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ISOLATION AND CHARACTERIZATION OF EXTENSINS FROM THE GRAMINACEOUS MONOCOT *ZEA MAYS*

by

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF EXTENSINS FROM THE GRAMINACEOUS MONOCOT *ZEA MAYS*

by

Marcia J. Kieliszewski

Graminaceous monocots generally contain low levels of hydroxyproline-rich structural glycoproteins (HRGPs). As HRGPs are at the cell surface, I used the intact cell elution technique (100 millimolar $AlCl_3$) to isolate soluble surface proteins from *Zea mays* cell suspension cultures. Further fractionation of the trichloroacetic acid-soluble eluate on the cation exchangers phospho-cellulose and BioRex-70 gave several retarded, hence presumably basic fractions, which also contained hydroxyproline (Hyp). These fractions yielded three HRGPs after a final purification step on Superose-6 gel filtration. As one of the HRGPs was unusually rich in threonine (25 mole%), it was designated a threonine-rich HRGP (THRGP). It contained about 27% carbohydrate occurring exclusively as O-arabinosylated Hyp, with 48% Hyp nonglycosylated. SDS-PAGE gave an apparent M_r of 72 kD for the THRGP and 52 kD for the deglycosylated THRGP. The other two HRGPs co-chromatographed, but resolved after HF deglycosylation, into two bands

(M_r = 68 & 70 kD) on SDS-PAGE. They were rich in histidine, thus designated HHRGPs, and contained 65% carbohydrate occurring as O-arabinosylated hydroxyproline (63 mole% Ara) and galactose (37 mole%) possibly occurring as polygalactose on threonine or serine. THRGP and HHRGP did not react with Yariv artificial antigen, or agglutinate rabbit reticulocytes, implying that the THRGP and HHRGPs are not arabinogalactan proteins or lectins. Furthermore, the THRGP and HHRGPs were not in the polyproline-II conformation, judging from circular dichroic spectra, although transmission electron microscopy (TEM) showed them as extended rods.

Quantitative ELISAs showed that antibodies raised against the THRGP cross-reacted with tomato extensins P1 and P2; conversely, antibodies raised against the tomato extensins cross-reacted with the THRGP and the HHRGPs, thus suggesting homology between tomato extensin and the maize HRGPs.

HF-solvolysis of cell walls from maize coleoptile, root, and root tip, released deglycosylated THRGP detected on SDS-PAGE immunoblots with the anti-THRGP antibodies raised against the intact THRGP.

Chymotryptic digestion of the deglycosylated HHRGPs gave a peptide map with 12 peptides, one of which contained the sequence:
Ser-Hyp-Hyp-Hyp-His-Ser-Hyp-Ser-Hyp, and three peptides containing the decameric sequence: Ala-Hyp-Hyp-Hyp-His-Phe-Pro-Ser-Hyp-Hyp. Both sequences share homology (88 and 66% respectively) with the N-terminal portion of the Type 3 dicot extensin domain:

Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-Tyr-Tyr-Tyr-Lys, thereby defining the HHRGPs as members of the extensin family.

Chymotryptic digestion of the deglycosylated THRGP gave a peptide map dominated by the hexadecapeptide TC5: Thr-Hyp-Ser-Hyp-Lys-Pro-Hyp-Thr-Pro-Lys-Pro-Thr-Hyp-Hyp-Thr-Tyr, which contains a specific tryptic-labile lysylproline bond, a noteworthy exception to the generally trypsin resistant Lys-Pro bond. Furthermore, the repetitive motif Ser-Hyp-Lys-Pro-Hyp-Thr-Pro-Lys is homologous with the decameric Type 1 domain of dicot extensins: Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys, modified by a Lys for Hyp substitution at residue 3, a Val-Tyr deletion at residues 8 and 9, and incomplete posttranslational modification of proline residues. One of the minor peptides contained the 8-residue sequence: Thr-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-Tyr which is homologous with the major repetitive motif of the Type 3 domain of dicot extensins. These results show that maize THRGP and HHRGPs are homologous with the dicot P1 and P3 extensins, and as such, are the first HRGPs and first extensins isolated from a monocot.

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LIST OF ABBREVIATIONS

Ara	Arabinose
AP	Alkaline phosphatase
AGP	Arabinogalactan protein
BSA	Bovine serum albumin
CAPS	Chromatography applications package software
CD	Circular dichroism
CP1, CP2, CP3	Cellex-P peak 1, 2, or 3
dHHRGP, dTHRGP, dP1, dP2	deglycosylated HHRGP, etc.
dw	Dry weight
EDTA	Ethylene-diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbant assay
FPLC	Fast protein liquid chromatography
GAX	Glucuronoarabinoxylan
GLC	Gas liquid chromatography
HC1, HC2 etc.	HHRGP chymotryptic peptide 1 etc.
HF	Anhydrous hydrogen fluoride
HFBA	Hepta-fluorobutyric acid
HHRGP	Histidine hydroxyproline-rich glycoprotein

HPLC	High pressure liquid chromatography
HRGP	Hydroxyproline-rich glycoprotein
Hyp	Hydroxyproline
IDT	Isodityrosine
IBM	International business machines
kD	Kilodalton
M_r	Relative molecular weight
OPA	Ortho-phthalaldehyde
P1	Tomato extensin precurser P1
P2	Tomato extensin precursor P2
PCV	Packed cell volume
PEGS	Polyethylene glycol succinate
PRP	Polystyrene reversed-phase
PTH	Phenylthiohydantoin
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid
TC1, TC2 etc.	THRGP chymotryptic peptide 1, etc.
TEM	Transmission electron microscopy
TPCK	L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone
TT1	THRGP tryptic peptide 1

Tris	Tris(hydroxymethyl)aminomethane

INTRODUCTION

Extracellular matrices play a profound role in the development and maintenance of cells and tissues. The plant extracellular matrix, its cell wall, defines cell size, shape, growth rate, and therefore morphology (Preston, 1974); it acts as a mechanical barrier against water loss and invading organisms, is involved in aspects of solute mobility, confers disease resistance (Preston, 1974; Darvill *et al.*, 1980a; Bell, 1981; Caplan, 1987), and may be intimately involved in cell surface interactions, developmental patterns, and mitotic activities (McNeil *et al.*, 1984; Tran Thanh Van *et al.*, 1985; Caplan, 1987; Keller & Lamb, 1989). An understanding of these phenomena requires a detailed understanding of the participating molecules. Thus, the structural elucidation of cell wall components assumes importance, as molecular structure is intimately related to molecular function, intermolecular associations, and ultimately, the realization of an accurate cell wall model.

I. Primary Plant Cell Wall Components

Generally, five components (in addition to water) comprise the bulk of the plant primary cell wall (see Table 1 for an historical perspective): pectin (5-35%), cellulose (17-30%), hemicellulose (1-30%), lipid (~1%) and protein (5-12%). However, the discovery in 1960 of wall protein was a concept that some were reluctant to accept, although evidence for the occurrence of wall protein had

accumulated since 1924, when Tupper-Carey and Priestley provided histochemical evidence for cell wall protein (Tupper-Carey & Priestley, 1924; Thimann & Bonner, 1933; Preston & Wardrop, 1949; Christiansen & Thimann, 1950; Tripp *et al.*, 1951). In fact, F.C. Steward, who first discovered hydroxyproline in plant tissues (and was told by R.D. Preston that it probably occurred extracellularly, as it showed no turnover; R.D. Preston to D. Lamport, personal communication), remained skeptical of the existence of wall protein (Steward *et al.*, 1967; Steward & Pollard, 1958; Steward & Thompson, 1954). Nevertheless, cell wall proteins do indeed exist as enzymes, lectins and structural proteins with diverse identities and incompletely defined functions.

Table 1. The Plant Cell Wall and Its Components: An Historical Perspective[a]

1661	Henshaw	First observation of the plant cell wall
1665	Hooke	Named cell: cella
1667		Micrographia
1682	Grew	First cell wall model
1825	Branconnot	Pectin
1839	Payen	Cellulose
1844		Lignin
1850	von Mohl	Concept of primary & secondary cell walls
1859	Fremy	Cutin
1885		Suberin
1891	Schulze	Hemicellulose
1934	Kerr & Bailey	Definition of primary & secondary cell wall
1960	Dougall & Shimbayashi Lamport & Northcote	Cell wall protein

[a] After Preston, 1974

II. The Extensins[1]: Structure, Regulation, and Function

The bulk of the wall's protein component consists of structural protein, the dicot extensins[2] being the first discovered (Dougall & Shimbayashi, 1960; Lamport & Northcote, 1960) and the best characterized. Extensins are a class of hydroxyproline-rich glycoproteins (HRGPs)[3] (Table 2) integral to the cell walls of higher plants; so integral, in fact, that in their "mature" wall-bound form, their complete insolublity in salts (Stuart & Varner, 1981), detergents (Fry, 1982), phenol (Fry, 1982), cold acids and alkalis (Blashek *et al.*, 1981), chelating agents (Muray & Northcote, 1978), and anhydrous HF (Mort, 1978) has been a major hindrance to their characterization. Nevertheless, a great deal of what we currently know about the chemistry and structure of extensin was obtained by characterizing isolated cell walls and glycopeptides released from enzyme-treated cell walls.

A. Extensin Structure

Analyses of peptides, and later of salt-extractable extensin monomers, confirmed the earlier cell wall amino acid analyses of Dougall & Shimbayashi (1960) and Lamport & Northcote (1960): extensin is basic, being rich in lysine, as

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[1] This section is a literature review as it pertains directly to this thesis. A detailed chronology of seminal experiments and concepts in extensinology is presented in Appendix A. Appendix B deals with the recently discovered Glycine-Rich Proteins.

[2] Extensin was so named because Lamport hypothesized a role for extensin in the regulation of cell extension growth (Lamport, 1963). To date, no one has proven his hypothesis wrong, or right.

[3] Although extensins are HRGPs, not all HRGPs are extensins. Two other classes of HRGPs are the solanaceous lectins, and the arabinogalatan proteins (AGPs), which are distinguishable from extensins by their chemical compositions (Table 2).

Table 2. Three Classes of Hydroxyproline-Rich Glycoproteins[a]

	Extensin	Arabinogalactan Proteins	Solanaceous Lectins
Solubility	Insoluble/Soluble	Freely soluble	Freely soluble
Location	Cell wall	Intra & Extracellular	Intra & Extracellular
Most common amino acids	Hyp, Ser, Lys Tyr, Val, His	Hyp, Ala, Ser Glu, Asp	Hyp, Ser, Gly, Cys
Glycopeptide linkages	Hyp-arabinose Ser-galactose	Hyp-arabinose Hyp-galactose	Hyp-arabinose Ser-galactose
Biological Activity	Structural wall component Disease resistance? Control of cell extension? Differentiation?	Cell-cell recognition? Glues? Anti-desiccants? Morphology?[b]	Cell-cell recognition? Sugar transport?

[a] Adapted from Lamport, 1980
[b] From Basile & Basile, 1989

well as hydroxyproline, serine, valine, tyrosine (Lamport, 1969), and sometimes histidine (Stuart & Varner, 1980)(Table 3).

Extensin is a structural protein. Structural proteins frequently exhibit short term amino acid sequence periodicities (North, 1968) which, for the recently isolated soluble monomers of extensin, is reflected in their peptide profiles: they are simple and dominated by a few major repeating peptides (Figure 1). Although the insolubility of wall-bound extensin initially precluded obtaining a truly representative peptide map, enzymic digestion of tomato cell walls released only 5 peptides, accounting for 1/3 of the wall hydroxyproline (Lamport, 1969; 1977); thus those peptides occur repeatedly in extensin. Further inspection of the peptide sequences (Table 4) reveals a "sub-periodicity" within the already periodic peptides as Ser-Hyp-Hyp-Hyp-Hyp occurs at least once in each peptide.

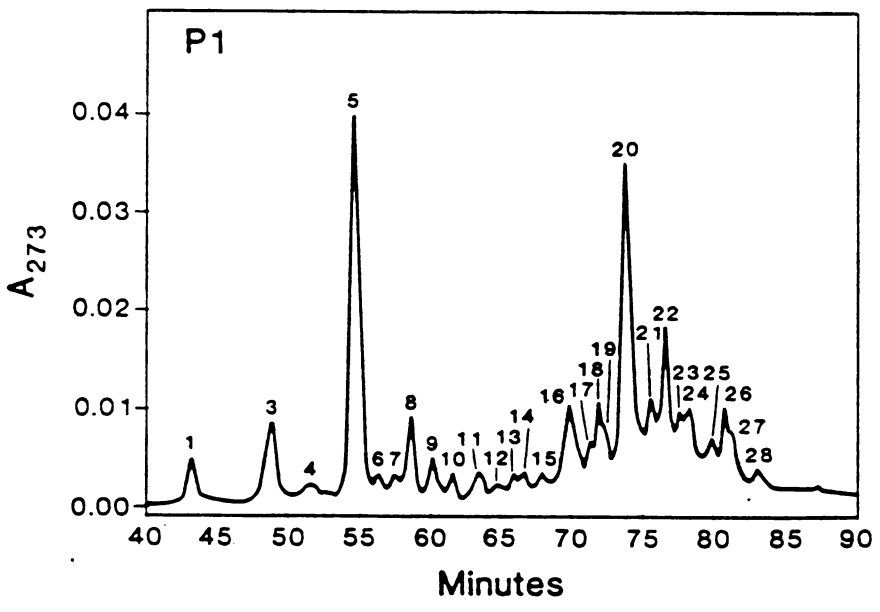

Figure 1. Tryptic peptide map of deglycosylated tomato extensin precursor P1 prior to insolubilization into the cell wall (Smith *et al.*, 1986).

Table 3. Amino Acid Compositions of Extensin Monomers[a]

Amino Acid	Carrot[b]	Melon[c]	Melon[c]	Potato[d]	Tobacco[e]	Tomato P1[f]	Tomato P2[f]
Hyp	45.5	38.0	43.0	41.7	42.2	32.7	41.8
Asx	0.3	2.7	3.2	0.7	0.4	1.4	0.7
Thr	1.2	1.2	2.7	3.2	4.9	6.2	1.0
Ser	14.0	7.0	12.3	9.4	8.3	9.8	12.1
Glx	0.3	2.7	2.4	1.1	0.6	1.5	0.3
Pro	0.9	6.2	2.1	9.2	6.8	9.6	0.8
Gly	0.4	5.2	8.6	1.1	0.4	1.7	0.3
Ala	0.4	1.7	3.8	0.9	1.9	2.9	0.5
Val	5.9	9.0	3.5	3.8	7.5	8.3	5.1
Cys	0.0	0.0	0.0	0.1	0.0	1.0	0.9
Met	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Ilu	0.3	1.1	1.2	0.3	0.3	1.0	0.9
Leu	0.3	1.5	1.9	0.2	0.0	1.0	0.2
Tyr	11.0	9.1	6.1	6.2	10.7	7.7	14.9
Phe	0.0	0.7	1.0	0.1	0.0	0.0	0.2
Lys	6.5	12.6	6.3	15.9	10.6	9.5	20.1
His	11.8	1.7	1.8	5.1	5.3	6.1	1.0
Arg	0.0	0.7	0.7	0.2	0.0	0.7	0.1
Trp	1.2	ND	ND	1.9	ND	ND	ND

[a] Expressed as mole %
[b] From Van Holst & Varner, 1984
[c] From Mazau *et al.*, 1988
[d] From Leach *et al.*, 1982
[e] Kieliszewski & Lamport, unpublished data
[f] From Smith *et al.*, 1984

Table 4. Amino Acid Sequences of Tomato Cell Wall Tryptic Peptides[a]

1.	Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-("U"-Tyr)-Lys[b]
2.	Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Lys
3.	Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys[c]
4.	Ser-Hyp-Hyp-Hyp-Hyp-Lys
5.	Ser-Hyp-Hyp-Hyp-Hyp-Val-("U"-Lys)-Lys[d]

[a] From Lamport, 1973

[b] "U" is Lamport's "unknown tyrosine derivative", isodityrosine (Fry, 1982) that occurs as an intramolecular crosslink (Epstein and Lamport, 1984). This peptide defines Type 3 extensins (Showalter & Rumeau, 1989)

[c] This peptide occurs as a major repeating peptide in tomato extensin precursor P1 (Smith *et al.*, 1986).

[d] This peptide occurs as a major repeating peptide in tomato extensin precursor P2 (Smith *et al.*, 1986).

Extensin is a glycoprotein having oligoarabinosides O-linked to hydroxyproline (Lamport, 1967), and α-O-linked galactosyl-serine (Allen *et al.*, 1978; Lamport *et al.*, 1973). In total, carbohydrate accounts for 40-65% of extensin's weight. The demonstration that hydroxyproline is O-glycosylated with arabinose oligosaccharides (1-5 residues)(Lamport & Miller, 1971; Mazau & Esquerre-Tugaye, 1986) is unique to plants, as hydroxyproline is not glycosylated in animal systems. The configuration of the tetra-arabinoside is:

α-L-Ara*f*(1-3)-β- L-Ara*f*(1-2)-β- L-Ara*f*(1-2)-β-L-Ara*f*(1-4)-Hyp

(Akiyama *et al.*, 1980), while the configuration of the other arabinosides is unknown (Figure 2).

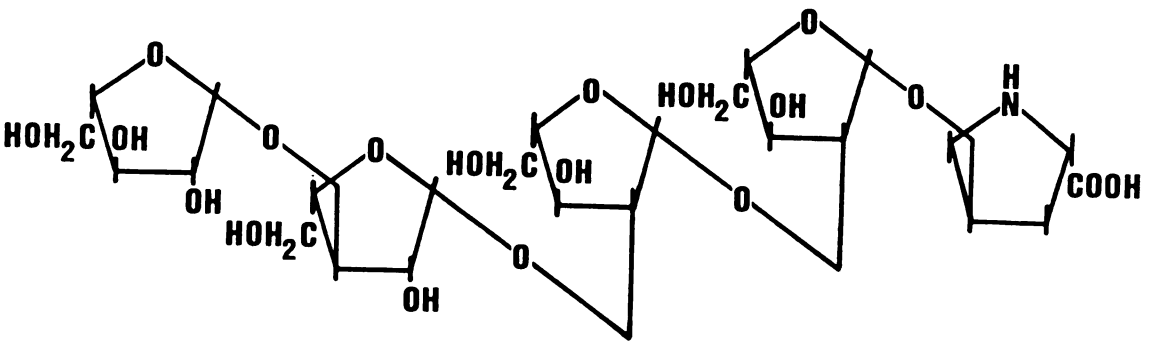

Figure 2. Hydroxyproline tetra-arabinoside

The general conformation of glycosylated extensin is a polyproline-II helix. Circular dichroic spectra, first of tomato extensin peptides, and later of carrot extensin monomers, is consistent with poly-L-hydroxyproline and poly-L-proline (a minimum at 205-206 nm and a maximum at 225-228 nm), which are both in a polyproline-II helix (3 residues/left-handed turn and 9.4 Å pitch) (Lamport, 1977; van Holst & Varner, 1984).

Extensin contains the unique amino acid isodityrosine (IDT) as an intramolecular crosslink. Lamport initially identified "an unusual modified tyrosine residue" in two extensin peptides isolated from tomato cell walls (Table 4)(Lamport, 1973). This observation eventually led to two elegant papers describing the chemical identity, localization and function of IDT. First, Stephen Fry demonstrated that cell wall hydrolysates contained a new amino acid, the diphenyl ether isodityrosine (Fig 3), and he speculated that it occurred in extensin as Lamport's "unknown" (Fry, 1982). Then, Epstein and Lamport (1984) showed that

the earlier extensin peptide "unknown" was indeed IDT (Table 4), and, surprisingly, that it served as an intramolecular crosslink rather than as an intermolecular crosslink as supposed.

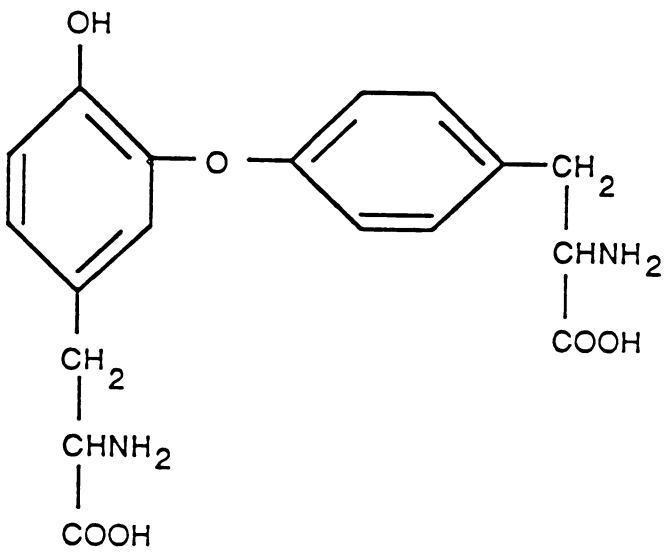

Figure 3. Isodityrosine (IDT)

Here the characterization of extensin may have stopped due to its unyielding insolubility, were it not for Maarten Chrispeels' interest in protein secretion. Employing [^{14}C] proline pulse-chase experiments, Chrispeels dubiously demonstrated that precursors of wall-bound extensin occur in the wall first as a salt soluble pool before insolubilization (Brysk & Chrispeels, 1972). Lamport initially dismissed Chrispeel's observation for three reasons: first, the pulse-chase kinetics, although suggestive, did not conclusively demonstrate turnover in the ionically-bound putative precursor; secondly, the amino acid and carbohydrate compositions of the putative precursor differed considerably from those of extensin wall peptides; thirdly, David

Pope, working with Lamport and (unfortunately) sycamore cell suspensions[4] could not repeat Chrispeels' experiments (Pope, 1977).

However, three years later Joseph Varner entered the cell wall protein field by purifying and partially characterizing the first extensin monomeric wall precursor (from carrot; Stuart & Varner, 1980). In a series of papers his laboratory not only corroborated much of Lamport's earlier wall peptide data but also made significant new contributions, most notably the cloning of the first extensin cDNA (a partial sequence from carrot)(Chen & Varner, 1985a) and genomic clone (carrot)(Chen & Varner, 1985b), thus providing information about extensin that is virtually impossible to obtain by protein sequencing: the complete sequence of the protein backbone (sans hydroxylation and IDT crosslinks). Furthermore, the clones proved that extensin is indeed a genuine protein. Some of Varner's other original contributions were the first visualization by transmission electron microscopy of extensin monomers as flexible 80 nm rods (van Holst & Varner, 1984), the demonstration that extensin mRNA levels rise in response to wounding (Chen & Varner, 1985a), and the observation that extensin monomer is insolubilized into walls concomitant with IDT formation (Cooper & Varner, 1984). Furthermore, Cassab *et al.* (1985) isolated the first putative extensin monomer from soybean seed coat and demonstrated via immunolocalization, its developmental and tissue-specific expression in the pallisade epidermal cells and hour glass cells of the soybean seed coat; and they developed a new method, tissue printing on nitrocellulose, for the

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[4] Frequently, the choice of a system can decide an experiment, as it did here. Sycamore was a poor system, as the cells have a very small pool of extensin precursors that is difficult to extract (Heckman *et al.*, 1987).

general immunological localization of soluble extensin in tissues (Cassab & Varner, 1987).

Varner's purification of a salt-soluble extensin monomer was a significant advance that prompted Lamport to reinvestigate his method for salt-elution of intact cell suspensions (Lamport, 1965), but this time using an optimal system, tomato suspensions, and (initially) an undergraduate lab aide[5]. In 1984 and 1986, Smith *et al.* rigorously demonstrated the precursor status of salt-elutable extensin monomers: ^{3}H-proline pulse-chase data clearly indicated turnover, restoration kinetics indicated precursor status, and the chemical compositions (Smith *et al.*, 1984) and, most convincingly, amino acid sequences of major peptides (Smith *et al.*, 1986) coincided with those isolated earlier from the insoluble tomato cell walls (Table 4)(Lamport, 1973). This was also the first demonstration that multiple extensins exist in a single plant system, implying a multigene family and subtle differences in function from one extensin to another. Thus Smith *et al.* (1984, 1986) suggested that "types" of extensin occur which are defined by their major repetitive sequence motifs.

Since Chen & Varner's cloning of the first extensin, numerous extensins from diverse species and tissues have been cloned, providing more details about the primary structure of the extensin polypeptides, the regulation of their expression, and therefore clues about their putative role(s) in the wall.

Combining extensin peptide and clone sequences, Showalter & Rumeau (1989) recently proposed two structural types of extensin. The types are defined,

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[5] Nathan Krupp, an undergraduate employee, did the initial precursor work.

as originally suggested by Smith *et al.* (1984,1986), by their major repeating peptides. One extensin type is predominantly composed of: Ser-Pro-Pro-Pro-Pro-Ser-Pro-Ser-Pro-Pro-Pro-Pro-Tyr-Tyr-Tyr-Lys[6] repeats (called "P3-type" extensin by Smith *et al.*, 1984, 1986); and the other type is composed of Ser-Pro-Pro-Pro-Pro-Thr-Pro-Val-Tyr-Lys repeats (called "P1-type" extensins by Smith *et al.*, 1984, 1986). Furthermore, extensins are organized into three domains distinguishable by their sequence motifs, as well as by their positions in the polypeptide: the C-terminus, the N-terminus, and the central polypeptide which is made up of the major repeating motifs (Showalter & Rumeau, 1989).

B. Regulation of Extensin Expression

Extensins and their mRNAs accumulate in tissues undergoing various stresses, such as: cell culture (Ecker & Davis, 1987; Lamport, 1965), infection (Esquerre-Tugaye & Mazau, 1974; Esquerre-Tugaye & Lamport, 1979; Showalter *et al.*, 1985), wounding (Chen & Varner, 1985a; Chrispeels *et al.*, 1974;), heat shock (Stermer & Hammerschmidt, 1987); and they accumulate in response to elicitors (Roby *et al.*, 1985, Showalter *et al.*,1985, Tierney *et al.*,1988), ethylene (Ridge & Osborne, 1970; Ecker & Davis, 1987), red light (Pike *et al.*,1979), gravity (Prassad & Cline, 1987), glutathione (Wingate *et al.*, 1988) and development (Cassab & Varner, 1985; Hong *et al.*, 1987; Franssen *et al.*, 1987; Keller & Lamb, 1989). Apparently, many of these responses are differentially regulated at a transcriptional level, however we lack the precise details about the regulation of extensin genes (Lawton & Lamb,

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[6] These sequences are derived from clones; actually, the Pro residues are Hyp as demonstrated by direct peptide sequencing (see Table 3).

1987; Wingate *et al.*, 1988; Keller & Lamb, 1989).

C. The Function of Extensin, and a Current Cell Wall Model

While structural in a general sense, the exact functions of extensin are unknown despite numerous clues pointing to their involvement in wall architecture, disease resistance, and development. The location of extensin in the extracellular matrix, its relative abundance in the wall, its insolubility, periodicity, hydroxyproline content[7], and apparent lack of enzymic activity led to a suggested role for extensin in cell wall architecture, and as an extension growth regulator via protein-carbohydrate and/or protein-protein crosslinks (Lamport, 1970). Several groups have demonstrated a positive correlation between HRGP levels and the cessation of cell elongation (Cleland & Karlsnes, 1967; Sadava *et al.*, 1973; Monro *et al.*, 1974; Klis & Eeltink, 1979).

A role for extensin in plant defense (first suggested by Esquerre-Tugaye & Mazau, 1974) is indicated by the differential accumulation of sets of extensins and their mRNAs in response to infection, elicitors, and wounding (Showalter *et al.*, 1985; Corbin *et al.*, 1987; Wingate *et al.*, 1988). Extensins are also known to agglutinate bacteria, probably due to their positive charge (Leach *et al.*,1982a), and may enhance the cell wall barrier and/or act as nucleation sites for lignin deposition (Whitmore, 1978).

Extensins may be involved in differentiation and tissue development. The distribution of extensin in different cells of the seed coat changes during seed

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[7] Hydroxyproline is a rare amino acid that usually only occurs in proteins of the extracellular matrix.

development, and extensin ultimately accumulates in the walls of the epidermal palisade and hourglass cells (Cassab & Varner, 1987). Furthermore, Hong *et al.* (1987) noted the differential expression of an extensin in the mature section of soybean hypocotyl tissue; and Keller & Lamb (1989) demonstrated the specific expression of a cell wall HRGP, probably a class of extensin, that is transiently induced in a small set of cells involved in initiation of lateral roots, and as such, may have a specialized structural function.

