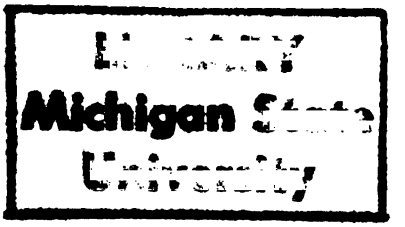

This is to certify that the

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ISOLATION AND CHARACTERIZATION OF EXTENSIN PRECURSORS FROM SUSPENSION CULTURED TOMATO CELLS

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James J. Smith

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Clifford A. Lolland

Major professor

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Daniel T. J. Lampard

ISOLATION AND CHARACTERIZATION OF EXTENSIN PRECURSORS FROM SUSPENSION CULTURED TOMATO CELLS

By

James J. Smith

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF EXTENSIN PRECURSORS FROM SUSPENSION CULTURED TOMATO CELLS

By

James J. Smith

Structural characterization of extensin, the hydroxyproline-rich glycoprotein (HRGP) component of the primary cell wall, has been difficult because extensin is insoluble, cannot be isolated without degradation, and presumably is covalently bound within the cell wall. Recent data from carrot roots support the idea that salt-soluble cell wall HRGP's (previously thought to be different from extensin) might be precursors of covalently bound extensin. Therefore, the objectives of the research reported in this dissertation were: 1) to determine if salt-soluble HRGP's were extensin precursors and 2) to characterize their structures.

HRGP's were salt-eluted from intact suspension-cultured tomato cells and purified by cation exchange chromatography of the trichloroacetic acid-soluble fraction. This yielded two pure hydroxyproline-rich glycoproteins (P1 and P2), as judged by gel filtration and gel electrophoresis. P1 and P2 had extensin-like amino

acid, sugar, and hydroxyproline-arabinoside profiles. In addition, kinetic data indicated that the P1 and P2 pools turned over, and supplied 50% (pulse-chase) and 98% (restoration kinetics) of the cell wall demand for hydroxyproline respectively. From these data I concluded that P1 and P2 were precursors of covalently-bound extensin.

Tryptic degradation of HF-deglycosylated P1 and P2 and HPLC peptide separation gave distinct tryptic peptide maps. P1 consisted largely of two peptides, Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys and Ser-Hyp-Hyp-Hyp-Hyp-Val-Lys-Pro-Tyr-His-Pro-Thr-Hyp-Val-Tyr-Lys, while P2 consisted almost entirely of the peptides, Tyr-Lys and Ser-Hyp-Hyp-Hyp-Hyp-Val-Tyr-Lys.

These peptide sequences showed that P1 and P2 are highly periodic structures with rigid domains (glycosylated Ser-Hyp-Hyp-Hyp-Hyp) separated by (non-glycosylated) flexible spacers. These non-glycosylated regions may crosslink extensin (via tyrosine) to itself or other wall polymers. Extensin crosslinkage could explain why extensin resists isolation and forms the basis of Lamport's recent "warp-weft" hypothesis, where cellulose microfibrils combine with extensin to form a molecular fabric. Cell-wall architecture might be controlled by the incorporation of

different extensin precursors to form walls of differing structure.

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LIST OF ABBREVIATIONS

AGP	arabinogalactan protein
CAPS	chromatography applications package software
CMC	carboxymethyl cellulose
dP1, dP2	HF-deglycosylated extensin precursors
HA_n	hydroxyproline with n arabinose residues
HCMM	hydroxyproline containing macromolecule
HF	anhydrous hydrogen fluoride
HFBA	heptafluorobutyric acid
HPGP	hydroxyproline-poor glycoprotein
HPLC	high performance liquid chromatography
HRGP	hydroxyproline-rich glycoprotein
Hyp	hydroxyproline
IBM	International Business Machines
IDT	isodityrosine
MGT	mean generation time
ODS	octadecylsilane
OPA	ortho-phthalaldehyde
P1, P2	glycosylated extensin precursors
PCV	packed cell volume
PRP	polystyrene reversed-phase
PTH	phenylthiohydantoin

RCW	residual cell wall
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SECW	salt-extractable cell wall
SEPS	sycamore-maple extracellular polysaccharides
TCA	trichloroacetic acid
TPCK	L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone

INTRODUCTION

A major goal of modern biology is to describe growth and differentiation at the molecular level. To understand a growth event on a molecular scale, we first need to identify what molecules exist in a given cell, tissue, organ, or organism, and then determine how they are arranged before and after growth. We can then approach the problem of how the change (i.e. growth) occurred.

When plant cells are treated with auxin (or low pH), their cell walls [the " wooden case" which surrounds them (Huxley, 1853)] elongate. The initial state is a rigid wall of one size; the final state is a rigid wall of a larger size. During elongation, the wall is temporarily "loosened", allowing the enlarging protoplast to enlarge the wall. Although this "auxin effect" (acid growth?) has provided the impetus for many experiments in cell wall biochemistry, it remains as unsolved mystery after 50 years.

