaceous (maize) or the dicot (tomato) cell wall HRGPs. Until now there has been no examination of non-graminaceous cell wall HRGPs. My goal was to determine the major characteristics through a biochemical approach. The biochemical approach to evolution is a relatively recent endeavor, yet the process has been ratified by molecular evidence. An excellent example of the parallel between "naturalist" and biochemical phylogenetic trees is the evolution of cytochrome c (Florkin, 1971). This approach provides additional criteria for evolutionary comparisons. Because of the direct involvement of the primary cell wall in growth and morphology, this is an excellent place to look for evolutionary change.

Two of the non-graminaceous monocots surveyed by Jarvis *et al.* (1988) were *Chlorophytum capense* (Asphodelaceae) and *Tulipa gesneriana* (tulip; Liliaceae). These two monocots both belong to the Superorder Liliiflorae—*C. capense* belongs to the Order Asparagales. Jarvis *et al.* showed these two monocots contain pectin in amounts comparable with dicots. Therefore, these monocots were favorable for comparison of wall HRGPs with graminaceous monocots and dicots. I obtained a suspension culture of asparagus (Superorder Liliiflorae, Order Asparagales). I utilized asparagus suspension cultures as a source of material (accumulated evidence supports similarity between cultured material and intact plant tissues; Darvill *et al.*, 1980) for examination of amino acid compositions, neutral

sugar compositions, and Hyp-arab profiles from both covalently bound wall fractions and salt-elutable HRGPs. I assayed for IDT in the covalently bound wall. And, I obtained sequence data from two individual Asparagus HRGPs, i.e. the first non-graminaceous monocot HRGPs to be isolated. These results provide characterization of extensins from the heretofore unrepresented non-graminaceous monocot.

MATERIALS AND METHODS

I. Methods for the Isolation and Purification of SA1 and SA2

A. Suspension Cultures

Suspension cultures of asparagus were started by Dr. Renate Desachs (cv. Jersey Giant; obtained from Dr. Ken Sink, MSU Dept. of Horticulture). I propagated these cultures in 1 liter erlenmeyer flasks containing approximately 550 ml of Murashige and Skoog medium (Murashige & Skoog, 1962) (+ 2mg/l 2,4-D). The flasks were shaken at 120 rpm on a gyrotory shaker at room temperature (27° C) under constant fluorescent lighting. Flasks were subcultured every 10 days to an initial packed cell volume of 5%. Cells were eluted between 10 to 12 days (PCV ~ 18%).

B. Intact Cell Elution and TCA Precipitation

I prepared crude HRGP by bulk elution with 100 mM $AlCl_3$. The culture medium was filtered from the cells through a coarse scintered glass funnel using vacuum. After rapidly washing with 2 to 3 volumes of distilled H_2O, elution of the cells was performed with 2 volumes of 100 mM $AlCl_3$. The $AlCl_3$ solution containing eluted HRGP was removed quickly by suction. The volume of the eluate was reduced to 100 ml at 30° C using a Buchi Rotovapor - R. TCA was added to a final concentration of 10% and the eluate was placed at 4°

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C for 18 hr. The precipitated protein was spun down (12,000 g, 60 min, SS-34 rotor) yielding a hydroxyproline-rich supernatant. Dialysis of the supernatant for 72 hr was followed by lyophilization and overnight desiccation (over P_2O_5). This 10% TCA soluble fraction has been designated "Crude HRGP" .

C. Superose-6 Gel Filtration

"Crude HRGP" was dissolved (20 mg/ml) in distilled H_2O and spun (10 min x max speed in a microfuge). I loaded 30 mg of this sample onto a Preparative Superose-6 cloumn (1.7 x 48 cm; 30 μm particle; Superose buffer = 0.2 M phosphate, pH 7.0). The column was eluted at a flow rate of 1 ml/min and the eluate was monitored at 220 nm. The Hyp content of each of the major fractions was determined by manual Hyp analysis (%Hyp w/w) and amino acid analysis (mole% Hyp). Each Hyp-containing fraction (fraction 2 and fraction 3&4) was pooled (~ 10 runs), concentrated by rotary evaporation, dialyzed 72 hr (4° C; against dH_2O), freeze-dried, and desiccated overnight (over P_2O_5).

Analytical Superose-6 gel filtration was used to check the quality of the bulk elutions. "Crude eluate" was dissolved (10 mg/ml) in distilled H_2O and 20 ul (200 μg) was loaded onto the column (1.0 x 30 cm; 14 μm particle). This analysis gave a general idea of the amount of extensin monomer in the crude eluates (mg crude monomer/mg crude).

D. PolySULFOETHYL Aspartamide Cation Exchange Chromatography

I dissolved Superose-6 fraction 3&4 (10 mg/ml) in buffer A (buffer A = 10 mM phosphate (9.2 g/l NaH_2PO_4 + 18.9 g/l Na_2HPO_4) (pH 3.0), 10% MeCN; buffer B = 10 mM $NaPO_4$ [pH 3.0], 10% MeCN, + 1 N NaCl) and loaded 5 mg maximum onto a PolySULFOETHYL Aspartamide column (9.4 x 200mm; 5 µm particle). The material was eluted with a gradient of 0 to 450 mM NaCl at a flow rate of 1.0 ml/min. The spectrum (200 - 600 nm) of the eluate was monitored with a diode array detector.

E. Microscale Succinylation

Deglycosylated extensin precursor (dSA1 or dSA2) was dissolved 1 mg/ml in pH 7.5 Phosphate Buffer (0.4 M phosphate). 100 µl (100 µg extensin) was transferred to a 1 ml microvial. 800 µg of solid succinic anhydride was added and the contents mixed well. The reaction was allowed to proceed for 30 minutes. 80 µl was loaded onto the analytical Superose-6 column.

F. Preparation of Cell Walls

Cells were frozen in liquid N_2 followed by 30 sec treatment in a Tekmar A-10 analytical mill. The powdered cells were immediately transferred to ~ 10 volumes of 0.5 N NaCl (cells were checked microscopically to check for complete breakage). Cytoplasmic debris was washed from the wall. Walls were suspended in 5 volumes of 1 M NaCl. The wall fragments were spun down leaving the cytoplasmic contaminants in the supernatant. The supernatant was decanted.

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Subsequently 5 washes with 2 volumes of dH_2O were performed in the same manner. After the final wash, the walls were resuspended in dH_2O (~ 30% suspension) then the fragments (clean as judged microscopically) were lyophilized and desiccated over P_2O_5. Alternatively, a 20% suspension of cells was ruptured by sonication on ice until the cells were completely broken (3 min bursts x max power, Braunsonic 1510). The walls were washed once in 0.5 N NaCl as described above (~ 5 vols.) and filtered through two layers of miracloth followed by 1 N NaCl (2 x 2 vols.) and water (5 x 2 vols.) washes. The walls were lyophilized and desiccated (over P_2O_5 overnight).

G. Cell Wall Pectin Estimation

25 mg of primary wall was heated at 120 °C for 1 hr in 50 mM KCHOOH buffer (pH 5.0) with occasional stirring. After cooling, 0.5 ml of 4 N K_2CO_3/0.3 M EDTA was added, and the mixture was stirred for 30 min. at 23 °C. The mixture was filtered through a scintered glass funnel by aspiration and washed with 2 x 1 ml dH_2O. The residue was recovered and desiccated overnight over P_2O_5. The desiccated residue was then weighed.

II. Methods for IDT Standard Preparation

A. Crude IDT Isolation

10 grams of tomato cell walls were refluxed in 500 mL of 6N HCl for 24 hr, then washed with distilled H_2O and filtered through

Whatman #1 paper until the pH was greater than 2.5. An initial clean up performed on Dowex-50 (H+) was followed by elution with 2 N NH_4OH to yield total amino acids. IDT was purified from total amino acids by chromatography on Aminex AG-50 X 4 (0.5 x 5 in). Elution was effected by a pyridine acetate gradient from pH 2.7 to 5.0. (Recovery of crude IDT was 30 mg.)

B. IDT Recrystallization

Further purification by recrystallization from hot water (x1) yielded distinctive needle-like crystals. The purified IDT was lyophilized and desiccated overnight (over P_2O_5). (The final yield was 1.4 mg.)

III. Methods for Determination of Composition and Purity

A. Amino Acid Analysis

Amino acid analysis employed a Pickering High Speed Sodium Cation Exchange column (3 x 150 mm) with buffers A, B, and C (A = Na^+ eluent, pH 3.15; B = Na^+ Eluent, pH 7.4, [Na^+] = 1.0 N; C = Na^+ Regenerant, [Na+] = 2.0 N). Post-column derivatization consisted of NaOCl oxidation followed by OPA coupling (allowing detection of secondary amino acids, Hyp and Pro) (Yokotsuka & Kushida, 1983). The reductant incorporated was 22.7 mM N,N-dimethyl-β-mercaptoethylamine HCl (Frister *et al.*, 1988). The eluate was monitored with a Gilson 3301 Spectra/Glo fluorometer (excitation at

360 nm, emission at 455 nm). Data were gathered via P.E. Nelson Turbochrom II software run on a Compaq 386.

B. Sugar Analysis

Neutral sugars were analyzed as their alditol acetates (Albersheim et al., 1967) on a Perkin-Elmer 910 Gas Chromatograph using a 2 mm i.d. x 6 ft PEGS 224 column (120-140 mesh) programmed from 130° to 180° C at 4° C/min. Data were recorded via P.E. Nelson Turbochrom II software run on a Compaq 386.

C. Hydroxyproline Assay

Samples were hydrolyzed (6 N HCl, 110° C, 18 hr), then the Hyp was measured by Kivirikko's method (Kivirikko & Liesma, 1959). This reaction involved hypobromite oxidation followed by coupling with Erlich's reagent (50 g of p-dimethylaminobenzaldehyde/1 l n-propanol). Quantitation was performed by monitoring at 560 nm.

D. Hydroxyproline Arabinoside Profiles

The first step in Hyp-Arab determination (Lamport, 1967) was alkaline hydrolysis (0.2 N $Ba(OH)_2$, 110° C, 18 hr) of the sample followed by neutralization with concentrated H_2SO_4, centrifugation (10 min x max speed in microfuge), and lyophilization of the supernatant. The sample was redissolved in 200 µl of distilled H_2O and 200 to 800 µg of Hyp was loaded onto a Technicon Chromobeads C (H+ form) column (0.6 x 60 cm). The Hyp-arabs were eluted with a linear 0 to 0.5 N HCl gradient and detected after automated post-column hydroxyproline assay (see Hydroxyproline Assay above).

E. Anhydrous HF Deglycosylation

Asparagus cell wall preparations were deglycosylated 2 hr at 0° C using 1 ml anhydrous HF (10% (v/v) dry methanol) per 20 mg material (Sanger & Lamport, 1983). The reaction was quenched by diluting 10 fold in ice cold distilled H_2O. The 10% HF preparation was spun down (15 min x max in clinical centrifuge), resuspended in distilled H_2O and respun (the preparation was washed with 10 volumes of water). The deglycosylated (HF insoluble) wall was lyophilized and desiccated overnight (over P_2O_5).

HRGPs were deglycosylated by the same procedure as the wall preparations, except without removal of the supernatant and washing of the insoluble pellet. The 10% HF preparation was dialyzed for 72 hr, then lyophilized and desiccated overnight (over P_2O_5).

F. Cell Wall IDT Estimation

The cell wall (HF insoluble) IDT content was estimated after acid hydrolysis (6 N HCl, 110° C, 18 hr) by reverse-phase HPLC using a Hamilton PRP-1 column (solvent A = 0.1% TFA, solvent B = 0.1% TFA/80% MeCN). The column was eluted with a gradient of 0-30% B in 30 min. As standards I ran 10 µg of L-tyrosine and 9.4 µg of IDT (previously prepared). L-tyrosine and IDT were previously seen to elute from this gradient at 15.1 min. and 27.8 min. respectively. The UV absorbance was recorded at 220 nm and 280 nm via diode array detection (Hewlett Packard 1040A).

G. SDS-PAGE Electrophoresis

The purity of the deglycosylated SA1 and deglycosylated SA2 HRGPs was assessed via 12% SDS-polyacrylamide gel electrophoresis (Laemmli & Favre, 1973). 10 μg of each protein was prepared in 10 ul sample buffer (10% glycerol; 62.5 mM Tris·Base; 0.01% Bromophenol Blue) and loaded onto a mini-gel (height x width x thickness = 6 cm x 8 cm x 0.75 mm). The proteins were stained with Coomassie Brilliant Blue R-250 in water:ethanol:acetic acid (25:25:10, v/v). Molecular weight standards were: ovalbumin = 42.1 kD, carbonic anhydrase = 30.4 kD, α-lactoglobulin = 18.2 kD, lysozyme = 13.7 kD, bovine trypsin inhibitor = 8.1 kD, and insulins A & B = 2.7 kD.

IV. Methods for Peptide Generation, Separation, and Sequencing

A. Tryptic Digestion

2 to 7 mg of deglycosylated SA1 or SA2 (1 - 4 mg/ml, 10 mM CaCl2) were denatured (boiled for 5 min and cooled on ice). The samples were brought to pH 8 with NaOH and TPCK-trypsin (Sigma,Type XIII) was added (enzyme:substrate ratio was 1:100). The trypsinolysis was monitored at pH 8 in a pH Stat (Radiometer - Copenhagen, Denmark).

B. HPLC Peptide Mapping

After spinning down the tryptic or pronase digests (10 min x max speed in microfuge), the supernatant was loaded onto a Hamilton PRP-1 (4.1 mm x 150 mm) and the peptides eluted via reverse-phase HPLC. The solvents in this system were: A = 0.1% TFA, B = 0.1% TFA/80% MeCN.