The periodic placement of active groups along the extensin peptide backbone raises possibilities of ionic and covalent interactions of extensin with extensin and other wall components, thus providing plausible mechanisms whereby extensin might fulfill its roles in wall architecture, disease resistance and differentiation. For example, the lysine ϵ-amino groups probably ionically pair with the pectic uronic acids, or they may form Schiff bases with the reducing ends of wall polysaccharides (but very few). Histidine imidazole rings, when protonated, may also ionically pair to negatively charged wall components, or form covalent crosslinkages, as is suggested to occur in collagen (Fujimoto *et al.*, 1982). Tyrosine residues and ferulate could oxidatively couple pectic polysaccharides (attached to ferulate by an ester linkage) to extensin (Neukom & Markwalder, 1978); and Whitmore (1978) hypothesized the *in vivo* formation of covalent bonds between coniferyl alcohol and the free hydroxyl of unsubstituted hydroxyproline.

Fry's 1982 discovery of the diphenyl ether crosslinked amino acid IDT in cell wall hydrolysates, and Epstein & Lamport's demonstration of IDT in extensin

(1984) provided a possible mechanism for extensin's insolubilization in the wall via peroxidase catalyzed IDT formation between two extensin molecules, making an intermolecular crosslink (Lamport, 1970; Fry, 1982). Thus Cooper *et al.*, (1984) and Lamport & Epstein (1983) proposed cell wall models wherein an extensin network is independently crosslinked by IDT, around cellulose microfibrils. Lamport & Epstein (1983) elaborated on the concept by likening the wall to a woven fabric; cellulose (anticlinal) constituting the fabric "warp" interpenetrating a transmural extensin (periclinal) "weft". This "warp-weft" model suggested that extensin couples the major load-bearing cellulose polymers into a defined network by IDT intermolecular crosslinks. So far, the evidence for this model is indirect:

1. Wall-bound extensin remains intact and insoluble after treatment with anhydrous HF, a reagent which specifically cleaves glycosidic linkages, leaving peptide bonds intact (Mort & Lamport, 1977).

2. IDT, extensin's unique crosslink amino acid, was discovered in cell wall hydrolysates (Fry, 1982) and extensin wall tryptides (Epstein & Lamport, 1984).

3. The demonstration of highly soluble salt-elutable extensin precursors to wall-bound extensin which contain little or no IDT (Cooper & Varner, 1984; Smith *et al.*, 1984, 1986).

4. Acid sodium chlorite oxidation of cell walls destroys phenolic rings, releasing fragments of wall-bound extensin (Mort, 1978; O'Neil & Selvendran, 1980).

5. Wall IDT content suggests a crosslink frequency sufficient to create pores large enough to enclose a cellulose microfibril (Lamport, 1986).

Thus, highly soluble extensin precursors containing virtually no IDT become insolubilized in the wall concomitant with the appearance of cell-wall IDT (Cooper & Varner, 1984). The possibility that extensin insolubility is due to covalent crosslinkage with wall polysaccharides is unlikely because extensin remains insoluble after all wall sugars are removed by anhydrous HF solvolysis. Finally, insoluble wall-bound extensin becomes soluble only after phenolic rings, such as those of IDT (and tyrosine), are destroyed by acid chlorite.

It is requisite that a cell wall model be generally applicable, at least to the primary cell walls of higher plants. Thus the question arises that begins to test the general applicability of the warp-weft model: Is extensin ubiquitous to higher plants?

D. Extensin in the Graminaceous Monocots

So far, the information known about extensin has been gathered exclusively from dicot systems with walls rich in hydroxyproline. The role of extensin (or of any HRGP) in the monocots has been largely ignored, probably because the walls of monocots, at least those of the graminaceous monocots, are hydroxyproline-poor (Burke *et al.*, 1974; Darvill *et al.*, 1980a; Lamport, 1965; Showalter & Varner, 1989).

Graminaceous walls are, in some respects, markedly different from, and yet in other respects, similar to, those of dicots. Both contain cellulose, pectins, hemicelluloses, and hydroxyproline-containing proteins; however, the quantities of

some of these components differ dramatically between the two groups (Figure 4)(Darvill *et al.*, 1980a; McNeil *et al.*, 1984; Fry, 1985). One major difference is in the acidic wall polysaccharides. The dicot wall is rich in the polyanionic gel-forming rhamnopolygalacturonans, or pectins, while graminaceous cell walls contain very little pectin (Talmadge *et al.*, 1973; Burke *et al.*, 1974). Instead, they contain the acidic polysaccharide glucuronoarabinoxylan, technically a hemicellulose, which however may have the same function in graminaceous walls as pectin does in the dicot wall (Darvill *et al.*, 1980b; Carpita, 1983, 1984; Carpita *et al.*, 1985; Fry, 1985). The graminaceous wall is also rich in "mixed-linkage" glucans (β-3 and β-4-linked), but xyloglucan-poor (1-5%), while the dicot wall contains little "mixed-linkage" glucans, but is 20-30% xyloglucan (McNeil *et al.*, 1984; Fry, 1985). Another major difference is in the protein component of the wall (Lamport, 1965; Burke *et al.*, 1974; Showalter & Varner, 1989). The dicot wall generally contains 10-20 times more hydroxyproline than the graminaceous wall[8]. However, evidence existed that the seed coat and pericarp of some monocots contained proteins possessing hydroxyproline (Van Etten *et al.*, 1963), hydroxyproline arabinosides (Lamport & Miller, 1971) and having vaguely extensin-like compositions (i.e. 11% Hyp, 10% Thr; 6% Lys)(Boundy *et al.*,1967). Also in contrast to dicot systems, hydroxyproline levels in the graminaceous monocots are apparently not related to disease resistence (Clarke *et al.*, 1981; Mazau & Esquerre-Tugaye, 1986). Thus, low levels of Hyp in

[8] Some dicot walls are Hyp-poor: for example, rose (Lamport, 1965) and sugar beet (Li *et al.*, 1989). However, dicot tissues are generally much more Hyp-rich than graminaceous tissues.

graminaceous walls, and its apparent non-involvement in disease resistance suggest, at best, graminaceous extensins which are probably structurally and functionally different from dicot extensins. This led me to ask the following questions:

1. Does extensin occur in the cell walls of the graminaceous monocots, specifically *Zea mays*?

2. Does the warp-weft cell wall model extend to the primary cell walls of the graminaceous monocots, specifically *Zea mays*?

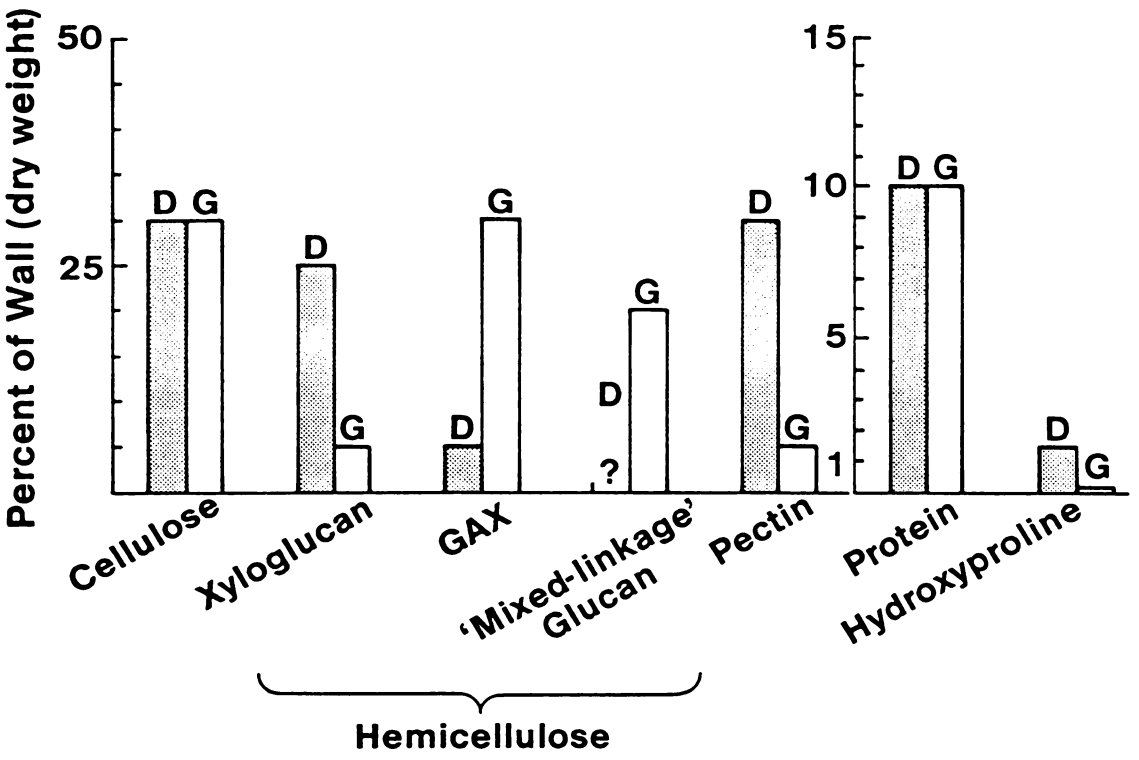

Figure 4. Dicot and graminaceous wall components compared.

MATERIALS AND METHODS

I. Methods for the Preparation of Cell Walls and Cell Wall Proteins

A. Cell Suspension Cultures

I grew maize cell suspensions (variety Black Mexican, a gift from Dr. Tom Hodges, Purdue University), in 1 L flasks containing 50 mL Murashige and Skoog medium (Murashige and Skoog, 1962) (2 mg/L, 2,4-D). They were shaken at 120 rpm on a gyrotary shaker at 27° C under 900 lux of constant fluorescent lighting, and subcultured every 11 days to an initial packed cell volume of 3%.

B. Intact Cell Elution

I prepared batches of crude HRGP from 11 day cultures (500 mL/1 L flask; 17 flasks/batch) harvested on a 2 L coarsely sintered funnel followed by a water wash, then gentle agitation of the cell pad (about 600 g fresh weight) in 1 L of 100 mM $AlCl_3$ (a nonplasmolyzing concentration) for 3 min before final suction. The eluate was reduced in volume to 100 mL at 32° C. After adding TCA (final concentration 10% w/v) to the concentrated eluate (18 hr, 4° C), centrifugation (13,000 g, 45 min, SS-34 rotor head) yielded a hydroxyproline-poor pellet (0.05% Hyp dw, discarded) and hydroxyproline-rich supernatant, which was dialyzed 72 hr at 4° C and then freeze-dried. I designate this TCA-soluble fraction 'crude HRGP'.

C. Cell Wall Preparation

Seeds of Sweet Sue sweet corn[9] (Harris Seeds) germinated for 4 days on germination paper (Anchor Paper) soaked in tap H_2O. Root tips, roots, coleoptiles with their primary leaf (henceforth I refer to this preparation as coleoptile), and whole cells of Black Mexican cell suspensions were separately frozen in N_2 (lq), ground to fine powder with a mortar and pestle, suspended in 1 M NaCl and sonicated for 10 min. The pelleted walls were washed with 5% w/v SDS followed by acetone washes (and pelleting) to remove SDS. Finally the walls were rinsed repeatedly with distilled H_2O until the walls were free of salt and cell debri, judging by microscopic examination, then the walls were freeze-dried.

D. Phosphocellulose Cation Exchange Chromatography

I dissolved crude HRGP (10 mg/mL) in 12 mM (pH 3.0) McIlvaine buffer (McIlvaine, 1921), and applied a maximum of 170 mg to a Bio-Rad Cellex-P phospho-cellulose column (15 mm i.d. x 200 mm) equilibrated with 12 mM McIlvaine buffer (pH 3.0). I eluted with a 3.0 to 6.8 pH gradient (100 mL of McIlvaine buffer) followed by a 200 mL 0 to 1 M NaCl gradient (200 mL) in 12 mM (pH 6.8) McIlvaine buffer at a flow rate of 19 mL/hr, monitoring the absorbance at 220 nm. Collected peaks were dialyzed 2 days against distilled H_2O and freeze-dried. I determined Hyp across Cellex-P peak 2 (CP2) and Cellex-P peak 3 (CP3) by collecting, concentrating and desalting 4 mL fractions in Amicon

[9] Sweet Sue was chosen because the seeds happened to be gardening "leftovers" and because they were NOT Black Mexican seeds.

centricon™ microconcentrators. Aliquots were blown down to dryness under N_2, hydrolyzed in 6 N HCl for 18 hr at 110° C, and then assayed for Hyp.

E. BioRex-70 Cation Exchange Chromatography

I dissolved freeze-dried Cellex-P Peak 2 in 2 mL 30 mM sodium phosphate buffer (pH 7.4) and applied a maximum of 20 mg to a Biorex-70 (100-200 mesh) column (8 mm i.d. x 100 mm) equilibrated with 30 mM sodium phosphate buffer (pH 7.4), and eluted with a 200 mL buffered 0 to 1 M NaCl gradient at a flow rate of 19 mL/hr, monitoring the absorbancy at 220 nm.

F. Superose-6 FPLC Gel Filtration

I injected 0.1-1 mg of dHHRGP or dTHRGP, or semi-purified HRGP in 250 μL 200 mM (pH 7.0), 0.02% azide-sodium phosphate buffer onto a Pharmacia Superose-6 FPLC gel filtration column, and eluted at a flow rate of 14 mL/hr, monitoring the absorbancy at 220 nm.

II. Methods for the Chemical Characterization of Cell Walls and Cell Wall Proteins

A. Neutral Sugar Analysis

I analyzed sugars as their alditol acetates (Albersheim *et al.*, 1967) on a Perkin-Elmer 910 Gas Chromatograph using a 6 foot x 2 mm i.d. PEGS224 column programmed from 130° to 180° at 4°/min. for neutral sugars and a 6 foot x 2 mm i.d. OV 275 column programmed from 130° to 230° at 2°/min for amino sugars, using an SP4100 computing integrator for data capture.

B. Anhydrous HF Deglycosylation

I deglycosylated 13 to 43 mg maize cell wall preparations (dw), or 3 to 13 mg maize HRGP, in a micro-apparatus containing 1 mL anhydrous HF/20 mg cell wall, and 10% (v/v) anhydrous methanol for 1 hr at 0° C (Sanger and Lamport, 1983). The reaction was quenched by pouring into stirred distilled H_2O at 2° C to a final concentration of 10% (v/v) HF, and then dialyzed for 48 hr at 4° C and freeze-dried. The HF-treated cell wall material was then resuspended in distilled H_2O and microcentrifuged to separate particulate (the HF-insoluble cell wall) from remaining HF-soluble (and H_2O soluble) large molecular weight wall components. Both the HF-insoluble wall pellet and the HF-soluble wall supernatant were then freeze-dried separately.

C. Hydroxyproline Assay

After hydrolysis (6 N HCl, 110° C, 18 h) of wall preparations, and of the HF-soluble[10] and HF-insoluble wall, I assayed Hyp content by Kivirikko's method (Kivirikko & Liesma, 1959) which involves alkaline hypobromite oxidation, subsequent coupling with acidic Ehrlich's reagent and monitoring at 560 nm.

D. Hydroxyproline Arabinoside Profile

After alkaline hydrolysis (0.44 N $Ba(OH)_2$ 18 hr, 105° C) of maize cell walls or HRGP, careful neutralization with concentrated H_2SO_4, followed by centrifugation and freeze-drying of the supernatant fraction, I determined

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[10] Henceforth, HF-soluble wall refers to the HF-soluble non-dialyzable large molecular weight wall components, and the HF-insoluble wall refers to the HF and H_2O-insoluble particulate that remains after dialysis.

hydroxyproline arabinosides (Lamport, 1967) by redissolving the lyophilate in distilled H_2O and applying 0.5 mL containing 100 to 200 μg hydroxyproline to a 75 x 0.6 cm column (H^+ form) of Technicon Chromobeads C washed with water, eluting with a 0 to 0.5 N HCl gradient, and monitoring the post-column hydroxyproline reaction at 560 nm.

E. Amino Acid Analysis

We used a Pickering High Speed Na^+ cation exchange column (3 mm i.d. x 150 mm) in series with a BX-8 cation exchange column (3.7 mm i.d. x 70 mm, Benson Co.) eluted by Pickering Buffers A (titrated to pH 3.1 with 6 N HCl), B and Sodium Regenerant. Post column fluorometric detection involved NaOCl oxidation and OPA coupling allowing Hyp and Pro detection (Yokotsuka & Kushida, 1983). I replaced β-mercaptoethanol (reductant) with 22.7 mM N,N-dimethyl-β-mercaptoethylamine HCl (Frister *et al.*,1988), and data capture was by a Compaq 386 with Nelson Turbochrom software.

F. Cell Wall Isodityrosine Estimation

I estimated maize cell wall (Black Mexican) IDT, after hydrolysis of the HF-insoluble wall in constant boiling HCl at 110° C for 20 hr, by reversed-phase on Hamilton PRP-1 column. Solvent A was 0.13% HFBA, and Solvent B was 0.13% HFBA in 80% CH_3CN(aq). The programmed gradient elution was: 0-30% Solvent B for 15 min, a 5 min hold at 30% Solvent B, then returned to 100% A in 5 min. Flow rate was 0.3 mL/min. Standards were 2 μg each of tyrosine, dityrosine, and isodityrosine chromatographed with and without the HF-insoluble maize wall

hydrolysate. Absorbance was monitored at 214 nm, and data recorded via IBM 9001 and CAPS. Sugar degradation products interfered with the assay therefore deglycosylation of the wall before IDT estimation was imperative.

G. SDS Gel Electrophoresis

This method is based on that of Laemmli & Favre (1973). I loaded 20 to 40 μg glycosylated THRGP or HHRGPs, and 10 to 15 μg deglycosylated THRGP (dTHRGP) or deglycosylated HHRGP (dHHRGP) in 10 to 25 μL sample buffer (Trizma base, 0.01 M; SDS, 1%; EDTA, 0.001 M; β-mercaptoethanol, 5%) onto a 15% polyacrylamide gel and ran the gels in pH 8.3 Tris-Gly buffer (Trizma base, 0.025 M; glycine, 0.192 M; SDS, 0.1%). Proteins were stained with 0.2% Coomassie Brilliant Blue R-250 in water:ethanol:acetic acid (25:25:10, v/v). Molecular weight standards (BRL) were: myosin H chain, 200 kD; phosphorylase b, 97.4 kD; BSA, 68 kD; ovalbumin, 43 kD; carbonic anhydrase, 29 kD; β-lactoglobulin, 18.4 kD; and lysozyme, 14.3 kD.

H. Sample Preparation for Transmission Electron Microscopy (TEM)

Dr. John Heckman (from Michigan State University's Center for Electron Optics) prepared the THRGP and HHRGP for TEM following the general methods of Tyler and Branton (1980): highly diluted samples (1-30 μg/mL) dissolved in 50% v/v aqueous glycerol were sprayed onto freshly cleaved mica chips with a modified airbrush. After drying the chips *in vacuo* on a rotary stage in a Balzers BAE 080 high vacuum evaporator, he shadowed the molecules at an angle of 5 to 6°, with Pt/C from a modified Balzers 052 twin-mantle electron-beam

evaporator. After backing with carbon at 90° C he floated the replicas on distilled H_2O, collected them on 300 mesh copper grids, and examined them in a JEOL 100 CX II transmission electron microscope operated at 100 kV.

I. Circular Dichroic Spectra

Mark Prairie and Dr. Bill Kreuger (of Upjohn Company, Kalamazoo, MI) recorded CD spectra of the THRGP, dTHRGP, HHRGP, poly-proline II and poly-hydroxyproline (1 mg protein/mL in 100 mM sodium phosphate buffer) on a Jasco J-600 spectropolarimeter in a 0.086 mm pathlength quartz cell. They scanned each sample from 260 to 178 nm four times. Molecular ellipticity [θ], has the dimensions of (degree-cm^2/dmol).

J. Precipitation with β-Glucosyl Yariv Antigen

I reacted 400 μg THRGP, HHRGP or sycamore arabinogalactan protein in distilled H_2O (1 mg/mL) with an equal volume of β-glucosyl Yariv Antigen (Jermeyn & Yeow, 1975)(1 mg/mL in 2% NaCl) for 1 hr at 27° C followed by pelleting in a bench-top centrifuge. The pellet was washed twice with 2 mL 2% NaCl, each wash was followed by pelleting in a bench-top centrifuge, and then dissolved in 2 mL 0.02 N NaOH. Absorbancy was read at 420 nm.

K. Assay of Agglutination

I assayed the agglutinating effect of a serial dilution of THRGP and HHRGP (100 to 10 ng/mL) on a 1% suspension of trypsinized rabbit erythrocytes in phosphate-buffered saline according to the method of Allen and Neuberger (1978).

L. Partial Acid Hydrolysis of HHRGP

I hydrolysed 4 samples of 0.5 mg HHRGP in 250 μL 0.1 N HCl (pH 1), for 0, 15, 30, or 60 min at 100° C, followed by microdialysis for 2 days, freeze-drying, and then alditol acetate derivatization after complete acid hydrolysis of the HHRGP. Then I assayed for neutral and amino sugars by gas chromatography.

III. Methods for Generating, Separating and Sequencing Peptides

A. Proteolysis with Trypsin and Chymotrypsin

I incubated 2 to 6 mg deglycosylated THRGP, HHRGP, or THRGP chymotryptic peptide (10 mg/mL) in freshly prepared 1% (w/v) NH_4HCO_3 (aq) containing 10 mM $CaCl_2$ with TPCK-trypsin or chymotrypsin (Worthington)(substrate:enzyme ratio was 100-200:1, w/w) at 27° C overnight.

B. Peptide Fractionation via Sephadex G-25 Superfine Gel Filtration

I injected 0.1 to 1 mg freeze-dried peptides (in 0.5 mL 0.1 N acetic acid) onto a 27.5 mm x 1.25 mm i.d. column of Sephadex G-25-80 (superfine) eluted with 0.1 N acetic acid at 10 mL/hr. Absorbancy was monitored at 220 nm on a Hewlett-Packard Photodiodearray spectrophotometer.

C. HPLC Peptide Mapping

I obtained peptide maps via reversed phase HPLC of tryptic/chymotryptic digests on a Hamilton PRP-1 (4.1 mm i.d. x 150 mm) column using programmed gradient elution (0.5 mL/min) with the following mobile phase solvents: A = 0.13% HFBA, and B = 0.13% HFBA in 80% (v/v) acetonitrile (aq). For resolution of peptides, the gradient began at 100% A and increased (0.5%/min) from 0 to 50%

B in 100 min. Absorbancy was monitored at 220 nm. I manually collected fractions for analyses. All peptides were rerun through the Hamilton column to assure purity before sequencing.

D. Automated Edman Degradation

Joe Leykam and Melanie Corlew (Michigan State University Macromolecular Facility) sequenced 2-200 nm HPLC-purified peptide via Edman degradation (Edman, 1970) on a 477A Applied Biosystems, Inc. gas phase sequencer.

IV. Methods for Immunological Characterization of THRGP and HHRGP

A. Generation of Rabbit Polyclonal Antibodies

Three 5-lb New Zealand white rabbits were from the Small Animal Care Facility, Michigan State University. The primary injection was 70 to 120 μg THRGP or dHHRGP in distilled H_2O emulsified in Freund's Complete Adjuvant (Cappel Laboratories), and injected subcutaneously into each shoulder and hip of the rabbit. When antibody titers fell to control serum levels, booster injections of antigen contained 50 to 100 μg protein in 500 μL water-in-oil emulsion of Freund's incomplete adjuvant injected subcutaneously over each hip.

B. Enzyme-Linked Immunoassay (ELISA)

Essentially, this is based on that of Engvall & Perlmann (1972). I coated each test well of 96 well polystyrene plates (Nunc, Thomas Scientific) with 20 or 200 ng antigen (antigens: THRGP, dTHRGP, HHRGP, dHHRGP, tomato extensin monomers P1 and P2, dP1 and dP2, tomato lectin, sycamore AGP) in 200 μL pH

9.6, 50 mM $NaHCO_3$ buffer, for 15 hr at 4° C; washed the plate once in H_2O and briefly dried it before blocking all remaining protein binding sites by addition of 200 μL BSA in PBS (final pH 7.5), for 30 min at 37° C; followed by washing twice with H_2O. Then I added 25 μL of the diluted sera (immune and control preimmune sera were diluted 1:400 to 1:256,000 in PBS) to the antigen-coated wells already containing 25 μL 1% BSA-Tween-20 (1 μL/mL) PBS at pH 7.5. After 1 hr at 37° C we washed the plate with distilled H_2O, added 50 μL goat anti-rabbit serum coupled to peroxidase (Cappel Laboratories) diluted 1:2000 in BSA/Tween-20/PBS to each well, incubated at 37° C for 30 min, washed the plate five times with distilled H_2O, then added 100 μL substrate to each well (11 mg ABTS and 15 μL 30% H_2O_2 in 50 mL of 50 mM citrate buffer, pH 4). After 30 min. incubation at 23° C, I added 100 μL NaF/EDTA stopping reagent (0.04% EDTA, 6 mM NaF, 2.5 mM HF) to each well, and then determined absorbance at 405 nm.

C. Immunoblotting

These methods are based on that of Laemmli & Favre (1974) and Towbin *et al.* (1979). I loaded 20 to 40 μg THRGP, 10 to 15 μg dTHRGP, and 50 μg HF-soluble maize cell wall preparations in 10 to 25 μL sample buffer onto a 15% polyacrylamide gel and ran the gels in pH 8.3 Tris-Glycine buffer as described earlier. After electrophoresis, the proteins were transferred to nitrocellulose sheets. The sheets were blocked with incubation buffer (10 mM Tris, pH 7.4, 0.9% NaCl, 3% BSA) for 18 hr at 4°C, then incubated 1 hr at 37° C with immune or preimmune sera diluted 1:5000 in incubation buffer. The nitrocellulose sheets

were washed three times with wash buffer (10 mM Tris [pH 7.4], 0.9% NaCl, 0.1% BSA) then incubated 1 h at 37°C with alkaline phosphatase conjugated goat anti-rabbit IgG diluted 1:2000 in AP buffer (100 mM Tris, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$) before washing three times in AP buffer, pH 7.5 and twice with AP buffer pH 9.5 (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). I added substrate (50 mL AP buffer, pH 9.5, with 16.5 mg nitroblue tetrazolium and 8.35 mg 5-bromo-4-chloro-3-indolyl phosphate) to cover the sheet and incubated in darkness at 27° C until color developed. The reaction was stopped with 10 mM Tris, pH 7.5, 1 mM EDTA.

RESULTS

I. Isolation of Maize HRGPs

A. Intact Cell Elution

The amount (mg/g cells dw) of elutable crude HRGP rose as a function of culture age. After subculture, total soluble eluate fell to a minimum at 2 days and rose to level off at 11 days (Figure 5). I used 100 mM $AlCl_3$ (a non-plasmolyzing concentration) to elute the cells. Thus, for bulk preparations I used 11 day cells (optimal age for recovery of maize HRGPs, judging by recoveries of HRGP from elutions of different aged cells) and 100 mM $AlCl_3$, the cells yielding 5.4 mg crude eluate/g cells dw. The crude eluate was 1.7% Hyp on a dw basis (*i.e.* 92 μg soluble Hyp/g dw cells).

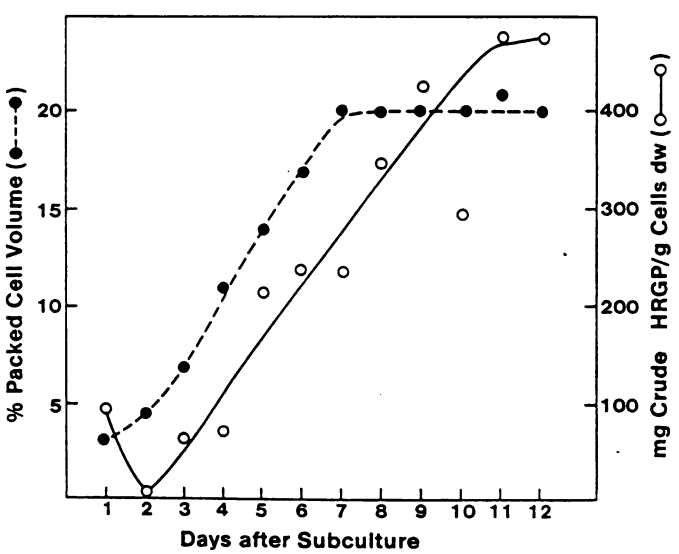


Figure 5. Yield of crude HRGP as a function of culture age.