Most of the chemical components of the primary cell wall* were discovered in the 19th century: pectin in

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* This dissertation deals exclusively with the primary cell wall and the term "cell wall", when used throughout the text, refers to the primary cell wall.

1825 (Braconnot), cellulose between 1838-1844 (Payen) and hemicellulose in 1891 (Schulze). The protein component however, was discovered relatively recently (1960) and we now must include protein in any description of the cell wall.

Unfortunately, we are still a long way from knowing what the cell wall looks like either before or after growth. We have a good idea of what most of the chemical components are, but the variations in their chemical linkages seem infinite and minor structural differences further confuse the picture. Thus, although several general models exist (Albersheim et al., 1973; Monro et al., 1976; Lamport & Epstein, 1983; Cooper et al., 1984), the in vivo arrangement of the different wall components is virtually unknown.

The goal of the research reported in this dissertation was to further elucidate the structure of the primary cell wall by studying the protein constituent. Using a structural approach, I hoped to learn how this protein interacts with other cell wall components and better understand how the cell wall changes during growth.

I. There is Protein in the Primary Cell Wall

The idea that protein exists in the cell wall was not readily accepted. Wiessner (1888), one of the first proponents of the existence of cell wall protein, declared that

the growing cell wall was a living structure that contained protein. Tupper-Carey and Priestly (1923) reported the presence of protein in isolated cell walls from Vicia faba meristem, but Wood (1926) refuted their claim on the basis of histochemical data which showed that not more than 0.001% of the total cellular protein was in the wall.

Through the years, however, data mounted in favor of the existence of wall protein as group after group reported the presence of nitrogen in isolated cell wall preparations (Thimann & Bonner, 1933; Preston & Wardrop, 1949; Christiansen & Thimann, 1950; Tripp et al., 1951; Northcote et al., 1958; Bishop et al., 1958; Ginzburg, 1958).

The breakthrough came in 1960 when two groups independently published amino acid compositions of isolated cell walls from tobacco callus (Dougall & Shimbayashi, 1960) and sycamore-maple (Acer pseudoplatanus) suspension cultures (Lamport & Northcote, 1960). These papers showed that hydroxyproline was associated almost exclusively with residual cell wall fractions. This not only showed that the wall had a characteristic composition, but also provided a unique marker (hydroxyproline) for cell wall protein.

Critics argued that the protein found in isolated cell walls was due to contamination by cytoplasmic proteins. Steward and colleagues had studied

hydroxyproline-rich protein from carrot root phloem discs in the 1950's and concluded that it was a cytoplasmic protein that was not metabolized (Steward & Thompson, 1954; Steward & Pollard, 1958; Pollard & Steward, 1959). The carrot discs incorporated ^{14}C-proline into protein as hydroxyproline and proline, and the radioactivity was found in the cytosolic fraction.

Lamport (1963a) addressed these criticisms by reporting that the bulk of the hydroxyproline was in the wall fraction. His report was based on experiments which demonstrated that hydroxyproline was neither associated with outer cell membrane which was firmly-bound to the wall nor adsorbed by the wall during cell breakage procedures.

But Steward persisted. He presented autoradiographic data which failed to note localization of hydroxyproline in the wall (Steward et al., 1967; Israel et al., 1968). Unfortunately, these results were questionable since Steward and his colleagues labelled their cells with 3,4-[^{3}H]-L-proline [this compound loses close to 50% of its label upon prolyl hydroxylation (Lamport, 1964; Oldham, 1968)]. When uniformly labelled [^{3}H]-L-proline was used and the cytoplasm was plasmolysed away from the cell wall after labelling, hydroxyproline was found mainly in the walls of both carrot root phloem explants (Sadava & Chrispeels, 1969) and suspension-cultured sycamore-maple cells (Roberts & Northcote, 1972). It is now generally

accepted that hydroxyproline-rich glycoprotein (HRGP) is a ubiquitous component of the primary cell walls of higher plants (Lamport & Miller, 1971).

II. Chemical Characteristics of Primary Cell Wall HRGP (Extensin)

While many workers tried to determine whether or not hydroxyproline-rich protein was in the wall, other workers concentrated on trying to determine the structure of the hydroxyproline-rich protein, how it gets into the wall, and what its function might be. Lamport (1963b) named this hydroxyproline-rich primary cell wall protein "extensin"** to reflect its hypothesized role in extension growth.

Insolubility is a most remarkable characteristic of "bulk" extensin. Extensin is insoluble in salt (Stuart and Varner, 1980), detergents, including 3% SDS in the presence of 1% beta-mercaptoethanol at 100 C (Fry, 1982), phenol/acetic acid/water (Fry, 1982), cold aqueous acids or alkalies (Blashek et al., 1981), chelating agents (Muray and Northcote, 1978) and anhydrous HF (Mort, 1978). Thus Lamport and others used biochemical and selective enzymic degradative methods to determine many of extensin's

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** All extensin is HRGP but not all HRGP is extensin. We now know there are at least three different types of HRGP and the term "extensin" is reserved for the insoluble, basic, cystine-poor HRGP firmly-bound to the primary cell wall (see Tables 1 and 2 for comparisons).

chemical characteristics.