Gradient for dSA1 peptide map:

time	flow	%A	%B
init	0.5	100	0
1.0	0.5	100	0
20.0	0.5	80	20
47.0	0.5	67	33

Gradient for dSA2 peptide map:

time	flow	%A	%B
init	0.5	100	0
1.0	0.5	100	0
15.0	0.5	94	6

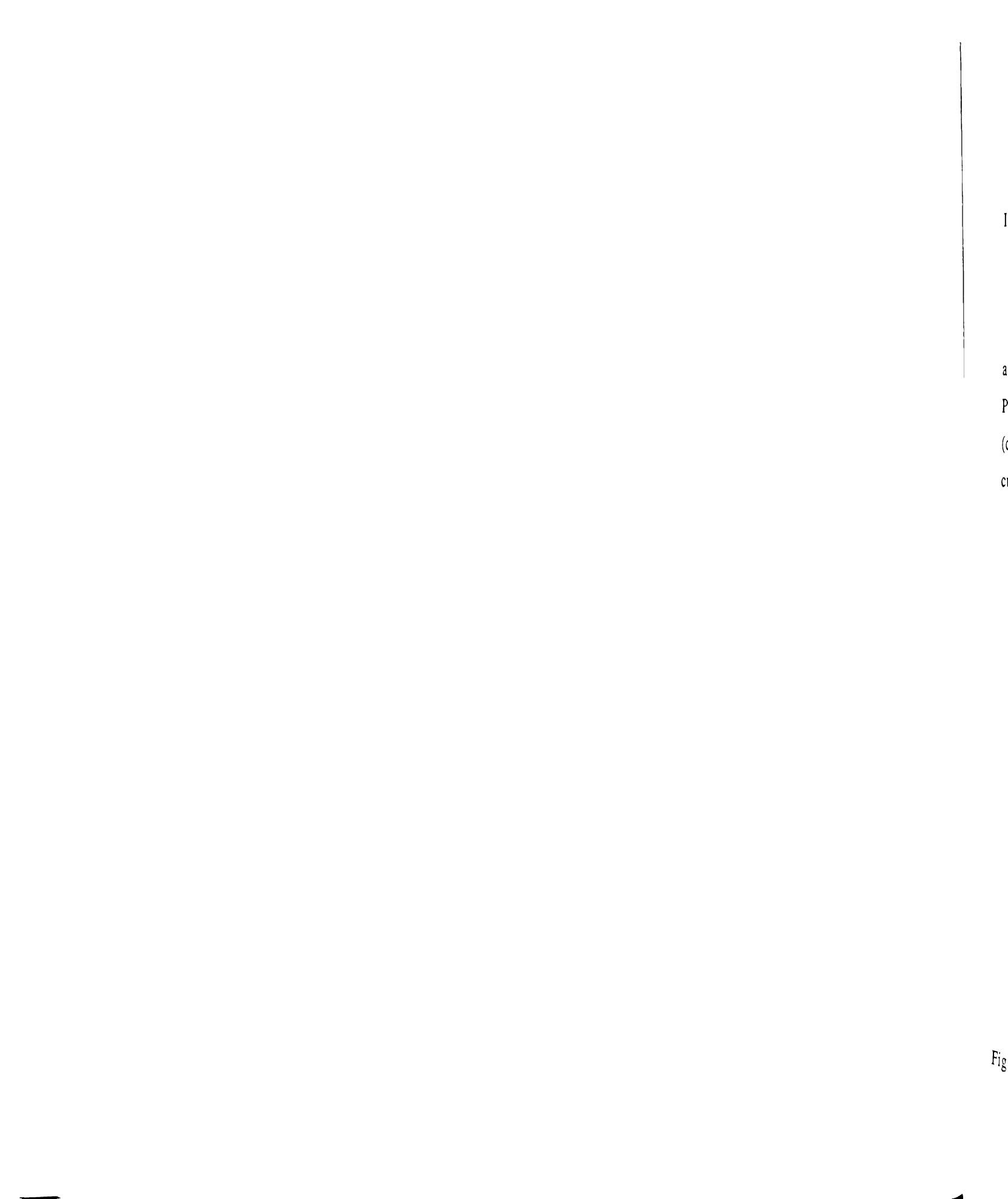
C. Peptide Purification

The major peptides obtained from the initial peptide maps of dSA1 and dSA2 could not be totally purified via Hamilton PRP-1 reverse-phase chromatography. A second column, PolyHYDROXY-ETHYL Aspartamide (PolyLC; 9.4 mm I.D. x 200 mm), took advantage of size exclusion chromatography (SEC) to effect fractionation of

peptides primarily on the basis of size. This column was eluted isocratically with a Na_2SO_4/KH_2PO_4/MeCN buffer (0.2 M Na2SO4; 5 mM KH2PO4; 25% MeCN; pH 3).

D. Automated Edman Degradation

Joe Leykam and Melanie Corlew (Michigan State University Macromolecular Facility) sequenced SA1 M6 and SA2 M4 peptides via Edman Degradation (Edman, 1970) on a 477A Applied Biosystems, Inc. gas phase sequencer.



RESULTS

I. Isolation of Asparagus HRGPs

A. $AlCl_3$ Elution of Intact Cells and Growth Curves

Growth curves showed the % packed cell volume (% PCV) of asparagus suspension cultures to plateau at a value of 18% to 20% PCV. I found no difference in the amount of TCA-soluble Crude HRGP (ca. 60 - 120 mg crude/ kg cells fw) based on the culture age. When cultures were inoculated at an initial 10% PCV, the optimum time of

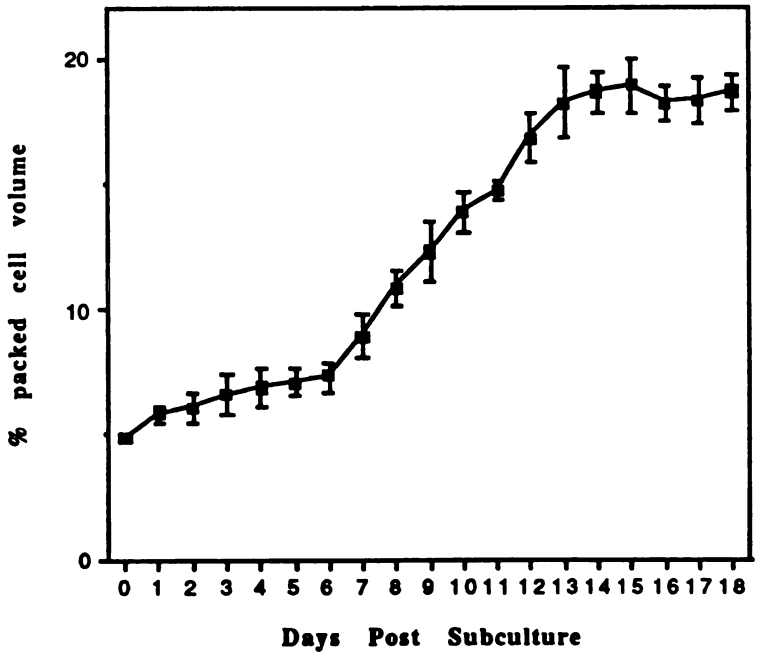


Figure 5. Asparagus suspension culture growth curves

harvest was 11 days (i.e. 18% PCV). Asparagus cell cultures were routinely eluted at 10 to 12 days with 100 mM $AlCl_3$. Figure 5 shows a growth curve with an initial 5% PCV inoculum. This growth curve demonstrates an initial lag phase until day 6 (~ 7% PCV). The growth rate is linear over the next 7 days (reaching ~ 18% PCV). A lower inoculum of 3% PCV resulted in virtually no growth within 15

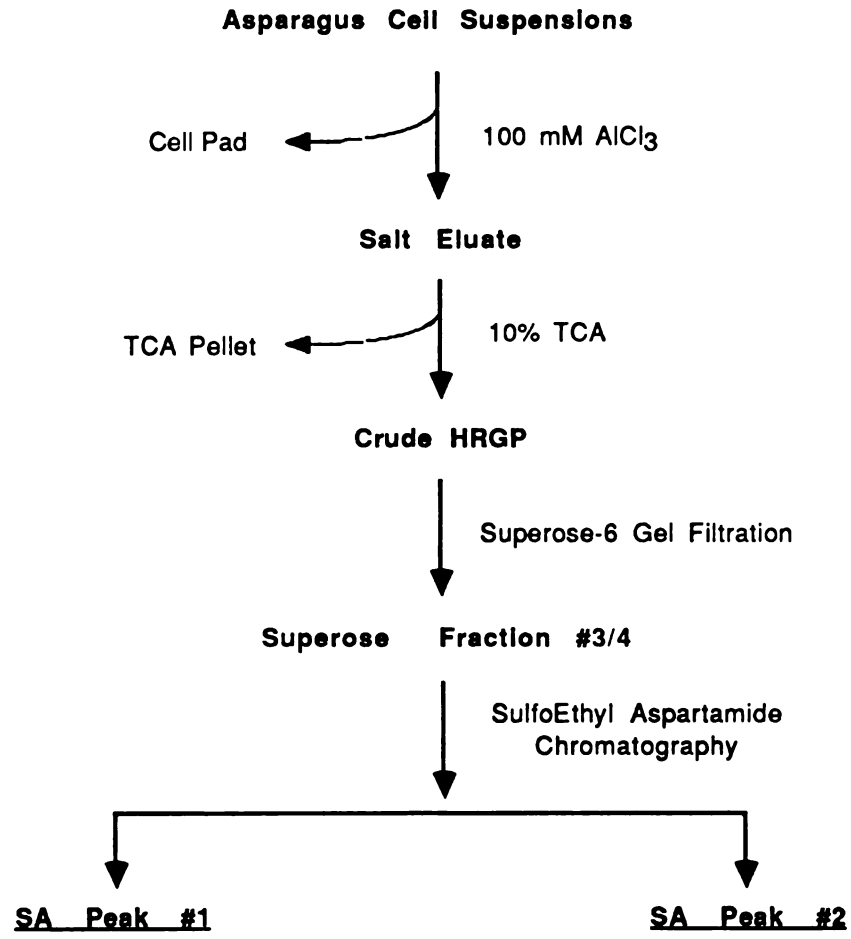


Figure 6. Fractionation scheme for asparagus TCA-soluble HRGPs

days. An initial inoculum of 10% PCV results in steady growth to ~ 20% PCV. Thus a 5% PCV inoculum allowed a reasonable timeframe for subculture and elution, and the cultures gave good yields.

B. TCA Precipitation of Crude Eluate

After precipitation for 18 hr at 4° C in 10% (w/v) TCA, insoluble protein was spun out leaving the Crude HRGP" in the supernatant. Lyophilization and desiccation yielded 7.2 (± 0.1) mg "Crude HRGP"/g cells dw. Hydroxyproline accounted for 2.8 (± 0.1)% (w/w) of the total cellular fraction. The TCA-soluble and insoluble material consisted of 7.0 (± 0.7)% and 2.8 (± 1.4)% (w/w) hydroxyproline respectively (Table 5). The TCA-soluble Crude HRGP was enriched in Hyp by 23-fold over the total cellular Hyp content. Analytical Superose-6 gel filtration, as a quality control step, showed the TCA-insoluble material to be poor in HRGP monomer—extensin monomers elute at ~ 2.1 V_o. Therefore the bulk of the HRGP monomer remained in the TCA-soluble fraction which accounted for ~ 70% of the elutable material).

C. Superose-6 FPLC Gel Filtration of Crude Eluate

I dissolved crude HRGP (20 mg/ml) in dH_2O and applied 30 mg to a preparative Superose-6 column. The crude HRGP was separated into 5 major fractions (Figure 7). Two of these fractions (3 and 4) were not resolved and were therefore pooled (fraction 3&4) for further fractionation. Manual Hyp analysis showed fractions 1 (void) and 5 to contain very little Hyp (< 0.4 % Hyp w/w). Fractions 2 and 3&4, on the other hand, contained 4.9 (± 0.9)% and 10.8 (± 1.8)% Hyp

dw respectively. Amino acid analysis revealed the presence of significant amounts of lysine and histidine which contribute to the basic nature of these glycoproteins (Table 3). Fraction 2 was not further analyzed.

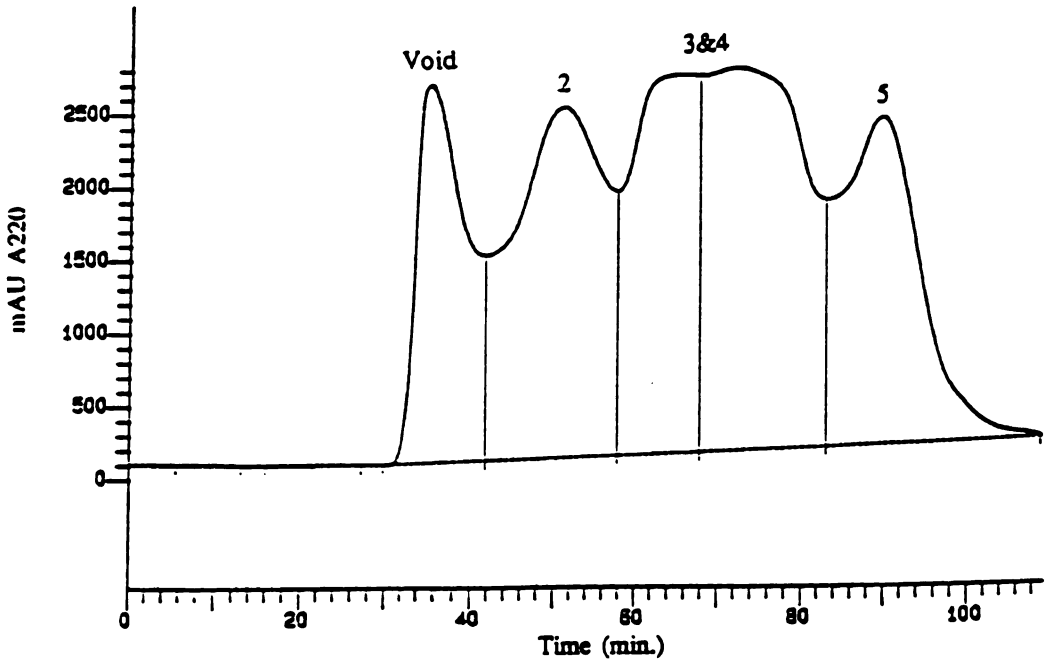


Figure 7. Superose-6 gel filtration of TCA-soluble crude HRGP.

D. PolySULFOETHYL Aspartamide Chromatography of Superose-6 Fraction 3&4

Cation exchange was performed on a PolySULFOETHYL Aspartamide (strong cation exchange) column (Figure 8). 5 mg maximum of Superose-6 fraction 3&4 (10 mg/ml in 10 mM $NaPO_4$,

10% MeCN) was loaded. The result was the separation of 2 major Hyp-rich components. The major fractions were designated SA1 and SA2.

Table 3. Amino Acid Compositions of Crude HRGP, and PreparativeSuperose-6 Fractions 2 and 3&4

Amino Acid	Crude HRGP[a]	Superose-6 Fraction #2	Superose-6 Fraction #3&4
Hyp	11.6	19.1	23.8
Asx	12.5	8.1	3.5
Thr	5.6	3.9	6.5
Ser	8.3	10.7	8.4
Glx	6.0	6.8	4.7
Pro	6.4	4.6	8.2
Gly	8.1	8.5	5.5
Ala	6.6	6.0	4.5
Val	6.1	4.4	6.2
Cys	n.d.	n.d.	n.d.
Met	0.0	0.0	0.0
Ile	4.5	3.0	3.5
Leu	7.4	6.0	5.2
Tyr	1.8	1.0	1.5
Phe	2.4	2.6	1.8
Lys	6.8	6.9	7.8
His	3.3	5.2	6.2
Arg	3.0	3.2	2.7

[a] Represented as Mole %

After PolySULFOETHYL Aspartamide fractionation, SA1 and SA2 were run on an analytical Superose-6 column. SA1 eluted at 2.1 V_o and appeared relatively pure. SA2 eluted at 2.2 V_o, however, it appeared that much of the material adsorbed to the Superose-6 column. After deglycosylation, both HRGPs were succinylated (to prevent non-specific adsorption to the column matrix) and run

through the same Superose-6 column. Both HRGPs eluted at 2.5 V_o. Thus it seems that SA2 did interact with the Superose-6 resin—possibly through lysine or arginine sidechains.