B. Treatment of the Crude Eluate with TCA

Overnight treatment with 10% (w/v) TCA at 4° C followed by centrifugation precipitated 50% by weight of the crude eluate. The TCA pellet was 72% protein and 0.5% Hyp dw, but the HRGP remained soluble. The yield of TCA-soluble crude HRGP was 2.7 mg/g cells dw. The crude HRGP was 60% protein and 3.5% Hyp dw, representing a 60-fold Hyp enrichment over the whole cell Hyp content of 0.06%. The crude HRGP was fractionated as shown in the flow chart (Figure 6).

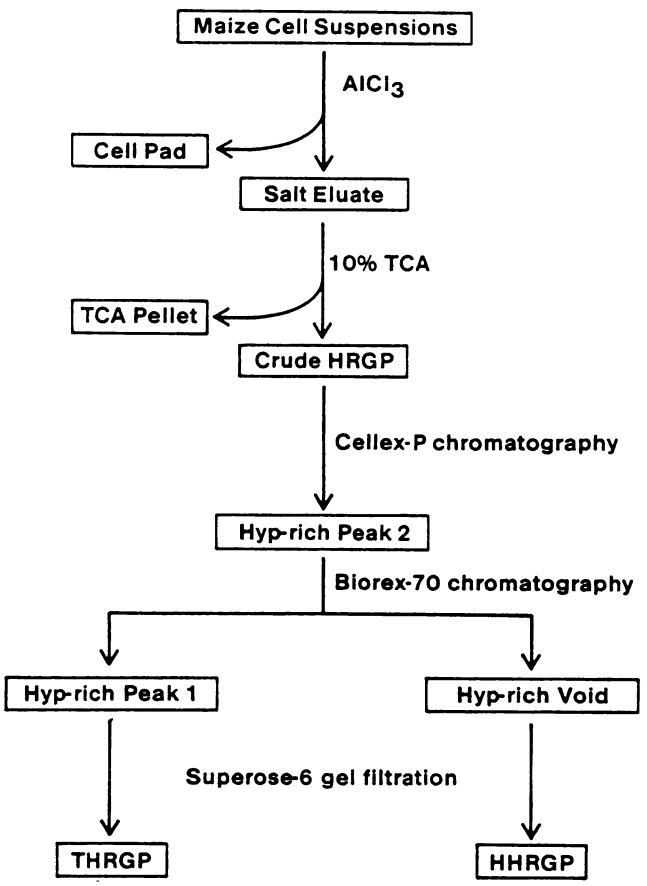


Figure 6. Maize HRGP fractionation flow chart

C. Phosphocellulose Chromatography of Maize Crude HRGP

Chromatography on phosphocellulose (Cellex-P, Biorad) yielded a void and three major protein peaks designated CP1, CP2 and CP3 respectively (Figure 7). CP1 eluted at pH 3.8 in the pH gradient, CP2 with 200 mM NaCl and CP3 at 450 mM NaCl. The void volume of the eluate and CP1 contained a trace of Hyp while CP2 contained 8.4% Hyp dw and CP3 contained 2.7% Hyp dw. The dry weight yields of the void volume plus CP1, CP2, and CP3 accounted for a 40% recovery of the material loaded on the column, and accounted for 76% recovery on a dry weight basis, of the starting hydroxyproline (Figure 8a). Figure 9 quantifies the μg of Hyp per each 4 mL fraction, beginning with fraction 35 of CP2 through fraction 53 of CP3. CP2, the shoulder following CP2, and CP3 contained significant amounts of Hyp.

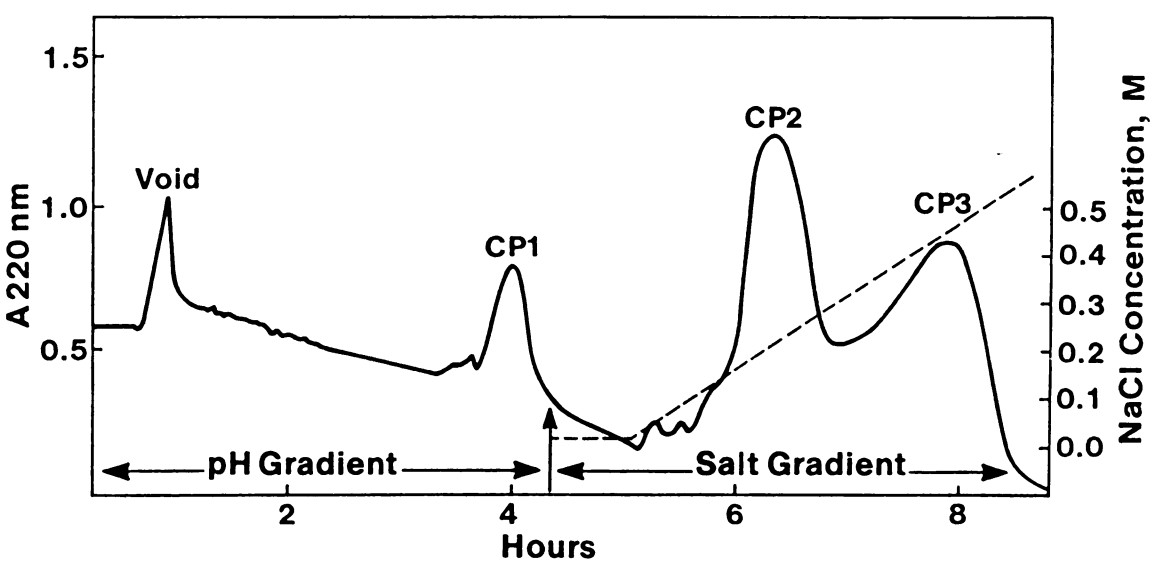
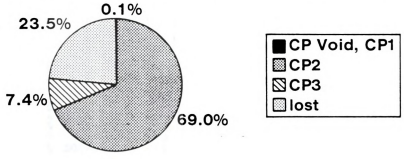
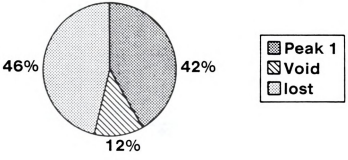


Figure 7. Phosphocellulose chromatography of maize crude HRGP

a) Hydroxyproline recovered from Cellex-P/dialysis



b) Hydroxyproline recovered from BioRex-70/dialysis



c) Hydroxyproline recovered from Superose-6/ultra filtration

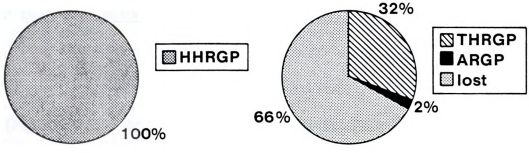


Figure 8. Recovery of hydroxyproline from a) Cellex-P b) BioRex-70 and c) Superose-6.

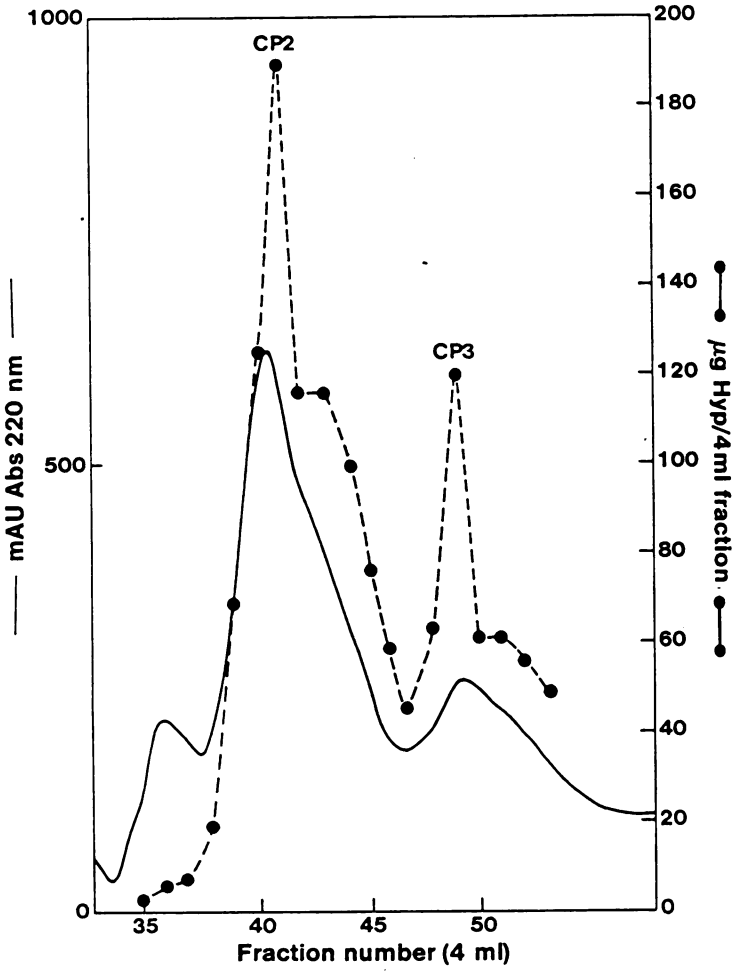


Figure 9. The hydroxyproline distribution in Cellex-P peaks CP2 and CP3

D. Biorex-70 Cation Exchange Chromatography of CP2

Further chromatography of CP2 on Biorex-70 gave a void (4% Hyp dw) and a Hyp-rich fraction (12% Hyp dw) designated Peak 1, which eluted at 200 mM NaCl (Figure 9). The dry weight yield of the void plus Peak 1 accounted for 54% of the weight and 54% of the Hyp (dw) loaded on the Biorex column (Figure 8b).

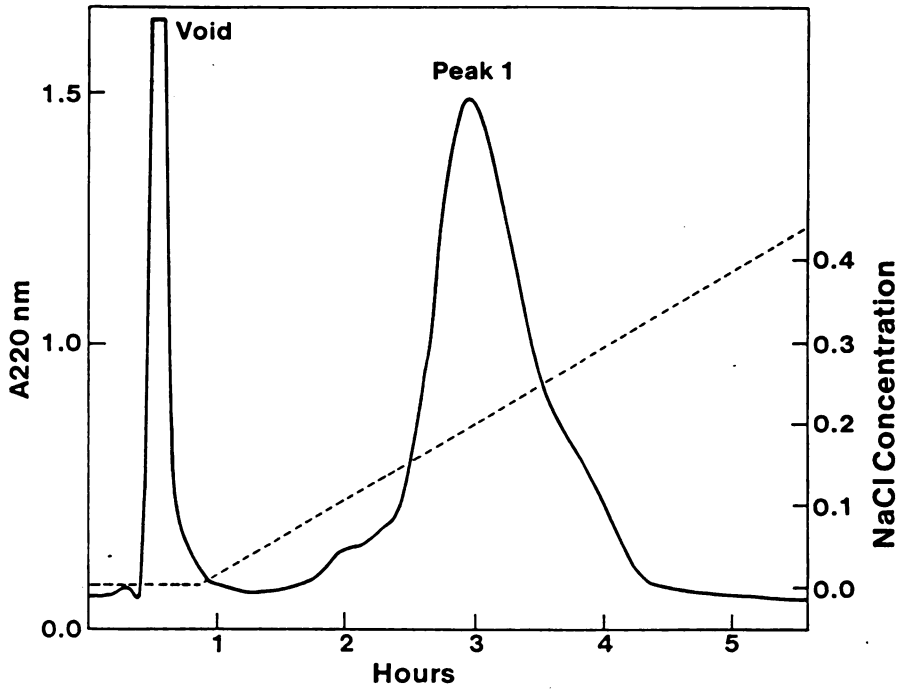


Figure 10. Cellex-P Peak 2 (CP2) on a Biorex-70 cation exchange column.

E. Superose-6 FPLC Gel Filtration of Glycosylated and Deglycosylated THRGP and HHRGP

Gel filtration of the Biorex Void gave a major Hyp-rich (8% Hyp dw), histidine-rich peak, designated HHRGP, at 1.8 V_o (Figure 11a). Gel filtration of Biorex Peak 1 on Superose-6 gave a major Hyp-rich (18% Hyp dw), threonine-rich peak, designated THRGP, at 2 x V_o, and a minor Hyp-containing peak (3% Hyp dw) that was also rich in alanine (16 mole% Ala, designated alanine-rich glycoprotein, or ARGP) eluted at 2.5 V_o (Figure 11b). The dry weight recovery of HHRGP was 52% of the starting Biorex void starting weight, and accounted for 100% recovery of Hyp loaded on the Superose-6 column (Figure 8c). Thus, after losses due to chromatography and dialysis, about 3.6% of the original TCA-soluble

crude HRGP dry weight (i.e. 3-4 mg HHRGP/100 mg crude eluate), and 8.4% (dw) of the starting Hyp was recovered. THRGP accounted for 1.8% and 9.4% of the crude HRGP dry weight and Hyp content respectively. Deglycosylated HHRGP eluted at 2.2 V_o, while the dTHRGP was retained on the column, probably due to ionic interactions with the Superose agarose matrix (data not shown).

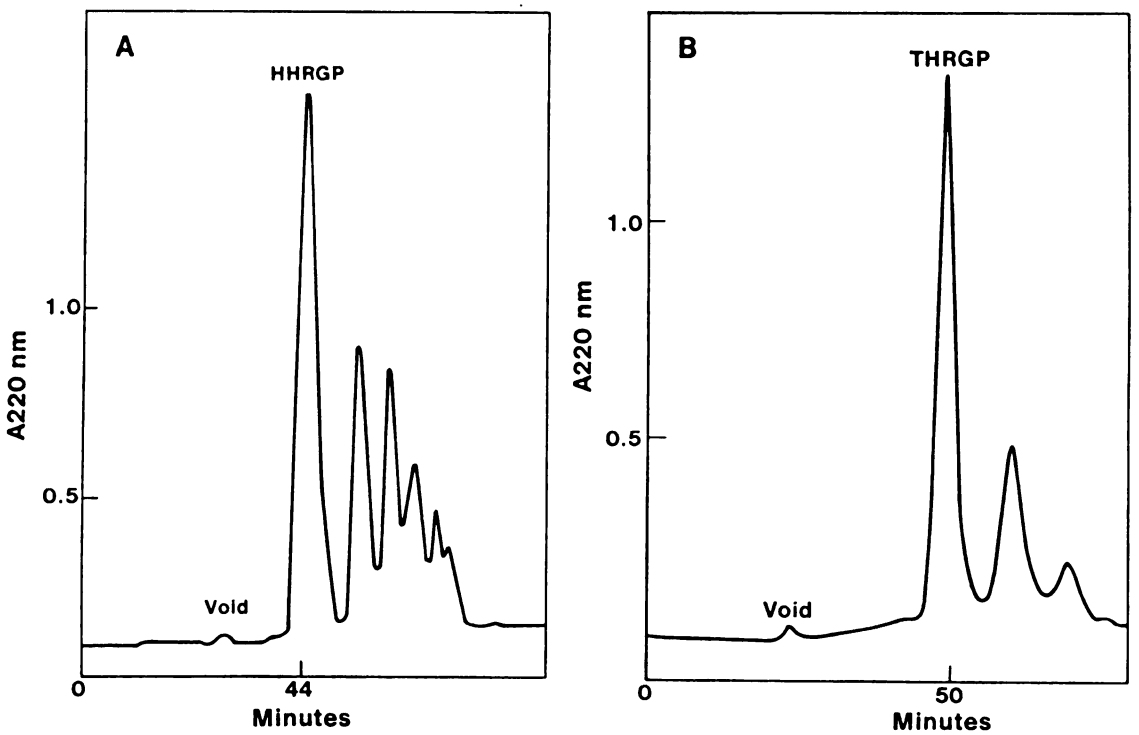


Figure 11. Superose-6 gel filtration of a) HHRGP and b) THRGP

II. Chemical and Structural Characterization of Maize HRGPs

A. Amino Acid Analyses of the THRGP and HHRGP

THRGP contained about 25 mole% Thr and Hyp, and was rich in Pro and Lys. HHRGP contained 35 mole% Hyp, and was rich in His and Ala (Table 5).

Table 5. Amino Acid Compositions of THRGP, HHRGP and Tomato Extensin P1

Amino Acid[a]	THRGP	HHRGP	Tomato Extensin P1[b]
Hyp	24.8	34.9	32.7
Asx	0.3	1.3	1.4
Thr	25.3	7.9	6.2
Ser	7.3	7.3	9.8
Glx	2.3	2.1	1.5
Pro	14.5	6.8	9.6
Gly	2.4	3.1	1.7
Ala	1.7	8.9	2.9
Val	0.7	1.5	8.3
Cys	0.0	0.0	0.0
Met	0.0	0.0	0.0
Ilu	0.1	0.0	1.0
Leu	0.2	0.0	1.0
Tyr	3.9	4.4	7.7
Phe	0.1	3.5	0.0
Lys	13.5	3.5	9.5
His	2.4	13.4	6.1
Arg	0.1	1.3	0.7

[a] Represented as Mole %
[b] From Smith *et al.*, 1984

B. HF-Deglycosylation of HRGPs

The THRGP lost 27-33% of its weight, and the HHRGP 60-70% of its weight, after HF-deglycosylation.

C. Neutral Sugar Analyses

Hydrolysis of THRGP in 2 N TFA followed by reduction with $NaBH_4$, alditol acetate derivatization, then gas chromatography showed arabinose as the only sugar substituent (Table 6). The arabinose:Hyp molar ratio was 1.44:1 and accounted for 27% by weight of the THRGP. Alditol acetate derivatization of the HHRGP showed galactose and arabinose as the only sugar substituents (Table 6).

The arabinose:Hyp molar ratio was 2.4:1, the galactose:Ser and galactose:Thr molar ratios were 5:1. Thus, arabinose and galactose accounted for 38% and 27% respectively, of HHRGP dry weight.

Table 6. Neutral Sugar Compositions of THRGP, HHRGP and Tomato Extensin P1

Neutral Sugar[a]	THRGP	HHRGP	Tomato Extensin P1[b]
Arabinose	100	63	91
Galactose	0	37	9

[a] Represented as Mole %
[b] From Smith *et al.*, 1984

D. Partial Acid Hydrolysis of the HHRGP

Treatment of HHRGP at pH 1 for 1 hr at 100° C removed all arabinose oligosaccharide substituents from the hydroxyproline residues (Figure 12). Very little galactose was hydrolysed, judging by alditol acetate derivatization and GLC of the HHRGP before and after hydrolysis.

E. Hydroxyproline Arabinoside Profiles of the THRGP and HHRGP

Hydroxyproline-arabinoside profiles of the THRGP (Figure 13) showed 48% nonglycosylated Hyp and Hyp-arabinoside 3 (Hyp-Ara_3) as the major glycosylated component (Table 7). The double peaks correspond to the *trans* and *cis* Hyp-arabinosides, the result of alkaline hydrolysis (Lamport & Miller, 1971). The Hyp-arabinoside arabinoside profile of the HHRGP showed 20% non-glycosylated Hyp and Hyp-arabinoside 3 as the major arabinoside component (Table 7).

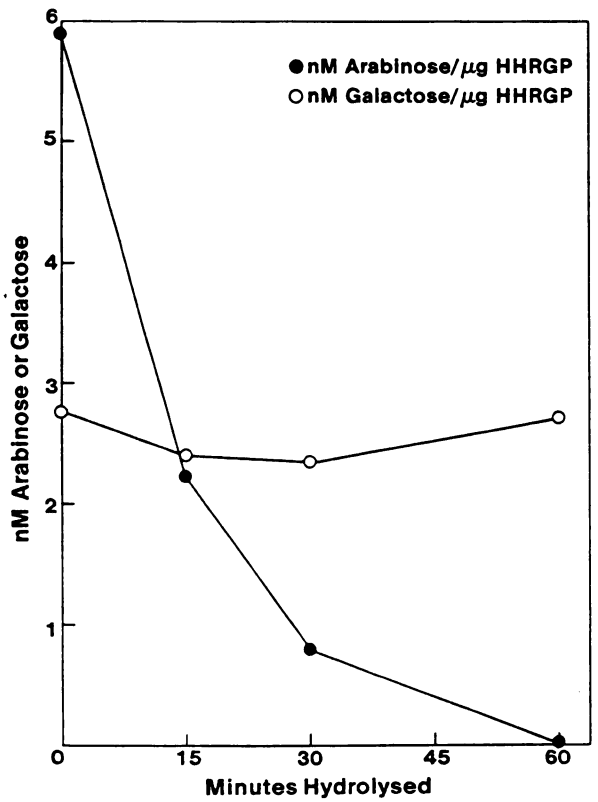


Figure 12. Partial acid hydrolysis of HHRGP at 100°C, pH 1

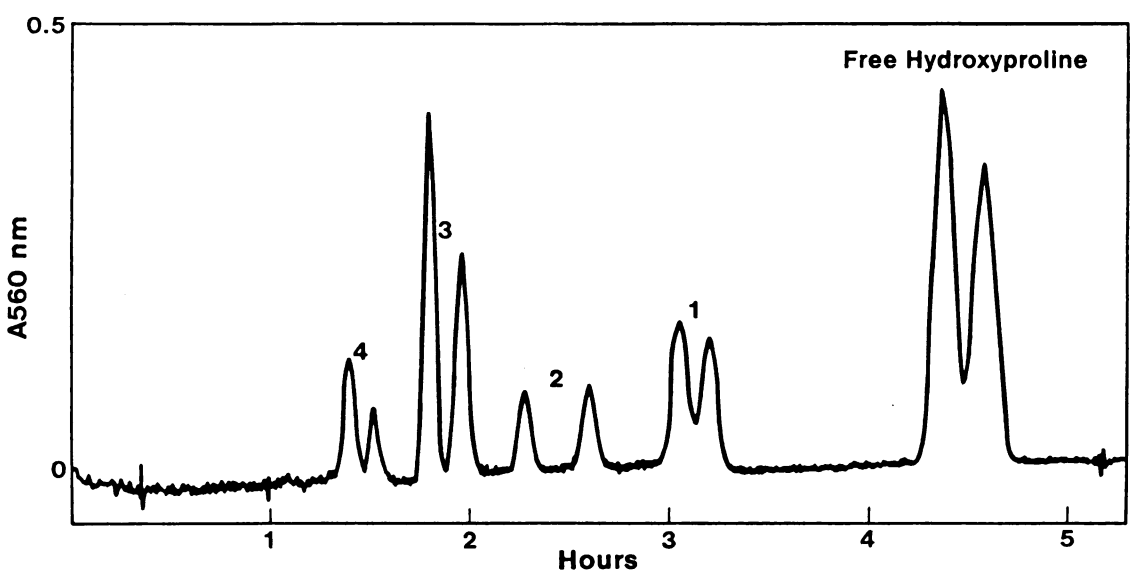


Figure 13. Hydroxyproline arabinoside Profile of THRGP. Numbers correspond to the Hyp arabinoside 1, 2, 3 or 4.

Table 7. THRGP, HHRGP and Tomato Extensin P1 Hydroxyproline Arabinosides[a]

Hyp Arabinoside	THRGP	HHRGP	Tomato Extensin[b] P1
Hyp-Ara	15	8	9
Hyp-Ara_2[c]	6	9	8
Hyp-Ara_3	25	42	33
Hyp-Ara_4	6	21	38
Free Hyp	48	20	12

[a] Represented as percent of total Hyp [c] Hydroxyproline di-arabinoside
[b] From Smith *et al.*, 1984

F. SDS-PAGE of the THRGP and HHRGP

The THRGP migrated as a fuzzy band with M_r range of 72-90 kD (Figure 14; lane 2). A high molecular weight band, probably a THRGP aggregate, migrated at about 200 kD; however the same preparation, after HF-deglycosylation (dTHRGP), lost the large molecular weight band and migrated with a M_r of 50 kD (Fig 14, lane 3), which was a 22 kD loss in M_r somewhat greater than predicted from the 30% arabinose content. The deglycosylated HHRGP (dHHRGP) at 5 μg/lane ran as two discreet bands with M_r 68 and 70 kD (Figure 15, lane 2) and at 15 μg, as a 68-70 kD smear (Figure 15, lane 3). Glycosylated HHRGP did not enter the SDS gel.

G. TEM Visualization of the THRGP and HHRGP

TEM shadowed preparations of the THRGP and HHRGP both showed rod-like molecules averaging 70 $\pm$ 3 nm in length (Figure 16 a & b, respectively).

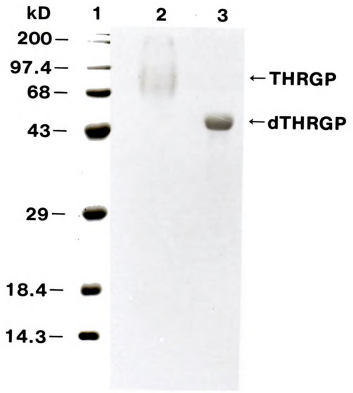


Figure 14. SDS-PAGE of THRGP (lane 2), dTHRGP (lane 3) and molecular weight standards (lane 1).

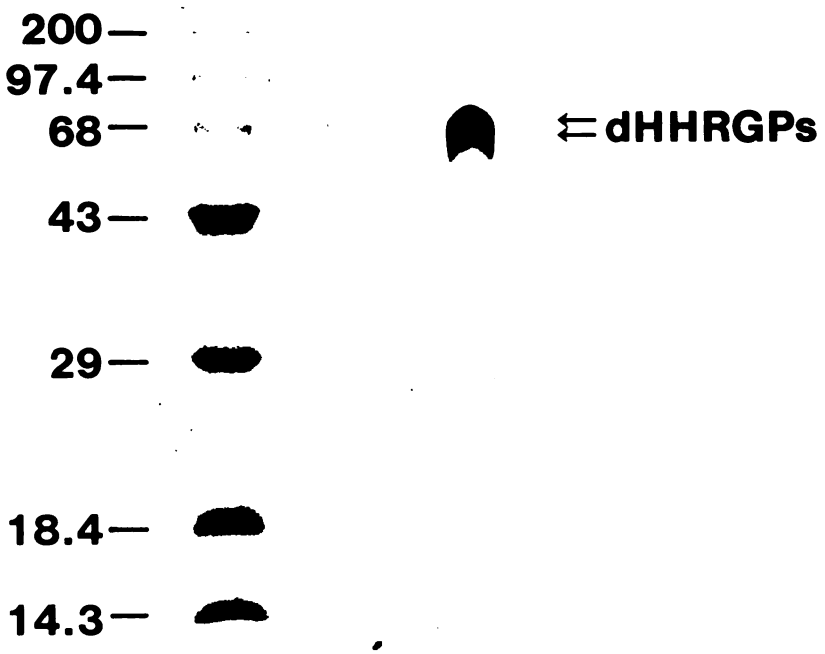


Figure 15. SDS-PAGE of dHHRGP (lanes 2 & 3) and molecular weight standards (lane 1).

H. Circular Dichroic Spectra of THRGP, dTHRGP and HHRGP

The CD spectra of the THRGP and dTHRGP showed no maximum and single broad minimums at 200 to 205 nm (Table 8)(Figure 17a), similar to that of a fibrous protein in an unordered, or "random coil" conformation (Tiffany & Krimm, 1969). The CD spectra of HHRGP showed a maximum at 224 nm (which indicates a more ordered structure for HHRGP than the THRGP (Tiffany & Krimm, 1969), possibly due to some polyproline II conformation; and HHRGP showed a minimum at 202 nm (Table 8) (Figure 17b).

Table 8. Features in the CD of THRGP, dTHRGP, HHRGP, Poly-L-Proline and Poly-4-Hydroxy-L-Proline in Order of Decreasing λ_{max}

Polymer	λ_{max}[a]	ΔE_{max}[a]	λ_{crs}	λ_{min}	ΔE_{min}
HHRGP	224	0.6	218	202	-3
THRGP	-	-	-	205	-3
dTHRGP	-	-	-	202	-3
Poly-L-Proline	228	1	222	206	-14
Poly(4-L-hydroxyproline)	225	2.2	219	205	-13
Random Coil[b]	-	-	-	190-200	varies
β-Sheet[c]	190-200	varies	205	210-220	varies
α-Helix[c]	190-195	varies	205	208+222	varies

[a] The subscripts max, crs, and min refer to observed maximum, crossover, and minimum. ΔE is the average CD/peptide bond; λ is the wavelength.
[b] From Tiffany & Krimm, 1969
[c] From Johnson, 1988

I. Assay of Agglutination

Serial dilutions of THRGP or HHRGP (100-10 ng/mL) did not agglutinate a 1% suspension of trypsinized rabbit erythrocytes.

Figure 16. Visualization of THRGP and HHRGP by transmission electron microscopy. Rotary shadowed A) HHRGP and B) THRGP were flexible rods about 70 nm long.