Amino acid analyses showed that both Lamport's (Lamport & Northcote, 1960) and Dougall & Shimbayashi's (1960) primary cell wall preparations were rich in serine, valine, tyrosine and lysine (in addition to hydroxyproline). By labelling suspension-cultured sycamore-maple cells with $^{18}O_2$, Lamport (1963b) also showed that extensin biosynthesis requires molecular oxygen. This involves the specific removal of the proline trans-4-proton (Lamport, 1964). Furthermore, Lamport showed that extensin consists largely of the repeating pentapeptide Ser-Hyp-Hyp-Hyp-Hyp and that L-arabinose oligosaccharides are O-glycosidically attached to most of the hydroxyproline residues (Lamport, 1967; 1969). Akiyama et al. (1980) determined the configuration of hydroxyproline-tetraarabinoside from suspension cultured tobacco cells as: alpha-L-Araf(1-3)-beta-L-Araf(1-2)-beta-L-Araf(1-2)-beta-L-Araf(1-4)Hyp. These hydroxyproline-rich regions exist in a "polyproline II" type conformation (Lamport, 1977; Homer & Roberts, 1979; Van Holst & Varner, 1984) stabilized by the arabinooligosaccharides. In addition, some (if not most) of the serine residues are galactosylated (Lamport et al., 1973) by a single alpha-linked galactose residue. Finally, one particularly interesting, but puzzling, characteristic of extensin was the reported existence of an "unknown" modified amino acid in tryptic peptides from partially

degraded tomato cell walls (Lamport, 1974).

III. Extensin Function and Cell Wall Models

The structural uniqueness and primary cell wall location of extensin had two major scientific consequences. First, the function of a hydroxyproline-rich cell wall protein was brought into question. Second, any existing model of the primary cell wall had to be modified to include extensin and to explain its resistance to extraction.

In 1963, Lamport suggested that the hydroxyproline-rich protein (extensin) might correspond to Bonner's "Haftpunkte" (hypothetical crosslinks between cellulose microfibrils that are responsible for changes in primary cell wall plasticity, and thus for the control of cell extension; Bonner, 1935). Lamport (1963) hypothesized that extensin controlled primary wall plasticity by providing a network of labile cross-linkages between the cellulose microfibrils. Lamport (1965) proposed that disulfide bonds held extensin together, based on his detection of cystine-bridged peptides via two-dimensional paper electrophoresis of chymotryptic digests from isolated sycamore-maple cell walls (Lamport, 1965). Thus wall extension growth could be controlled by making and breaking disulfide bonds and auxin would act to "loosen" the cell wall by triggering their cleavage. However, no peptides or glycopeptides were ever isolated which contained both

hydroxyproline and sulfur-containing amino acids (Lamport, 1970), so this hypothesis was rejected.

The discovery of hydroxyproline-arabinosides (Lamport, 1967; 1969) inspired a new hypothesis. Lamport (1970) thought that the arabinosides represented the beginnings of much larger polysaccharide chains which were alkali-labile 2 to 4 arabinose residues away from hydroxyproline. In this model, (based on data collected from a Hyp-rich, TCA soluble macromolecule of MW 230 kDa which contained 95% carbohydrate and 5% protein) the cellulose microfibrils were linked together by extensin via beta-1,3-galactans attached to the hydroxyproline-arabinosides. In keeping with the "extensin hypothesis" (Lamport, 1965), these labile linkages would break during growth thereby allowing cell extension.

Shortly thereafter, Albersheim proposed a comprehensive cell wall model based on degradation and chemical analyses of suspension-cultured sycamore-maple cell walls (Keegstra et al., 1973; Talmadge et al., 1973; Bauer et al., 1973). This model correctly asserted that the cellulose microfibrils were "coated" with xyloglucan which was held in place only by hydrogen bonds. The rest of the wall components in this model existed as one huge covalently bound macromolecule with xyloglucan bound to pectin (through the arabinan and 4-linked galactan side chains of the pectic polymer), and pectin bound to extensin

(through a 3,6-linked arabinogalactan attached to extensin serine residues). Albersheim ruled out glycosidic (alkali-stable) attachment of wall polysaccharides to arabinosyl tetrasaccharides; his proposed extensin-polysaccharide link was alkali-stable, but the proposed linkage to the hydroxyproline arabinosides was alkali-labile (Lamport, 1970).