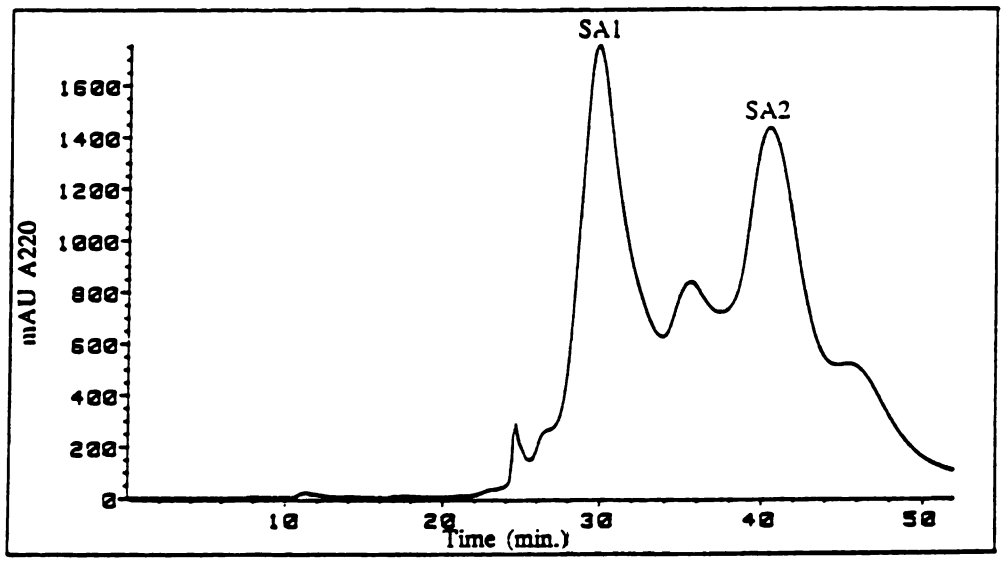


Figure 8. PolySULFOETHYL Aspartamide cation exchange chromatography of Superose-6 fraction 3&4

II. Chemical and Structural Characterization of Salt-elutable Asparagus HRGPs

A. Amino Acid and Manual Hyp Analyses of SA1 and SA2

Amino acid analysis showed SA1 and SA2 to be proline and serine-rich as well as Hyp-rich (Table 4). SA1 is also rich in histidine (9.7 mole%). SA2 on the other hand contains larger amounts of valine (8.2 mole%), and lysine (9.6 mole%). These asparagus HRGPs do not exhibit an extreme bias toward a few amino acids which is characteristic of these other extensins; and they do contain amino

acids which are rare in previously studied extensins. Aspartic acid/asparagine, glutamic acid/glutamine (amino acid analysis of acid hydrolysates cannot discriminate between these two pairs of related amino acids), isoleucine, leucine, and arginine are more abundant in these non-graminaceous HRGPs than in maize and tomato extensins (also these amino acids are less common in sugar beet P1 and Douglas Fir SP1; Table 20, Appendix).

Table 4. Comparison of Asparagus HRGPs with Maize[b] and Tomato[c] HRGPs

Amino Acid	SA1[a]	(+/-)	SA2	(+/-)	Maize HHRGP	Tomato P1
Hyp	27.8	0.8	21.4	1.7	34.9	32.7
Asx	4.1	0.4	3.4	0.7	1.3	1.4
Thr	4.4	0.1	6.9	0.7	7.9	6.2
Ser	8.5	0.3	9.2	0.6	7.3	9.8
Glx	5.1	0.6	3.9	0.6	2.1	1.5
Pro	8.7	0.5	8.0	0.5	6.8	9.6
Gly	5.1	0.5	6.6	1.4	3.1	1.7
Ala	3.0	0.3	5.1	0.6	8.9	2.9
Val	1.7	0.5	8.2	1.0	1.5	8.3
Cys	n.d.		n.d.		n.d.	n.d.
Met	0.0	0.0	0.1	0.1	0.0	0.0
Ile	3.4	0.1	2.7	0.5	0.0	1.0
Leu	4.4	0.4	4.5	0.7	0.0	1.0
Tyr	5.0	0.8	2.5	0.6	4.4	7.7
Phe	1.2	0.4	2.1	0.3	3.5	0.0
Lys	5.7	0.4	9.6	1.1	3.5	9.5
His	9.7	1.0	2.4	0.8	13.4	6.1
Arg	2.3	1.0	3.4	0.6	1.3	0.7

[a] Represented as Mole %
b Kieliszewski M, 1989
c Smith *et al.*, 1986

The Manual Hyp method was used to follow the fractionation of Hyp throughout the purification of the HRGPs (Table 5). Although a

more crude estimation of the Hyp levels, these data provide a method for corroboration of amino acid analysis data. By this method SA1 and SA2 contain 11.4 (±1.5)% and 13.1 (±0.2)% Hyp dw respectively.

Table 5. Manual Hydroxyproline Analyses *: Steps to Extensin Purification and Wall Fractionation

Extensin Purification		Wall Fractionation	
	Total Cell Hyp 0.3 (± 0.1)		
TCA Insol.	2.8 (± 1.4)	Intact Wall	0.5 (± 0.1)
TCA Soluble	7.0 (± 0.7)	HF Deg.-Sol.	0.4
Sup-6 F. 2	4.9 (± 0.9)	HF Deg.-Insol.	3.7 (± 1.3)
Sup-6 F. 3&4	10.8 (± 1.8)		
SA1	11.4 (± 1.5)		
SA2	13.1 (± 0.2)		

* % Hyp dw

B. Neutral Sugar Analyses of SA1 and SA2

Quantative analysis of neutral sugars via their alditol acetates showed the major components to be arabinose and galactose (Table 6). 200 ug of each HRGP was hydrolyzed in 2 N TFA followed by derivatization of the sugars to their alditol acetates by $NaBH_4$

reduction. Figure 9 shows the gas chromatogram of SA1 alditol acetates. Arabinose and galactose together equal 54% (w/w) of SA1 and account for 94 mole% of the total sugar. These components make up 45% (w/w) and account for 93 mole% of the total sugar of SA2. SA1 contains 78 mole% arabinose and 16 mole% galactose. SA2 contains 86 mole% arabinose and 7 mole% galactose. Both HRGPs contain ~4 mole% glucose. Xylose, mannose, and rhamnose may be present in very small quantities. Ara:Hyp and Gal:Ser ratios of SA1 are 2.6:1 and 1.3:1; Ara:Hyp and Gal:Ser ratios for SA2 are 3.3:1 and 0.6:1 respectively.

C. Hydroxyproline Arabinoside Profile of SA1 and SA2

Hydroxyproline-arabinoside profiles of SA1 and SA2 showed the majority of arabinose to be attached to Hyp as tetra- and tri-arabinosides (Table 7). Figure 10 shows the Hyp-arab profile of SA1. 34% of arabinose in SA1 is in the form of tri-arabinoside and 28% in the form of tetra-arabinoside. SA2 is composed of 32% tri- and 21% tetra-arabinoside. These compositions are intermediate to those of Tomato P1 and P2 HRGPs, and the maize HHRGP.

D. HF Deglycosylation of SA1 and SA2

Amino acid analysis of SA1 showed a protein content of ~ 41 (±3.8)% (w/w). Deglycosylation of SA1 resulted in recovery of 43% to 55% (w/w) of the original material. Accordingly, amino acid analyses of HF deglycosylated SA1 (dSA1) indicated 80% to 100% (w/w) protein. Amino acid analysis of SA2 showed a protein content of ~ 60 (±5.5)% (w/w). Deglycosylation of SA2 resulted in recovery of 73%

(w/w) of the original material (deglycosylation of SA2 appears to have been incomplete). Therefore the data corroborate a protein content of ~ 45% (w/w) for SA1 and ~ 60% (w/w) for SA2. Table 8 summarizes these data along with neutral sugars data.

E. SDS-PAGE of dSA1 and dSA2

SA1 and SA2, when loaded onto a 12% SDS-polyacrylamide gel, did not enter into the gel as might be expected considering the high degree of glycosylation. After deglycosylation, dSA1 migrated with an apparent molecular weight (M_r) of ~ 44 kD (Figure 11, lane 2). dSA2 ran with a Mr of ~ 37 kD (Figure 11, lane 3). Due to the rodlike nature of these proteins these molecular weights can only be taken as rough approximations.

F. Tryptic Digestion, Peptide Mapping, and Peptide Sequencing

Tryptic digestion of dSA1 and mapping via reverse phase on a Hamilton PRP-1 column gave 8 major peptides (Figure 12) —an unusually complicated peptide map for repetitive proteins such as extensins. Further purification of these peptides was attempted through a second run over the Hamilton PRP-1. Peptide M2 appeared pure judging by the second PRP-1 run; Peptides M4 and M5 each resolved into 2 separate peaks (collected as M4a, M4b, M5a, and M5b); and peptides M6 and M7 both appeared as two or more unresolved peaks. Size exclusion chromatography via a PolyHYDROXYETHYL Aspartamide (PolyLC) column run in SEC

Table 6. Sugar Compositions of Asparagus, Tomato[b], and Maize[c] HRGPs

Neutral Sugar [a]	SA1	SA2	Tomato avg. P1/P2	Maize THRGP	Maize HHRGP
Rhamnose	1	1	0.2	0	0
Fucose	0	0	0.1	0	0
Arabinose	78	86	90.1	100	63
Xylose	2	2	0.3	0	0
Mannose	1	1	0.8	0	0
Galactose	16	7	6.5	0	37
Glucose	4	4	2.2	0	0
Ara:Hyp	2.6:1	3.3:1	2.77:1	1.44:1	2.4:1
Gal:Ser	1.3:1	0.6:1	n.d.	*	5:1

[a] Represented as Mole% of sugar
[b] Smith JJ, 1985
[c] Kieliszewski M, 1989
* no Gal:Ser in THRGP

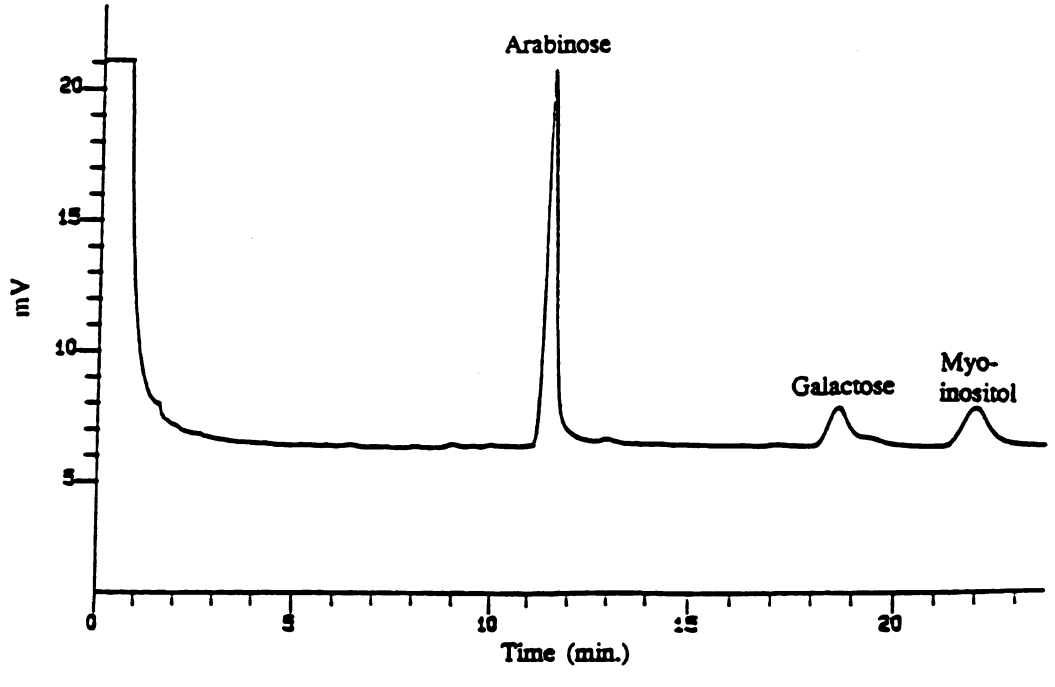


Figure 9. Gas chromatography of neutral sugar alditol acetates from SA1

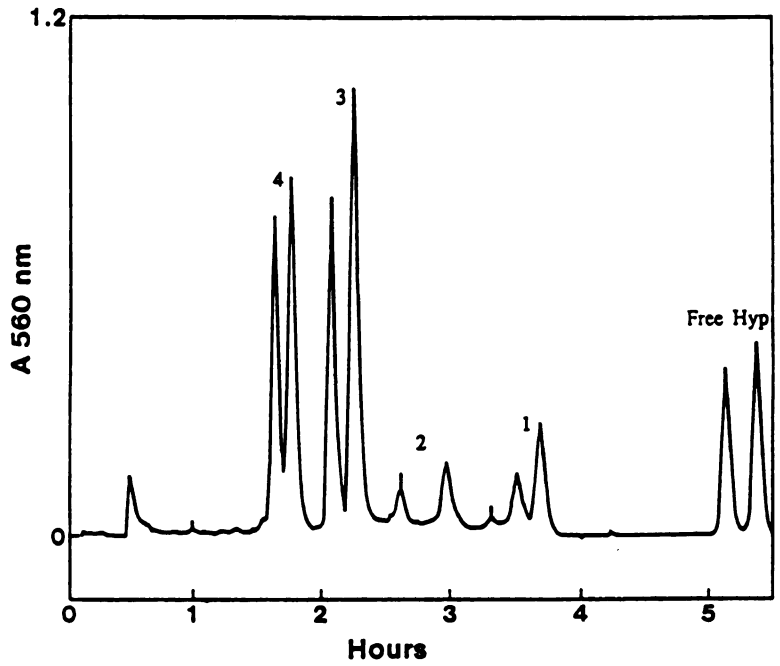


Figure 10. Hydroxyproline-arabinoside profile of SA1.

Table 7. Hydroxyproline-arabinoside Profiles of Asparagus, Tomato[a], and Maize[b] HRGPs

	SA1	SA2	Tomato avg. P1/P2	Maize THRGP	Maize HHRGP
Free Hyp *	17	28	9.5	48	20
Hyp-Ara$_1$	15	13	7.6	15	8
Hyp-Ara$_2$	6	6	7.9	6	9
Hyp-Ara$_3$	34	32	28.7	25	42
Hyp-Ara$_4$	28	21	46.3	6	21

* Expressed as % of total Hyp [a] from Smith *et al.*, 1984
[b] from Kieliszewski M, 1989

Table 8. SA1 and SA2 Protein/Carbohydrate Compositions

	SA1	SA2
HF Insoluble	43-55%	73%
% Protein [a]	41 (± 3.8)%	60 (± 5.5)%
% Carbohydrate [b]	54%	45%

[a] based on amino acid analysis data
[b] based on neutral sugars data (Ara + Gal)

(size exclusion chromatography) mode was the next purification step. One of these major peptides, designated SA1 M6, provided a single major component which was purified and sequenced. The sequence of this peptide was His-Lys-Pro-Hyp-Hyp-Ser-Ser-His-Leu-Pro-Hyp-Hyp-Ile-Tyr. (This C-terminal tyrosine may be due to chymotryptic contamination of trypsin.) The two subsequences of this peptide Lys-Pro-Hyp-Hyp and Ser-Ser-His-Leu-Pro are significant (see Discussion I., E.). Amino acid compositions of M4a, M4b, M5b, M6, and M7 are given in Table 9. The remaining peptides (M4a, M4b, M5b, and M7) proved to be heterogeneous (containing 3 or more major components) when run over the PolyLC column. These peptides have not been further analyzed Tryptic digestion of dSA2 gave 4 major peptides (Figure 13). This peptide map conforms to the simplicity normally seen in extensins due to their repetitive nature. Further purification was again attempted with a second .