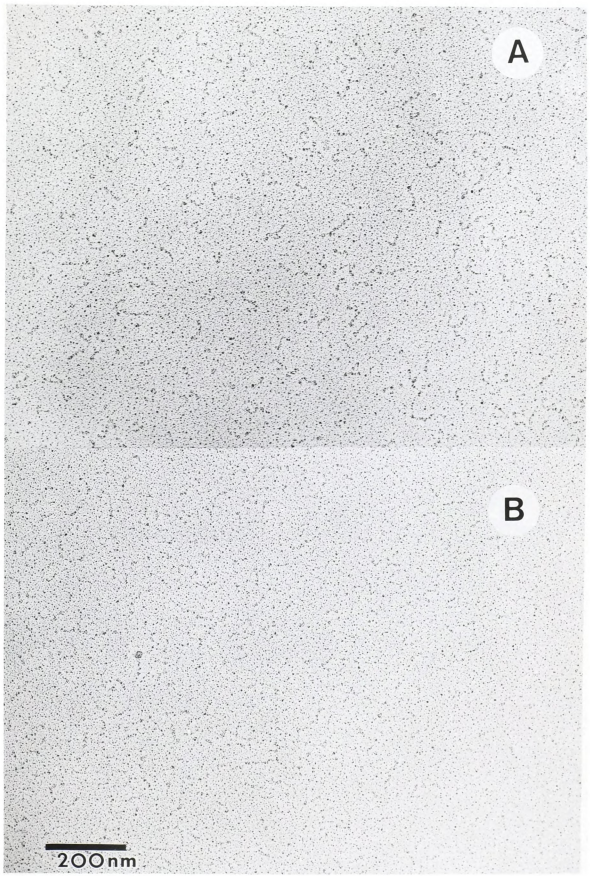
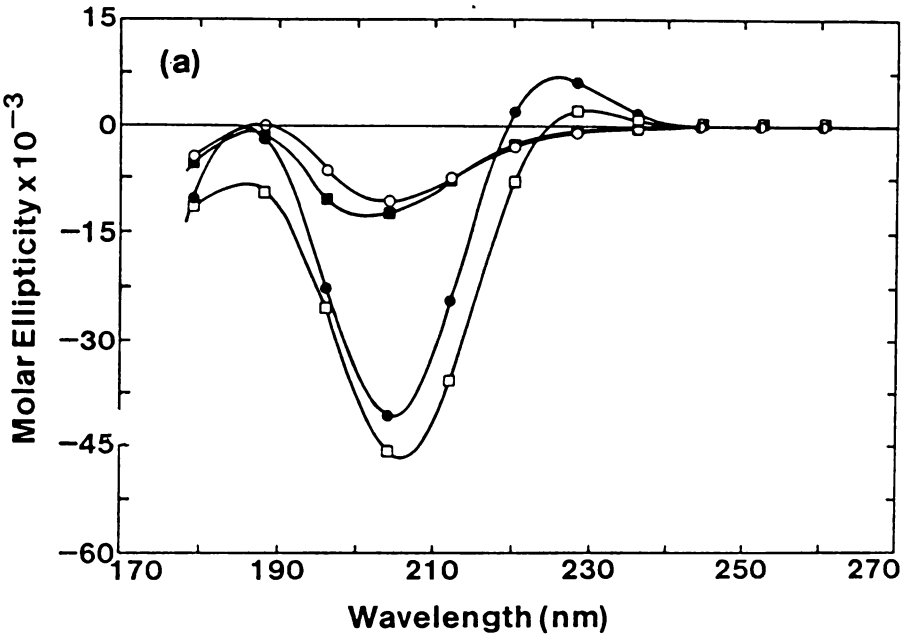
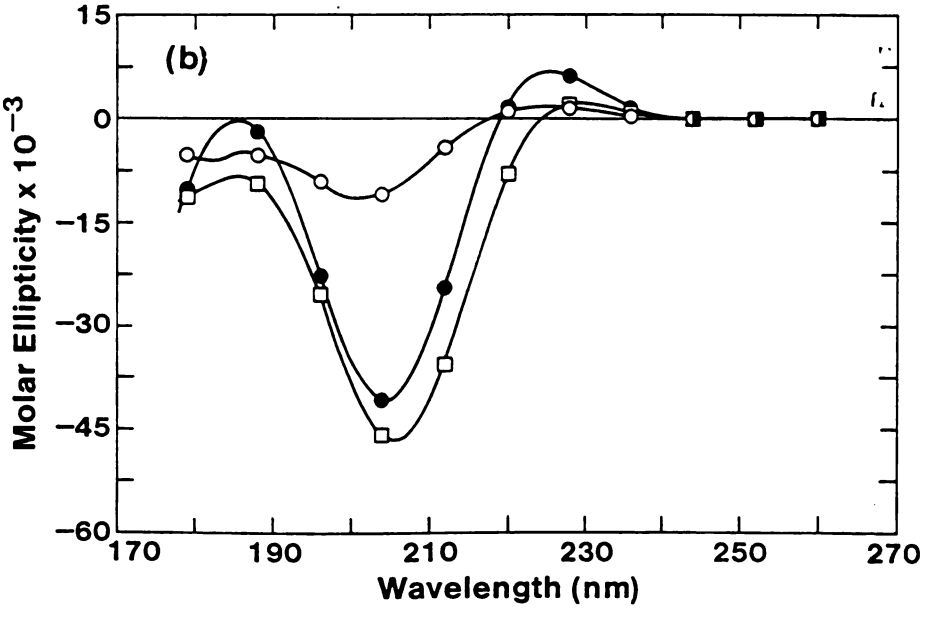

A
B
200nm

Figure 17. Circular dichroic spectroscopy of a) THRGP, deglycosylated THRGP (dTHRGP), and b) HHRGP.

a) CD spectra of poly-L-proline II (□—□), poly-4-hydroxy-L-proline (●—●), THRGP (○—○), and dTHRGP (■—■), showed the THRGP secondary structure is not a polyproline II helix.
b) CD spectra of polly-L-proline II (□—□), poly-4-hydroyx-L-proline (●—●), and HHRGP (○—○) showed that HHRGP secondary structure was more ordered than the THRGP, possibly due to some polyproline II conformation, however, still mainly random coil.


(a)
15
0
−15
−30
−45
−60
170
190
210
230
250
270
Molar Ellipticity x 10^{-3}
Wavelength (nm)


(b)
15
0
−15
−30
−45
−60
170
190
210
230
250
270
Molar Ellipticity x 10^{-3}
Wavelength (nm)

J. Reaction of THRPG and HHRGP with β-Glucosyl Yariv Antigen

Neither THRGP nor the HHRGP reacted with Yariv Antigen, even at the relatively high level of 0.5 mg/mL where a standard AGP (sycamore) gave a absorbance of 2.36 at 420 nm.

K. Proteolysis of dTHRGP with Chymotrypsin and/or Trypsin, Sephadex G-25 Gel Filtration, HPLC Peptide Mapping and Edman Degradation

Chymotryptic digestion of dTHRGP yielded peptides of four size classes, judging by Sephadex G-25 gel filtration (Figure 18). Fractionation of the chymotryptic digest by HPLC gave a peptide map (Figure 19) consisting of a relatively few peptides dominated by a single major component, TC5 (See Table 9 for the amino acid molar ratios of the 8 peptides). Automated Edman degradation gave a 16-residue sequence for TC5: Thr-Hyp-Ser-Hyp-Lys-Pro-Hyp-Thr-Pro-Lys-Pro-Thr-Hyp-Hyp-Thr-Tyr which, as the dominant peptide, therefore exists as a repetitive unit of the THRGP. Furthermore, residues 3 through 10 of TC5 and TC4 constitute 8-residue sequences highly homologous with tryptic peptide H5 from tomato extensin P1: Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys (Smith *et al.*, 1986), and tryptic peptide H5 from sugar beet extensin P1: Ser-Hyp-Hyp-Val-His-Glu-Tyr-Pro-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys (Figure 29) (Li *et al.*, 1990). The minor maize peptides sequenced were also related, including TC1 which contained the only Ser-Hyp-Hyp-Hyp-Hyp sequence in the THRGP (Table 10). Peptides TC1, TC2, TC4 and TC5 were sequenced a minimum of twice (from different peptide preparations), and peptides TC6, TC7 and TT1, the

tryptic peptide, were sequenced once. The typical peptide repetitive yield, i.e. for TC5, was 77%, vs. 97% for standard proteins. The weight percent distribution of recovered peptide was: TC1, 10%; TC2 and TC3 combined for 11%; TC4, 18%; TC5, 43%; TC6, TC7 and TC8 combined for 18%. Thus the total dTHRGP peptide recovered from the PRP-1 column was approximately 57% of the material loaded.

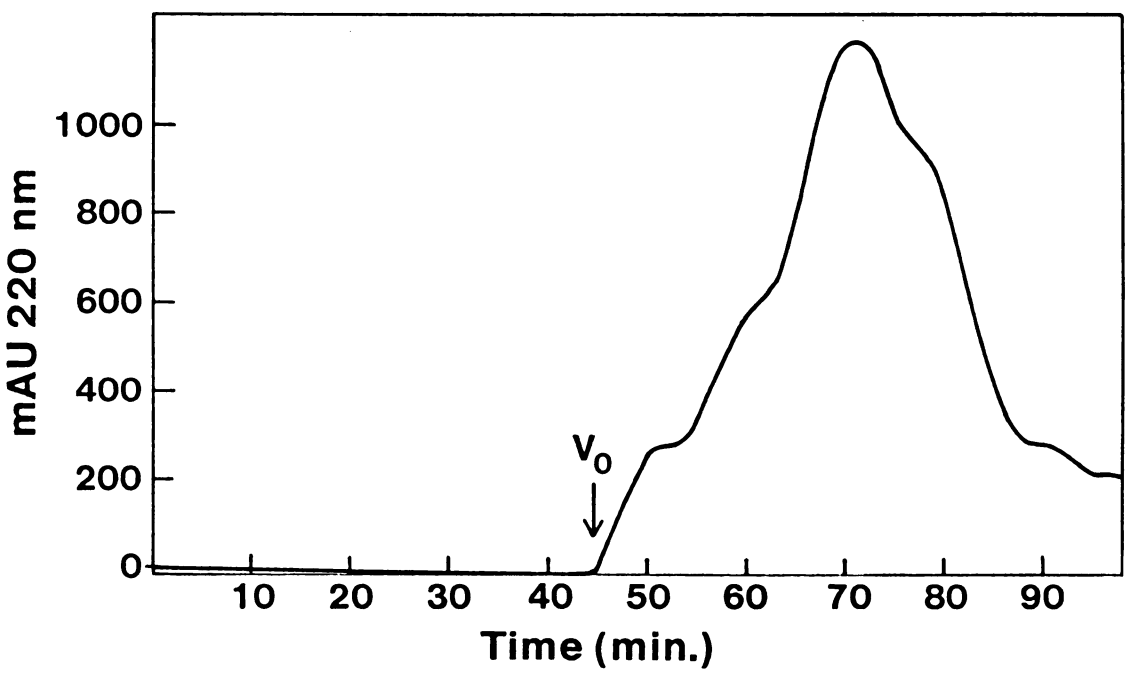


Figure 18. Sephadex G-25 gel filtration of dTHRGP chymotryptic peptides.

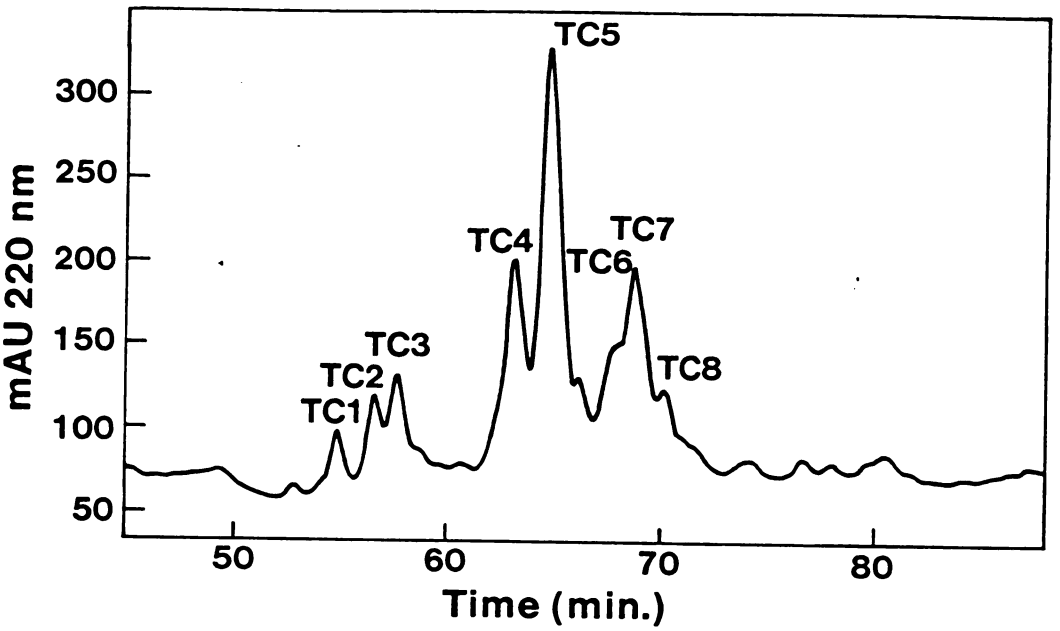


Figure 19. Chymotryptic peptide map of dTHRGP.

Table 9. Amino Acid Compositions of dTHRGP Chymotryptic Peptides[a]

Amino Acid	TC1	TC2	TC3	TC4	TC5	TC6	TC7	TC8
Hyp	7.0	4.1	6.8	4.5	5.2	5.7	7.0	6.9
Asx								
Thr	5.2	3.5	5.6	3.8	4.4	5.0	6.8	6.7
Ser	3.4	0.8	1.3	1.1	1.1		1.3	1.5
Glx								
Pro	1.7	1.0	2.2	2.3	2.9	3.2	4.4	4.6
Gly								
Ala						1.0		1.0
Val	1.1							
Ilu								
Leu								
Tyr	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Phe								
Lys		0.7	1.9	1.9	2.0	2.9	2.6	1.2
His	1.2						0.9	
Arg								

[a] Represented as Molar Ratios

Table 10. Amino Acid Sequences of dTHRGP Tryptic and Chymotryptic Peptides

Chymotryptic Peptide	Sequence
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
TC3	ND
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)-
TC8	ND
Tryptic Peptide	**Sequence**
TT1	Pro-Thr-Hyp-Hyp-Thr-Tyr-Thr-Hyp-Ser-Hyp-Lys-Pro-Hyp-Thr-Pro-Lys

L. Tryptic Digestion of dTHRGP and Chymotryptic Peptide TC5, HPLC Peptide Mapping, and Edman Degradation

A major peptide, TT1, recovered from tryptic digestion of dTHRGP (See Figure 20 for tryptic peptide map) showed an apparently anomalous N-terminal proline residue (conventionally, trypsin does not cleave lysylproline), and whose sequence overlapped with chymotrytic peptide TC5 (Table 10). Figure 21a shows the amino acid sequence and chromatographic profile (220 and 280 nm) of dTHRGP major chymotryptic peptide TC5. Although this 16-residue peptide has two Lys-Pro peptide bonds (Ser-Hyp-Lys-Pro-Hyp and Thr-Pro-Lys-Pro-Thr), only the latter was cleaved by trypsin. Thus, in tryptic digestions of chymotryptic peptides of the deglycosylated THRGP, or in tryptic digestions of deglycosylated THRGP, the Lys-Pro linkage in the Thr-Pro-Lys-Pro-Thr sequence was slowly

cleaved. There was no evidence of other Lys-Pro cleavage in the THRGP. Figure 21b shows the peptide profile of the partially digested chymotryptic peptide after a 12-hr incubation with TPCK trypsin. Two new peptides appeared. The first peptide (at 50 min on the map) was a 6-residue peptide beginning with the proline of the Lys-Pro cleavage and ending with the tyrosine residue that terminated TC5, Pro-Thr-Hyp-Hyp-Thr-Tyr. The second peptide (at 58 min) was a 10-residue peptide ending with the lysine residue of the lysyl-proline bond from TC5, Thr-Hyp-Ser-Hyp-Lys-Pro-Hyp-Thr-Pro-Lys. The third peptide (67 min) was intact TC5. The presence or absence of tyrosine in the peptides was corroborated by absorbance, or lack of it, at 280 nm.

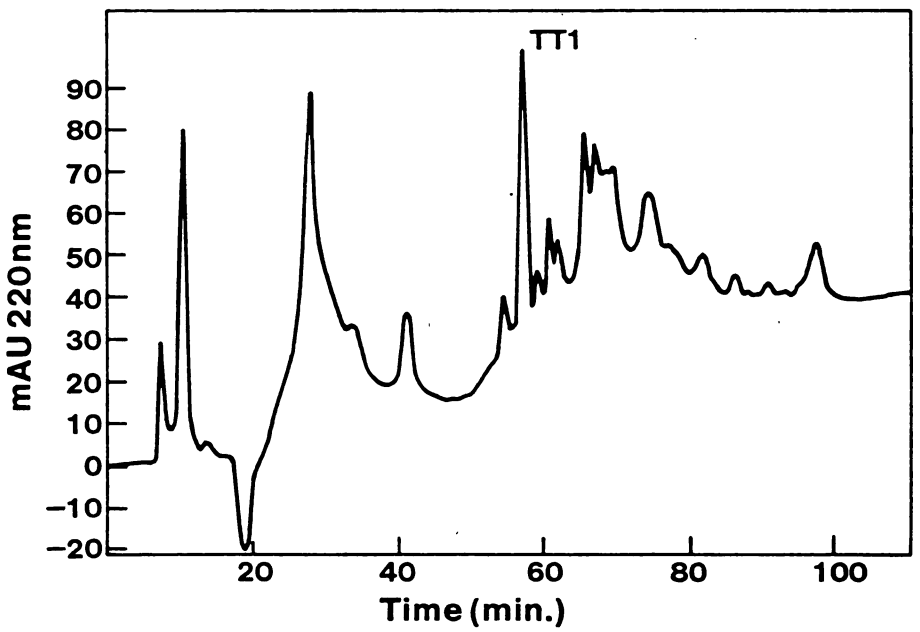


Figure 20. Tryptic peptide map of dTHRGP[11].

[11] The tryptic peptide map was done only once on impure THRGP therefore many minor peptides appear.

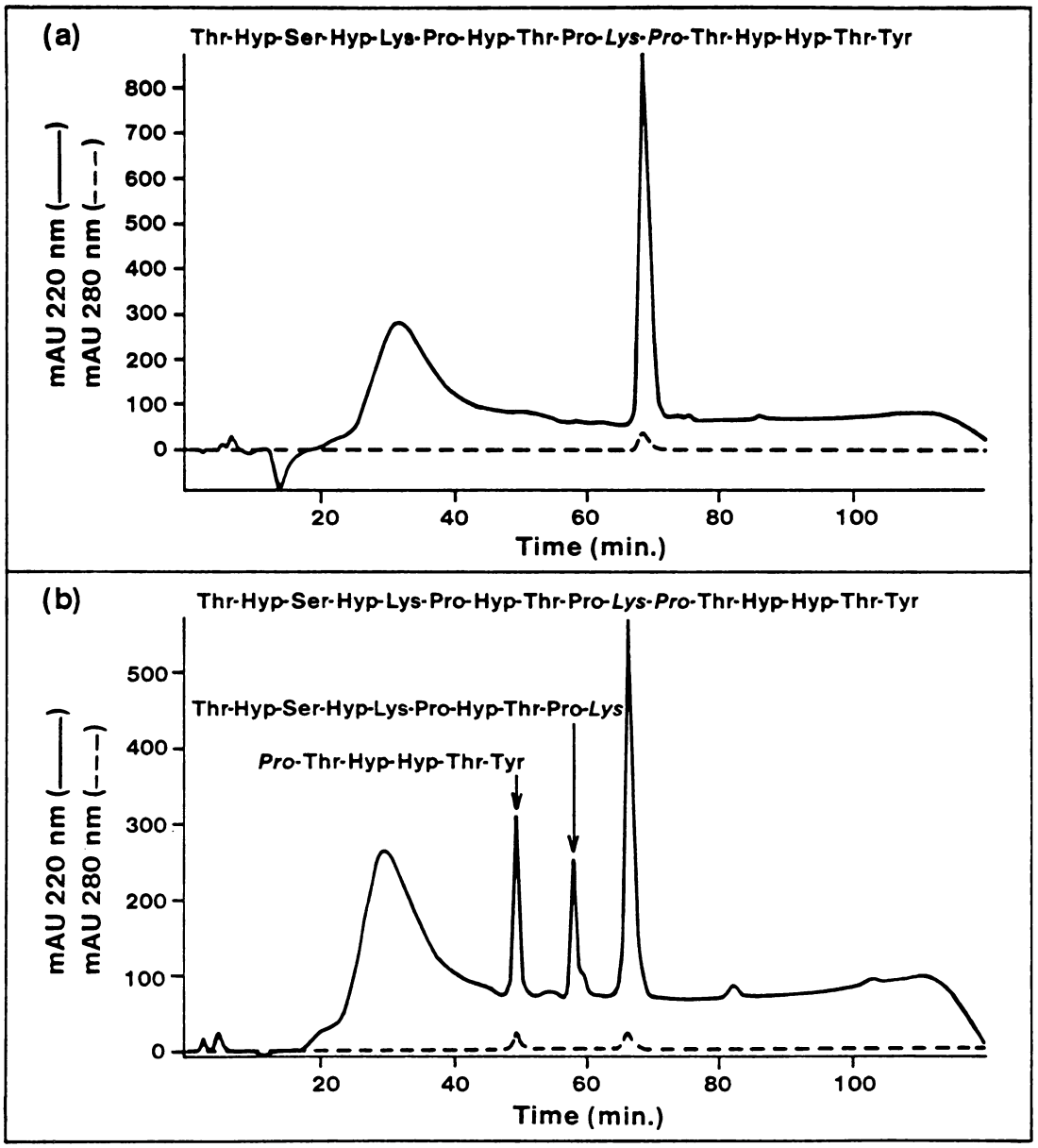


Figure 21. Chymotryptic peptide TC5 a) before and b) after tryptic hydrolysis

M. Chymotryptic Digestion of dHHRGP, Sephadex G-25 Gel Filtration, HPLC Peptide Mapping, and Edman Degradation of dHHRGP

Judging by Sephadex G-25 Gel Filtration (Figure 22), chymotryptic digestion of dHHRGP yielded peptides of several size classes, from large peptides that eluted immediately behind the G-25 void, to very small peptides (i.e about 6 residues; Table 12) eluting at 90 min. HPLC fractionation of the complete chymotryptic dHHRGP digest gave a peptide map consisting of 4-5 minor peptides and 7-8 major peptides (Figure 23) The amino acid molar ratios of the dHHRGP peptides do not match exactly the amino acid sequences of the peptides, especially HC1, whose amino acid composition (Table 11) predicts a serine residue which does not appear in the sequence (Table 12). However, many of the sequences are incomplete (HC1, HC4, HC6, and HC12); furthermore, the dHHRGP peptide map, amino acid compositions and sequences were determined only once for the HHRGPs, and therefore should be considered preliminary data. Judging by Edman degradation, the tetrapeptide Ala-Hyp-Hyp-Hyp occured in peptides HC3, HC4, HC10, HC11, and HC12; and comprised a "sub-periodicity" in the decapeptide: Ala-Hyp-Hyp-Hyp-His-Phe-Pro-Ser-Hyp-Hyp... that occurred in HC10, HC11 and HC12 and as a nine-residue variant in HC4 (a Tyr for His substitution at residue 5 and deletion of the serine residue). Thus, both the Ala-$(Hyp)_3$ tetramer and the decamer: Ala-Hyp-Hyp-Hyp-His-Phe-Pro-Ser-Hyp-Hyp are repetitive units in at least one of the HHRGPs. Edman degradation of the major peptide HC6 did not give a complete sequence (Table 12), however, HC6 was

homologous (89%) with the first 9 amino acids of "P3-type" extensin peptide (Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-Tyr-Tyr-Tyr-Lys)(Smith *et al.*, 1986; Showalter & Rumeau,1989), except for the single substitution of His for Hyp at residue 5. Furthermore, peptides HC10, HC11, and HC12 are 66% homologous with the first 10 amino acids of P3 type extensin, having Ala and Phe substitutions for Ser at residues 1 and 6, and a His for Hyp substition at residue 5. HC10 contained an asparagine-centered 9 residue palindrome: Hyp-Hyp-Ala-Ala-Asn-Ala-Ala-Hyp-Hyp. Chymotryptic peptide HC2 showed a C-terminal histidine residue, indicating that chymotrypsin may be selectively cleaving after some histidine residues. Chymotryptic cleavage of His-Thr and His-Ser bonds occurs in Azurin (Ambler & Brown, 1967).

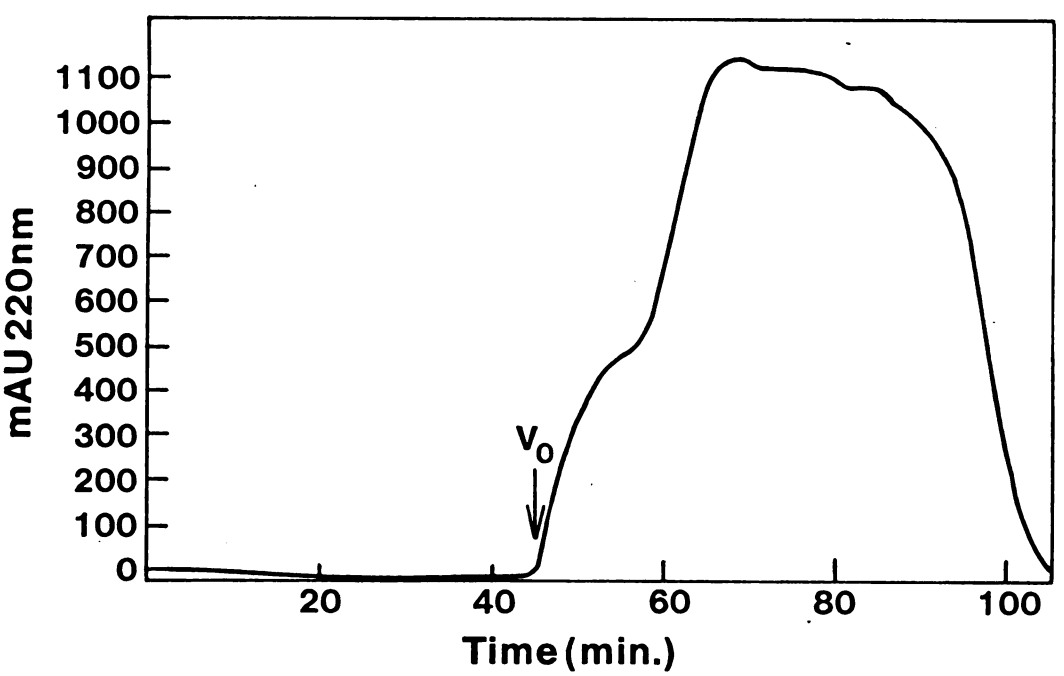


Figure 22. Sephadex G-25 gel filtration of dHHRGP chymotryptic peptides.

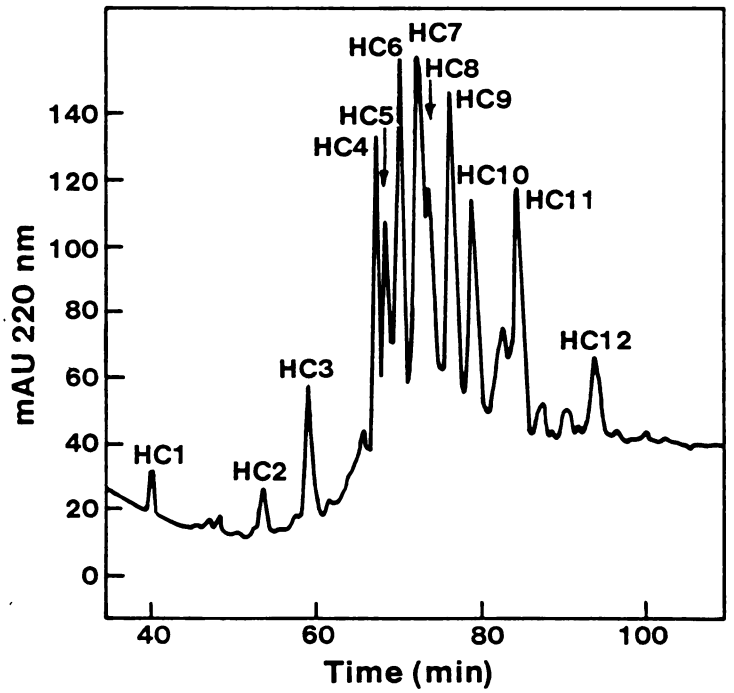


Figure 23. Chymotryptic HPLC peptide map of dHHRGP

III. Immunological Characterization of THRGP

A. Cross-Reactivity of Anti-Tomato Extensin Polyclonal Antibodies with THRGP and HHRGP

Rabbit polyclonal antibodies raised against tomato extensin monomers P1 and against the protein backbone of HF-deglycosylated P1 and P2 cross-reacted ca. 40% and 18% respectively with the THRGP and HHRGP (Figure 24), indicating common antigenic epitopes between the tomato extensins and the maize HRGPs.