Monro, Penny & Bailey (1976) argued against the Albersheim model based on the following results from alkaline extractions of lupin hypocotyls (Monro et al., 1972; 1974): 1) conditions which removed polyuronide did not remove extensin (this would not be possible given Albersheim's model); and 2) 10% KOH at 20-24 C removed hemicellulose without extracting polyuronide (removal of hemicellulose should release both extensin and polyuronide according to Albersheim's model). Monro et al. (1976) suggested that linkages other than glycosidic bonds were involved in the cohesion of matrix polymers, and conjectured that certain polymers were not glycosidically interconnected.

Who was right? Why the discrepancy? Monro et al. were correct. Both Lamport and Albersheim (Keegstra) had been led astray by arabinogalactan protein (AGP). In 1974, reports began to appear showing that arabinogalactans were associated with hydroxyproline-rich protein moieties different from extensin (Fincher et al., 1974). These

arabinogalactan proteins (AGP's) are acidic, have MW's 100 kDa, contain 90% carbohydrate (vs. 50% for extensin) and 10% protein. AGP's are secreted into the culture medium of sycamore-maple suspension cultures (Pope 1977) and are probably not covalently incorporated into the wall matrix. Their exact structure and function remain unknown (for reviews see Clarke, 1979; Fincher et al., 1983).

Lamport's 1970 model was almost certainly based on an AGP (see above) and Albersheim based much of his 1973 model on the macromolecules of the sycamore-maple extracellular polysaccharide (SEPS) fraction. At that time there was no reason to believe that this fraction did not represent the wall (cf. Miller et al., 1974). But Pope (1977), working in Lamport's laboratory, showed that the SEPS fraction had a hydroxyproline-arabinoside profile different from that of the cell wall, that it probably contained different molecular species, and thus it could not accurately be used to model the cell wall. Hydroxyproline linked O-glycosidically to polysaccharide (Hyp-X) existed only in the SEPS fraction and not in the wall. Pope also proposed that the arabinogalactan polysaccharide, attached to serine in Albersheim's model, was actually attached to hydroxyproline. These results greatly aided in the disentanglement of the growing number of conflicting wall models.

In the meantime, many workers had noted a correlation

between extensin deposition and cessation of elongation growth (Cleland & Karlsnes, 1967; Winter et al., 1971; Bailey & Kauss, 1974; Sadava & Chrispeels, 1973). Bailey & Kauss (1974) obtained more alkali "extractable extensin" in growing mung bean hypocotyl tissue than in non-growing tissue, while Sadava & Chrispeels (1973) correlated hydroxyproline-rich protein with wall inextensibility at the end of elongation growth. Thus, some workers left the field after having convinced themselves that the secretion mechanism and function of hydroxyproline-rich protein had been established, and that this protein had nothing to do with wall loosening and auxin-induced growth.

When Andrew Mort joined the Lamport laboratory, he quite correctly reasoned that if extensin was held in the wall by polysaccharide, or any other glycosidic links, then anhydrous HF (which completely dissolves and depolymerizes polysaccharides but leaves peptide bonds intact, Mort & Lamport, 1977) should bring extensin into solution. These attempts were unsuccessful, and this failure led Mort (1978) to write, "perhaps the only way to obtain intact extensin will be to extract it from cells before it is put into the wall."

But these negative results were very important because they implied that extensin was held in the wall by something other than glycosidic linkages. Mort (1978) was able to solubilize some extensin using an acidified

chlorite oxidation procedure commonly used in delignification. Selvendran (1975) had used acidified chlorite to isolate "non-diffusable hydroxyproline-containing glycoprotein(s)" from runner beans and later modified the procedure (O'Neill & Selvendran, 1980) to reduce amino acid destruction during protein isolation. Partial solubilization of extensin by chlorite strongly implicated phenolic crosslinkages as the reason for extensin insolubility. Lamport suggested that peroxidase could catalyze the formation (involving the "unknown" tyrosine derivative) of an insoluble crosslinked extensin network (Lamport, 1980; Lamport & Catt, 1981).

Fry (1982) strengthened the phenolic crosslink hypothesis when he identified isodityrosine (IDT) as the "unknown tyrosine derivative" in hydrolysates from suspension-cultured sycamore-maple cells. Epstein and Lamport (1984) identified IDT in the previously-isolated (Lamport, 1969) tomato tryptic peptide S2A11 and crystallized pure IDT from a sycamore-maple cell wall hydrolysate. This verified Fry's identification.

These data inspired Lamport to envision the extensin network as a meshwork of defined porosity, and he proposed a new model portraying the primary cell wall (Lamport & Epstein, 1983) as a woven structure consisting of two concatenated polymers. In this model, cellulose microfibrils penetrate the mesh of an extensin net, and both of these

polymers are suspended in the hydrophilic pectin-hemicellulose gel. Microfibrillar slippage could then be regulated by controlled incorporation of extensin into this molecular fabric, thus controlling the "tightness" of the weave (or the plasticity of the wall). This model is currently being tested in the Lamport laboratory.