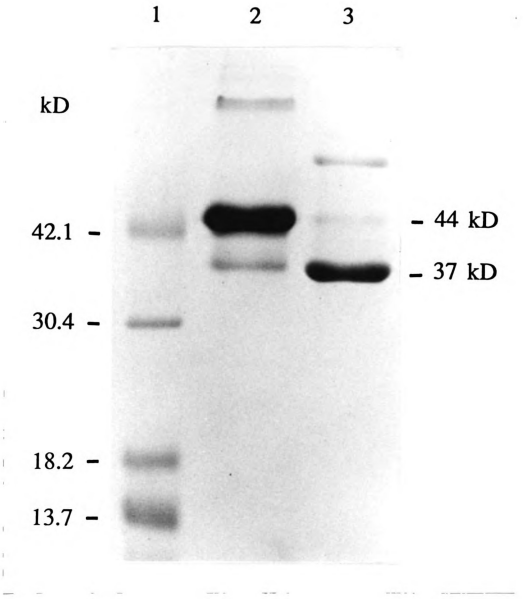


Figure 11. SDS-PAGE analysis of dSA1 and dSA2.

Hamilton PRP-1 run. The major portion of each of these fractions was collected. Although only M4 appeared pure, the amino acid compositions were analyzed for each. M1 showed only histidine. The amino acid compositions of the other major fractions are shown in Table 10. One of these peptides, SA2-M4 consisted of the following sequence: Ser-Hyp-Hyp-Hyp-Ser-Hyp-Val-Lys-Pro-Thr-Pro-Arg. This sequence matched perfectly the proposed empirical formula deduced from amino acid analysis and proved to be a very interesting sequence (see Discussion I., E.)). Further purification via PolyLC size exclusion chromatography showed heterogeneity among M1, M2, and M3. These peptides were not further analyzed.

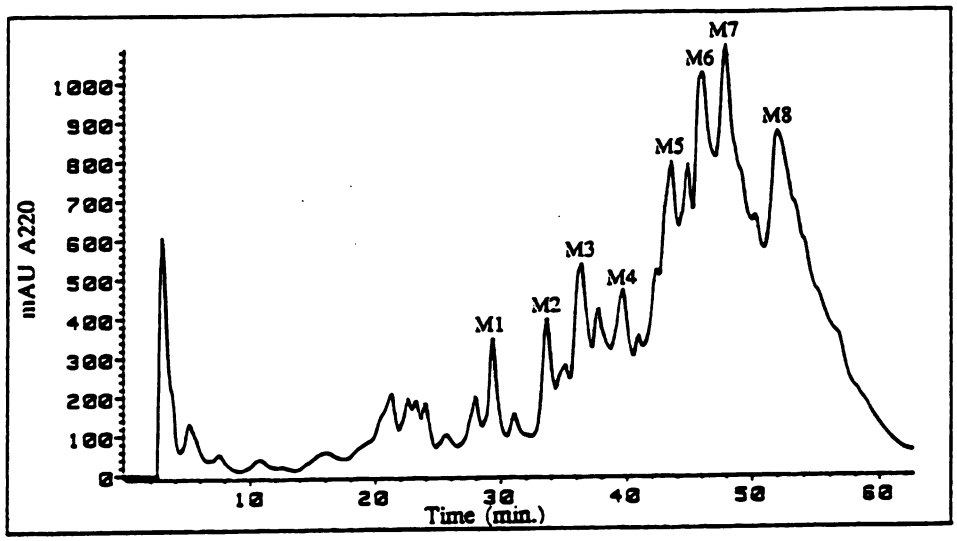


Figure 12. Tryptic peptide map of HF deglycosylated SA1.

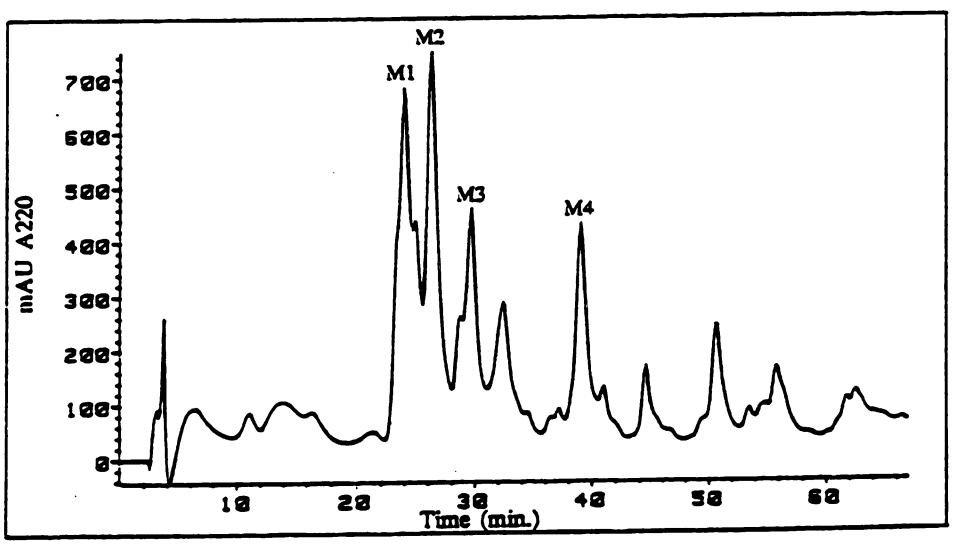


Figure 13. Tryptic peptide map of HF deglycosylated SA2.

Table 9. Amino Acid Composition of Major Peaks from dSA1 Tryptic Peptide Map

Amino Acid[a]	M4a	M4b	M5	M6	M7
Hyp	19.8	26.2	47.9	15.0	22.7
Asp	5.8	1.8	0.0	3.3	0.6
Thr	8.2	5.6	6.0	3.3	4.7
Ser	23.5	7.1	10.1	8.3	6.8
Glu	8.3	4.4	1.5	5.4	5.2
Pro	1.6	6.7	4.8	12.6	8.5
Gly	10.8	5.0	0.7	8.0	3.9
Ala	4.1	4.1	6.3	2.4	1.8
Val	3.8	9.8	8.0	1.8	1.3
Cys	n.d.	n.d.	n.d.	n.d.	n.d.
Met	0.0	0.0	0.0	0.0	0.0
Ile	1.0	1.3	0.0	4.7	3.6
Leu	1.0	4.8	0.0	4.8	3.9
Tyr	0.7	0.7	1.2	3.5	4,2
Phe	0.6	0.6	0.0	1.2	1.3
Lys	8.8	5.7	11.9	12.7	6.5
His	1.6	10.3	0.0	11.7	9.0
Arg	0.6	0.8	1.7	1.4	1.4

[a] Represented as Mole %

Table 10. Amino Acid Composition of Major Peaks from dSA2 Tryptic Peptide Map

Amino Acid[a]	M2	M3	M4
Hyp	19.8	47.9	34.8
Asp	5.8	0.0	0.0
Thr	8.2	6.0	5.6
Ser	23.5	10.1	14.1
Glu	8.3	1.5	0.7
Pro	1.6	4.8	17.6
Gly	10.8	0.7	0.0
Ala	4.1	6.3	0.0
Val	3.8	8.0	5.8
Cys	0.0	0.0	0.0
Met	0.0	0.0	0.0
Ile	1.0	0.0	1.1
Leu	1.0	0.0	0.0
Tyr	0.7	1.2	0.0
Phe	0.6	0.0	0.0
Lys	8.8	11.9	9.7
His	1.6	0.0	0.0
Arg	0.6	1.7	10.6

[a] Represented as Mole %
() = deduced empirical formula

III. Chemical and Structural Characterization of the Asparagus Cell Wall

A. Estimation of Asparagus Wall Pectin Content

A crude estimate of the primary cell wall pectin content gave a value of 20% (dw). This value is similar to the dicot (~35%) (Darvill *et al.*, 1980). The pectin content of graminaceous primary cell walls varies between 1.3% to 6% (dw) (Ray & Rottenberg, 1974; Darvill, 1976; Dever *et al.*, 1978).

B. Amino Acid and Manual Hyp Analyses of Wall Fractions

Amino acid analysis of asparagus cell wall showed similarity in composition with maize cell wall (Table 11). These analyses revealed 20.1 (± 2.8)% protein. The asparagus cell wall (2.1 ± 0.5 mole% Hyp) contained twice as much Hyp (mole% basis) as the maize wall. Manual Hyp analyses also showed the asparagus wall to contain more Hyp than maize (maize wall = 0.07% to 0.2% Hyp w/w; asparagus wall = 0.45% to 0.57% Hyp w/w). On the other hand, the amino acid composition of the tomato cell wall is significantly different from these monocot walls. Table 12 shows Hyp analyses of various wall fractions (intact, HF-soluble, and HF-insoluble).

Amino acid analyses of the HF-insoluble wall showed a 2-fold increase in Hyp (mole%). Otherwise the amino acid composition is similar to the intact wall. Protein comprised 38.9 (± 2.6)% of this material. Manual Hyp assays showed an increase from 0.5% Hyp dw to 3.7 (±1.3)% Hyp dw when intact and HF-insoluble wall fractions

were compared (Table 5). The HF-soluble was also higher in Hyp (4.5 mole%, one analysis) than the intact wall. This fraction is also glycine-rich (Table 12) according to the analysis. Because of the low recovery of HF-soluble material (~ 3% dw of the intact wall) and low protein and Hyp contents (~ 11% and 0.4% dw respectively), this material was not further analyzed

C. Hydroxyproline Arabinoside Profile

The hydroxyproline-arabinoside profile of the asparagus cell wall proved to be very similar to that of the tomato cell wall (Table 13). 32% of the arabinose was in the form of tri-arabinoside while 50% was in the form of tetra-arabinoside—in both walls the tetra-arabinoside predominates.

D. HF Deglycosylation

After deglycosylation of asparagus cell wall (20.1 ± 2.8% protein), 14.6 (± 1.5)% of the material (HF-insoluble) was recovered while the remaining components were solubilized (~ 85%). Tables 12 and 5 show the amino acid compositions and Hyp contents (dw) respectively. The recoverable Hyp containing protein/glycoprotein was predominantly covalently bound in the wall. Table 14 shows % recoveries, % protein, and % Hyp contents (dw) of the HF-insoluble and HF-soluble fractions.

Table 11. Amino Acid Compositions of Asparagus, Maize[b], and Tomato[c] Cell Walls

Amino Acid [a]	Asparagus Wall	(+/-)	Maize Wall	Tomato Wall
Hyp	2.1	0.5	1.1	28.5
Asp	8.7	0.5	10.4	4.0
Thr	4.9	0.1	5.1	4.6
Ser	7.0	0.7	6.9	14.2
Glu	10.1	0.6	9.3	2.8
Pro	5.8	0.3	3.7	3.9
Gly	13.2	1.1	10.7	3.3
Ala	10.4	1.6	10.6	3.2
Val	5.0	0.4	6.4	7.0
Cys	n.d.		n.d.	n.d.
Met	0.8	0.2	1.7	0.3
Ile	4.1	0.4	4.2	1.8
Leu	7.6	0.6	10.3	2.5
Tyr	1.7	0.1	1.9	6.3
Phe	3.2	0.5	4.0	1.3
Lys	7.0	0.4	6.2	10.5
His	2.9	1.5	2.1	2.7
Arg	4.9	0.3	4.7	1.2

[a] Represented as Mole % [b] from Kieliszewski M, 1989
[c] from Smith et al., 1984

Table 12. Amino Acid Composition of Intact, HF-Soluble[b] and HF-Insoluble Asparagus Cell Wall Fractions

Amino Acid [a]	Intact Wall	(+/-)	HF Soluble Wall	HF Insoluble Wall	(+/-)
Hyp	2.1	0.5	4.5	4.8	2.2
Asp	8.7	0.5	6.4	8.2	1.2
Thr	4.9	0.1	4.3	4.5	0.4
Ser	7.0	0.7	9.7	6.3	0.2
Glu	10.1	0.6	8.8	8.7	0.6
Pro	5.8	0.3	5.9	6.6	0.3
Gly	13.2	1.1	19.7	12.7	1.2
Ala	10.4	1.6	9.6	9.1	0.7
Val	5.0	0.4	4.5	5.1	0.5
Cys	n.d.		n.d.	n.d.	
Met	0.8	0.2	0.4	1.2	0.2
Ile	4.1	0.4	3.1	4.2	0.4
Leu	7.6	0.6	4.6	8.3	0.8
Tyr	1.7	0.1	2.0	1.4	0.4
Phe	3.2	0.5	2.4	3.5	0.5
Lys	7.0	0.4	7.6	6.3	0.6
His	2.9	1.5	2.7	3.7	1.7
Arg	4.9	0.3	3.8	5.2	0.3

[a] Represented as Mole %
b analyzed once

E. Enzymic Digestion of HF Deglycosylated Asparagus Wall

Tryptic digestion solubilized 64 (± 9)% dw of the HF deglycosylated wall. Peptide maps of the trypsin solubilized material via Hamilton PRP-1 reverse phase chromatography gave irreproducible results. Pronase digestion solubilized 61 (± 10)% dw of the deglycosylated wall. Table 15 compares the amino acid compositions of the HF-insoluble, trypsin-insoluble, and pronase-insoluble wall fractions (note the similarity). Pronase generated peptide maps reproducibly showed three major peptides which I designated Pro1, Pro2, and Pro3. Amino acid analyses indicated that methionine and isoleucine make up ~ 54 mole% of Pro1, and phenylalanine makes up ~ 50 mole% of Pro2. Pro3 contained ~ 21 mole% Hyp and appeared to contain IDT. Table 15 also shows the amino acid composition of Pro3. Figure 14 shows the pronase peptide map and the spectrum of Pro3. Also, a 26.1 minute peak—the retention time for IDT—was seen when an acid hydrolysate of Pro3 was run on the Hamilton PRP-1 and eluted by the gradient described by J.J. Smith (see Materials & Methods III., F.). Based on these data, it was proposed that Pro3 likely contained IDT. Problems with solublity of this peptide led to an alternative approach for assaying IDT in the bound wall.

F. IDT Detection in Asparagus Cell Wall

I initially began work on the covalently bound wall protein. This resulted in the isolation of the pronase peptide, Pro3. Amino acid analyses of Pro3 showed negligible tyrosine and ~ 5 mole% lysine

(Table 15); Epstein and Lamport (1984) had previously seen IDT co-chromatograph with lysine on a cation exchange based amino acid analyzer; Pro3 showed a spectrum reminiscent of IDT (Figure 14).