B. Generation of Antibodies against THRGP and Characterization via ELISA

Three weeks after challenging 2 rabbits with THRGP, an immunogenic reaction was apparent as determined by ELISA assays. Titer rose until week 5 and remained high for more than 17 weeks after the primary injection. Figure 25

Table 11. Amino Acid Compositions of dHHRGP Chymotryptic Peptides[a]

Amino Acid	HC1	HC2	HC3	HC4	HC5	HC6	HC7	HC8	HC9	HC10	HC11	HC12
Hyp	2.8	2.1	5.7	4.9	3.9	5.2	ND	ND	7.2	10.0	9.7	5.3
Asx			0.9							1.0		
Thr				1.6							2.1	
Ser	0.8	1.1		0.9	0.9	2.2			1.4	1.5	1.9	0.8
Glx												
Pro				0.9					1.1	1.3	1.3	
Gly						1.1						
Ala	1.0	0.9	2.1	1.3	1.5	0.8			2.6	4.7	2.6	1.3
Val												
Ilu												
Leu												
Tyr			1.0	1.0		1.0			1.0	1.0	1.0	
Phe				0.8						1.3	1.2	1.0
Lys												
His	1.0	2.0	1.4	1.0	2.0	2.2			4.5	4.7	4.9	2.3
Arg												

[a] Represented as molar ratios based on His, Tyr, or Phe, depending upon the peptide composition

Table 12. Amino Acid Sequences of dHHRGP Chymotryptic Peptides

Chymotryptic Peptide	Sequence
HC1	Ala-Hyp-Hyp-Ala-Pro-Ala-Pro-
HC2	Ser-Hyp-Hyp-Ala-His-His
HC3	N/Phe-Ala-Hyp-Hyp-Hyp-Ala-His-Tyr
HC4	Ala-Hyp-Hyp-Hyp-Tyr-Phe-Pro-Hyp(Thr)-Hyp-
HC5	ND
HC6	Ser-Hyp-Hyp-Hyp-His-Ser-Hyp-Ser-Hyp-Gly-
HC7	ND
HC8	ND
HC9	ND
HC10	Ala-Hyp-Hyp-Hyp-His-Phe-Pro-Ser-Hyp-Hyp-Ala-Ala-Asn-Ala-Ala-Hyp-Hyp-Hyp-Ala-His-Tyr
HC11	Ala-Hyp-Hyp-Hyp-His-Phe-Pro-Ser-Hyp-Hyp-Thr(Hyp)-His-His-His-Hyp-Hyp-Hyp-Tyr
HC12	Ala-Ala-Hyp-Hyp-Hyp-His-Phe-Pro-Ser-Hyp-Hyp-

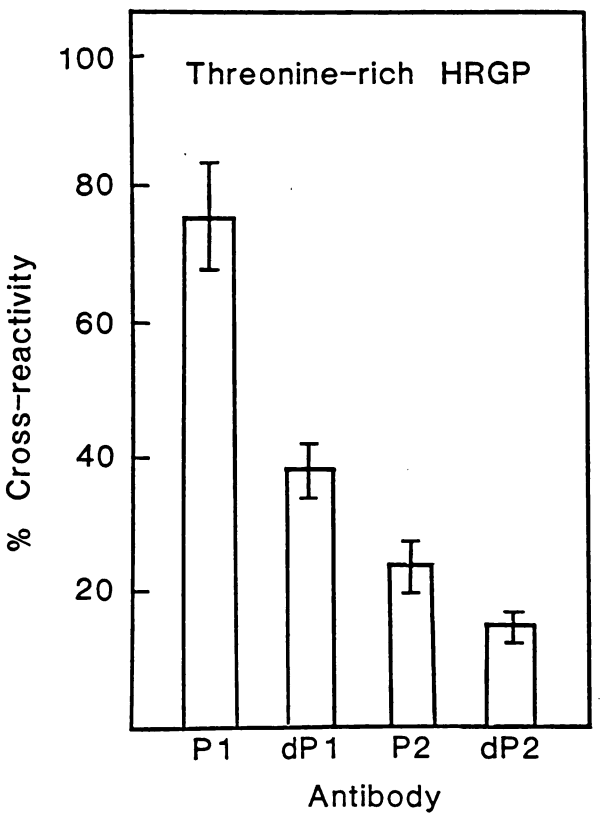


Figure 24. Reactivity of THRGP with antibodies raised against tomato extensin precursors.

a detectable reaction to 20 ng THRGP/microtiter well and antisera dilutions as high as 1:64,000. We routinely worked with primary antisera dilutions of 1:5000. Figure 26 shows the cross-reactivity of THRGP antibodies with antigens: dTHRGP, HHRGP, dHHRGP, tomato extensin monomers P1 and P2, deglycosylated P1 and P2 (dP1, dP2). The antibodies did not react with tomato lectin or sycamore AGP (data not shown). Pre-immune control serum did not react with any of the antigens.

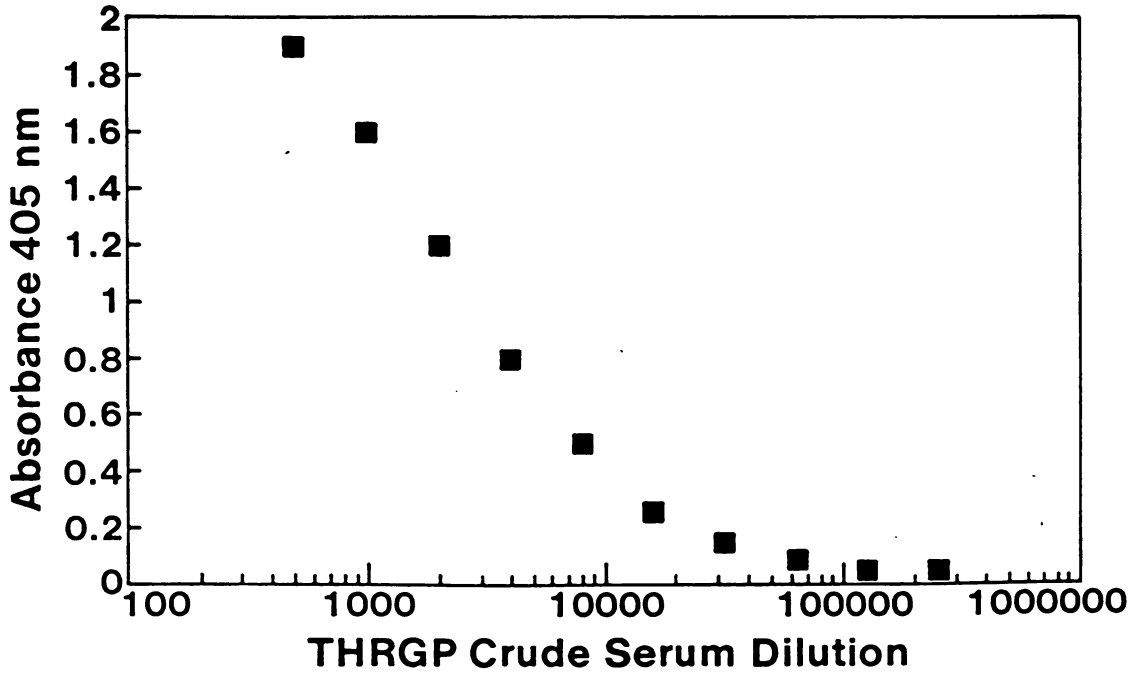


Figure 25. Serial dilution of anti-THRGP antiserum with THRGP antigen at 20 ng/ELISA microtiter well.

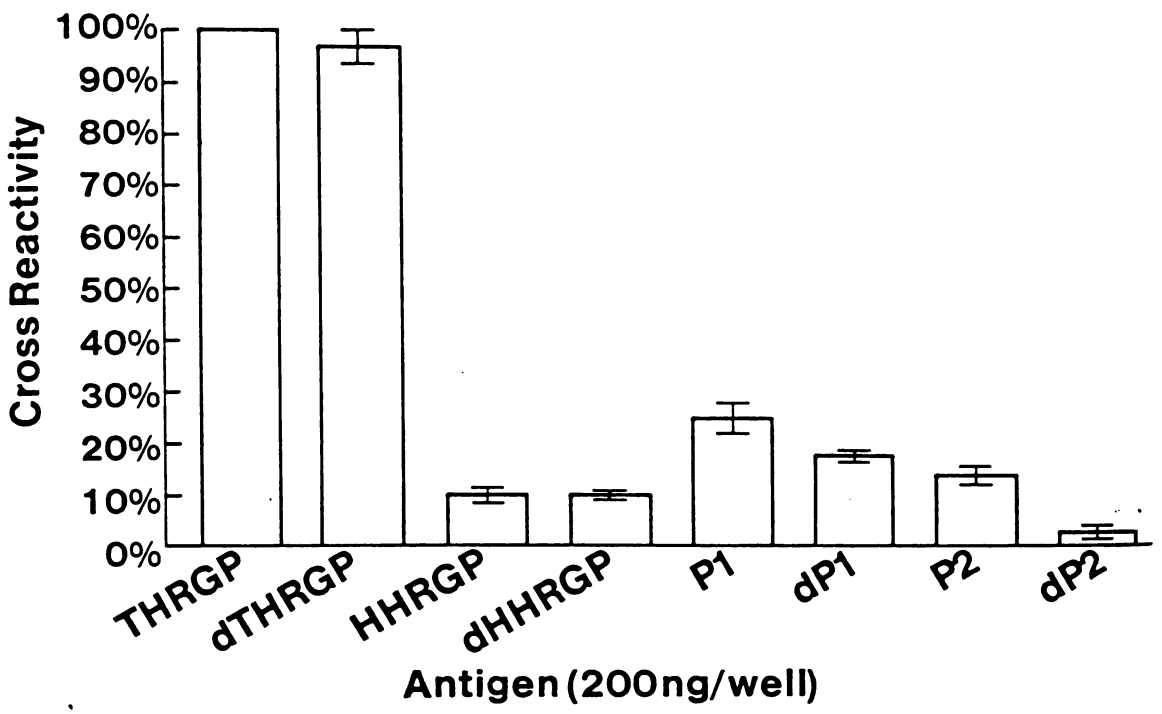


Figure 26. Cross-reactivities of anti-THRGP antibodies with dTHRGP, HHRGP, dHHRGP and tomato extensins P1, dP1, P2, dP2.

IV. Hydroxyproline Arabinoside and Protein Profile of the Maize Cell Wall

A. Hydroxyproline Arabinoside Profile of the Maize Cell Wall

Hydroxyproline-arabinoside profiles of the maize cell wall showed 24% nonglycosylated Hyp, and hydroxyproline tri-arabinoside as the major hydroxyproline arabinoside component (Table 13).

Table 13. Hydroxyproline Arabinoside Profiles[a] of Maize and Tomato Cell Walls

Hyp Arabinoside	Black Mexican	Maize Pericarp[b]	Tomato[c]
Hyp-Ara	9	15	10
Hyp-Ara_2[d]	6	2	9
Hyp-Ara_3	41	13	28
Hyp-Ara_4	10	4	48
Free Hyp	24	66	5

[a] Expressed as percent of total Hyp
[b] From Lamport & Miller, 1971
[c] From Smith *et al.*, 1984
[d] Hydroxyproline di-arabinoside

B. HF Deglycosylation and Hydroxyproline Content of the Maize Cell Wall

Cell walls prepared from maize (Black Mexican) cell suspensions and maize (Sweet Sue) coleoptile, root, and root tip contained bound hydroxyproline, mostly HF-soluble, although some (from a trace to 17.4%) remained associated with the HF-insoluble residual wall fraction (Table 14). Significant amounts of Hyp (~40%) and protein (~25%) were lost during dialysis, possibly as dialyzable molecules, or by adsorption to dialysis membranes.

C. Amino Acid Analyses of the Black Mexican Maize Cell Wall, the HF-Soluble Wall and the HF-Insoluble Wall

Judging by recoveries after amino acid analyses, the Black Mexican cell wall before deglycosylation was 10% protein, the HF-solubilized wall 20% protein, and the HF-insoluble wall fraction was 50% protein. The protein component of the HF-solublized wall fraction was enriched in Hyp and His, while the HF-insoluble wall fraction retained a trace of Hyp (Table 15).

Table 14. Hydroxyproline Distribution in the HF-Soluble and HF-Insoluble Black Mexican Maize Cell Wall

	Cell Wall Dry Weight		HF-Soluble High Molecular Weight		HF-Insoluble Residue	
	mg	μg Hyp	mg	μg Hyp	mg	μgHyp
Root Tip	100	144	10	63	1	trace
% Hyp distribution		100%		43.8%		ND[a]
Root	100	70	8.9	30.4	4.1	12.2
% Hyp distribution		100%		43.4%		17.4%
Coleoptile	100	200	10.5	104.7	5.1	12.3
% Hyp distribution		100%		52.3%		6.2%
Black Mexican Cultures (11 d)	100	150	9.5	76	5	15
% Hyp distribution		100%		44%		10%

[a] recovery too low for accurate assay

D. Black Mexican Cell Wall Isodityrosine

The HF-insoluble Black Mexican maize cell wall contained tyrosine, but no dityrosine or IDT, as assayed on a Hamilton PRP-1 column; however, an unknown "phenolic" compound eluted at 21.7 min, between dityrosine (20.7 min) and IDT (23.6 min). Figure 27a shows the Tyr (13.7 min), Dityr, and IDT standards, and Figure 27b shows the hydrolyzed HF-Insoluble maize cell wall on PRP-1. IDT, dityrosine and tyrosine standards combined with the HF-Insoluble wall hydrolysate and chromatographed on PRP-1 showed that the maize unknown eluted between dityrosine and IDT (data not shown).

Table 15. Amino Acid Compositions of the HF-Deglycosylated Maize Cell Wall

Amino Acid[a]	Intact Cell Wall	HF-Soluble Wall	HF-Insoluble Wall
Hyp	1.1	3.5	trace
Asx	10.4	8.5	10.6
Thr	5.1	6.0	5.3
Ser	6.9	9.8	6.3
Glx	9.3	8.7	10.8
Pro	3.7	3.5	4.7
Gly	10.7	11.8	10.8
Ala	10.6	12.8	10.8
Cys	0.0	0.0	0.3
Val	6.4	4.9	7.6
Met	1.7	0.2	0.1
Ilu	4.2	3.1	0.3
Leu	10.3	6.5	9.0
Tyr	1.9	1.1	2.0
Phe	4.0	6.1	4.8
Lys	6.2	2.9	7.0
His	2.1	8.6	2.6
Arg	4.7	4.7	6.0

[a] Represented as Mole %

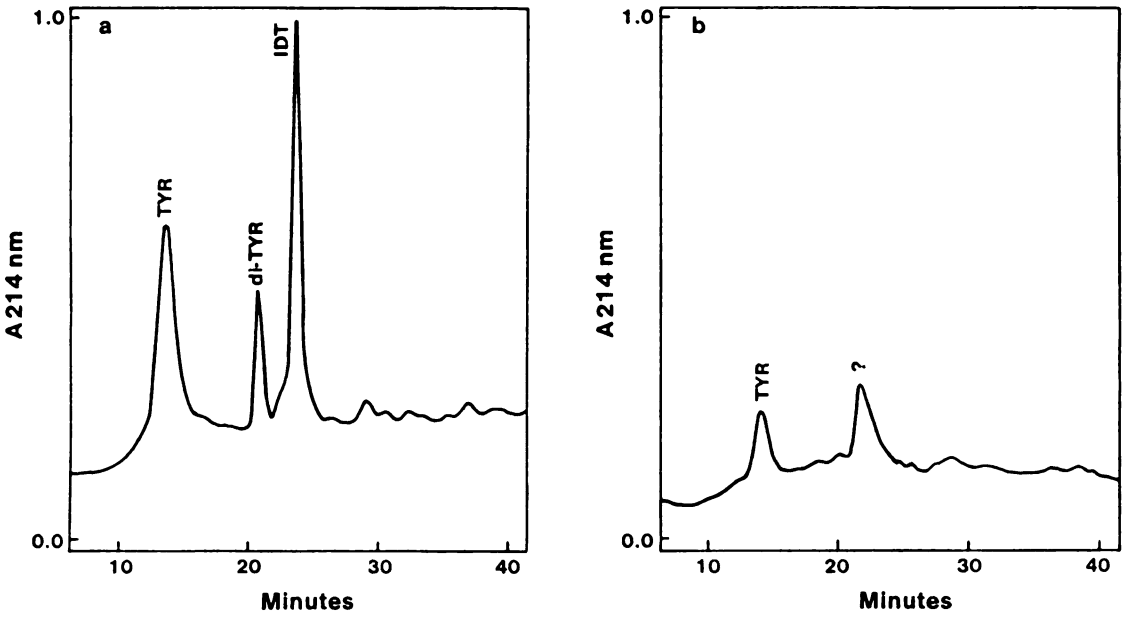


Figure 27. Assay of IDT in the Black Mexican HF-insoluble cell wall. a) Tyrosine, dityrosine and IDT standards. b) The HF-insoluble cell wall contains tyrosine and and unknown (?).

E. Immunoblot Analysis of the Maize Cell Wall

Anti-THRGP antibodies detected an immunoreactive protein in SDS-PAGE immunoblots of the HF-solubilized wall fractions of Sweet Sue root, root tip, and coleoptile: in each instance, the antibodies detected a major band that migrated with the same M_r as the dTHRGP (Figure 28). Preimmune control serum did not react with the blots. All cell wall preparations showed a Coomassie Blue-stained smear of HF-soluble cell wall proteins whose sizes on SDS-PAGE ranged from 200 kD to very small components that migrated with the marker dye (data not shown).

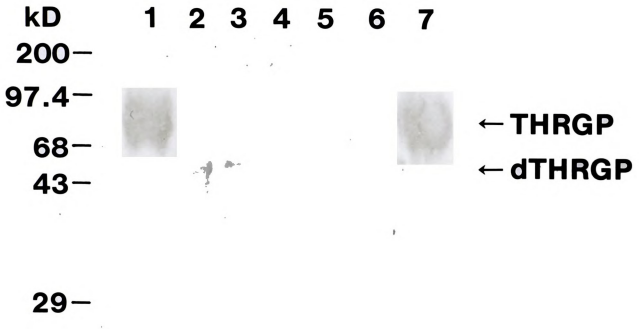


Figure 28. Immunoblot of THRGP (lanes 1 & 7), dTHRGP (lanes 2 & 6), and the HF-soluble cell wall fractions from Sweet Sue coleoptile (lane 3), root (lane 4), and root tip (lane 5).

DISCUSSION

Monocots are, by comparison with dicots, relatively poor in hydroxyproline, although it has been clear for some time that monocot Hyp-containing proteins do exist, both in the grasses and other monocot families (van Etten *et al.*, 1963; Lamport, 1965; Boundy *et al.*, 1967; Burke *et al.*, 1974).

Much of this work implicitly assumes that easily soluble HRGPs correspond to arabinogalactan proteins, while the insoluble HRGPs correspond to extensin. The latter hypothesis is difficult to test. However, recent reinvestigation of the 'intact cell elution' technique (Lamport, 1965) showed that under optimal conditions, soluble monomeric extensin precursors to wall-bound extensin can be ionically desorbed directly from the cell surface of intact tomato cells grown in suspension culture (Smith *et al.*, 1984). Smith *et al.* (1984 & 1986) and others have characterized soluble extensin monomers chemically (Chrispeels, 1969; Stuart & Varner, 1980; van Holst & Varner, 1984; Cassab *et al.*, 1985), immunologically (Leach *et al.*, 1982b; Kieliszewski & Lamport, 1986; Cassab & Varner, 1987), and electron microscopically (Heckman *et al.*, 1988; Stafstrom & Staehelin, 1986; van Holst & Varner, 1984), and thereby provided the tools to determine whether or not extensin occurred in graminaceous monocots, a question relevant to current ideas about the control of cell extension (*cf.* oat coleoptiles) and the proposed

model for the primary cell wall of dicots, which invokes an extensin 'weft' to mechanically couple the load-bearing microfibrillar polymer 'warp'-cellulose (Lamport & Epstein, 1983).

First however, we must summarize the criteria which enable us to classify an HRGP as a member of the extensin glycoprotein family localized in the primary cell wall. These criteria involve primary, secondary and tertiary structure and, therefore, include posttranslational modifications (for the extensins this can account for more than 40% of the amino acid residues) by hydroxylation and glycosylation, which dramatically alter properties of the unadorned polypeptide backbone. Generally, extensins are defined as hydroxyproline-rich glycoproteins that are insolubilized in the cell walls of higher plants. They are basic, rodlike macromolecules with a polyproline-II helical conformation arising in part from the characteristic repetitive pentapeptide Ser-$(Hyp)_4$. Many of the Hyp residues are glycosylated by short oligoarabinosides, while the Ser residues are often galactosylated by a single residue. However, one must remember that these criteria are based on knowledge of a very few dicot extensins, from only three of which (tomato, Smith *et al., 1986;*, sugar beet, Li *et al.*, 1990; melon, Esquerre-Tugaye & Lamport, 1979) do we have direct, rather than cDNA-derived primary sequence information. It would therefore be dangerous to elevate any single characteristic of a dicot extensin to the level of dogma and demand that all extensins subscribe to a pattern which might simply represent extensins from advanced herbaceous dicots. Thus, peptide sequences from sugar beet extensin (Li *et al.*, 1989)

(Figure 29) show that the Ser-Hyp-Hyp-Hyp-Hyp pentameric motif, common in other dicots, can no longer be considered a diagnostic *sine qua non* of extensin (Franssen *et al.*, 1988; Hood *et al.*, 1988; Kieliszewski & Lamport, 1988). In addition, we must also realize that fibrous proteins have their own rules which frequently differ from those formulated for globular proteins (Tiffany & Krimm, 1969; Doolittle, 1986), where folding is of paramount importance.

I. The Isolation and Characterization of *Zea mays* Extensins

A. Elution of Intact Maize Cells and Preparation of HRGPs

My data show the successful application of the intact cell elution technique to maize cell suspension cultures. Here an HRGP monocot/dicot difference occurs. In Hyp-rich cultures of dicots, like tomato, the pool of monomeric extensin peaks during rapid cell growth (Smith *et al.*,1984), while in cultured maize cells the elutable crude HRGP pool rises only slowly during rapid cell growth, and peaks at day 11 after subculture, long after the cells have (abruptly) ceased expansion growth (Figure 5)(Kieliszewski & Lamport, 1987), pointing to possible functional differences between dicot and monocot extensins. More than 30 proteins appeared in the crude HRGP eluate (judging by SDS-PAGE and staining with Coomassie Blue), but only four or five occurred as major components, of which at least three were HRGPs, one of them being unusually rich in threonine (hence a threonine-rich HRGP, or THRGP), and the other two rich in histidine (HHRGPs)(Table 5).

Like dicot extensins, the THRGP and HHRGPs are basic[11] proteins, the THRGP being rich in lysine and the HHRGPs rich in histidine. The THRGP and HHRGPs co-chromatograph on phosphocellulose, as both THRGP and HHRGP are positively charged at pH 3 (Figure 7). However, BioRex-70 exploits the pK_a's of lysine ($pK_a \sim 10.5$) and histidine ($pK_a \sim 6$) to effect a separation of THRGP from HHRGP as HHRGP is mainly uncharged at pH 7 and voids the Biorex-70 column, while the THRGP at pH 7 is highly basic and binds to the matrix (Figure 10).

B. Amino Acid Compositions of THRGP and HHRGP

Ten amino acids accounted for 98 mole% of the THRGP amino acid residues, being richest in threonine and hydroxyproline, each accounting for about 25 mole%, with a high proline, lysine, and serine content, and lesser but significant amounts of tyrosine, histidine, alanine, and valine (Table 5)(Kieliszewski & Lamport, 1987). The THRGP amino acid composition is consistent with the amino acid compositions of a putative THRGP cDNA clone recently isolated from maize coleoptile (Stiefel *et al.*, 1988) and a related glycine-rich THRGP isolated from maize pericarp (Hood *et al.*, 1988) (Table 16).

Eleven amino acids accounted for 95 mole % of HHRGP amino acid residues. The HHRGPs are richer in hydroxyproline ($\sim$34 mole%) than either the THRGP ($\sim$25 mole%) or tomato extensin P1 ($\sim$32 mole%), they are histidine-rich like carrot extensin, and they are relatively rich in alanine ($\sim$9 mole%)(Tables 3 & 5)(Kieliszewski & Lamport, 1988). Such biased compositions are typical of

[11] In the wall microenvironment the pH $\sim$3, judging by the pH of the growth medium, therefore the HHRGPs are positively charged.

HRGPs in general, and extensin in particular, although the threonine-rich feature of the THRGP and the alanine-rich feature of the HHRGPs are novel.

Table 16. Amino Acid Compositions of Three Maize THRGPs

Amino Acid	THRGP	PC-1[a]	MC56 Clone[b]
Hyp	24.8	21.9	
Asx	0.7	2.1	0.4
Thr	25.3	17.5	22.8
Ser	7.3	5.5	6.7
Glx	2.3	2.5	0.0
Pro	14.5	13.5	45.7
Gly	2.4	7.1	0.0
Ala	1.7	5.2	2.2
Val	0.7	2.7	0.4
Cys	0.0	n.d.	0.4
Met	0.0	n.d.	0.4
Ilu	0.1	0.4	0.4
Leu	0.0	0.2	0.4
Tyr	3.7	4.6	6.4
Phe	0.1	0.1	0.1
His	2.4	3.6	0.7
Lys	13.5	11.3	11.2
Arg	0.1	0.7	0.7

[a] From Hood *et al.*, 1988
[b] From Stiefel *et al.*, 1988

C. Glycosylation Profiles of THRGP, HHRGP, and the Maize Cell Wall

The THRGP, HHRGP and maize cell wall Hyp arabinoside profiles are consistent with typical extensin glycosylation patterns, and corroborate earlier work (Lamport & Miller, 1971) which showed a high proportion of nonglycosylated Hyp residues in the monocots (Tables 7 & 13)(Kieliszewski & Lamport, 1987). However, the absence of galactose from the THRGP, as well as its high threonine

content, distinguish it from dicot extensins (Table 6). Although the HHRGP Hyp arabinoside profile (Table 7) is consistent with dicot extensins, its high galactose content (37 mole% of the total sugars) is unique (Table 6), and considering the HHRGPs are rich in alanine, it suggests that they may be graminaceous arabinogalactan proteins (AGPs). However, the very large heterooligosaccharide characteristic of AGPs (an arabinogalactan O-linked to hydroxyproline; Pope, 1977) did not appear in the Hyp arabinoside profile of the HHRGPs. Furthermore, a 60 min partial acid hydrolysis removed all arabinose from the HHRGPs, but left the galactose residues virtually intact (Figure 12), indicating that galactose exists as pyranosides probably independent of the arabinosides, i.e. directly attached to an amino acid other than hydroxyproline. Thus, as serine and threonine are the prime candidates for O-glycosylation (possibly tyrosine), and judging by the molar ratios of galactose to either serine or threonine (5:1) (the molar ratio of Gal:Tyr is about 9:1), it is likely that polygalactose occurs on one or both of the HHRGPs. Although there has been no demonstration of polygalactosyl O-serine/threonine or arabinosyl-polygalactosyl serine/threonine as a component of glycoproteins, Desai *et al.* (1981) presented evidence that digalactosyl-serine occurs in a Hyp-rich lectin from *Datura stramonium*.