IV. Extensin Precursors

Although much information was gathered via analyses of cell wall preparations, the insolubility of the wall protein remained a major obstacle to its study (Lamport & Catt, 1981). Brysk and Chrispeels (1972) reasoned that the usually degradative direct chemical assault on extensin structure "precludes the possibility of characterizing intact extensin molecules as they probably exist before their incorporation into the wall matrix." Chrispeels and colleagues subsequently published a series of nine reports*** on the "Synthesis and Secretion of Hydroxyproline Containing Macromolecules in Carrots" (HCMM's). Chrispeels (1969) demonstrated the synthesis and secretion of carrot HCMM's by measuring the kinetics of ^{14}C-proline and hydroxyproline incorporation into

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*** Chrispeels (1969, 1970), Doershug and Chrispeels (1970), Sadava and Chrispeels (1971a, 1971b), Brysk and Chrispeels (1972), Chrispeels, Sadava and Cho (1974), Gardiner and Chrispeels (1975), Cho and Chrispeels (1976).

and chase from different cytoplasmic and cell wall fractions [for biosynthetic studies in sycamore-maple suspension cultures, see Dashek (1970)]. Chrispeels showed that the TCA-soluble HCMM's associated with the membranous organelles rapidly turned over and were transferred to salt-extractable (SECW) and residual (RCW) cell wall fractions at different rates.

However, when Brysk & Chrispeels (1972) characterized the glycoprotein (HCMM) common to both the membranous organelles and the SECW fraction, they reported an amino acid composition incompatible with the known composition of the cell wall or isolated extensin fragments. Furthermore, given the kinetic data presented (Chrispeels, 1969), the SECW pool described by Chrispeels' group could not account for all of the hydroxyproline known to be in the wall. However, and perhaps most significantly, suspension-cultured sycamore-maple cells did not contain similar salt-extractable HCMM's (Pope, 1977). Lamport did not believe Chrispeels' HCMM was a true precursor to covalently bound extensin.

In 1974, Roberts showed that several HRGP's were the main structural components of the Chlamydomonas cell wall (Roberts 1974). This provided evidence for the hypothesized structural role of HRGP in higher plants and piqued the interest of Varner's group in St. Louis. Stuart & Varner (1980) began studying hydroxyproline-rich

protein synthesis in elongating pea and lettuce hypocotyls, but noticed that most of the hydroxyproline accumulated adjacent to the cut ends of the tissue.

At about this time, HRGP involvement in plant-pathogen interactions began attracting much attention (Sequeira & Graham, 1977; Esquerre-Tugaye et al., 1979). Infection of melon hypocotyls with Colletotrichum lagenarium induced extensin accumulation (Esquerre-Tugaye & Mazau, 1974; Esquerre-Tugaye & Lamport, 1979) while wall HRGP levels increased upon infection of bean leaves with southern bean mosaic virus (Kimmins & Brown, 1975) or infection of cucumber hypocotyls with cucumber scab fungus (Cladosporium cucumerinum; Hammerschmidt et al., 1984). In addition, heat shock-induced resistance in cucumbers was associated with enhanced levels of cell wall hydroxyproline (Stermer, 1984). Though believed to be a general resistance phenomenon, an increase in hydroxyproline was only observed in susceptible varieties of wheat when challenged with Erisyphe graminis DC. f. sp. tritici (Clarke et al., 1981).

To better understand the accumulation of wound-related HRGP, Stuart and Varner (1980) returned to the carrot root phloem discs of Chrispeels and presented methods for the identification, electrophoresis and purification of a single salt-extractable HRGP. They also characterized its molecular weight, determined the amino

acid composition, and measured the time course of its incorporation into the cell wall. Most significantly, this protein had an extensin-like amino acid composition.

M.A. Smith (1981a) repeated Chrispeels' radiolabelling experiments and found that 20-30% of the radioactivity from labelled proline was incorporated into the RCW fraction. He also corroborated Holleman's (1967) observation that alpha,alpha-prime dipyridyl (an inhibitor of prolyl hydroxylase) did not alter the incorporation of ^{14}C-proline counts into the RCW fraction. This indicated that hydroxyproline-arabinosides are not necessary for transport or binding of HRGP to the cell wall (Smith, 1981b). Cooper and Varner (1983b) later studied prolyl hydroxylase inhibition using the more specific inhibitor 3,4-dehydroproline, and Cooper (1984) was able to show that hydroxyproline synthesis was required for plant cell wall regeneration in isolated protoplasts.

Cooper and Varner (1983a) demonstrated that much of the carrot HRGP arrives at the wall in soluble form and gradually becomes insoluble. They further noted that treatment of carrot with ascorbate resulted in increased extractibility of carrot HRGP. This may have been due to inhibition of peroxidase mediated crosslinking of extensin. In addition, Varner initiated experiments designed to isolate and sequence the genes encoding extensin (Stuart et al., 1982).