Table 13. Hydroxyproline-arabinoside Profiles of Asparagus, Tomato[a], and Maize[b] Cell Walls

	Asparagus	Tomato	Maize (pericarp)	Maize Black Mexican
Free Hyp *	8	5.3	66	24
Hyp-Ara_1	5	9.9	15	9
Hyp-Ara_2	4	9.1	2	6
Hyp-Ara_3	32	27.5	13	41
Hyp-Ara_4	50	48.3	4	10

* Expressed as % of total Hyp
a from Smith *et al.*, 1984
b from Kieliszewski M, 1989

Table 14. HF Deglycosylation Data from Asparagus Walls

	% Recovery (w/w)	% protein (w/w)	% Hyp (w/w)
Asparagus Wall	NA	20.1 ± 2.8	0.45-0.57
Asparagus HF-insol.	14.6 ± 1.5	38.9 ± 2.6	3.7 ± 1.3
Asparagus HF-sol.	~3	~11	0.4

Table 15. Amino Acid Compositions of HF-insoluble Wall, Trypsin-insoluble Wall, Pronase-insoluble Wall, and Pro3

Amino Acid [a]	HF Insol. Wall	(+/-)	Tryp. Insol. Wall	(+/-)	Pron. Insol. Wall	(+/-)	Pro3	(+/-)
Hyp	4.8	2.2	4.8	0.6	4.5	2.1	20.6	0.1
Asp	8.2	1.2	8.5	2.4	8.7	0.9	7.2	0.8
Thr	4.5	0.4	4.1	0.4	4.3	0.1	3.6	0.3
Ser	6.3	0.2	8.1	2.1	5.8	0.8	8.4	0.5
Glu	8.7	0.6	8.3	2.5	7.2	0.1	7.8	0.4
Pro	6.6	0.3	6.6	0.4	7.3	1.1	16.4	1.2
Gly	12.7	1.2	13.8	0.8	15.6	4.2	9.3	1.4
Ala	9.1	0.7	8.2	1.1	8.6	0.8	3.0	0.1
Val	5.1	0.5	3.5	1.0	5.2	2.3	3.1	0.4
Cys	n.d.		n.d.		n.d.		n.d.	
Met	1.2	0.2	0.8	0.1	0.8	0.3	0.0	0.0
Ile	4.2	0.4	3.0	1.1	4.6	1.0	2.4	0.0
Leu	8.3	0.8	7.2	0.8	7.5	1.2	4.2	0.3
Tyr	1.4	0.4	1.5	0.1	1.5	0.2	0.7	0.0
Phe	3.5	0.5	2.8	0.4	3.3	0.1	4.1	0.4
Lys	6.3	0.6	6.5	2.1	6.4	0.7	4.9	0.2
His	3.7	1.7	3.0	0.8	4.0	0.4	2.5	0.0
Arg	5.2	0.3	4.8	2.3	4.9	1.1	2.1	0.4

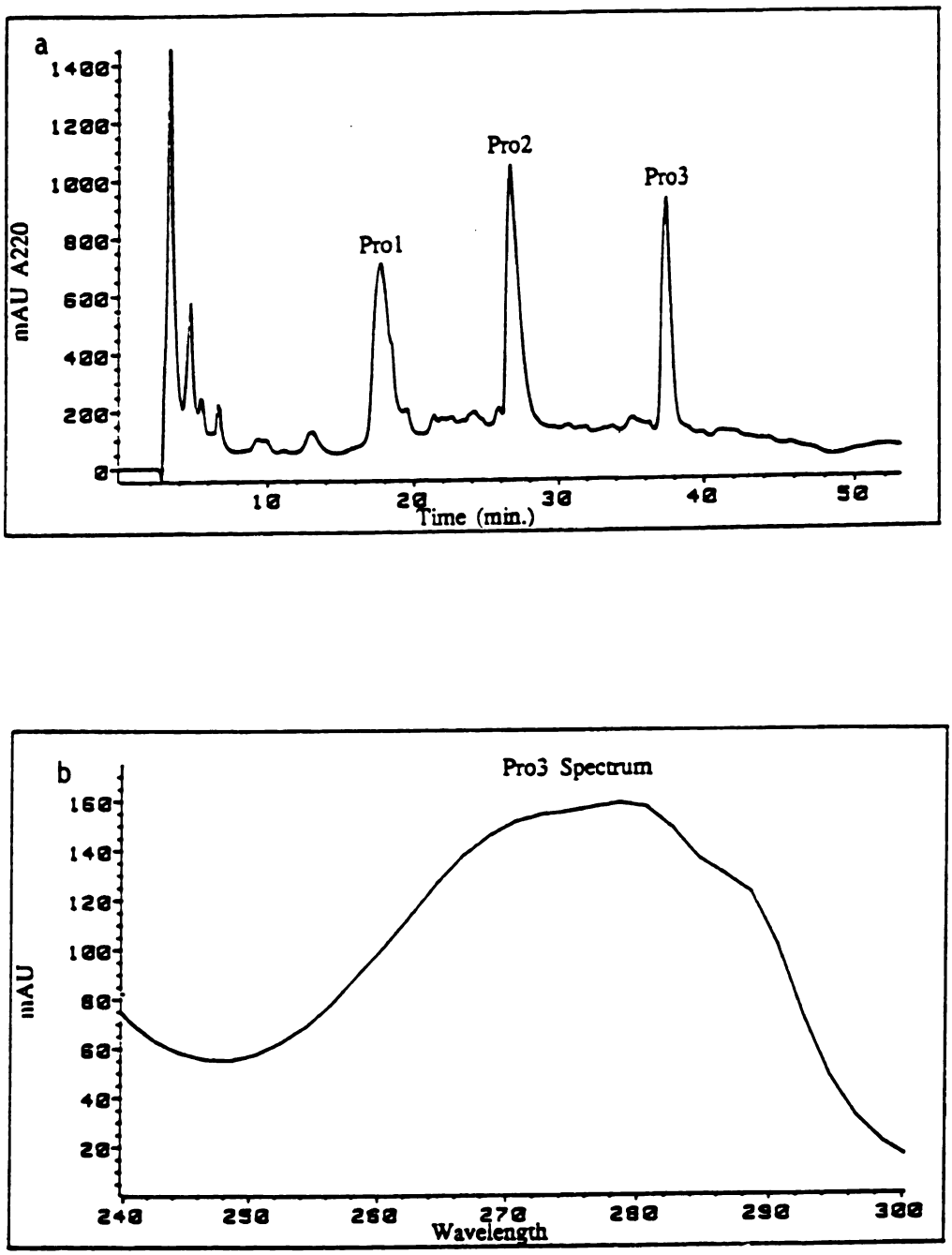
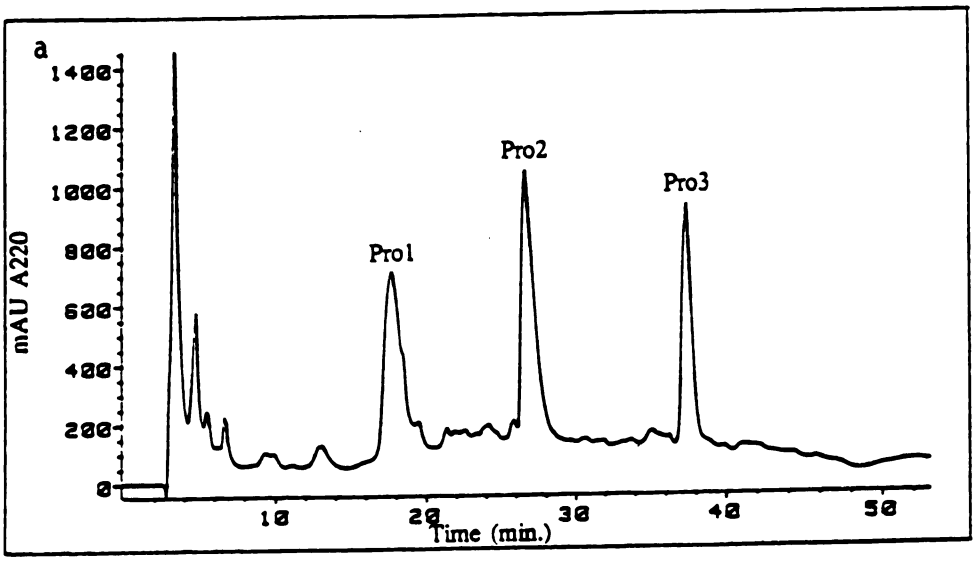


Figure 14. a) Pronase peptide map of HF deglycosylated asparagus wall.
b) Spectrum of Pro3 in 0.1% TFA.



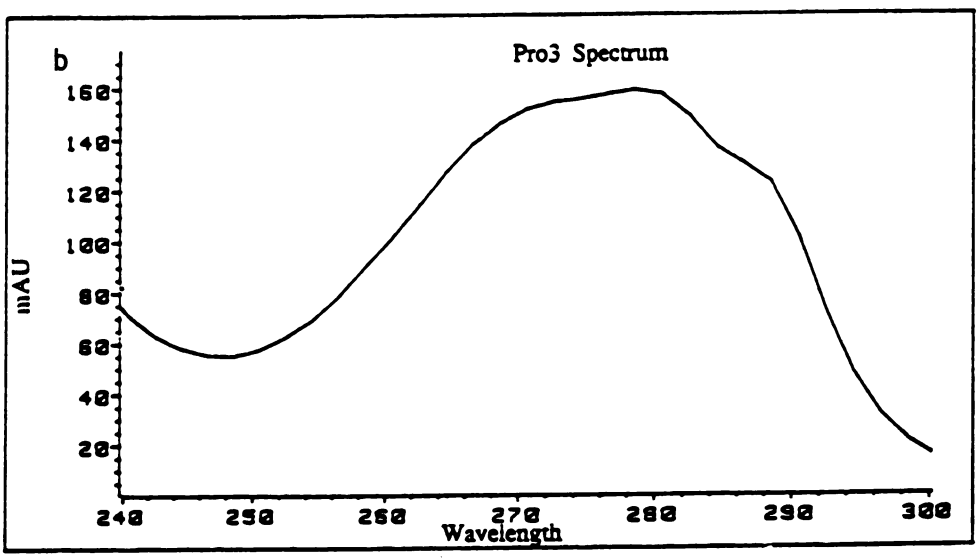


Figure 14. a) Pronase peptide map of HF deglycosylated asparagus wall.
b) Spectrum of Pro3 in 0.1% TFA.

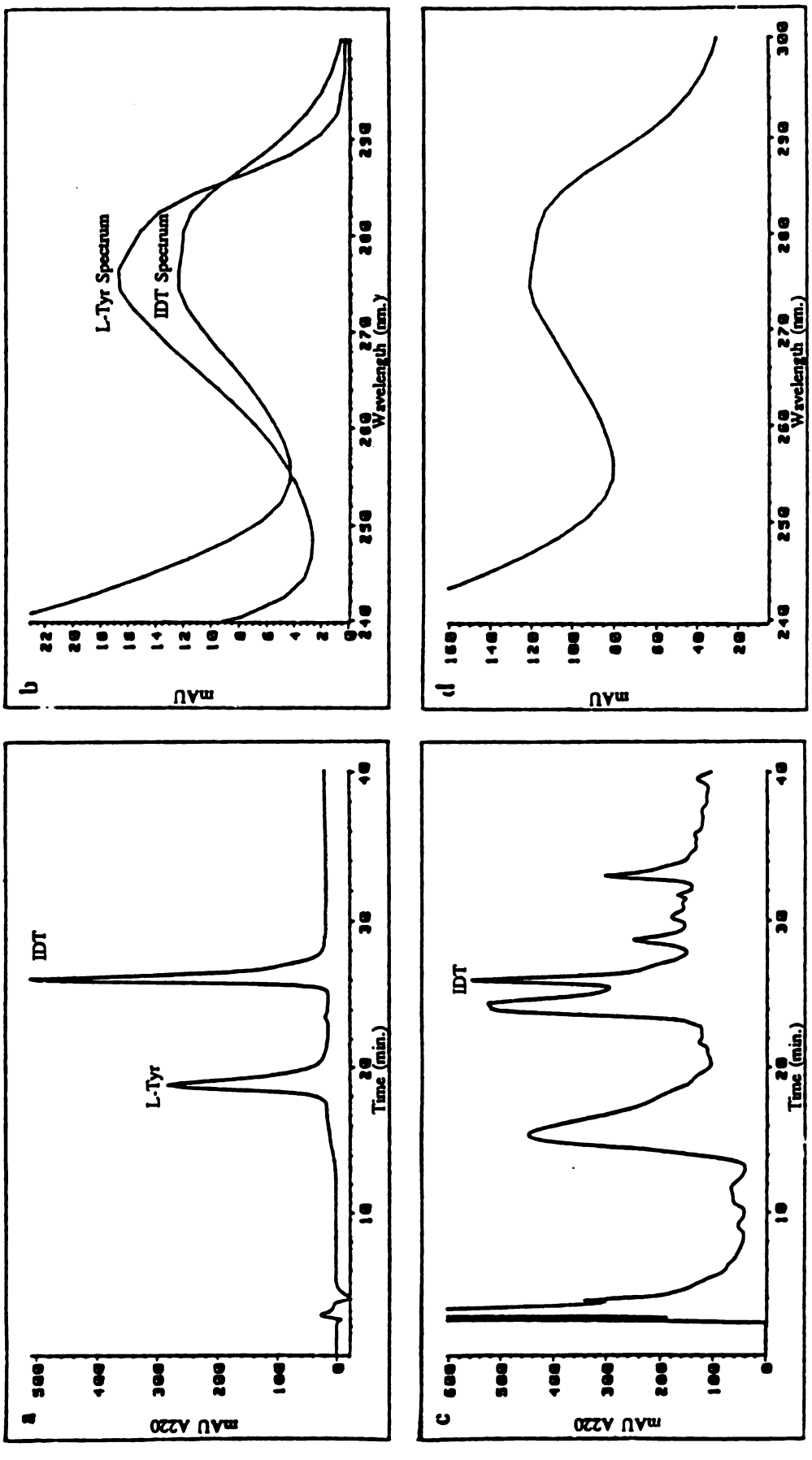
I ran an aliquot of acid hydrolyzed, HF deglycosylated asparagus cell wall on the Hamilton PRP-1 (Figure 15). The result was the detection of a peak which eluted at 26.1 minutes (authentic IDT eluted at 26.2 min). The spectrum of this peak matches that of the IDT standard. Subsequent runs were performed and the 26.1 minute peak was collected. The spectrum from A_{240} to A_{350} in acid (0.1 N HCl, pH 1.7) and in alkali (0.1 N NaOH, pH 13) was plotted (Figure 16). The maxima and minima (pH 1.7 max = 273 nm and 279 nm, min = 254 nm; pH 13 max = 284 nm and 297 nm; min = 268 nm) of these spectra match those reported by Epstein & Lamport for IDT. Peak to valley ratios (A_{273}/A_{254} at pH 1.7) and (A_{297}/A_{268} at pH 13) were 1.3 and 1.1 respectively (see Discussion II., E.). I calculated the Hyp:IDT ratio for asparagus wall to be 66:1.

IV. Preparation and Characterization of IDT from Tomato Cell Wall

A. Isolation of IDT via Aminex AG-50 X 4 Chromatography

After acid hydrolysis of tomato cell wall and an initial clean up of the hydrolysate by NH_4OH elution, IDT was isolated from the mixture of amino acids by chromatography on Aminex AG-50 x 4 resin (Figure 17). Peak #3 of the chromatogram proved to be IDT. The recovery of crude IDT was 30 mg/10 g (0.3%) of tomato wall. After recrystallization in water, I recovered a final yield of 1.4 mg IDT (0.014% w/w of the primary wall).

Figure 15. Assay for IDT in asparagus HF deglycosylayed wall hydrolysate
a) L-Tyr and IDT standards
b) spectra of L-Tyr and IDT standards
c) asparagus wall hydrolysate
d) spectrum of asparagus IDT (rt. = 26.2 min.)


a
mAU A220
IDT
L-Tyr
Time (min.)
b
mAU
L-Tyr Spectrum
IDT Spectrum
Wavelength (nm.)
c
mAU A220
IDT
Time (min.)
d
mAU
Wavelength (nm.)