D. SDS-PAGE of THRGP and HHRGP

The THRGP's status as a monomer is suggested by its behavior on Superose-6 gel filtration and SDS gel electrophoresis (Figure 11b). The THRGP migrated as a smear on SDS-PAGE with M_r of 72-90 kD (Figure 14, lane 2), an overestimate judging by its contour length and glycosylation profile, and probably

due, in part, to its cationic nature and glycosylation (Segrest *et al.*, 1971). SDS-PAGE indicated the apparent M_r of THRGP as 50 kD after deglycosylation (Figure 14, lane 3), or roughly twice that predicted from the related MC56 THRGP cDNA clone (Stiefel *et al.*, 1988). Yet this anomalous M_r agrees with the M_r of a putative MC56 THRGP protein (after HF deglycosylation) isolated from maize cell walls and identified by immunoblotting by Stiefel *et al.* (1988). SDS-PAGE also overestimates the M_r of deglycosylated tomato extensins P1 and P2 (Smith *et al.*, 1984). Possibly SDS-PAGE does not provide a reliable estimate of molecular weight for deglycosylated HRGPs due their high content of secondary amino acids and charged amino acids which may interfere with their ability to bind SDS (Takano *et al.*, 1988). Like dicot extensins, glycosylated HHRGP did not enter an SDS-PAGE gel. After deglycosylation HHRGP, which had appeared homogeneous by Superose-6 gel filtration (Figure 11a), resolved into two bands (68 & 70 kD) on SDS-PAGE (Figure 15, lane 2). Both bands are HHRGPs judging by their identical behaviour on two cation exchange columns and gel filtration (Figures 7, 10 (the void) & 11a), their simple amino acid composition, carbohydrate profile (Tables 5 & 6), and peptide amino acid compositions and sequences (Tables 11 & 12).

E. Immunological Characterization of THRGP and HHRGP

I recently raised and characterized antibodies against tomato extensin monomers P1 and P2, and against the protein backbone of these two monomers after stripping the carbohydrate off the proteins via anhydrous HF (Kieliszewski & Lamport, 1986). The two antibodies raised against the glycosylated extensins

P1 and P2 cross-reacted with the THRGP and HHRGPs indicating common antigenic epitopes between the glycosylated tomato extensins and the glycosylated maize HRGPs (Figure 24). Some of the common epitopes probably involve the Hyp arabinosides, which are common to both maize and tomato HRGPs. Furthermore, antibodies raised against deglycosylated extensin P1 crossreacted 27% with the THRGP (Figure 24), indicating common antigenic epitopes between tomato extensin backbone and the THRGP protein backbone. Thus, the polyclonal antibodies raised against tomato extensins proclaim the THRGP and HHRGPs at least 'extensin-like' (Kieliszewski & Lamport, 1988)

The THRGP is much more immunogenic than either tomato extensin P1 (and dP1) or P2 (and dP2), judging by the titer of the anti-THRGP antibodies (Figure 25): quantitative ELISAs using anti-THRGP antibodies showed a positive response of 1.2 Abs_{405} to 20 ng THRGP antigen, with antiserum dilutions of 1:2000; whereas a comparable reaction with anti-tomato extensin antibodies required dilutions of 1:200 to 1:800 and 200 ng antigen/microtiter well (Kieliszewski & Lamport, 1986; Kieliszewski *et al.*, 1990).

Judging from the high cross-reactivity (97%) of anti-THRGP antibodies (from two rabbits) with deglycosylated THRGP, the peptide backbone is highly antigenic, while the epitopes contributed by the hydroxyproline arabinosides are much less (Figure 26). The low cross-reactivities with other HRGP antigens, including glycosylated and deglycosylated HHRGP, confirm that the antibodies are quite specific for the THRGP backbone. Consistent with the cross-reactivities of anti-tomato extensin antibodies with THRGP, antigens tomato P1 and dP1 showed the

most cross-reactivity with the anti-THRGP antibodies (Fig 26), again indicating common antigenic epitopes, and implying some homology between the maize THRGP and tomato P1.

F. Transmission Electron Microscopy and Circular Dichroic Spectra of THRGP and HHRGP

Transmission electron microscopy of low-angle rotary-shadowed material visualized THRGP and HHRGP molecules as flexuous rods (Figures 16a & b) similar to those of dicot extensins (van Holst & Varner, 1984; Stafstrom & Staehelin, 1986; Heckman *et al.*, 1988) although somewhat shorter (70 nm contour length vs 80 nm for dicot extensins) (Kieliszewski & Lamport, 1988), but consistent with their elution position on Superose-6 gel filtration close to dicot extensin monomers. Furthermore, TEM visualization of the THRGP and HHRGPs, combined with their Hyp and Pro content of 39-41% suggested a polyproline-II conformation (3 residues/turn with 9.4 A pitch) similar to extensin. However, CD data indicate that the THRGP and dTHRGP exist in an "unordered" or "random coil" conformation (Table 8; Figure 17a), although the HHRGPs show a broad positive band at 210 nm indicating a more ordered structure, possibly having some polyproline II conformation (Table 8; Figure 17b)(Tiffany & Krimm, 1969). Although this lack of polyproline II conformation seemed anomalous in view of the elevated proline/hydroxyproline content of THRGP and HHRGP, the dispersion of proline and hydroxyproline residues (Tables 10 & 12) partially resolves the anomaly, as CD spectra of synthetic polypeptides show that nucleation of the polyproline II helix usually requires at least four contiguous proline residues

(Okabayashi & Isemura, 1968; Deber *et al.*, 1970). Indeed, a tetrahydroxyproline block occurs only in the minor THRGP peptide TC1 (Table 10), in agreement with a single occurrence of tetraproline at the C-terminus of the cDNA clone MC56 (Stiefel *et al.*, 1988)(cf Figure 30), while the HHRGPs contain repeating tri-hydroxyproline blocks (Table 12). The anomaly may also be a product of the poorly defined term "random coil" which clearly does not preclude the THRGP or HHRGP secondary structure deduced by TEM (Tiffany & Krimm, 1969), or the presence of secondary structure in other "random coil" proteins. For example there is even a report of a monoclonal antibody that can recognize a conformational epitope in a random coil protein (Saad *et al.*, 1988).

In contrast to dicot extensin, wherein the carbohydrate apparently helps to maintain the secondary polyproline-II structure (van Holst & Varner, 1984), the Hyp arabinoside moieties of the THRGP have little influence on the secondary structure of the THRGP, judging by its CD spectra before and after HF deglycosylation (Table 8; Figure 17a). The minimum at 205 nm increased slightly in its intensity (ΔE_{min} increased from -3 to -3.6) and was blue shifted by 3 nm to 202 nm (Kieliszewski *et al.* 1990).

At this point, there is sufficient structural and chemical similarity with the extensins to consider the THRGP and HHRGPs as analogous proteins, although their lack of polyproline-II helix might argue against homology.

G. dHHRGP Peptides Share Homology with P3-Type Dicot Extensins

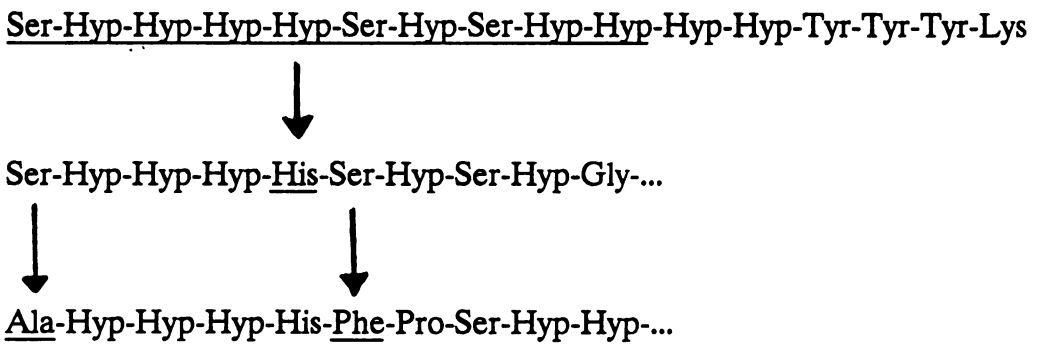
Chymotryptic digestion of dHHRGPs generated peptides, none of which voided the G-25 gel filtration column (Figure 22), hence the absence of a protease-

resistant core and an essentially complete digestion.

HPLC of the digestion produced a peptide map with 12 peptides (Figure 23), all of them containing Hyp, Ala and His (except HC7 & HC8, whose compositions and sequences are not yet determined) and reflecting the simple amino acid analyses of the proteins (Table 11). Thus both bands resolved on SDS-PAGE are HHRGPs.

Although no tetra(hydroxy)proline occurs in any of the sequenced dHHRGP peptides, four peptides (Table 12) do share significant homology with the N-terminus of the P3-type extensin repetitive hexadecamer:

Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-Tyr-Tyr-Tyr-Lys (Table 4), deduced as follows: Chymotryptic peptide HC6 has only a His for Hyp substitution at position 5, and therefore shows 89% homology with the above hexadecamer terminus: Ser-Hyp-Hyp-Hyp-His-Ser-Hyp-Ser-Hyp-Gly. Peptides HC10, HC11 & HC12 each have one repeat of the decameric sequence: Ala-Hyp-Hyp-Hyp-His-Phe-Pro-Ser-Hyp-Hyp which is 88% homologous with HC6 (Ala for Ser at residue 1; Phe for Ser at residue 6, and unhydroxylated Pro at residue 7), and therefore 66% homology with the N-terminal portion of P3 type extensin domain. Thus, at least one of the HHRGPs has a (repeating) decamer sharing 66% homology with P3-type extensin, thereby defining the HHRGP(s) as members of the extensin family (Doolittle, 1981; Dayhoff *et al.*, 1983; Marchelonis *et al.*, 1984). One can then trace a possible divergence from a Type P3 domain through the HC6 sequence to the common repeat of HC10, HC11 & HC12 as follows:



Alternatively, the Ala-Hyp-Hyp-Hyp repeat might be a primitive feature, as HRGPs from *Chlamydomonas* are frequently rich in alanine, and a recently cloned cDNA for a *Chlamydomonas* cell wall HRGP demonstrates X-Pro-Pro-Pro repeats, although only one is Ala-Pro-Pro-Pro (U. Goodenough to D. Lamport, personal communication).

Two intriguing sequences occur in the N-terminal portions of peptides HC10 & HC11 (Table 12). HC10 contains a 9 residue palindrome (residues 9-17) centered around an asparagine residue: Hyp-Hyp-Ala-Ala-Asn-Ala-Ala-Hyp-Hyp, that is reminiscent of an alanine-rich 7 residue palindrome (of unknown function) that occurs in Chlamydomonas (U. Goodenough to D. Lamport, personal communication). The other unusual HHRGP sequence occurs in the N-terminal portion of HC11: Thr(Hyp)-Hyp-Hyp-His-His-His-Hyp-Hyp-Hyp-. The occurrence of 3 consecutive histidine residues is a rare event, as histidine is one of the four least frequently occurring amino acids (Doolittle, 1981), and may be involved in HHRGP function in the maize cell wall. Furthermore, if residue 11 of TC11 is hydroxyproline rather than threonine, the histidine triplet exists as the center of another palindrome: Hyp-Hyp-Hyp-His-His-His-Hyp-Hyp-Hyp. Thus palindromes

sandwiched between a decameric repeating motif may define at least one of the HHRGPs. Likewise, P3-type extensins from tomato, petunia and bean (Showalter & Rumeau, 1989) are defined by an 11-residue repeating palindrome: Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp followed by a tetramer containing a tyrosine triplet: Tyr-Tyr-Tyr-Lys in which the outer two tyrosines are ultimately enzymically modified to form isodityrosine as an intramolecular crosslink (Epstein & Lamport, 1984)(Table 4). However, there is no evidence to suggest the three consecutive histidines of HC11 undergo any modification analogous to crosslinking.

H. dTHRGP Peptides Share Homology with P1 and P3-Type Dicot Extensins

Chymotryptic digestion of the dTHRGP generated peptides, none of which voided the G-25 gel filtration column, indicating the absence of a protease-resistant core and an essentially complete digestion (Figure 18).

HPLC of the complete chymotryptic digest produced a simple peptide map with only 8 peptides (Fig 19). Despite the occurrence of only a single tetrahydroxyproline in the THRGP (Table 10, TC1), homology exists between the THRGP repetitive sequences (Table 10, TC5 and TC4) and tomato P1 extensin as follows: the decameric motif Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys occurs as tryptic peptides from tomato P1 (Smith *et al.*, 1986) and tobacco (M. Kieliszewski and D. Lamport, unpublished data), as cDNA and DNA sequences from tobacco, Petunia and carrot (Chen & Varner, 1985a & b; Showalter & Rumeau, 1989), and also as a modified peptide sequence in sugar beet (Figure 29)(Li *et al.*, 1990). However, the modified decamer is also discernible in THRGP

chymotryptic peptides TC5 and TC4 as the repetitive motif: Ser-Hyp-Lys-Pro-Hyp-Thr-Pro-Lys which differs from the tomato P1 decamer essentially by a Lys for Hyp substitution at residue 3 and a Val-Tyr deletion at residues 8 and 9 (Figure 29)(Kieliszewski *et al.*, 1990). The corresponding derived sequence occurs seven times in cDNA clone MC56 (Stiefel *et al.*, 1988) (Figure 30) pointing to

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

[X] = Val His Glu Tyr Pro

[Y] = Val Lys Pro Tyr His Pro

[Z] = Ala Thr Lys Pro Pro

Figure 29. The decameric motif of P1-type extensins.

significant homology with the isolated THRGP glycoprotein. Futhermore, like a typical dicot extensin, MC56 THRGP is organized into three distinct domains: the C and N-termini and the central domain composed of the major repeating motifs (Figure 30). Further homology with dicot extensins from tomato (Smith *et al.*, 1986), petunia and bean (Showalter & Rumeau, 1989) occurs in chymotryptic

peptide TC1 (Table 10) and the corresponding C-terminal sequence of clone MC56 (Figure 30). The TC1 octapeptide Thr-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-Tyr (Table 10), and MC56 cDNA sequence Thr-Pro-Ser-Pro-Pro-Pro-Pro-Tyr-Tyr (Stiefel *et al.*, 1988) are homologous with the dicot extensin Type 3 domain: Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-Tyr-Tyr (except for a Thr for Ser substitution in position 1). The single substitution in maize of Thr for the dicot Ser in the first position is effected by a single nucleotide base change. Thus the maize THRGP is a fusion of two dicot general extensin types into one protein: a modified tomato Type 1 extensin peptide backbone, with a C-terminal tail homologous with the repeating peptide of tomato Type 3 extensin. From both peptide and cDNA sequences I conclude therefore, that in maize a Ser-Hyp-Lys-Pro-Hyp pentamer replaces the dicot Ser-$(Hyp)_4$ pentamer throughout the THRGP molecule except for a single occurrence of Ser-$(Hyp)_4$ in peptide TC1 (Table 10) which from MC56 (Stiefel *et al.*, 1988) corresponds to the C-terminal tail (Figure 30). Thus, a single Ser-$(Hyp)_4$ in an advanced graminaceous monocot is probably an evolutionary remnant, hence the C-terminal tail may, like some others, represent a vestigial condition. This retention of 70% homology (allowing for a Val-Tyr "gap") in a repetitive motif, and almost 90% homology in the C-terminal tail establishes membership of maize THRGP in the extensin family (Doolittle, 1981), albeit a member with some unique characteristics, especially a distinctive and quite selective proline hydroxylation pattern with rather subtle determinants.

Figure 30. Proposed primary amino acid sequence of a THRGP encoded by cDNA clone MC56 isolated from maize coleoptile. Peptides common to the Black Mexican THRGP and MC56-THRGP are labeled on the right as TC1, TC5, TC6 and TC7. Tryptic peptide TC1 is underlined. Residue 160 may be Hyp rather than Pro.

1
Thr Hyp Hyp Thr Tyr

21
Thr Hyp Ser Hyp Lys Pro Hyp ----------Thr Pro Lys Pro Thr Hyp Hyp Thr Tyr (TC5)

22 42
Thr Hyp Ser Hyp Lys Pro Hyp Ala Ser Lys Pro Pro Thr Pro Lys Pro Thr Hyp Hyp Thr Tyr

58
Thr Hyp Ser Hyp Lys Pro Hyp ---------- Thr Pro Lys Pro Thr Hyp Hyp Thr Tyr (TC5)

59 79
Thr Hyp Ser Hyp Lys Pro Hyp Ala Thr Lys Pro Pro Thr Pro Lys Pro Thr Hyp Hyp Thr Tyr

95
Thr Hyp Ser Hyp Lys Pro Hyp ----------Thr Pro Lys Pro Thr Hyp Hyp Thr Tyr (TC5)

113 116
Thr Hyp Ser Hyp Lys Pro Hyp Ala Thr Lys Pro Pro Thr Pro Lys Pro Thr Hyp Hyp Thr Tyr

117 132
Thr Hyp Ser Hyp Lys Pro Hyp ----------Thr Pro Lys Pro Thr Hyp Hyp Thr Tyr (TC5)

148
Thr Hyp Ser Hyp Lys Pro Hyp ----------Thr Pro Lys Pro Thr Hyp Hyp Thr Tyr (TC5)

167
Thr Hyp Ser Hyp Lys Pro Hyp ------Thr His Pro-----Thr (Pro) Lys Pro Thr Hyp Hyp Thr Tyr (TC7)

183
Thr Hyp Ser Hyp Lys Pro Hyp ----------Thr Pro Lys Pro Thr Hyp Hyp Thr Tyr (TC5)

199
Thr Hyp Ser Hyp Lys Pro Hyp ----------Thr Pro Lys Pro Thr Hyp Hyp Thr Tyr (TC5)

220
Thr Hyp Ser Hyp Lys Pro Hyp Ala Thr Lys Pro Pro Thr Pro Lys Pro Thr Hyp Hyp Thr Tyr

234
Thr Hyp Thr Hyp Lys Pro Hyp Ala Thr Lys Pro Pro Thr Tyr ---------- (TC6)

236 251
Thr Hyp Thr Hyp Hyp Val Ser His Thr Hyp Ser Hyp Hyp Hyp Hyp Tyr Tyr (TC1)

I. The Specificity of Prolylhydroxylase

Combining the THRGP peptide and MC56 cDNA sequence information (Table 10 & Figure 30) we see that the MC56 THRGP contains about 121 proline residues of which approximately 74 are candidates for posttranslational hydroxylation and glycosylation. The major repetitive chymotryptic peptide TC5 contains five Hyp residues, at least two being glycosylated (on average) based on the hydroxyproline glycoside profile showing 48% Hyp glycosylation of the intact THRGP (Kieliszewski & Lamport, 1987). Thus TC5 corresponds to 14 potential glycosylated domains which alternate regularly with non-glycosylated domains (Figures 30 & 31), the latter occurring mainly as the pentapeptide palindrome Thr-Pro-Lys-Pro-Thr. But what determines which proline residues are hydroxylated and which are not? Despite the reported preference of plant prolylhydroxylases for (artificial) substrates with a polyproline-II secondary structure (Tanaka *et al.*, 1981), the CD data indicate that the polyproline-II conformation may not be a requirement for hydroxylation. Because two thirds of the THRGP proline residues are hydroxylated (Table 5) with a high degree of specificity (Table 10), a hydroxylation code seems possible, although it is not immediately self-evident. "Windows" of one, two, or three contiguous residues do not yield an exclusive hydroxylation code; for example, Thr-Pro-Lys is generally unhydroxylated although Thr-Hyp-Lys also occurs in peptide TC6 (Table 10 and residues 223 to 225 in Figure 30). Assuming the predicted hydroxylations of MC56 THRGP are correct, there are five occurrences of Lys-Pro-Pro-Thr but seven Lys-Pro-Hyp-Thr (See Figure 30). Interestingly Lys-Pro

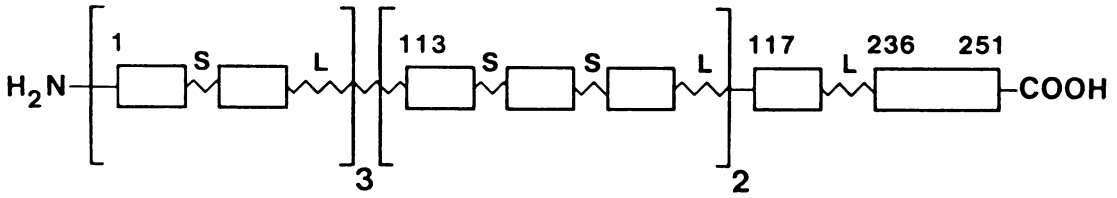


Figure 31. Schematic block diagram for a THRGP based on combined direct peptide sequencing and cDNA clone MC56 (Stiefel *et al.*, 1988). The blocks represent the repetitive eleven-residue sequence: Hyp-Hyp-Thr-Tyr-Thr-Hyp-Ser/Thr-Hyp-Lys-Pro-Hyp in which 2 or 3 of the 5 Hyp residues are glycoslated. These domains are separated by short (S) or long (L) non-glycosylated domains. The last block corresponds to the C-terminus of the molecule. Superscripts correspond to the residue numbers of Figure 30. Subscripts denote the number of pattern repeat units.

and His-Pro are probably never hydroxylated here or in any of the known dicot extensin peptides. However, a window of four contiguous residues does identify four exclusive sequences (Figure 32) which account for every hydroxyproline residue in the THRGP molecule, except for the final Pro residue in TC4. Each window contains two candidates for hydroxylation: I. X-Pro-Pro-X

II. X-Pro-X-Pro III. Pro-X-X-Pro and IV. Pro-Pro-Pro-Pro, where X refers to specific residues (Table 10, Figures 30 & 32). A single prolylhydroxylase would have to recognize subtle differences in peptide conformation; for example, the major nonhydroxylated THRGP domain occurs as the repetitive pentapeptide palindrome: Thr-Pro-Lys-Pro-Thr whose conformation might not allow hydroxylation of its Thr-Pro, while Lys-Pro is never hydroxylated.

Window:	Hydroxylation Substrate Sequences	Hydroxylated Product Sequences	Non-hydroxylated Related Sequences
I.	Thr-Pro-Pro-Thr	----> Thr-Hyp-Hyp-Thr	
II.	Thr-Pro-Ser-Pro	----> Thr-Hyp-Ser-Hyp	
III.	Pro-Lys-Pro-Pro	----> Hyp-Lys-Pro-Hyp	Pro-Lys-Pro-Thr Hyp-Lys-Pro-Pro Thr-Lys-Pro-Pro
IV.	Pro-Pro-Pro-Pro	----> Hyp-Hyp-Hyp-Hyp	

Figure 32. Suggested four-residue windows recognized by THRGP prolyl hydroxylase.

An alternative hypothesis postulates the existence of three prolyl hydroxylase isozymes, each specific for its own tetrapeptide window, I, II, or III (Figure 32) and requiring specific non-proline residues within that window, while IV might be a special case involving recognition by I and III, which is not unreasonable considering the multimeric character of the enzyme (Bolwell *et al.*, 1985) and that different catalytic subunits occur in animal systems (Kivirikko, 1989).

Compared to the THRGP hydroxylation profile, the HHRGP peptide hydroxylation profile is straightforward: all proline residues are hydroxylated with two general exceptions. First, Phe-Pro is not hydroxylated. Thus the bulky side chains of Phe-Pro, Lys-Pro and His-Pro in the HHRGP, THRGP and dicot extensins (Smith *et al.*, 1986) may sterically hinder prolyl hydroxylase. Secondly,

the HHRGP sequences contain two instances of the THRGP hydroxylation window X-Pro-X-Pro, but in only one sequence are the prolines hydroxylated: Ala-Pro-Ala-Pro occurs in HC1,and Ser-Hyp-Ser-Hyp in HC6. Thus X must be a specific residue for hydroxylation to occur. Alternatively, the peptide conformation surrounding the sequence may dictate hydroxylation. Like the THRGP, a definite polyproline-II conformation is apparently not required for extensive hydroxylation of HHRGP proline residues[12]. This implies that the use of natural rather than artificial substrates to assay prolyl hydroxylase(s) would facilitate its isolation and characterization in higher plants.

J. Further Comparison of Black Mexican THRGP and MC56 THRGP

Further comparison of the Black Mexican THRGP with MC56 THRGP clone (Stiefel *et al.*, 1988) shows that some differences exist between the two THRGPs: MC56 THRGP lacks at least 2 small minor peptides, TC2: Thr-Hyp-Ser-Hyp-Lys-Pro-Thr-Hyp-Hyp-Thr-Tyr, and TC4: Thr-Hyp-Ser-Hyp-Lys-Pro-Hyp-Thr-Hyp-Lys-Pro-Pro..., which occur in the Black Mexican THRGP (Table 10), while a major 21-residue peptide(s) of MC56 THRGP is missing from the Black Mexican (the peptide represented by residues 22 to 42 in Figure 30: Thr-Hyp-Ser-Hyp-Lys-Pro-Hyp-Ala-Ser-Lys-Pro-Pro-Thr-Pro-Lys-Pro-Thr-Hyp-Hyp-Thr-Tyr). However, Black Mexican peptide TC8 (Table 9) has an amino acid composition resembling that of the missing peptide, therefore the peptide may be present in lesser amounts in the Black Mexican THRGP, or

[12] This assumes the THRGP and HHRGP *in vitro* random coil conformations, as assayed by CD, are identical with their *in vivo* conformations.

in view of its more hydrophobic nature, it is retained on the reversed phase column used for peptide separations. Alternatively, the two THRGPs may be encoded by separate but homologous genes.

K. Tryptic Hydrolysis of Lysylproline in dTHRGP

Although trypsin was the obvious choice for peptide mapping of the THRGP, which contained 12 mole% lysine, I recovered few peptides after tryptic digestion of THRGP (Figure 20). A peptide initially purified from the tryptic digest showed an N-terminal proline residue (Peptide TT1 of Table 10):

Pro-Thr-Hyp-Hyp-Thr-Tyr-Thr-Hyp-Ser-Hyp-Lys-Pro-Hyp-Thr-Pro-Lys which was originally dismissed as an artifact, because it is generally believed that Lys-Pro bonds are not hydrolyzed (Hill, 1965; Kasper, 1975). However, chymotryptic digestion of the THRGP yielded the major peptide TC5 which overlapped with the tryptic peptide TT1, and contained two Lys-Pro bonds, thus strengthening the suspicion that trypsin cleaved a specific Lys-Pro bond. Further tryptic cleavage of chymotryptic peptide TC5 showed that only the latter of the two Lys-Pro linkages, Hyp-Lys-Pro-Hyp and Pro-Lys-Pro-Thr, was labile (Table 17). One other example of a trypsin-sensitive Lys-Pro was reported in proline-rich proteins from human saliva (Table 17) (Wong *et al.*, 1979; Wong & Bennick, 1980; Schlesinger & Hay, 1986). Thus for Lys-Pro cleavage, an extended polypeptide backbone (characteristic of a proline-rich polypeptide) is probably a necessary, but not sufficient, condition, because trypsin-resistant Lys-Pro bonds occur in the same proteins and also in another HRGP isolated from tomato (Smith *et al.*, 1986). Inspection of the available sequences around susceptible and resistant Lys-Pro

bonds (Table 17) suggests a second condition for cleavage, namely, backbone flexibility flanking the susceptible Lys-Pro bonds. Table 17 shows that a susceptible Lys-Pro bond occurs in sequences which have N-terminal flexibility at residue -3 (where Lys-Pro = -1 and +1) and C-terminal flexibility at residue +2. Peptides with resistant Lys-Pro fail to meet the required flanking flexibility, because proline or hydroxyproline residues at -3 or +2 constrain rotation around the phi (peptide N to alpha C) bond. Thus the seemingly antithetical requirements for specific flexibility, together with the highly constrained but extended backbone conformation of a proline-rich protein, may account for the rarity of Lys-Pro cleavage (Kieliszewski *et al.*, 1989).