Informed of these observations, Lamport rethought his position on Chrispeels' HCMM. Perhaps it was an extensin precursor. So Lamport had Nathan Krupp (working on a summer (1982) undergraduate project) try to elute soluble extensin precursors from sycamore-maple and tomato (Lycopersicon esculentum) suspension cultures. Krupp was successful in eluting a putative extensin precursor directly from the intact cell surface of tomato cells without the use of ascorbate.

This raised the possibility of obtaining large quantities of soluble extensin precursors which could be sequenced and manipulated, a goal that had been sought for twenty years. Thus, I set out to do two things:

1. To determine if the HCMM's Krupp eluted from tomato cells were indeed precursors of the tightly-bound extensin network; and

2. To begin primary sequence determination via enzymic peptide generation, peptide mapping, and automated Edman degradation.

Briefly, the soluble HCMM's eluted from the cell wall of intact tomato cell suspensions yielded two components (P1 & P2) displaying kinetic and chemical properties that indicated their role as precursors of firmly-bound extensin.

I characterized P1 and P2 by tryptic degradation of the HF-deglycosylated polypeptides, dP1 and dP2. This was

followed by HPLC peptide mapping and automated Edman degradation of the purified peptides. The tryptic peptide maps were dominated by a very few major peptides, indicating a repetitive and therefore highly periodic extensin polypeptide backbone.

MATERIALS AND METHODS

I. Methods For Isolation And Purification Of P1 And P2

A. Suspension Cultures

I grew tomato cell suspension cultures (derived from a callus culture of the variety "Bonnie Best" donated to Dr. D.T.A. Lamport by Dr. H. Murakishi in 1967) in 1 liter flasks containing 550-600 ml M6E medium. The cells were shaken at 120 rpm on a gyrotory shaker at 27 C under subdued fluorescent lighting and subcultured, except where noted, every 7 days to an initial packed cell volume of 1-5%. For some experiments cells were grown as described but on MET medium.

The M6E medium consisted of sucrose and salts as follows (all as g/l of medium): sucrose (20); $Ca(NO_3)_2 \cdot 4H_2O$ (0.242); KNO_3 (0.085); KCl (0.061); $MgSO_4 \cdot 7H_2O$ (0.042); KH_2PO_4 (0.020); $FeCl_3 \cdot 6H_2O$ (0.025); and 2,4-dichlorophenoxyacetic acid (0.002). In addition, each liter of medium contained the 70% EtOH soluble fraction of 1.25 g Difco yeast extract (dissolved in H_20).

The MET medium consisted of sucrose and salts as follows (all as mg/l of medium): sucrose (18000); $Ca(NO_3)_2 \cdot 4H_2O$ (242); KNO_3 (85); KCl (61); $MgSO_4 \cdot 7H_2O$ (210);

NH_4NO_3 (83); KH_2PO_4 (170); NaFeEDTA (37); H_3BO_3 (6.2); $MnSO_4 \cdot H_2O$ (22); $ZnSO_4 \cdot 7H_2O$ (8.6); KI (0.83); $Na_2MoO_4 \cdot 2H_2O$ (0.25); $CuSO_4 \cdot 5H_2O$ (0.025); $CoCl_2 \cdot 6H_2O$ (0.025); and myo-Inositol (60); Thiamine·HCl (3); and 2,4-dichlorophenoxyacetic acid (1). Each liter of MET medium also contained the 70% ethanolic yeast extract described above.

B. Cell Columns

I prepared cell columns by pouring 10-20 ml of the appropriate living cell suspension into a glass column (8 x 100 mm). I allowed the cells to settle and then washed them briefly with 10 ml distilled water. They were then eluted with a 0-100 mM $CaCl_2$ gradient (total volume 50 ml) at a flow rate of 15-20 ml/hr. I collected 24-2 ml fractions, dialysed each fraction in a multiple sample microdialysis apparatus (Bethesda Research Laboratories) and then took aliquots for the hydroxyproline assay.

C. Assay Of Peroxidase

The peroxidase assay involved spectrophotometric determination of tetraguaiacol formation from guaiacol monitored at 470 nm (Maehly & Chance, 1954). The assay mixture contained 8 mM guaiacol in 10 mM NaPi buffer, pH 6.1, (2.0 ml), 5 ul 3% H_2O_2, and 50 ul aliquots taken from the cell column fractions before dialysis. Peroxidase activity is expressed as ΔA_{470} after 5 min reaction.

D. Assay Of Hydroxyproline

I determined the hydroxyproline content by the method of Kivirikko and Liessma (1959) involving acid hydrolysis (6 N HCl, 110 C, 18 hr) followed by alkaline hypobromite oxidation and coupling with acidic Ehrlich's reagent.