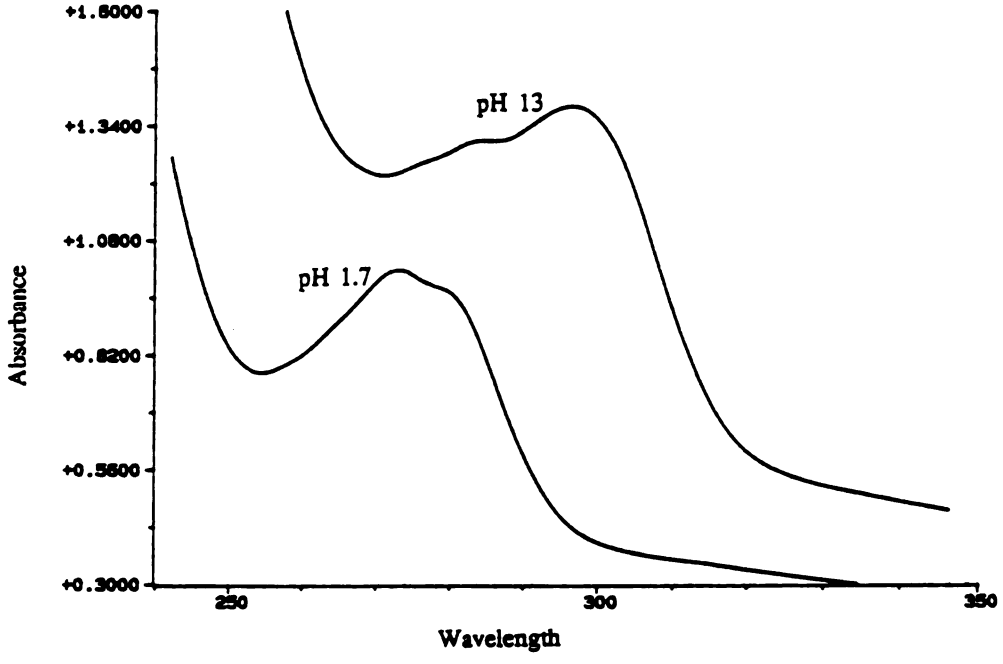


Figure 16. Spectra of IDT from asparagus in acid and alkali.

B. Characterization of IDT via:

i) Hamilton PRP-1 Chromatography: IDT was previously reported to elute from a Hamilton PRP-1 column at 27.8 min. (A = 0.1% TFA, B = A + 80% MeCN; gradient = 0-30% B in 30 min. at 0.5 ml/min.). IDT isolated from the tomato wall eluted from this gradient at 26.2 minutes.

ii) Acid/Alkali Spectral Shift and Molar extinction Coefficient: IDT has characteristic spectra in acid vs alkali. In acid (0.1 N HCl) two maxima occur at 273 and 279 nm respectively. In alkali (0.1 N

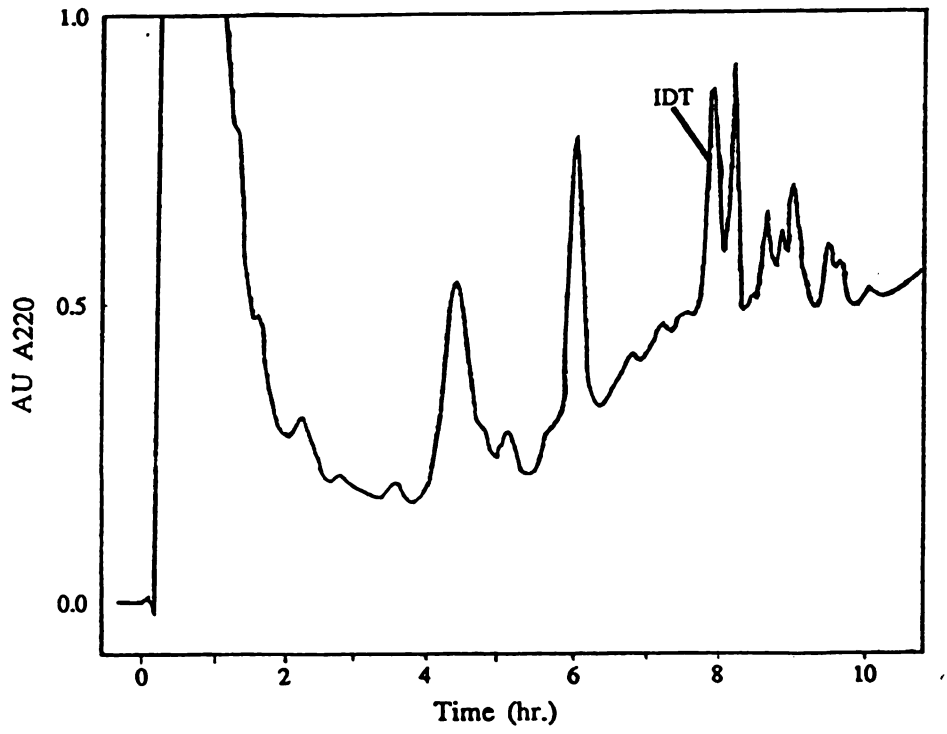


Figure 17. Isolation of IDT from tomato cell walls via Aminex AG-50 X 4 chromatography.

NaOH) these maxima shift to 284 and 297 nm. The minimum also shifts from 254 nm to 268 nm when comparing acid vs alkali spectra. The peak to valley ratios were similar to those previously reported. The peak to valley ratio (297 nm/268 nm) at pH 13 = 1.3 and the ratio (273 nm/254 nm) at pH 1.7 = 2.2. These results are consistent with those of Epstein & Lamport (1984). Using the molar extinction

coefficient reported (4.3 x 10^3 at A_{297} nm), I was able to calculate back to the measured concentration within ~ 20%.

DISCUSSION

Monocot, compared with dicot, cell wall protein is relatively Hyp-poor (Lamport, 1965) which explains the lack of study (until recently) of monocot cell wall HRGP, extensin. Structural characterization of graminaceous HRGPs has been performed in maize. The THRGP and HHRGP are very different from advanced dicot extensins: 1) the amino acid compositions are unique, 2) neutral sugar compositions are unlike tomato, and 3) Hyp-arab profiles resemble primitive dicots and gymnosperms more than advanced dicots. Maize wall amino acid composition and Hyp-arabs also resemble the more primitive species, and the maize wall lacks IDT (Kieliszewski, 1989).

Previous work has shown that HRGPs can be eluted from the surface of suspension cultured tomato and maize cells (Smith, 1985; Kieliszewski & Lamport, 1987). Precursor status of the tomato P1 and P2 (and therefore identification as extensins) was shown through kinetic studies and sequence comparisons with wall-bound protein. Inclusion of the maize THRGP among extensins was based on homology of this HRGP to tomato P1 determined by direct sequence analysis (Kieliszewski *et al.*, 1990) and immunological data (Kieliszewski & Lamport, 1987). Here I have presented the isolation and partial characterization of two HRGPs from the wall of a pectin-rich (the Graminae are pectin-poor; McNeil *et al.*, 1984), non-graminaceous monocot. Based on protein sequence data and

glycosylation profiles, these HRGPs are members of the extensin family.

I. Isolation and Characterization of Asparagus Extensins

A. Purification of Asparagus HRGPs, SA1 and SA2

Separation of HRGPs after salt elution and TCA precipitation was an empirical process. Biorex-70 and Cellex-P cation exchangers used in the purification of sugar beet (Li *et al.*, 1990) and maize (Kieliszewski, 1989) HRGPs were unsuccessful for asparagus HRGP separation (carboxymethyl cellulose, used for tomato HRGPs [Smith, 1985], was not tried). In order to simplify this task I decided to first separate the crude HRGP by gel filtration on preparative grade Superose-6. This fractionation removed 30% to 40% of the material (Hyp-poor).

I concentrated on further fractionation of the major Superose-6 fraction (fraction 3&4, two unresolved peaks). This fraction also contained the highest concentration of hydroxyproline. PolySULFOETHYL Aspartamide chromatography separated two major Hyp-rich fractions from Superose-6 fraction 3&4 which I designated SA1 and SA2. These two proteins co-chromatographed on analytical Superose-6 gel filtration eluting at 2.2 x V_o like tomato P1. After HF deglycosylation, SDS-PAGE resulted in a band of M_r ~ 44 kD for dSA1 and a band of M_r ~ 37 kD for dSA2 (tomato dP1 ~ 55 kD, tomato dP2 ~ 53.5 kD [Smith, 1985], maize dTHRGP ~ 50 kD, maize dHHRGPs ~ 68

kD and 70 kD [Kileiszewski, 1989]). These are only apparent molecular weights as flexuous, rod-like molecules run anomalously through porous gel matrices (Heckman *et al.*, 1988). These data showed SA1 and SA2 to be monomers. My visual assessment of purity from these gels was > 90% for each HRGP.

B. Amino Acid Analyses of SA1 and SA2

As seen in maize (Kieliszewski & Lamport, 1988) and Douglas Fir (Kieliszewski *et al.*, to be submitted), species more removed from advanced herbaceous dicots often have individual pecularities in their HRGP amino acid compositions. Maize yielded a THRGP (25.3 mole% threonine) (Kieliszewski & Lamport, 1987; Kieliszewski *et al.*, 1990) and HHRGPs (16.0 mole% histidine) (Kieliszewski, unpublished data). Kieliszewski (unpublished data) showed Douglas Fir (a gymnosperm) to contain a PHRGP (21.3 mole% proline) (Table 22, Appendix). Asparagus HRGPs SA1 and SA2 do not contain such high amounts of any one particular amino acid, but do contain several amino acids which are rare among extensins, notably aspartic acid (or asparagine),glutamic acid (or glutamine), isoleucine, leucine, and arginine. No clear parallel in amino acid composition exists between asparagus HRGPs and any other particular extensin(s) studied (Table 4, Results; Table 20, Appendix).

The presence of hydrophobic amino acids in the wall could possibly prevent loss of water. Isoleucine and leucine were found in higher amounts in monocot (asparagus, maize, and rice),

gymnosperm (Douglas Fir), and primitive dicot (sugar beet) walls than in the advanced dicot wall (tomato) (Table 20, Appendix). Perhaps this represents adaptation to drier climates. The presence of these amino acids in salt-elutable HRGPs is unique to asparagus. Possibly, both Hyp-rich and Hyp-poor components have independently evolved to contain these hydrophobic amino acids. Alternatively there may be an evolutionary relationship between these structural proteins.

C. Sugar Analyses and Hyp-arab Profiles of SA1 and SA2

Comparison of neutral sugars of SA1 and SA2 with the sugar compositions of tomato HRGPs (avg. of P1 and P2), and maize HRGPs (Table 6) showed the asparagus extensins to resemble dicot extensins more than maize HRGPs. Like the tomato P1 and P2 extensins, SA1 and SA2 have more diverse sugar compositions than the maize HRGPs. The THRGP (100 mole% arabinose) and HHRGP (63 mole% arabinose; 37 mole% galactose) sugar compositions are extremely simple. On the other hand, asparagus and tomato HRGPs contain 80 to 90 mole% arabinose, 7 to 16 mole% galactose, and 2 to 4 mole% glucose. There may also be trace amounts of xylose, rhamnose, and mannose.

The Hyp-arabinoside profile of SA1 shows a close resemblance to that of dicot species (Table 7; Table 21, Appendix). The Hyp-arab profile of SA2 also resembles those from dicot species, but begins to also resemble that of the maize HHRGP (the Hyp-ara_3:Hyp-ara_4 ratio is more like the HHRGP). The ~ 3:1 Ara:Hyp ratios of SA1 and SA2

are characteristic of dicot extensins, whereas the maize THRGP and HHRGP show less substituted Hyp—Ara:Hyp ratios of ~ 1:1 and ~ 2:1 respectively (Table 6). Overall, the sugar compositions and Hyp-arab profiles of asparagus HRGPs are more like the dicot than the graminaceous monocot. Also interesting are the Gal:Ser ratios of SA1 vs SA2. We do not know which or how many serine residues are galactosylated. It has been previously seen that serine residues are attached to a single galactose residue (Lamport *et al.*, 1973). Based on my data it appears that approximately half of the serine residues of SA2 are glycosylated (Gal:Ser ~ 0.6:1). SA1, on the other hand, has a Gal:Ser ratio of ~ 1:1. This suggests that possibly all serine residues of SA1 are galactosylated or that some of the Ser-gal is in di-galactoside or polygalactoside form. Desai *et al.* (1981) presented evidence of di-galactosyl-serine in a Hyp-rich lectin from *Datura stramonium*. The maize HHRGP Gal:Ser ratio of 5:1 suggests the occurrence of polygalactosyl-serine (Kieliszewski, 1989). If this is the case, it would not be unexpected to see some of this component in other monocot walls.

D. Crosslinking with Tomato Acidic Peroxidase

Another focus in the lab is on the crosslink in the primary cell wall. Tomato acidic peroxidase crosslinks monomers of tomato P1 and P2 extensins, carrot extensin, and Ginkgo (a primitive gymnosperm) (D.T.A. Lamport & B. Upham, personal communication). An assay was developed, using Superose-6 gel filtration (Everdeen *et al.*, 1988), which shows loss of the monomeric component with concommitant increase of higher molecular weight material upon

incubation of the monomeric tomato P1 and P2, and the Ginkgo HRGP with this peroxidase. Several additional substrates (maize THRGP and HHRGP; sugar beet P1; Douglas Fir PHRGP; and asparagus SA1 and SA2) have also been assayed and showed no crosslinking (B. Upham, personal communication). Without knowledge of the actual crosslink occurring in vitro and in muro it is impossible to know why some extensins crosslink and others do not. It is notable that these HRGPs which do not crosslink are from walls which are Hyp-poor—tomato, carrot, and Gingko have Hyp-rich walls.

In asparagus some Hyp remains insoluble after HF deglycosylation. The HF deglycosylated wall is enriched in Hyp (dw and mole%) compared with the intact wall (Tables 12 & 14). In maize, it appears that most of the HRGP component is solubilized after deglycosylation (Kieliszewski, 1989). Amino acid compositions of HF deglycosylated Douglas Fir, and sugar beet walls have not been examined. Likewise there are no amino acid compositions available for any Gingko wall fractions—intact walls, HF deglycosylated walls, or HRGPs. Due to crosslinking of Ginkgo HRGP by tomato acidic peroxidase, study of the Gingko wall could be especially enlightening in the search for a crosslink domain.

Lack of crosslinking of asparagus HRGPs in vitro, but presence of IDT in the wall suggests intramolecular IDT—but how are these HRGPs bound in the wall? On the other hand, there may be a different enzyme—perhaps another peroxidase—which crosslinks asparagus HRGP monomers. Perhaps IDT is an intermolecular crosslink in these other species (asparagus, maize, sugar beet, and

Douglas Fir), but narrow substrate specificity of tomato acidic peroxidase prevents the enzyme from crosslinking these other HRGPs. Since asparagus contains IDT, this offers another system in which to study crosslinking (perhaps other species mentioned here also contain IDT). At this time there is no evidence of intermolecular IDT—even in tomato where most of this study has focused. Perhaps one of these other species may provide a better system to study intermolecular crosslinking or to look for intermolecular IDT.