L. Some Evolutionary Implications of Graminaceous Extensins

Characterization of the graminaceous THRGP & HHRGP extensins also have some interesting evolutionary implications, as we can now directly relate three widely different genera to two repetitive elements (typified by the tomato P1 and P3 decamers) which contain tetrahydroxyproline (tomato) or variants of tetra(hydroxy)proline: i) split by an insertion sequence (sugar beet) or ii) a Lys or His for Hyp substitution (maize)(Figure 29). While it is not clear which condition is primitive, the Hyp-Lys-Pro-Hyp and Hyp-Hyp-Hyp-His of maize is an advanced feature judging from the relatively recent origin of the graminaceous monocots, and the single vestigial Ser-$(Hyp)_4$ of maize THRGP. On the other hand split tetrahydroxyproline (or proline) is widespread in advanced dicots (Franssen *et al.*, 1987; Hong *et al.*, 1987), but also occurs in primitive dicots represented by the chenopod, sugar beet (Li *et al.*, 1990). Thus one possible

Table 17. Trypsin Labile and Trypsin Stable Lys-Pro Bonds

Threonine Hydroxyproline-Rich Glycoprotein:										
Trypsin labile:	Pro	Hyp	Thr	Pro	Lys	Pro	Thr	Hyp	Hyp	Thr
Trypsin stable:	Thr	Hyp	Ser	Hyp	Lys	Pro	Hyp	Thr	Pro	Lys
Position	-5	-4	-3	-2	-1	+1	+2	+3	+4	+5
Tomato Hydroxyproline-Rich Glycoprotein*:										
Trypsin stable:	Hyp	Hyp	Hyp	Val	Lys	Pro	Tyr	His	Pro	Thr
Position	-5	-4	-3	-2	-1	+1	+2	+3	+4	+5
Proline-Rich Phosphoproteins:**										
Trypsin labile:	Pro	Pro	Gln	Gly	Lys	Pro	Gln	Gly	Pro	Pro
Trypsin stable:	Pro	Pro	Pro	Gly	Lys	Pro	Gln	Gly	Pro	Pro
Position	-5	-4	-3	-2	-1	+1	+2	+3	+4	+5

* From Smith *et al.*, 1986
** From Wong *et al.*, 1979; Wong & Bennick, 1980; Schlesinger & Hay, 1986

evolutionary progression is:

Hyp-Hyp-[X]-Hyp-Hyp ---> Hyp-Hyp-Hyp-Hyp ---> Hyp-Lys-Pro-Hyp and Hyp-Hyp-Hyp-His. However, divergence from tetrahydroxyproline seems more likely, especially as sequences of HRGPs from *Volvox* and *Chlamydomonas reinhardtii* show repeating clusters of X-Pro-Pro-Pro (*Chlamydomonas* cDNA clone, U. Goodenough, personal communication) and Ser-$(Hyp)_{>6}$ (*Volvox*; Schlipfenbacher *et al.*, 1986). Resolution of this problem clearly requires sequence information from non-graminaceous monocots, primitive dicots and pre-angiosperms. Because the wall is so intimately involved in the creation of plant form, the evolution of structural wall proteins, such as extensin, must be coupled to and should therefore parallel, the evolution of structures *per se*.

Finally, there is the question of extensin function. While structural in a general sense, no discrete function is assigned to any extensin, therefore it is not possible to discuss functional homologies between dicot and monocot extensins; however, numerous clues point to fundamental roles for dicot extensins in growth, development and stress response (Showalter & Varner, 1989; Showalter & Rumeau, 1989). Isolation of multiple extensins (Smith *et al.*, 1984; Smith *et al.*, 1986) and extensin cDNAs (Corbin *et al.*, 1987) shows that a small glycoprotein family exists, which, by analogy with collagen, may be tailored to the tissue. For example, of the twelve collagen types, four occur exclusively in cartilage (Piez, 1987). Thus, a systematic approach to function demands a classification of extensin types, starting with the most highly expressed, best-characterized and most easily recognized. Two easily recognizable decameric motifs (and variants) identify P1 and P3 type extensins which include the maize THRGP and HHRGPs, whose functions probably differ from dicot P1 and P3. For example, the maize HRGPs are expressed at a significantly lower level than dicot extensins. Furthermore, HF treatment of salt-washed walls solubilized the bulk of the HRGPs (Figure 28 & Table 14); this is quite unlike the dicots where covalently bound extensin is generally HF-insoluble (Smith *et al.*, 1984). Interestingly, MC56 mRNA is actively expressed in the root tip and coleoptile, although much less in the root, suggesting a possible tissue specificity for the HRGP (Stiefel *et al.*, 1988); but this is only apparent, as significant amounts of THRGP occurred in all maize tissues examined, notably, 4 day coleoptile, root, root tip cell walls (Table 14), and also in maize pericarp from which a related glycine-rich THRGP was recently isolated

(Hood *et al.*, 1988). This was corroborated by the cell wall hydroxyproline content for each tissue (Table 14, Hood *et al.*, 1988). The presence of THRGP protein in the virtual absence of THRGP mRNA simply confirms that structural cell wall proteins do not turnover. Other features, such as the exceedingly high structural periodicity which even includes a repetitive seven residue palindrome in the THRGP, and at least one (repetitive?) palindrome in one or both HHRGPs, point to a special function for the maize HRGPs, perhaps involving self-assembly by specific interactions (e.g. the ϵ-amino groups of lysine) with the major acidic polysaccharide components which are glucuronoarabinoxylans in the graminaceous monocots rather than the rhamnopolygalacturonans characteristic of the dicots and non-graminaceous monocots (Figure 4)(Burke *et al.*, 1974; Darvill *et al.*, 1980b; Carpita, 1983, 1984, 1985; Jarvis *et al.*, 1988). Also, the unusual lability of the palindromic lysyl residue in TC5 (Table 17) could imply a cleavage site possibly enabling cell expansion by relaxing the network. Significantly, the THRGP and HHRGPs apparently do not contain potential IDT-forming sequences like those that occur in dicot extensins. For example, neither Tyr-Tyr-Tyr-Lys nor Tyr-Lys-Tyr-Lys occurred in any sequenced maize peptide (Tables 10 & 12). Furthermore the tyrosine-containing putative intermolecular crosslink sequence of P1 (the Val-Lys-Pro-Tyr-His-Pro insertion sequence of Figure 29) is absent from the maize HRGP sequences. And although THRGP contains a version of the P1 type extensin decamer Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys, the deletion of Val-Tyr in the THRGP eliminates a putative intermolecular IDT crosslink site, which may explain its solubility in HF. Finally, THRGP structural periodicity

involving regularly alternating glycosylated and non-glycosylated regions (Figure 32), may, as previously suggested, be related to the insertion mechanism for a transmural protein whose reptation into the wall would be aided by the glycosylated "thread" of a molecular screw (Lamport, 1989).

II. The Maize Cell Wall

Primary cell walls of higher plants contain both ionically-bound and covalently-bound protein components, the bulk of which consists of structural glycoproteins. In dicots, many of these proteins are rich in hydroxyproline, most of it firmly associated with the wall matrix; however, the cell walls of the graminaceous monocots are Hyp-poor which implies that the graminaceous walls contain significantly less structural protein than dicots (Carpita & Kanabus, 1988), or that structural protein alternatives to extensin occur in the cell walls of some higher plants.

Although the maize cell wall is like the dicot wall in that it accounts for 40% of the cell and is about 10% protein, judging by protein recoveries from amino acid analyses, it is only 0.7-0.2% hydroxyproline on a dry weight basis (vs. 1-2% in dicots)(Figure 4). Furthermore, another graminaceous monocot/dicot difference shows up on treatment of the wall with anhydrous HF. Although most of the wall-bound dicot extensin remains insoluble in HF, suggesting covalent extensin-extensin crosslinks, possibly by IDT, as a mechanism for insolubilization of extensin monomers into the dicot cell wall, the maize cell wall generally retains little Hyp after treatment with HF (Tables 14 & 15), and the HF-insoluble wall contains no

IDT (Figure 27b). Clearly, if there is an HRGP network *in muro*, then HF cleaves the intermolecular crosslinks, presumably because they differ from the dicot crosslinks. Nevertheless, an HF-insoluble residual wall protein remains that accounts for the bulk of the wall protein, and is therefore probably structural rather than enzymic protein. Furthermore, fractionation of maize cell wall hydrolysates yielded a UV-absorbing peak eluting (from the PRP-1 column; Figure 27b) between dityrosine and IDT, suggesting another tyrosine derivative (assuming it is indeed an amino acid), and raising the possibility of another protein crosslink. Clearly, extensin is not the only structural cell wall protein and any cell wall model must take that into account. Thus the framework of the warp-weft model could be the same but with some differences in detail. These important monocot/dicot differences could well reflect the essential dichotomy between these two groups of Angiosperms and their growth habit, which especially in the dicots, relies on turgidity and for support, while silica plays a large role in the grasses (Stebbins, 1974). This together with a radically different arrangement of meristems (Table 18) may reflect a possible fundamental difference in primary wall organization (in addition to the switch from pectin to glucuronoarabinoxylans) between dicots and graminaceous monocots, which diverged >150 million years ago. Nevertheless, it is conceivable that some generally accepted monocot/dicot differences (Table 18) may not hold in some closely related groups. Stebbins (1974) considers the Chenopodiaceae as fairly close to the monocot line of evolution, noting that: "the first-formed vascular bundles may either form a circle of widely separated units or be scattered through the stem, giving a superficial

Table 18. Six Key Structural Characteristics of Dicots and Monocots[a]

	Dicots	Monocots
Leaf Veins	Net-like	Parallel
Cotyledons	two	one
Flower Parts	In multiples of 4 or 5	In multiples of 3
Vascular Bundles	Cylindrical arrangement Vascular Cambium Produced	Scattered No Vascular Cambium
Roots	Tap roots	No tap root

[a] cf. Figure 4 for a cell wall comparison of dicots vs. graminaceous monocots

resemblance to monocotyledons." Furthermore, the remarkable resemblance between the amino acid profiles of sugar beet and maize cell walls (both from cultured cells)(Table 19) seem highly significant rather than superficial, especially as the hydroxyproline arabinoside profiles of the two walls are also similar (Li *et al.*, 1990).

If we assume that extracellular matrices are *a priori* network structures, then new "Hyp-less" structural proteins in monocots raise questions about the sort of network these structural proteins may create: the kind of crosslinks involved; how they are formed; and of course the role played by small amounts of HRGPs.

Table 19. Amino Acid Compositions of Black Mexican Maize and Sugar Beet Wall

Amino Acid	Maize	Beet[a]
Hyp	1.1	0.4
Asx	10.4	12.0
Thr	5.3	5.1
Ser	6.9	7.3
Glx	9.3	12.0
Pro	3.7	5.1
Gly	10.7	9.5
Ala	10.6	8.7
Cys	0.0	0.0
Val	6.4	6.5
Met	1.7	1.6
Ilu	4.2	4.8
Leu	10.3	9.7
Tyr	1.9	1.3
Phe	4.0	3.8
Lys	7.0	7.1
His	2.1	2.2
Arg	4.7	3.3

[a] From Li *et al.*, 1990

IDEAS FOR FUTURE WORK[13]

To ascribe a precise function to purely structural proteins, which lack enzymic activity, demands several different approaches; especially as the existence of several extensin types (in tomato, P1a, P1b, P2 & P3, Smith *et al.*, 1986; and in maize, THRGP and multiple HHRGPs) probably reflects a diverse functionality. For example, the analogous Hyp-rich structural (glyco)protein of animal extracellular matrices, collagen (Types I-XII), serves very different functions which, according to Eyre (1980), include i. ropes (tendons and ligaments) ii. woven sheets (skin and facia) iii. filtration membranes (glomeruli) iv. supporting skeleton reinforced with mineral salts (bone and dentin) v. tissue organization and vi. mediation of the interactions between specific cell layers (Bornstein & Sage, 1980).

A few of the different approaches toward determining extensin function might involve: the determination of *in situ* tissue distributions of particular extensin types, manipulation of extensin's post-translational modifications, *in vitro* binding experiments involving particular extensins and other wall polymers (e.g. GAX or pectins), isolation of extensin/polysaccharide heteropolymers that interact *in vivo*,

[13] The data presented in this section is very preliminary, therefore it is not presented in the Results section of this thesis.

crystallization of extensins for X-ray diffraction, the isolation of extensin mutants, and finally, a comparison of diverse species to determine which features are rigorously conserved (e.g. Hyp-arabinosides, P1 and P3-type domains). But first we need a precise structural model of the THRGP, HHRGPs, and other structural wall proteins. This entails a detailed chemical and structural characterization of these proteins coupled with macromolecular modeling experiments in order to define domains that might confer function.

I. Characterization of Maize Hyp-Containing Wall Proteins

So far, the two HHRGPs have been characterized as one protein because they co-chromatograph on the ion exchangers Cellex-P, BioRex-70 and Superose-6 gel filtration. One approach to characterizing the individual HHRGP polypeptide backbones[14] is to isolate and characterize cDNA clones (via oligonucleotide probes based on dHHRGP peptide sequences or antibodies raised against the dHHRGPs) from a Black Mexican Maize cDNA library constructed recently for me by Stratagene. Sequences of the HHRGP cDNA clones combined with the HHRGP peptide sequences to identify Hyp residues will allow the determination of the complete primary amino acid sequence of the HHRGPs (this is virtually impossible by peptide sequencing alone because of the repetitive nature of the proteins). Clones will also detail the differences in the HHRGP polypeptide backbones, thus providing clues for separating the intact HHRGPs from each other

[14] *In vitro* translation of Black Mexican mRNAs (using rabbit reticulocyte lysate) yielded two proteins (with $M_r \sim 68$ & 70 kD) identified by immunoblotting using anti-dHHRGP antibodies; thus the differences between the HHRGPs are in the polypeptide backbones, rather than post-translational modifications.

for further chemical characterization as individual proteins. Separation of the two HHRGPs is desirable (but not absolutely necessary) before characterization of their carbohydrate components, especially the galactose moieties. Once separated, mild acid treatment of each HHRGP at pH 1 for 1 h at 100° C will remove all arabinosides on the hydroxyproline hydroxyl groups, leaving galactopyranosyl residues mainly intact[15] (Lamport *et al.*, 1973). Then NaOH/borohydride catalyzed β-elimination, will yield alanine from O-glycosyl serine or α-aminobutenoic acid from O-glycosyl-threonine (Aminoff *et al.*, 1980). The eliminated carbohydrate moieties can be detected by fluorometry after end labeling via pyridinylation with 2-aminopyridine and detection and characterization after HPLC by the methods of Maness & Mort (1989) or Seto & Shinohara (1989).

As well as the THRGP and the two HHRGPs already characterized from the Black Mexican cell suspensions, the crude eluate also contains at least two other Hyp-containing proteins that are candidates for characterization; One is another HHRGP, but more basic than the other two judging by its elution on Cellex-P in CP3 (Figures 7 & 9) and a preliminary amino acid analysis. The other Hyp-containing protein in the crude eluate is about 4 mole% Hyp and rich in Ser, Gly and Ala (I designated it an alanine-rich glycoprotein, or ARGP)(Figure 8c), and separates from THRGP only during gel filtration on Superose-6 (Figure 11). I have purified this the ARGP to constant composition and have several mg set

[15] In dicot extensins, the arabino-oligosides ionize at high pH and hence electrostatically shield the galactosylserine linkage from β-elimination. Therefore the arabinosides must first be removed from the protein before proceeding.

aside for future characterization (including peptide mapping and sequence analysis). Others have reported Hyp-poor proteins as wall components (Kimmins & Brown, 1975), however, because the small amounts of Hyp in these proteins may result from contamination with Hyp-rich proteins, proof that such proteins exist requires the isolation of Hyp-poor peptides.

A complete structural characterization of the THRGP and the HHRGPs also requires the assignment of carbohydrate to particular amino acid residues. Drs. Dan Kassel and Klaus Biemann (M.I.T.) have recently offered to do GC-Mass Spectroscopy of THRGP and HHRGP glycopeptides in order to identify the exact locations and nature of the glycosides on the peptide backbones.

II. The Major Protein Component (non-HRGP) of the Maize Cell Wall

Unlike the major protein component of many dicot walls, the major protein component of the maize cell wall is not an HRGP. Nor is this component extremely glycine-rich (i.e. >60 mole%), and therefore it is not directly comparable to glycine-rich wall proteins such as those associated with seed coat walls (Varner & Cassab, 1986) or the glycine-rich proteins from petunia (see Appendix B)(Condit & Meagher, 1986, 1987) and bean (Keller *et al.*,1989 a,b). I have isolated three peptides from the firmly bound non-HRGP protein component of the wall. These peptides contain no Hyp, and together with amino acid analyses of the maize cell wall confirm that the major structural protein component of the maize cell wall is not an HRGP (Table 20). My goal is to purify and characterize a soluble precursor to the insoluble wall-bound protein, by analogy with work on dicot extensins from tomato, sycamore-maple, tobacco, sugar beet and maize. This

assumes that, like extensin, an elutable pool of soluble precursors to the insoluble protein exists in the maize cell wall. This is a reasonable assumption in the light of current views of the wall as consisting of subunits which are assembled *in muro*. As the the HF-insoluble wall protein residue contains no obvious "marker" like the hydroxyproline of extensin, how does one unequivocally identify a protein that has no known enzymic activity and apparently no unusual component? Peptide sequences of seven or more residues are generally considered as statistically unique and therefore provide absolute identification. I have two strategies for purification and characterization of the major maize wall protein component(s). First, fractionate tryptic or chymotryptic digests of all eluted proteins (after HF deglycosylation) and sequence peptides to identify peptides homologous to those isolated from wall digests. This "brute force" approach virturally assures results, but is labor intensive. A second potentially quicker method is to raise antibodies against major wall peptides and use the IgGs to identify a precursor via immunoblot analysis, immunoprecipitation, and/or immunoaffinity chromatography. Because trypsin solubilizes about 30% of the HF-insoluble wall residue (yielding at least three major peptides >20 residues, with compositions which are consistent with the overall composition of the insoluble protein) antibodies raised against the tryptic peptides will facilitate identification of the soluble "precursor" non-HRGP in salt eluates of intact cells. This approach assumes the wall peptides are antigenic and the antibodies specific.

Table 20. Amino Acid Compositions of the Black Mexican Maize HF-Insoluble Cell Wall and Tryptic Peptides Isolated from the HF-Insoluble Wall

Amino Acid	HF-Insoluble Cell Wall	Peptide 1	Peptide 2	Peptide 3
Hyp	trace	0.0	0.0	0.0
Asx	10.6	8.0	8.1	13.2
Thr	5.3	7.3	3.4	5.6
Ser	6.8	15.5	9.0	9.9
Glx	11.3	12.1	7.0	12.9
Pro	4.7	3.4	6.3	2.2
Gly	9.6	17.6	14.9	13.3
Ala	10.4	4.0	7.3	9.0
Cys	0.0	0.0	0.0	0.0
Val	7.2	9.0	18.4	9.8
Met	1.5	0.0	0.0	0.0
Ilu	3.5	1.5	2.8	1.0
Leu	8.8	5.1	4.5	2.6
Tyr	1.9	2.8	0.0	0.8
Phe	3.9	3.1	1.4	1.2
His	2.5	1.4	3.2	3.0
Lys	6.4	3.8	8.6	10.4
Arg	5.6	5.4	5.1	5.0

Part of the characterization of the maize major structural (non-HRGP) wall protein is identification of crosslink amino acids. The presence of an "unknown phenolic" in the HF-insoluble maize wall hydrolysate (Figures 27 a & b) raises the possibility of another crosslink, possibly an amino acid, that may be analogous to IDT or dityrosine. HPLC of wall hydrolysates will separate the unknown from other wall components, after which it can be characterized (cf. Fry, 1982).

Another approach to defining roles for the maize HRGPs and other structural proteins is to attempt to localize wall proteins to their respective tissues. Are they restricted? If so, to what tissues? And what properties do they impart to those

tissues? A first step is to achieve specific cytolocalization.

III. Immunocytochemical Localization of Maize HRGPs

In 1985, I raised and characterized antibodies against two dicot extensins (P1 and P2 from tomato), including their deglycosylated forms (Kieliszewski & Lamport, 1986). The existence of these antibodies made it possible for the first time to determine whether cross-reactivities arise from carbohydrate or from polypeptide epitopes. This is important because antibodies must be specific to be useful for cytolocalization, i.e. show minimal cross-reactivities. Therefore I raised and characterized two sets of rabbit polyclonal antibodies, one set against the THRGP, whose polypeptide backbone is the major antigenic epitope (Figure 26), and the other set against the two deglycosylated HHRGPs (glycosylated HHRGP apparently is not antigenic, as two attempts to raise antibodies to HHRGP failed) (Figures 33 & 34) Although the cross-reactivity of the two sets of antibodies with ARGP and poly-L-proline still needs to be determined, the antibodies for each maize HRGP show little cross-reactivity with the other maize HRGP (Figures 26 & 34). Furthermore, the anti-THRGP antibodies do not cross-react with solanaceous lectins or AGPs (from dicots), and will probably therefore be specific for THRGP in intact tissues. However, only the anti-THRGP antibodies will be useful for cytolocalization because they react with glycosylated THRGP, while the anti-dHHRGP antibodies are not of immediate use for immunolabeling as they do not react with glycosylated HHRGP (Figure 34)(See section V for use of anti-dHHRGP antibodies to screen for cell wall mutants. Dr. Keith Roberts (John Innes Institute, U.K.) has begun TEM immunogold labeling of maize sections using

the anti-THRGP antibodies; hopefully this work will provide details about the tissue and cell specificity (if any) of THRGP.

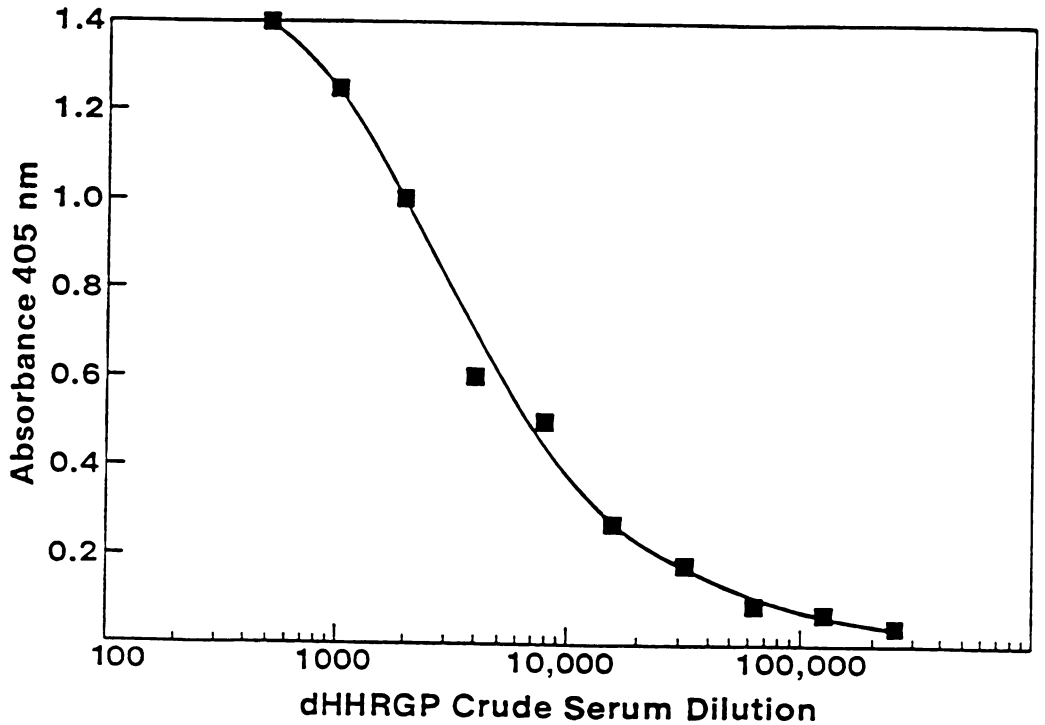


Figure 33. Serial dilution of anti-dHHRGP antiserum with dHHRGP antigen at 20 ng/ELISA microtiter well.

IV. Enzymes that Catalyze the Post-Translational Modifications of Extensin

Although posttranslational modifications account for as much as 40% of extensin's amino acid residues (via hydroxylation, crosslink formation and glycosylation) and from 30-65% of extensin's dry weight (by glycosylation) we know very little about the roles the modifications play in the function of extensin, and the enzymes that catalyze those modifications. Nor are we likely to soon learn anything about those enzymes or the roles of extensin's post-translational

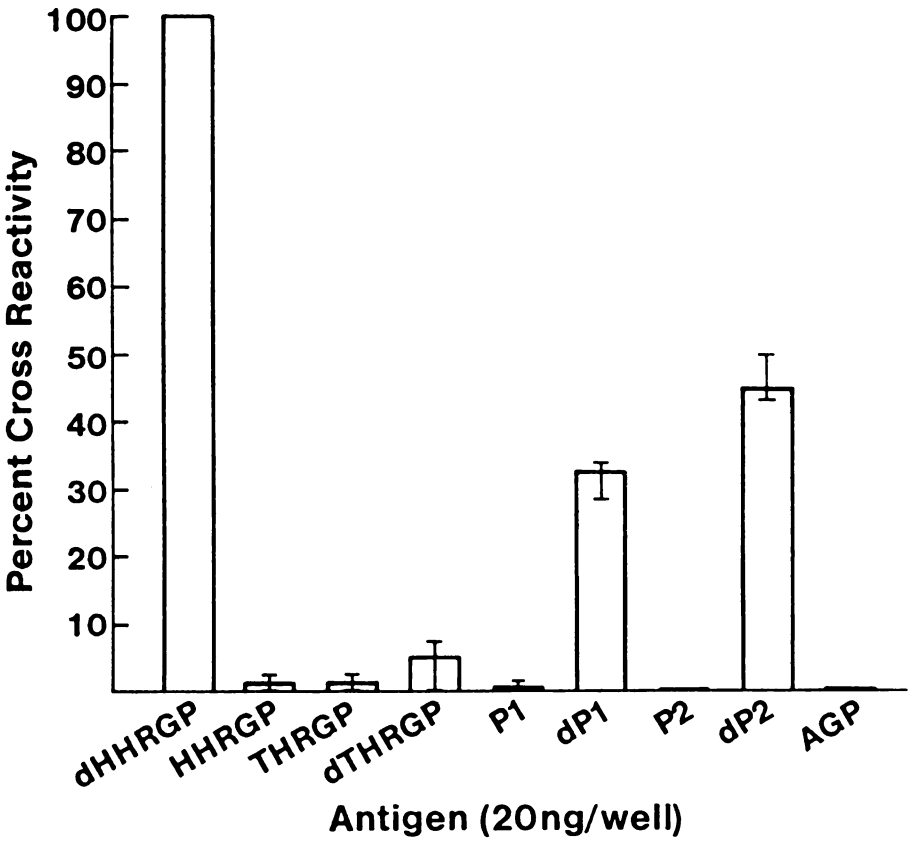


Figure 34. Cross-reactivities of anti-dHHRGP antibodies with HHRGP, THRGP, dTHRGP, P1, dP1, P2, dP2, and sycamore AGP.

modifications, as most current extensin research is focused on the extensin polypeptide backbone as it is derived from clones. Yet the function of extensin within the wall matrix probably has as much to do with its posttranslational modifications as it does with the unmodified polypeptide backbone *per se*. For example, most of the extensin protein backbone is wrapped in carbohydrate (except for the THRGP which is about 30% carbohydrate, dw) with very few peptide sequences presented to the wall matrix. This is evidenced by the very limited digestibility of glycosylated extensin with proteolytic enzymes (Lamport, 1973; Smith *et al.*, 1986). Furthermore, many extensins become crosslinked

intramolecularly by IDT, and are probably crosslinked intermolecularly by unidentified links. Therefore, one step toward determining the function of extensin as dictated by its posttranslational modifications, is to isolate and characterize the enzymes involved. Unfortunately, projects aimed at isolating and characterizing the arabinosyl and galactosyl transferases that catalyze the glycosylation of extensin are very difficult (and therefore high risk) because the enzymes are membrane-bound (Karr, 1972). Furthermore, obtaining substrate quantities of carbohydrate-stripped extensin peptides was, until recently, a problem. However, the generation of substrate for *in vitro* glycosylation is no longer an obstacle in view of the "intact cell elution" technique and HF-deglycosylation which allows the facile preparation of several milligrams of deglycosylated extensin monomer in a relatively short time.

Two "posttranslational" enzymes that may be easier to isolate and characterize than the arabinosyl or galactosyl transferases are 1) the enzyme that catalyzes IDT formation[16] and 2) the enzyme that hydroxylates peptidyl proline to form 4-hydroxyproline, prolyl 4-hydroxylase (Kivirikko, 1989).

The only demonstration of the specific location of IDT is as an intramolecular crosslink in two tomato extensin peptides (Epstein & Lamport, 1984). The Type-3 domain:

Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-Tyr-Tyr-Tyr-Lys occurs as a tomato wall tryptic peptide (with IDT)(Table 4), and as a major repeat in some extensin clones (sans IDT)(Showalter & Rumeau, 1989); however, an

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[16] The isolation of an IDT-forming enzyme applies only to dicot and monocot walls that contain IDT. The maize cell wall apparently contains no IDT.

extensin precursor containing this repeat has never been isolated. The other IDT-containing sequence: Ser-Hyp-Hyp-Hyp-Hyp-Val-Tyr-Lys-Tyr-Lys occurs as a major repeat in tomato wall peptides (Table 4) and in tomato extensin precursor P2 (Smith *et al.*, 1986). The isolation, characterization, and manipulation of an IDT-forming enzyme will have a significant impact on our current thinking about mechanisms for extensin's wall insolubilization and cell wall models which hypothesize IDT as an intermolecular crosslink responsible for the incorporation of extensin into the wall.