E. Isolation Of Crude HRGP's

I initially prepared 'crude' HRGP's from one culture flask (650 ml, 0-14 days post-subculture) by rapid filtration on a 600 ml coarse sintered-glass funnel. After a brief water wash, the cells were resuspended in 50 mM $CaCl_2$ for 5 min. The filtrate was collected after suction. Later, I prepared crude precursors in bulk from cultures of the desired age (4-6 days for high P1 yield, 7 days or later for high P2 yield, when grown on M6E) by filtration of cells in 15-30 1 l flasks on a large 120 um polypropylene filter and then washed them with 2 l of water. The cell pad was then suspended twice in 2 l 25mM $AlCl_3 \cdot 6H_2O$ (or 50 mM $CaCl_2 \cdot 2H_2O$) for 5 min and the eluate suctioned off. The 4 l eluate was reduced in volume to approximately 100 ml using a Buchi Rotavapor 150 at 40 C. Addition of TCA to a final concentration of 10% (w/v) in the eluate yielded a precipitate after 18 hr at 4 C. Centrifugation of the TCA-treated eluate (at 9000 rpm, 1 hr) yielded a hydroxyproline-poor pellet and a hydroxyproline-rich supernate. The latter was dialysed 48 hr at 4 C and then

freeze dried. The yield of crude HRGP was 80-200 mg/ 30g dry weight cells (avg. batch size), depending upon the eluting salt.

F. Ion Exchange Chromatography

1. Carboxymethyl cellulose

I dissolved crude HRGP's (10 mg/ml) in 30 mM NaPi buffer, pH 7.8, applied a maximum of 15 mg to a Whatman CM-52 carboxymethyl cellulose column (8 x 100 mm) equilibrated with 30 mM NaPi buffer, pH 7.8, and then eluted P1 and P2 with a 0-1.0 M NaCl gradient (in buffer) at a flow rate of 10 ml/hr. The mixing chamber contained 50 ml 30 mM NaPi buffer, pH 7.8, and the reservoir contained 50 ml 30 mM NaPi buffer containing 1M NaCl, pH 7.8. The flow was monitored at 280 nm using an ISCO UV monitor and chart recorder. To separate larger quantities of crude (100-200 mg) I used a 2.5 x 20 cm column of CM-52 and eluted P1 and P2 with the same gradient at 60 ml/hr.

2. BioRex 70

Jim Willard optimized the bulk separation and routinely fractionated 100-200 mg crude TCA-soluble HRGP's (10 mg/ml in 30 mM NaPi buffer, pH 7.6) via gradient elution of a 1.5 x 90 cm column of BioRex 70 (100-200 mesh): the mixing chamber contained 300 ml 30 mM NaPi buffer, pH 7.6, and the reservoir contained 300 ml 30 mM NaPi buffer containing 1M NaCl, pH 6.1. The flow was

60 ml/h (6 ml fractions), and monitored at 280 nm with an ISCO Model UA-4 absorbance detector.

II. Methods For Composition And Purity Determinations

A. Amino Acid Analysis

Amino acid compositions were determined using a modified Dionex system fitted with a 16 cm DC5A micro-column, eluted with Dionex Hi Phi Buffers A and B (Buffer A was adjusted to pH 3.05 to effect Hyp/Asp separation), and Benson's (Box 12812, Reno, NV 89510) buffer C. A Spectra-Physics SP4100 computing integrator integrated and identified component peaks. Whenever possible, the eluent was monitored at 570 nm and 440 nm for accurate estimation of Hyp and Pro. IDT estimations in isolated cell walls [involving accurate determination of a ninhydrin response factor (Epstein & Lamport, 1984)] were obtained by additional amino acid analyses with buffer C alone, which improved the Lys/IDT resolution.

We later changed over to B-X8 resin (Benson Co.) eluted by Pickering Buffers A and B, and Benson's buffer C. Fluorometric detection after NaOCl oxidation and o-phthalaldehyde coupling allowed Hyp and Pro detection (Yokotsuka & Kushida, 1983). Data capture was by IBM 9001 computer with IBM CAPS software.

The dP2 Tyr/IDT ratio was estimated by reverse phase

HPLC on Hamilton PRP-1 (4.1 x 150 mm) using an SP8000 liquid chromatograph. 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 the first 15 min, then a 5 min hold at 30% B, followed by a return to 100% A in the next 5 min. Absorbance was monitored at 273 nm (the IDT absorbance maximum in acid). IDT and tyrosine were quantitated by comparison of the sample peak areas with the areas given by known amounts of previously purified IDT (Epstein and Lamport, 1984) and reagent grade L-Tyrosine. Data capture was via the IBM 9001 and CAPS. Deglycosylation was imperative, otherwise sugar degradation products appeared in the chromatograms preventing accurate Tyr and IDT estimation.

B. Sugar Analysis

Sugars were analyzed as their alditol acetates (Albersheim et al., 1967) on a Perkin-Elmer 910 Gas Chromatograph using a 2 mm x 6 ft PEGS 224 column (polyethylene glycol succinate) programmed from 130-180 C at 1 C/min and using an SP4100 computing integrator for data capture.