E. Peptide Sequence Data: SA1 M6 and SA2 M4

Two interesting peptides have been sequenced from the salt-elutable HRGPs of asparagus. The major tryptide, M6, from asparagus SA1 gave the following sequence: His-Lys-Pro-Hyp-Hyp-[Ser-Ser-His-*Leu-Pro*]-*Hyp*-*Hyp*-Ile-Tyr. Three features of this sequence are of interest. First, the Lys-Pro-Hyp-Hyp sequence recurs twice in the forty-five residue peptide from the Douglas Fir PHRGP (Lys-Pro-Hyp occurs two additional times) (Kieliszewski, unpublished data). Significance of this sequence is unknown, but from its repetitiveness in the PHRGP it is likely that there is some structural importance. Another tetra-peptide sequence in this tryptic peptide is *Leu-Pro-Hyp-Hyp* . X-Pro-Pro-Pro (where Pro can also be Hyp) proves to be a common motif. This motif also occurs in SA2 M4 as Ser-Hyp-Hyp-Hyp, in maize HHRGP as Ala-Hyp-Hyp-Hyp and Ser-Hyp-Hyp-Hyp, in sugar beet as Tyr-Pro-Hyp-Hyp, and in Douglas Fir as Lys-Pro-Hyp-Hyp and Ile-Pro-Pro-Hyp. Lack of these tetra-peptide sequences distinguishes the advanced dicot wall from

Table 16. X-Pro-Pro-Pro Motifs of Asparagus, Maize[a], Sugar Beet[b], and Douglas Fir[c]

Asparagus:	SA1M6	Lys-Pro-Hyp-Hyp Leu-Pro-Hyp-Hyp
	SA2M4	Ser-Hyp-Hyp-Hyp
Maize:	HHRGP "	Ser-Hyp-Hyp-Hyp Ala-Hyp-Hyp-Hyp
Sugar beet:	P1	Tyr-Pro-Hyp-Hyp
Douglas Fir:	PHRGP	Ile-Pro-Pro-Hyp Lys-Pro-Hyp-Hyp

[a] Kieliszewski, 1989
[b] Li *et al.*, 1990
[c] Kieliszewski, unpublished data

the other walls studied. Evolution of extensins is a major focus in this lab, particularly identification of primitive extensin repetitive motifs. Table 24 (Appendix) shows peptide sequence data from tomato, maize, asparagus, sugar beet, and Douglas Fir. How has this wall component evolved? What are the essential characteristics of extensins? Additional sequence data from these HRGPs should aid answering these questions.

Another interesting sequence within M6 is [Ser-Ser-His-Leu-Pro]. Lamport has described "a split block extensin" (Li *et al.*, 1990). This describes the splitting of the Ser-Hyp$_4$ motif with an

insertion/deletion sequence either between the second and third Hyp residues or after the Ser-Hyp_4 block. Tomato, maize, and sugar beet each exhibit this phenomenon with their own specific insertion sequence (Figure 2, Introduction, II). The characteristics common to each of these sequences are (a) short length (5 or 6 residues), (b) termination with proline, and (c) location within or after the Ser-Hyp_4 block. This five-residue asparagus sequence ascribes to these characteristics, suggesting that SA1 may be a non-graminaceous "split block extensin".

The second peptide, SA2 M4, is: Ser-Hyp-Hyp-Hyp-Ser-Hyp-Val-Lys-Pro-Thr-Pro-Arg. This is a very interesting peptide. Below, this sequence is aligned with sequences of a tomato peptide (P1 H_{20}) and also a maize peptide (THRGP TC2).

Tomato H_{20}: Ser—Hyp—Hyp—Hyp — Hyp—Val—Lys—Pro

Asp. SA2 M4: Ser-Hyp-Hyp-*Hyp-Ser-Hyp-Val-Lys-Pro-Thr-Pro*—Arg

Maize THRGP TC2: *Hyp-Ser-Hyp — Lys-Pro-Thr-Hyp*

SA2 M4 shows homology with both of these sequences from tomato and maize. The tomato sequence shares eight residues in common with SA2 M4 and the maize sequence shares seven amino acids. Only the insertion/deletion of a single serine residue differentiates asparagus and tomato sequences. Similarly, only an insertion/deletion of a valine residue differentiates asparagus and

maize sequences (hydroxylation of proline is a post-translational event). Since extensins frequently contain insertions and deletions, we ignore these single amino acid insertions or deletions when considering homology. The odds against eight amino acids being identical by chance is 8^{20}—for seven amino acids, 7^{20}. Thus this non-graminaceous sequence appears to bridge these dicot and graminaceous sequences.

These asparagus HRGPs appear to be less repetitive than other HRGPs (amino acid compositions are not extremely biased, dSA1 tryptic peptide map is relatively complex). Although both were major peptides, whether SA1 M6 and SA2 M4 represent major repetitive motifs will be unknown until further sequence data are obtained.

II. Analyses of Covalently Bound Wall Glycoprotein

A. Amino Acid Analysis of Asparagus Wall

Amino acid analyses of the asparagus cell wall showed a very similar composition to the graminaceous cell wall (Table 12, Results; Table 18, Appendix). The amounts of Hyp, Asx, Ser, Glx, Gly, Ala, Ile, Leu, and Tyr are very different compared with the advanced dicot, tomato (sugar beet and Douglas Fir also have amino acid compositions similar to these monocots). Not as drastically different, but still distinguishable, are the amounts of Phe and Lys. The only amino acids which are in comparable amounts in the tomato and

these other cell walls are Thr, Pro, Val, His, and Arg. These data show Hyp-rich walls of the advanced dicots to be exceptional. The Hyp-poor protein which dominates these other walls differs greatly in composition from extensins. More data from this Hyp-poor (glyco)protein is required before a model of these other primary cell walls can be constructed.

B. Protein and Hydroxyproline Content of the Asparagus Wall

The protein content of the asparagus cell wall was a surprisingly high ~ 20% (dw). The hydroxyproline content was 0.45% - 0.57% Hyp (dw) (Table 14, Results III.,E.). A survey of several (graminaceous) monocots showed protein and Hyp contents of 7% - 17% (dw) and < 0.05% - 0.16% (dw) respectively (Burke *et al.*, 1974). Dicot cell walls generally contain 5% to 10% protein (dw) (Darvill *et al.*, 1980) and ~ 0.2% to 2.7% Hyp (dw) (Showalter & Varner, 1989). Though there is a wide variability within both the graminaceous monocots and the dicots surveyed with respect to protein and hydroxyproline contents, these results in combination with HF-insoluble wall data (Table 14, Results III.,E.) indicate that more HRGP is bound into the asparagus wall than into the maize (graminaceous) wall. The function of HRGP in these Hyp-poor walls is unknown. The small amount suggests that they may play other than a structural role—perhaps a stress or disease related function?

C. Hydroxyproline Insolubilization in the Wall

Additional evidence from HF deglycosylation of the asparagus wall supports the observation of HRGP being covalently bound. The existence of Hyp-rich and Hyp-poor wall (glyco)protein networks is seen by the extremes of tomato and maize wall-bound proteins respectively (Table 11, Results; Table 20, Appendix). Deglycosylation of the maize wall resulted in loss of Hyp on a mole% basis (on a dw basis, the Hyp content remains approximately the same) (Kieliszewski, 1989) and three tryptic peptides from HF-insoluble maize wall are devoid of Hyp (Table 17). Deglycosylated asparagus wall, on the other hand, was enriched in Hyp (mole% and % dw bases) (Tables 12 & 14, Results) though the content was far less than in tomato walls. Also, Pro3 (a pronase cleaved peptide from the asparagus cell wall) contains ~ 20 mole% Hyp (Table 17; Table 15, Results). Therefore, the covalently bound asparagus wall contains components of both types of wall network. A question remaining is whether or not the components are from two independent or one integrated network(s).

D. The HF-Soluble Wall

The HF-soluble asparagus wall was not extensively studied. Amino acid analysis (performed once) of this fraction (Table 12, Results) showed 19.7 mole% glycine. The maize HF-soluble wall contained ~ 12 mole% glycine (Kieliszewski, 1989); the same amount as seen in the intact and HF-insoluble fractions. In some plants with low amounts of hydroxyproline, there are glycine-rich proteins

Table 17. Comparison of Peptides from Asparagus and Maize[b] Walls

Amino[a]	Asparagus				Maize			
	HF-Insol. Acid	(+/-)	Pro3 Wall	(+/-)	HF-Insol. Wall	Peptide 1	Peptide 2	Peptide 3
Hyp	4.8	2.2	20.6	0.1	trace	0.0	0.0	0.0
Asx	8.2	1.2	7.2	0.8	10.6	8.0	8.1	13.2
Thr	4.5	0.4	3.6	0.3	5.3	7.3	3.4	5.6
Ser	6.3	0.2	8.4	0.5	6.3	15.5	9.0	9.9
Glx	8.7	0.6	7.8	0.4	10.8	12.1	7.0	12.9
Pro	6.6	0.3	16.4	1.2	4.7	3.4	6.3	2.2
Gly	12.7	1.2	9.3	1.4	10.8	17.6	14.9	13.3
Ala	9.1	0.7	3.0	0.1	10.8	4.0	7.3	9.0
Val	5.1	0.5	3.1	0.4	7.6	9.0	18.4	9.8
Cys	n.d.		n.d.		0.3	0.0	0.0	n.d.
Met	1.2	0.2	0.0	0.0	0.1	0.0	0.0	0.0
Ile	4.2	0.4	2.4	2.4	0.3	1.5	2.8	1.0
Leu	8.3	0.8	4.2	0.3	9.0	5.1	4.5	2.6
Tyr	1.4	0.4	0.7	0.0	2.0	2.8	0.0	0.8
Phe	3.5	0.5	4.1	0.4	4.8	3.1	1.4	1.2
Lys	6.3	0.6	4.9	0.2	7.0	3.8	8.6	10.4
His	3.7	1.7	2.5	0.0	2.6	1.4	3.2	3.0
Arg	5.2	0.3	2.1	0.4	6.0	5.4	5.1	5.0

[a] Represented as Mole %

[b] Kieliszewski M, 1989

(GRPs) located in the wall (Condit & Meagher, 1986; Keller *et al.*, 1989[b]). These proteins (> 60 mole% glycine) are presumed to be structural and may take the place of HRGPs. The asparagus wall appears likely to contain a GRP(s). Interestingly, proline and glycine are encoded by complementary codons: proline predominantly encoded by CCA, and glycine predominantly encoded by GGT. GRPs have been cloned from maize (Gomez *et al.*, 1988) and rice (Mundy & Chua, 1988). The glycine codons specified for the maize GRP clone are mainly GGC while the proline codons for the maize THRGP are predominantly coded by CCG. Therefore it could be possible that through gene duplication and inversion the noncoding strand for HRGPs has given rise to GRP genes (or vice-versa) (Keller *et al.*, 1988). To date no extensin gene has been found to be transcribed in the reverse orientation (Showalter & Rumeau, 1990). Whether or not there is any relationship between these proteins will require additional study.

E. IDT Detection in the Asparagus Wall

The absence (or extremely low level) of IDT and loss of HRGP seen in maize with HF treatment (Kieliszewski, 1989) indicate non-IDT crosslinks in the Hyp-poor wall (glyco)protein. This also suggests that Hyp-rich and Hyp-poor components comprise two different networks which are not covalently crosslinked with each other. Why is HRGP crosslinked into asparagus walls and not into maize walls? A possible explaination lies in the amount of HRGP present. Perhaps the higher amount of HRGP in asparagus walls allows formation of a network which cannot be achieved by the lower amount found in

maize. Possibly maize HRGPs do not crosslink! If they do not crosslink, what is their function? Again, a suggestion is that these HRGPs may be involved in stress response or disease resistance.

The Hyp:IDT ratio (66:1) of asparagus is higher than that of tomato walls. The location of IDT, whether in Hyp-rich, Hyp-poor, or both (glyco)protein components, is unknown. Indication of its presence in Pro3 (pronase wall peptide #3) supports the expectation of IDT in the HRGP component, while lack of IDT in the Hyp-poor protein of the maize cell wall suggests that the asparagus wall Hyp-poor protein probably does not contain IDT. (Kieliszewski [1989] reports another possible "tyrosine derivative" in the maize wall) Supposing IDT to be an intermolecular crosslink, the higher Hyp:IDT ratio could reflect a less dense HRGP network. The presence of IDT in both asparagus and tomato, and absence in maize (wall and salt-elutable HRGPs) suggests that the asparagus wall is more closely related to the dicot wall than is the graminaceous maize wall. The greater insolubilization of HRGP into the asparagus wall (than into the maize wall) also supports this relationship. The asparagus wall also contains a component which elutes from the Hamilton PRP-1 with approximately the same retention time of this unknown component from the maize wall. Perhaps this is another candidate for a Hyp-poor/IDT-poor wall crosslink.

Upon analysis of the IDT obtained from the asparagus wall, there are some slight discrepancies with the data from Epstein & Lamport (1984). The spectral data show identical maxima and minima in acid and alkali, but when the same amount of material is

examined (from asparagus) the magnitudes of the two spectra differ proportionately from the previous data. Also, the peak/valley ratio reported by Epstein & Lamport (1984) in acid is approximately twice that which I observed. I used the same concentration of material when plotting acid and alkali spectra. I assumed that Epstein also used the same concentration, but this was not explicitly stated, so I cannot be positive. The difference in the peak/valley ratios indicates that I might have some contaminant in the asparagus IDT which absorbs at A_{254} (This contaminant does not appear to be present in the IDT which I prepared from tomato walls).

F. Hyp-arab Profile of the Asparagus Wall

The tomato wall Hyp-arab profile was very similar to the average of tomato P1 and P2. Sequences from tomato P1, P2, and P3 have all been found in the deglycosylated tomato cell wall indicating their covalent crosslinkage. On the other hand, the maize wall profile is very much like that of the HHRGP, suggesting that the HHRGP is the major wall-bound HRGP (not the major protein component!) in maize. Unfortunately, no sequence data are available from the maize wall to confirm this suggestion.

The Hyp-arab profile of the asparagus wall is very similar to that from the tomato cell wall. However, the average of SA1 and SA2 profiles does not equal that of the asparagus wall. I propose two explanations: 1) these profiles have only been performed once and it is possible that the amounts of Hyp-Ara_4 and Hyp-Ara_3 are

underestimated due to cleavage of glycosidic bonds (this would indicate that SA1 and SA2 are even more dicot-like than the data suggest), or 2) there is at least one additional HRGP (preparative Superose-6 fraction 2) which may be more dicot-like and might be preferrentially crosslinked into the wall. Sequence data from the wall, and characterization of this third HRGP will be required to resolve the wall HRGP composition.

Table 18. Comparison of Asparagus Wall Hyp-Arab Profile with Averaged Values from SA1 and SA2

	Average (SA1+SA2)	Wall	difference (x HRGPs - wall)
Free Hyp	23	8	+15
Hyp-Ara1	14	5	+9
Hyp-Ara2	6	4	+2
Hyp-Ara3	33	32	+1
Hyp-Ara4	25	50	-25

Little is known about the glycosyl transferases which attach arabinose and galactose residues to hydroxyproline and serine respectively. This posttranslational modification occurs in the Golgi, and at least three different arabinosyl transferases are presumed to sequentially add arabinose residues to peptidyl hydroxyproline (Karr, 1972; Owens & Northcote, 1981; Sadava & Chrispeels 1978;

Showalter & Varner, 1989). Since the arabinosylation profile is one of the major differences between the graminaceous monocots and the dicots, one might expect some differences in the activity and/or specificity of these glycosyl transferases. The graminaceous monocot may have lower arabinosyl transferase and higher galactosyl transferase activities. Asparagus would seem to have glycosyl transferase activities more similar to the dicots. The apparent importance of posttranslational modification of extensins warrants further study of these systems.