Thus there are two approaches to extensin crosslinking: a) isolate an enzyme that catalyzes an extensin intermolecular crosslink (which may or may not be IDT), and b) isolate the enzyme that catalyzes the formation of intramolecular IDT. The first approach is currently in progress by Derek Lamport (Everdeen *et al.*, 1988). The second approach aims specifically at isolation of the IDT-forming enzyme. Here generation of substrate is crucial; specifically, the production of monomeric extensin or extensin peptides containing either of the two potential intramolecular crosslink sequences, but without IDT:

Ser-Hyp-Hyp-Hyp-Hyp-Val-Tyr-Lys-Tyr-Lys or

Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-Tyr-Tyr-Tyr-Lys. Then the (poly)peptides can be used as substrate to assay *in vitro* IDT formation using crude (and later, not-so-crude) wall enzyme preparations[17]. Supposedly, the

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[17] The IDT-forming enzyme is a wall enzyme, judging by increases in wall IDT concomitant with extensin precursor insolubilization (Cooper & Varner, 1985)

addition of peroxidase inhibitors and free radical scavengers inhibits IDT formation in the cell wall (Cooper & Varner, 1981), thus addition of peroxidase inhibitors and free radical scavengers to cell suspensions followed by isolation of P2, hopefully without IDT, may be one way to generate substrate for the IDT-forming enzyme. Alternatively, Joseph Leykam (Macromolecular Facility, M.S.U. Biochemistry Dept.) can synthesize substrate quantities of the substrate peptides. One drawback is the assay of IDT *per se*, as it requires an 18 hr acid hydrolysis followed by HPLC and spectrophotometric IDT detection. However, recent developments in microwave-driven acid hydrolysis
(5 min/hydrolysis) followed by HPLC and spectrophotometric IDT detection, may expedite the assay (Choiu & Wang, 1989).

Of the enzymes that catalyze the posttranslational modifications of algal and higher plant HRGPs, prolyl hydroxylase is the best characterized, although nothing is known about the active site of the plant enzyme. Futhermore, all of the "characterization" of the plant enzyme has been done using artificial substrate (i.e. polyproline II and protocollagen) (Andreae *et al.*, 1988; Bolwell *et al.*, 1985; Cohen *et al.*, 1983; Kaska *et al.*, 1987a & b). This has lead to the dubious assumption that prolyl hydroxylase of higher plants prefers a polyproline II substrate conformation (Tanaka *et al.*, 1981) although the enzyme also hydroxylates protocollagen (also in a PPII conformation) but at a very low rate (Andreae *et al.*, 1988; Cohen *et al.*, 1983; Sadava & Chrispeels, 1971; Sauer & Robinson, 1985). The very specific hydroxylation of the THRGP and the extensive hydroxylation (34 mole%) of

HHRGP, neither of which are in an obvious polyproline II conformation, suggests that a) this assumed specificity of prolyl hydroxylase for polyproline II is incorrect, and b) that multiple forms of prolyl hydroxylase exist, each with a defined substrate specificity. Furthermore, the existence of the THRGP extensin, with 1/2 of its Hyp residues nonglycosylated, suggests that non-glycosylated Hyp, as well as glycosylated Hyp, may impart some function to extensin in general and to the THRGP in particular, possibly, by loose analogy with collagen[18], in its association with other wall polymers. Therefore, Hyp may play a dual role in the function of extensins, both as the major site of glycosylation, and as a hydrogen bond donor; and the use of natural substrate (i.e. non-hydroxylated extensins generated and purified from cells treated with the suicide inhibitor, 3,4-dehydroproline; Fowden *et al.*, 1963; Myllylla *et al.*, 1979) should facilitate the purification and accurate characterization of plant prolyl hydroxylase(s).

A complementary approach to isolating, characterizing and manipulating the enzymes that catalyze the post-translational modifications of extensin involves the generation of extensin mutants involving those enzymes.

V. Extensin Mutants: A Positive Screen

The isolation and characterization of cell wall mutants involving extensin is an obvious approach to defining the function of extensin *in muro*. The question

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[18] Hydroxyproline plays a critical role in the assembly of animal extracellular matrices, as its hydroxyl groups are essential for the folding of procollagen polypeptide chains into a triple helix at body temperature. Thus prolyl hydroxylase is a target enzyme for therapeutic intervention in fibrotic disorders (Kivirikko, 1989).

arises: How does one screen for mutants in a non-enzymic structural protein? A possible positive screen for "posttranslational" mutants (e.g. arabinosyl and galactosyl transferases and/or prolyl hydroxylase(s)) could utilize the anti-dHHRGP antibodies which only recognize the polypeptide backbone epitopes normally obscured by glycosylation. For example, dHHRGP antibodies cross-react 2% with HHRGP and THRGP, 1% with tomato extensin P1, 0% with tomato extensin P2 and AGP (Figure 34). Furthermore, *in vitro* translation of Black Mexican mRNA followed by SDS-PAGE and immunoblotting using anti-dHHRGP antibodies[19] showed that the antibodies recognize both of the HHRGP polypeptide backbones without hydroxylation of the proline residues (data not shown). Thus, mutants in the glycosyl transferases or prolyl hydroxylase (resulting in underglycosylated or nonglycosylated extensins, respectively) should be detectable in both tomato and maize walls by using the anti-dHHRGP antibodies.

For example, a mutagenized suspension culture could be plated and grown as a colonies on a solid medium. Then one could use a replica filter screening technique (applying Varner's tissue-blotting technique, Cassab & Varner, 1985), using $CaCl_2$-soaked nitrocellulose "lifts" to screen for non-glycosylated extensins by immunodetection with the dHHRGP antibodies. Mutants detected would likely be mutants in prolyl hydroxylase or the glycosyl transferases responsible for most of extensin's post-translational modifications. Controls would involve immunoblots

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[19] Immunoscreening for mutant glycoproteins has precedence in animal systems i.e. the detection of abberant underglycosylated breast mucins associated with breast cancer using antibodies raised against the HF-deglycosylated mucin (Gendler *et al.*, 1987)

of cells grown on medium supplemented with 3,4-dehydroproline.

A logistical drawback is that our suspension cultures are diploid, do not undergo meiosis, and therefore any recessive mutations would not be detected by this approach. On the other hand, the alternative of immunoscreening individual seedlings for extensin mutants is overwhelmingly labor intensive. One solution is to mutagenize and culture haploid cells (Catt, 1981), (e.g. pollen-derived cultures or anther cultures of maize, rye, *Arabidopsis thaliana*, tomato, tobacco, petunia or beet; Sangwan & Norreel, 1975; Bajaj, 1983). This approach is particularly attractive because frequently plants can be regenerated from such cultures. Alternatively, the immunoscreening of individual seedling might be feasible if one could first select a phenotype associated with extensin mutants. Thus, a crucial preliminary experiment would involve germinating "wild type" seeds on medium containing 3,4-dehydroproline which should inhibit virtually all prolyl hydroxylase activity (Cooper & Varner, 1983) and result in non-hydroxylated non-arabinosylated extensin "mutants." If such a "mutation" has profound effects on the cell wall it is likely to have profound effects on plant morphology, therefore a certain phenotypic subpopulation could be selected for immunoscreening.

VI. Macromolecular Associations of Extensin

It is likely that extracellular matrices must self-assemble *in situ*, the assembly being regulated primarily by the physical and chemical properties of the macromolecular monomers (Eyre, 1980). An elegant example is the alga *Chlamydomonas reinhardtii* whose lattice-like crystalline cell wall self-assembles

in vitro (Roberts, 1974; Goodenough *et al.*, 1987). Significantly, three of the four molecules involved are hydroxyproline-rich glycoproteins, and the fourth is a glycine-rich species. Assuming that the cell wall of higher plants, like the *Chlamydomonas* cell wall, also largely self-assembles, the precise interactions and "structures" that occur between extensin and the other extracellular macromolecules, in part, define the function of extensin in the cell wall.

An obvious extensin-polysaccharide interaction likely occurs between the positively-charged extensin lysine or histidine residues and the negatively-charged GAX (in maize) or pectins (in dicots). One method for isolating putative interacting GAX with THRGP or HHRGP involves using cross-linking agents *in vitro* and *in vivo*. For example, if Schiff bases form between the lysine residues of THRGP and aldehyde groups of other wall components, cyanoborohydride reduction should stabilize the Schiff base. Or, if the THRGP lysine residues ionically interact with the glucuronic acid carboxyl groups of GAX, a "zero length" isopeptide bond between the two might be formed by reaction with carbodiimide (Vandekerckhove *et al.*,1989). One should then be able to isolate crosslinked dimer fragments after degradation or fragmentation of the cell wall (for *in vivo* experiments) or of *in vitro* crosslinked polymers, i.e. to distinguish a random from an orderly pattern of ionic interactions.

Another possibility is the use of extensin or extensin peptides coupled to a solid support, e.g. affinity chromatography of cell wall molecules (pectins, GAX) or fragments of molecules. These experiments are difficult because they require the isolation and assay of wall polysaccharides or their fragments (a miserable

job!). Controls for the binding experiments would include preincubation of the extensin-matrix with anti-extensin antibodies to inhibit binding, derivatization of extensin active R-groups (acetylation of lysines, destruction of histidine imidazole rings with diethylpyrocarbonate), and competing reactions with free lysine, histidine, uronic acids, or even Ca^{++}[20].

A biochemical approach to cell wall regeneration will also help to elucidate a possible role of cell wall proteins as "organizers" of cell wall assembly, as suggested by experiments with 3,4-dehydroproline, which inhibited dicot wall regeneration . Evidently dicot protoplasts cannot regenerate their walls using posttranslationally defective HRGPs (unhydroxylated) and therefore underglycosylated)(Cooper & Varner, 1984). This experiment bears repeating and can be extended to the monocot *Zea mays*, where only the Hyp-containing protein components should be affected by dehydroproline; the major non-HRGP structural components should remain unaffected as dehydroproline is highly specific for prolyl hydroxylase. This approach will therefore help distinguish between the roles of HRGPs versus the non-HRGPs. For example, dehydroproline inhibition of maize protoplast wall regeneration would suggest an "organizer" role for the minor HRGP components of a monocot cell wall. A critical control experiment will involve addition of exogenous maize HRGPs, which should overcome the inhibiting effect of

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[20] Calcium may have a dual function in pectin-extensin interactions. It probably forms salt bridges between pectic carboxyls, furthermore calcium "melts" polyproline II conformations (Tiffany & Krimm, 1969); thus calcium may alter the secondary structure of dicot extensins in the wall. Some potentially useful experiments would be CD of dicot extensins plus and minus Ca^{++}, or of pectin and extensin plus or minus Ca^{++}, and especially as a funtion of pH and ionic strength.

dehydroproline on protoplast wall regeneration.

Presumably, the various types of extensin evolved to perform different functions in different tissues with different mechanical and physiological properties. For example, the maize cell wall has low levels of extensins which are also HF-insoluble; this contrasts dramatically with the large amounts of HF-insoluble extensins in dicot cell walls. These differences may be related to the very different growth habits of grasses (silica support) and herbaceous dicots (turgor support). Despite extensive data on the primary structure of extensins, (posttranslational modifications and regulation of expression, etc.) the discreet function of any extensin type remains unknown, probably because we don't know how extensin interacts specifically with other wall macromolecules. Yet its role in wall self-assembly and disease resistance, the roles of its post-translational modifications, and its function in morphogenesis, all depend on intermolecular reactions such as those suggested above. Future tests of these working hypotheses demands a combination of chemistry and molecular biology. For example, peptide sequences hypothetically involved as functional domains could be altered by site-directed mutagenesis with predictable (?) results. And highly expressed extensins could be down-regulated by antisense RNA. Ultimately, all these questions involve an understanding of cell wall chemistry, and the ability to model that chemistry in four dimensions.

APPENDICES

APPENDIX A

A CHRONOLOGY OF SEMINAL EXPERIMENTS AND CONCEPTS IN EXTENSINOLOGY

Date	Scientist(s)	Discovery/Suggestion
1924	Tupper-Carey & Priestley	Provided histochemical evidence for protein in cell walls of *Vicia faba*.
1933	Thimann & Bonner	Nitrogen occurs in cell wall preparations.
1951	Tripp *et al.*	Identified Asp, Ser and Gly in cotton cell wall hydroylysates.
1954	Steward & Thompson	Discovered Hyp in carrot disc hydrolysates.
1960	Dougall & Shimbayashi	Discovered Hyp in tobacco callus cell wall hydrolysates.
	Lamport & Northcote	Produced a pipettable sycamore cell suspension culture for biochemical use.
	Lamport & Northcote	Discovered Hyp in sycamore suspension cell wall hydrolysates and inferred existence of cell wall protein.
1961	Van Etten *et al.*	Hyp occurs in seed coats and pericarp, including *Zea mays*.
1963	Lamport	Hyp biosynthesis in plants requires molecular O_2. Named extensin.
1964	Lamport	The hydroxylation of Pro to form Hyp involves the displacement of the proline trans-4-proton.
1965	Lamport	Hyp accumulates in cell walls of cultured cells.
1967	Lamport	Isolated a new carbohydrate-protein linkage: arabinosyl-O-hydroxyproline.
	Cleland & Karlsnes	Hyp-containing proteins may have a role in the cessation of elongation growth.
	Holleman	Hydroxylation and subsequent arabinosylation of extensin is NOT required for insolublization into cell wall.
	Boundy *et al.*	Partially purified a Hyp-containing "mucopolysaccharide" from maize pericarp.
1969	Chrispeels	Initiated the carrot root disc system; suggested the 10-fold increase in cell wall Hyp was a wound response, and that the TCA soluble cytoplasmic Hyp is precursor for wall-bound Hyp.
	Lamport	Isolated first extensin cell wall glycopeptides (tomato).
1970	Ridge & Osborne	A correlation exists between ethylene and the production of HRGPs & increased peroxidase.

Appendix A.... continued

	Lamport	Proposed a cell wall model in which cellulose microfibrils are linked together by extensin via β-1,3-galatans attached to extensin's hydroxyproline arabinosides.
1971	Lamport & Miller	Hydroxyproline arabinosides are common in the plant kingdom.
1972	Brysk & Chrispeels	Partially purified a salt-extractible putative extensin precursor from carrot cell walls. Suggested a pool of precursors to wall-bound extensin occurs in the wall.
1973	Lamport *et al.*	Galactosyl-serine occurs in tomato extensin wall peptides.
	Lamport	First sequences of extensin peptides. Ser-$(Hyp)_4$ subperiodicity is evident.
	Keegstra *et al.*	Suggested a primary cell wall model in which pectin is covalently linked to extensin.
1974	Monro *et al.*	Extensin is probably NOT covalently bound to pectin in *Lupinus* hypocotyls. Suggested that NON-glycosidic bonds link the wall together.
	Esquerre-Tugaye & Mazau	Hyp-arabinosides increase 10-fold in fungus-infected melon cell walls; suggested induction of a special "modified" extensin during infection.
	Lamport	Noted an "unknown tyrosine derivative" in tomato cell wall tryptic peptides.
	Burke *et al.*	Cell walls of suspension cultured gramineae contain the same amount of wall protein as dicot walls but $< 10\%$ the amount of Hyp. Suggest the monocot structural protein is "very different from that found in dicots". Showed glucuronoarabinoxylans are the principal wall component in graminaceous monocots.
1975	Selvandran	Used acidified chlorite, an oxidant that destroys sulfur and aromatic amino acids, to extract extensin from cell walls.
	Gardiner & Chrispeels	Golgi apparatus is major site of extensin glycoprotein transport.
1977	Mort & Lamport	First used anhydrous HF to deglycosylate glycoproteins. Showed that wall-bound extensin is insoluble in HF while wall polysaccharides are soluble. HF-insoluble wall is 50% protein & 50% unknown phenolic" (?) component.
	Lamport	First CD data of extensin cell wall peptides; showed polyproline-II conformation.
	Pope	Demonstrated a polysaccharide linked to Hyp (nicknamed "Big Hyp-X) in arabinogalactan protein.
1978	Whitmore	Observed polyphenolics bonded via peroxidase to Hyp-containing wall proteins. Suggested such protein crosslinking is involved in the early stages of lignification and that Hyp is the amino acid involved.
1979	Esquerre-Tugaye *et al.*	Showed inverse relationship between accumulation of Hyp and fungal pathogen compatibility.
	Esquerre-Tugaye & Lamport	First sequences of extensin tryptides isolated from walls of fungus-infected melon.

Appendix A... continued

	Basile	Suggested HRGPs regulate aspects of plant morphogenesis.
	Pike *et al.*	Hyp accumulates in cell wall in response to red light.
1980	Lamport	Suggested extensin is peroxidatically crosslinked in the wall via the "unknown tyrosine derivative". There may be two independent networks, one of protein, one of carbohydrate.
	O'Neil & Selvendran	Suggested extensin held in wall by phenolic crosslinks.
	Stuart & Varner	Purified the first extensin monomer (from Chrispeels carrot disc system).
1981	Cooper & Varner	Showed the removal of H_2O_2 and inhibition of peroxidase increased the amount of soluble extensin in carrot cell walls.
	Clarke *et al.*	Hyp levels increase in cell walls of fungus-infected wheat ONLY in susceptible varieties.
	Catt	Used haploid *Datura innoxia* cultures to screen for cell wall mutants in alkaline phosphatase.
	Lamport & Catt	Noted that peroxidase activity is low in regions of highest growth rates.
1982	Fry	Identified the new crosslink amino acid, isodityrosine, in cell wall hydrolysates and suggested it occured in extensin as Lamport's "unknown tyrosine derivative". Showed IDT is split by acidified chlorite and suggested extensin is held in wall by intermolecular IDT crosslinks formed by peroxidase.
	Leach *et al.*	Raised first antibodies against a purified plant cell wall component (extensin). First immunological localization of extensin in cell walls of sectioned tissue.
	Leach *et al.*	Extensin agglutinates bacteria.1983
	Lamport & Epstein	Identified "unknown tyrosine derivative" in wall peptides as being IDT, and crystallized the amino acid. Proposed a cell wall model comprised of a concatenated extensin-cellulose network. Demonstrated that IDT serves as an intramolecular crosslink in extensin wall peptides.
	Cooper & Varner	Insolubilization of extensin into the wall is inhibited by free radical scavengers and peroxidase inhibitors.
1984	Smith *et al.*	Pulse-chase and restoration kinetics show monomeric salt-extractable extensin is precursor to the insoluble extensin network, and they contain no IDT. Showed extensin monomers can be eluted from the cell surface without cytoplasmic contamination. First demonstration of multiple extensins in a single tissue. Suggested "types" of extensin occur.
	Cooper	Inhibition of proline hydroxylation prevented wall regeneration in protoplasts.
	van Holst & Varner	First electron micrographs visualize carrot extensin monomer as a rod 80 nm long. CD data of carrot monomer confirm it has a polyproline-II conformation, although less polyproline-II after deglycoyslation suggesting that arabinose oligomers stabilize the rod.

Appendix A....continued

	Cooper & Varner	Carrot cell walls insolubilized extensin monomers concomitant with IDT formation; yet *in vitro* crosslinking using peroxidase and H_2O_2 formed dityrosine, not IDT.
	Hammerschmidt *et al.*	Disease resistant varieties of cucumber showed rapid increases wall Hyp levels, whereas susceptible varieties did not.
1985	Chen & Varner	Isolated the first partial cDNA extensin clone (carrot) and partial cDNA clone for cell wall protein P33. Extensin mRNA is wound-inducible.
	Chen & Varner	Isolated first extensin genomic clone (carrot).
	Cassab *et al.*	Demonstrated, via immunolocalization, the developmental and tissue specific expression of a soybean extensin.
	Roby *et al.*	Plant fungal elicitors trigger synthesis of ethylene and cell wall Hyp.
	Showalter *et al.*	Fungal elicitors and infection cause accumulation of HRGP mRNAs as an early response in non-susceptible plants and as a late reaction in susceptible plants. Data also implies intercellular transmission of elicitation signal.
1986	Smith *et al.*	First direct amino acid sequences of extensin precursors; sequencing corroborates some of the sequences determined earlier from wall peptides.
	Condit & Meagher	Isolated first genomic clone of a putative cell wall Glycine-Rich Protein.
	Stafstrom & Staehelin	Electron micrographs of extensin "oligomers". Suggested intramolecular IDT introduces "kinks" into monomers and that oligomers form via preferable crosslinking at the ends of monomers. Detected IDT in monomeric extensin. Claimed deglycosylation of monomeric extensin causes the rod to "collapse".
	Mazau & Esquerre-Tugaye	Hyp accumulates in virus infected cell walls.
	Kieliszewski & Lamport	Raised first antibodies against the protein backbone of a completely deglycosylated glycoprotein (tomato extensins).
1987	Stermer & Hammerschmidt	Hyp accumulates in cell walls of heat-shocked plants.
	Corbin *et al.*	A particular set of HRGP genes are induced by elicitor. Suggested intercellular stress signalling occurs in higher plants.
	Cassab & Varner	Developed method, tissue printing on nitrocellulose, for immunological localization of soluble extensin in plant tissues.
	Lawton & Lamb	Extensin genes activated in response to wounding and fungal elicitors.
	Prasad & Cline	Hyp accumulates in response to gravity.
	Kieliszewski & Lamport	First purification of an HRGP (THRGP) from a monocot (*Zea mays*).

Appendix A....continued

1988	Heckman *et al.*	Succinylation of deglycosylated extensin monomers reversed the net charge and enhanced their EM visualization as flexible rods, contrary to the conclusions of Stafstrom & Staehelin (1986).
	Wingate *et al.*	Glutathione stimulates transcription of genes encoding HRGPs.
	Stiefel *et al.*	Isolated first cDNA clone for a monocot HRGP (*Zea mays*, THRGP).
	Hood *et al.*	Isolated a THRGP from maize pericarp.
1989	Kieliszewski *et al.*	Demonstrated trypsin labile lysylproline in maize HRGP.
	Keller & Lamb	A specific extensin is transiently induced in a small set of cells involved in lateral root initiation; best evidence for role of extensin in development.
1990	Kieliszewski *et al.*	First amino acid sequences of maize extensin THRGP show P1 & P3-type extensin homology with dicots, and CD spectra of THRGP indicate its conformation is not polyproline II.
	Li *et al.*	First amino acid sequences of sugar beet extensin. Ser-Hyp-Hyp-Hyp-Hyp appears as a split block.

APPENDIX B

GLYCINE-RICH PROTEINS (GRPs)

GRP Structure

In 1965, Lamport surveyed the amino acid compositions of nine dicots, and a primitive gymnosperm (Gingko), and concluded from the wide ranges present in the value of any particular amino acid of one wall compared to another, that structural wall protein alternatives to the Hyp-rich extensins were likely (Lamport, 1965). Burke *et al.* (1974) reached a similar conclusion about graminacous monocot walls after analyzing four species. For several years, rumors circulated about glycine-rich hydroxyproline-poor cell walls and wall protein fractions in some plant species and tissues (Rackis *et al.*, 1961; Melin *et al.*,1979; Dreker *et al.*, 1980; Varner & Cassab, 1986). Finally, in 1986, Condit & Meagher serendipitously[1] isolated a petunia gene encoding a glycine-rich (67 mole %) putative cell wall protein (GRP) composed of $(Gly\text{-}X)_n$ repeats, which make it structurally analogous to silk fibroin (Pauling & Corey, 1951; Condit & Meagher, 1986). Keller *et al.*

[1] The proline residues in extensin are primarily encoded by CCA, while glycine in the GRPs is primarily encoded by GGT; thus extensin probes can be used to isolate GRP genes, because their mRNAs are encoded by opposite strands of similar sequences. In fact, Condit was originally probing for an extensin when she unexpectedly recovered her glycine-rich gene (C. Condit to M. Kieliszewski, personal communication).

(1988) have since used extensin clones to isolate clones for two distinct glycine-rich cell wall[2] proteins from bean. The three GRP clones encode proteins with a range of sizes and having similar, but distinctive, amino acid compositions (Table 1). All three proteins are likely β-pleated sheets, the two larger GRPs (from petunia and bean GRP 1.8) consisting of 8 anti-parallel strands with charged residues along the edges of the sheet, bulky side chains occurring regularly along one face, while the opposite face holds the hydrogens of the glycine R-groups (Condit & Meagher, 1986; Keller *et al.*, 1988). Like extensins, the GRPs are organized into three distinct domains: the C-terminus, the N-terminus, and the middle section composed of the major repeating polypeptides (Condit & Meagher, 1986, 1987).

Regulation of GRP Expression.

Like some extensins, the GRPs are apparently developmentally regulated and induced by wounding (Condit & Meagher, 1986; Keller *et al.*, 1988, 1989a). The two GRPs from bean, GRP1.8 and GRP1.0, although encoding proteins of similar

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[2] Keller *et al.* (1988) demonstrated GRP localization in the cell wall by immunolocalization, using polyclonal antibodies raised against a fusion protein, however, the data supporting their conclusion is not entirely convincing. The anti-GRP 1.8 antibodies also reacted with two proteins in the cytoplasm. The authors give three explanations: the cytoplasmic GRPs are GRP 1.8 in transit to the wall; the cytoplasmic GRPs are actually contaminants from the cell wall; or GRP 1.8 does indeed occur as a *bone fide* cytoplasmic component but with another function in the cytoplasm. Unfortunately, Keller presents no quantitative data about the titer, dilution, and specificity of their antibodies. For instance, do they cross-react with other GRPs such as collagens, the structurally analogous fibroins, or poly-glycine? These are important controls considering the very high background contributed by the control rabbit serum.

may accumulate in different cell types (Keller *et al.*, 1989b). GRP 1.8 shows cell-type specificity, as it is specifically expressed in the protoxylem of bean hypocotyl vascular tissue; thus the GRP1.8 promoter is probably regulated by specific developmental and environmental signals (Keller *et al.*, 1989a & b).

Table 21. Glycine-Rich Proteins of Dicots

	Petunia GRP[a]	Bean GRP 1.8[b]	Bean GRP 1.0[b]
Estimated Size:			
Protein by Clone	25 kD	36.7 kD	19.9 kD
Protein by SDS-PAGE	ND	53 kD	ND
Abundant Amino Acids[c]:			
Gly	67	58	63
Ala	8	8	9
Tyr	0	7	5
pI:	ND	ND	ND
Postranslation Modifications:	ND	None?	ND

[a] From Condit & Meagher, 1986, 1987
[b] From Keller *et al.*, 1988, 1989b
[c] Represented as Mole %

Function of GRPs

There exists an obvious relationship between GRPs and the development of vascular systems, implying a specific role for GRPs in the functional specialization of vascular tissue. Furthermore, GRP is insolubilized in the vascular tissue concomitant with the cessation of cell extension growth and lignification of the vascular tissue. Because the bean GRPs are fairly rich in tyrosine, the tyrosine residues may serve as a "substratum" for lignin deposition in protoxylem and/or be

the vehicle for GRP wall insolubilization via the formation of IDT intermolecular crosslinks (Keller *et al.*, 1989b).

GRPs in the Graminaceous Monocots

GRPs occur in two graminaceous monocots: maize and rice; however, they, they are smaller than the dicot GRPs and, except for being Gly-rich, are compositionally distinct (Table 2). The maize GRP has a repeating peptide (Gly-Gly-Tyr-Gly-Gly) (Gomez *et al.*, 1988), while the rice GRP has no definite repeating motif (Mundy & Chua, 1988). Furthermore, both monocot GRPs are rapidly induced by ABA and water stress (Gomez *et al.*, 1988; Mundy & Chua, 1988).

Table 22. Glycine-Rich Proteins of Graminaceous Monocots

	Maize GRP[a]	Rice GRP[b]
Estimated Size:		
Protein by Clone	15.4 kD	16.5 kD
Protein by SDS-PAGE	ND	21 kD
Abundant Amino Acids[c]	Gly (37%), Arg (9%) Glu (6%), Ala (6%)	Gly (26%), Thr (14%) Lys (8%), Gln (7%)
pI:	5.7	9.4
Post-Translational Modifications:	ND	ND

[a] From Gomez *et al.*, 1988
[b] From Mundy & Chua, 1988
[c] Represented as Mole %

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