III. Summary

I used three criteria to relate the asparagus (non-graminaceous), maize (graminaceous), and tomato (dicot) wall HRGPs: 1) wall Hyp-arab profiles, 2) extensin Hyp-arab profiles, and 3) extensin neutral sugar compositions. Table 19 shows a rating scheme for these characteristics. On a scale of 1 to 5, 1 being tomato-like and 5 being maize-like, I rated asparagus (also Douglas Fir, and sugar beet where data were available) by this system. In the cases of wall Hyp-arabs and neutral sugar composition, asparagus was definitely more dicot-like. (Douglas Fir had a wall Hyp-arab profile intermediate to that of the dicot and graminaceous monocot. Sugar beet had a Hyp-arab profile much more graminaceous-like.) Extensin Hyp-arab profiles showed a gradual variation between the extremes of advanced dicot and graminaceous monocot profiles. SA1 and SA2 again resembled more closely the dicot extensins, though SA2 shows a transition toward the maize HHRGP Hyp-arab profile. The sugar beet P1

extensin Hyp-arab profile was more like that of the THRGP. The Douglas Fir PHRGP profile was even more extreme than the graminaceous profiles— due to complete lack of Hyp-ara$_4$ and very high amount of free Hyp, the PHRGP was rated a 6 (comparison of Hyp-arab profiles was based on the difference between the combined mole% of Hyp-ara$_3$ and Hyp-ara$_4$, and the mole% free Hyp).

A difficulty with comparisons of the dicot wall with these other walls is that the dicot wall protein is predominantly HRGP, whereas the major protein component of the other walls is Hyp-poor. My specific goal was to compare HRGPs. Based on the HRGP and IDT data, the asparagus primary cell wall resembles the tomato (advanced dicot) wall much more than the maize (graminaceous monocot) wall. On the other hand the major (glyco)protein component of the asparagus wall much more resembles these other walls (graminaceous, gymnosperm, and primitive dicot). Overall the asparagus (non-graminaceous) primary cell wall resembles the dicot primary cell wall more than does the maize (graminaceous) primary cell wall. The occurrence of the Hyp-poor protein in walls of such diversified (and primitive) species indicates that the monocots and dicots split very long ago—possibly as far back as the gymnosperms. This favors Martin *et al.*'s theory of the divergence occurring ~ 320 million years ago. Progenitors of the Graminae may have split early from the dicot line while other monocot lines (e.g. Liliiflorae) split later after attaining some of the more dicot-like

Table 19. Rating of the Carbohydrate Characteristics of Wall Components.

	1 (Dicot-like)	2	3	4	5 (Graminaceous monocot-like)	6
Wall Hyp-arabs	Tomato, Asparagus		D. Fir		Maize Sugar beet	
Neutral Sugars	Tom. P1, P2, Asp. SA1, SA2				Maize THRGP, HHRGP	
Extensin Hyp-arabs	Tom. P1,P2	Asp. SA1	Asp. SA2	Maize HHRGP	Maize THRGP Sugar beet P1	D. Fir PHRGP

characteristics (e.g. high pectin content, highly arabinosylated HRGPs, and IDT). For the sake of simplicity, most theories favor a monophyletic origin for monocot and dicot lines (i.e. monocots and dicots being derived from a singular common ancestor), but a polyphyletic origin for these species is within reason. For example, different monocot and dicot lines may have arisen from different gymnosperm ancestors.

It is commonly believed that dicots represent the main lineage of plants. Alternatively, monocots may represent the main lineage—dicots having split-off and replaced Hyp-poor with Hyp-rich structural protein. In either event, the asparagus HRGPs more closely resemble dicot HRGPs and appear to bridge graminaceous and dicot extensins. These wall data indicate that asparagus may represent a group of monocots evolutionarily intermediate to the Graminae and advanced dicot lineages. Finally, graminaceous and non-graminaceous monocot wall compositions are different and argue strongly against using graminaceous data exclusively in describing monocots.

IV. Future

This work points to several avenues for future exploration. First, additional sequence data from SA1 and SA2 would be valuable. Also, there is the third HRGP component (Superose-6 fraction 2) which should be analyzed. These data would determine the major repetitive motif(s) of asparagus extensins.

A second area for research is the crosslink in the Hyp/IDT-poor walls. The other Hyp-poor walls mentioned here (i.e. rice, Douglas Fir, and sugar beet) should be assayed for IDT. This assay should show whether IDT and/or the second putative "tyrosine-derivative" (Kieliszewski, 1989) are present in these walls. This additional putative "tyrosine-derivative" should be characterized to determine whether it might qualify as a crosslink.

Another area to be explored more broadly is extensin crosslinking. Do these HRGPs not crosslinked by tomato acidic peroxidase crosslink at all? A starting point would be to isolate another peroxidase from one of the species not crosslinked by tomato acidic peroxidase (e.g. asparagus). Then try crosslinking these other HRGPs. Is crosslinking prevented by substrate specificity? Eventually it would be beneficial to isolate peroxidases from several species and "cross-check" them with the various HRGPs, then characterize the peroxidases of interest.

Study of the major Hyp-poor wall component (already proposed by Kieliszewski, 1989) will be a major area of future research. This Hyp-poor wall will have to be characterized before any speculation toward wall models for these Hyp-poor species can be made. If Hyp-rich walls turn out to contain some of this Hyp-poor material, this research may also aid in clarifying current wall models.

Finally, more study of glycosyl transferases is warranted. The high degree of post-translational modification of extensin attests to the importance of these systems. Little is known about these

enzymes. It is presumed that there are at least three arabinosyl transferases (Showalter & Varner, 1989) due to the number of different linkages (Figure 3, Introduction II.). The possibility of di- and/or polygalactosyl-serine in maize and asparagus indicates that there may also be a complex system of galactosyl transferases. Since Hyp-arab profiles and neutral sugar compositions of dicot, non-graminaceous monocot, and graminaceous monocot HRGPs are all different, understanding the glycosyl transferase systems is important in understanding the evolution of these HRGP components.

APPENDIX

Table 20. Amino Acid Compositions of Cell Walls from Asparagus, Tomato[b], Maize[c], Sugar Beet[d], and Douglas Fir[e]

Amino Acid	Asparagus[a] Culture	(+/-)	Maize Culture	Maize Coleoptile	Sugar Beet Culture	Douglas Fir Culture	Tomato Culture
Hyp	2.1	0.5	1.1	2.6	0.4	0.8	28.5
Asx	8.7	0.5	10.4	8.9	12.0	9.4	4.0
Thr	4.9	0.1	5.1	5.2	5.1	5.3	4.6
Ser	7.0	0.7	6.9	7.4	7.3	7.5	14.2
Glx	10.1	0.6	9.3	10.2	12.0	11.8	2.8
Pro	5.8	0.3	3.7	5.0	5.1	5.4	3.9
Gly	13.2	1.1	10.7	10.5	9.5	9.5	3.3
Ala	10.4	1.6	10.6	11.6	8.7	9.0	3.2
Val	5.0	0.4	6.4	6.8	6.5	6.7	7.0
Cys	n.d.		n.d.	n.d.	n.d.	n.d.	n.d.
Met	0.8	0.2	1.7	0.4	1.6	0.7	0.8
Ile	4.1	0.4	4.2	4.4	4.8	5.0	1.8
Leu	7.6	0.6	10.3	10.2	9.7	10.6	2.5
Tyr	1.7	0.1	1.9	1.0	1.3	1.9	6.3
Phe	3.2	0.5	4.0	3.2	3.8	3.5	1.3
Lys	7.0	0.4	6.2	6.1	7.1	6.6	10.5
His	2.9	1.5	2.1	2.3	2.2	1.6	2.7
Arg	4.9	0.3	4.7	4.3	3.3	4.6	1.2

[a] Represented as Mole %

[b] Smith *et al.*, 1986

[c] Kieliszewski et al., 1989

[d] Li *et al.*, 1990

[e] Kieliszewski M, unpublished data, unpublished data

Table 21. Hydroxyproline Arabinoside Profiles of Cell Walls from Asparagus, Maize[a], Tomato[b], Sugar Beet[c], and Douglas Fir[d]

	Asparagus	Maize	Tomato	Sugar Beet	Douglas Fir
Free Hyp	8	24	5	27	10
Hyp Ara_1	5	9	10	14	6
Hyp Ara_2	4	6	9	18	6
Hyp Ara_3	32	41	28	35	45
Hyp Ara_4	50	10	48	6	20

[a] from Kieliszewski *et al.*, Kieliszewski, unpublished
[b] from Smith *et al.*, 1986
[c] from Li *et al.*, 1990
[d] from Kieliszewski *et al.*, submitted

Table 22. Amino Acid Compositions of Extensins from Asparagus, Maize[a], Tomato[b], Sugar Beet[c], and Douglas Fir[d]

Amino Acid	Asparagus		Maize		Tomato	Sugar Beet	Douglas Fir	
	SA1	SA2	THRGP	HHRGP	P1	P1	PHRGP	SP1
Hyp	27.8	21.4	24.8	34.9	32.7	33.8	28.1	29.3
Asx	4.1	3.4	0.3	1.3	1.4	0.6	0.0	0.0
Thr	4.4	6.9	25.3	7.9	6.2	6.7	1.5	4.5
Ser	8.5	9.2	7.3	7.3	9.8	9.6	2.5	6.8
Glx	5.1	3.9	2.3	2.1	1.5	3.4	0.0	2.9
Pro	8.7	8.0	14.5	6.8	9.6	6.1	21.3	14.2
Gly	5.1	6.6	2.4	3.1	1.7	1.4	0.6	2.0
Ala	3.0	5.1	1.7	8.9	2.9	1.5	0.0	4.1
Val	1.7	8.2	0.7	1.5	8.3	10.8	17.3	6.5
Cys	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Met	0.0	0.1	0.0	0.0	0.0	0.3	0.0	0.0
Ile	3.4	2.7	0.1	0.0	1.0	0.5	8.9	1.8
Leu	4.4	4.5	0.2	0.0	1.0	0.6	0.0	1.8
Tyr	5.0	2..5	3.9	4.4	7.7	9.4	4.9	4.6
Phe	1.2	2.1	0.1	3.5	0.0	0.0	0.0	0.7
Lys	5.7	9.6	13.5	3.5	9.5	9.6	11.0	14.9
His	9.7	2.4	2.4	13.4	6.1	5.6	1.2	6.0
Arg	2.3	3.4	0.1	1.3	0.7	0.1	1.9	0.6

[a] from Kieliszewski *et al.*, Kieliszewski, unpublished
[b] from Smith *et al.*, 1986
[c] from Li *et al.*, 1990
[d] from Kieliszewski *et al.*, submitted

Table 23. Hydroxyproline Arabinoside Profiles of Extensins from Asparagus, Maize[a], Tomato[b], Sugar Beet[c], and Douglas Fir[d]

	Asparagus		Maize		Tomato	Sugar Beet	Douglas Fir
	SA1	SA2	THRGP	HHRGP	P1	P1	PHRGP
Free Hyp	17	28	48	20	12	32	73
Hyp Ara_1	15	13	15	8	9	31	9
Hyp Ara_2	6	6	6	9	8	17	5
Hyp Ara_3	34	32	25	42	33	16	11
Hyp Ara_4	28	21	6	21	38	4	0

[a] from Kieliszewski *et al.*, Kieliszewski, unpublished
[b] from Smith *et al.*, 1986
[c] from Li *et al.*, 1990
[d] from Kieliszewski *et al.*, submitted

Table 24. Peptide Sequences from Tamato[a], Sugar Beet[b], Maize[c], Asparagus, and Douglas Fir [d]

Source	Peptide	Sequence
Tomato P1:	H5	Ser Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys
	H20	Ser Hyp-Hyp-Hyp-Hyp-[Val-Lys-Pro-Tyr-His-Pro]-Thr-Hyp-Val-Tyr-Lys
Sugar Beet P1:	H1	Ser-Hyp-Hyp-Val-His-Lys
	H2	Tyr-Pro-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys
	H4	Ser Hyp-Hyp-[Val-His-Glu-Tyr-Pro]-Hyp-Hyp-Thr-X-Val-Tyr-Lys
	H5	Ser Hyp-Hyp-[Val-His-Glu-Tyr-Pro]-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys
Maize THRGP:	TC1	Thr-Hyp-Thr-Hyp-Val-Ser-His-Thr-Hyp-Ser Hyp-Hyp-Hyp-Hyp-Tyr
	TC2	Thr-Hyp-Ser-Hyp-Lys-Pro-Thr-Hyp-Hyp-Thr-Tyr
	TC4	Thr-Hyp-Ser-Hyp-Lys-Pro-Hyp-Thr-Hyp-Lys-Pro-Pro
	TC5	Thr-Hyp-Ser-Hyp-Lys-Pro-Hyp-Thr-Pro-Lys-Pro-Thr-Hyp-Hyp-Thr-Tyr
	TC6	Thr-Hyp-Thr-Hyp-Lys-Pro-Hyp-Ala-Thr-Lys-Pro-Pro-Thr-Tyr
	TC7	Thr-Hyp-Ser-Hyp-Lys-Pro-Hyp-Thr-His-Pro-Thr-(Pro)
Maize HHRGP:	HC1	Ala-Hyp-Hyp-Ala-Pro-Ala-Pro
	HC2	Ser-Hyp-Hyp-Ala-His-His
	HC3	Phe-Ala-Hyp-Hyp-Hyp-Ala-His-Tyr
	HC4	Ala-Hyp-Hyp-Hyp-Tyr-Phe-Pro-Hyp(Thr)-Hyp
	HC6	Ser-Hyp-Hyp-Hyp-His-Ser-Hyp-Ser-Hyp-Gly
	HC10	Ala-Hyp-Hyp-Hyp-His-Phe-Pro-Ser-Hyp-Hyp-Ala-Ala-Asn-Ala-Ala-Hyp-Hyp-Hyp-Ala-His-Tyr
	HC12	Ala-Ala-Hyp-Hyp-Hyp-His-Phe-Pro-Ser-Hyp-Hyp
Asp. SA1:	M6	His-Lys-Pro-Hyp-Hyp-Ser-Ser-His-Leu-Pro-Hyp-Hyp-Ile-Tyr
SA2:	M4	Ser-Hyp-Hyp-Hyp-Ser-Hyp-Val-Lys-Pro-Thr-Pro-Arg
Douglas Fir PHRGP:		Lys-Pro-Hyp-Val-Hyp-Val-Ile-Pro-Pro-Hyp-Val-Val-Lys-Pro-Hyp-Hyp-Val-Tyr-Lys-Pro-Hyp-Val-Hyp-Val-Ile-Pro-Pro-Hyp-Val-Val-Lys-Pro-Hyp-Hyp-Val-Tyr-Lys-Pro(Ile)-Pro-(Hyp)-Hyp-Val-Ile-Lys-Pro

[a] Smith *et al.*, 1986 [b] Li *et al.*, 1990 [c] Kieliszewski *et al.*, 1990; Kieliszewski, unpublished data
[d] Kieliszewski *et al.*, submitted

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