C. Hydroxyproline Arabinoside Profiles

Hydroxyproline arabinoside profiles were determined by Pat Muldoon after alkaline hydrolysis of appropriate samples, careful neutralization, and separation of the

arabinosides on Technicon Chromobeads C via elution with a pH gradient and monitoring as described by Lamport & Miller (1971).

D. Sepharose CL-6B Gel Filtration

I injected 1-5 mg P1, P2, dP1, or dP2 (10 mg/ml in 1 M NaCl) onto a 1.25 x 100 cm column of Sepharose CL-6B-200, eluted the proteins with 1 M NaCl at 13 ml/h using a syringe pump, and collected 2 ml fractions whose absorbance was read at 280 nm.

E. ZORBAX GF-250 HPLC Gel Filtration

HRGP's separated on the BioRex 70 cation exchanger were dissolved at 10 mg/ml in 0.2 M NaPi buffer containing 0.005% NaN_3, pH 7.0. I injected 200 ug samples onto a 9.4 x 250 mm DuPont GF-250 HPLC column (GF = gel filtration) and eluted at 1.0 ml/min with the sample buffer using an LDC Model I Constametric Pump (Milton Roy Co.). Absorbance was monitored at 254 nm using an ISCO Model 1870 Absorbance detector. Data capture was via an SP4100 computing integrator.

F. HF Deglycosylation

I deglycosylated 5-50 mg glycosylated P1a,P1b, or P2 in a micro apparatus containing 2-4 ml anhydrous HF and 5-10% (v/v) anhydrous MeOH for 1 hr at 0 C as described by Sanger & Lamport (1983). The reaction was quenched by

pouring into stirred H_2O at 2 C to a final concentration of 5-10% (v/v) HF, and then dialysed for 16-24 hr at 4 C and freeze dried. Yields were typically 35-40% of the original weight for P1 and P2.

G. Gel Electrophoresis

For SDS-PAGE, 5 mg precursor material were deglycosylated, the HF removed by evacuation, and the residue immediately dissolved in 1 ml H_2O. Aliquots of 50 and 100 ul were blown dry with N_2, the residue dissolved in 25 ul sample buffer [0.01 M Trizma base (pH 10.0), 1% SDS, 0.001 M EDTA, and 5% beta-mercaptoethanol] and applied to the 'sepracomb' of commercially prepared 10-20% acrylamide gradient Sepra-Gels (Separation Science Inc.). Gels were run in Tris-Gly buffer [0.025 M Trizma base (pH 9.8), 0.192 M glycine, 0.1% SDS] for 3 hr at a constant power of 15 watts. Bromophenol blue was used as a tracking dye.

III. Kinetic Methods

A. Short-term Pulse Labelling

Carrier-free L-[5-^{3}H]proline (40 uCi; Amersham, 21 Ci/mmol) in 1 ml H_2O was added aseptically (via syringe fitted with a 0.2 u millipore filter) to a 1 l flask containing 650 ml cell suspension (6 day old, 10% packed cell volume). At various times after the pulse (see Figure 15), I took 100 ml aliquots of cell suspension, prepared

and separated P1 and P2 via the small CMC column as described above. I monitored the tritium content of each CMC fraction in aquasol or ACS (1 ml in 10 ml) using a Beckman LS 133 or LS 7500 scintillation counter and determined CPM per mg glycoprotein using 0.46 and 0.56 absorbance units at 280nm = 1mg glycoprotein/ml for P1 and P2 respectively.

B. Pulse Chase

Carrier-free L-[U-^{3}H]proline (75 uCi; Amersham, 653 mCi/mmol) was added to a 1 l flask containing 650 ml cell suspension (4 day cells, 7% packed cell volume) as described above. After 2 hr, I added 1 g unlabelled proline as chase, and took 50 ml aliquots of cell suspension at various time intervals (Figure 16). P1 and P2 were eluted from the cells with $CaCl_2$, separated via CMC, the counts monitored and specific activities calculated as described above.

C. Restoration Kinetics

The rate of pool repletion after an initial depletion was determined as follows: 650 ml of a 4 or 8 day old culture were filtered on a coarse sintered-glass funnel, the cells washed briefly with water, and then extracted for 5 min with 50 mM $CaCl_2$ (100 mM for 8 day cells; eluates saved for Hyp assay). After a brief water wash, the cells were resuspended in their original growth medium. At

various times, 50 ml aliquots of the depleted cells were taken and assayed for the reappearance of salt-elutable Hyp.

IV. Methods For Peptide Generation, Separation, And Sequencing

A. Tryptic Digestion

HF-deglycosylated extensin precursors (0.2-20 mg) were dissolved at 10 mg/ml in freshly prepared 2% (w/v) NH_4HCO_3 (aq) containing 10 mM $CaCl_2$ (minimum total volume=100ul). I added TPCK-trypsin (Worthington) to this solution giving a final substrate:enzyme ratio of 100:1 and incubated the solution at 30-35 C with constant stirring.

In some experiments the time course of tryptic cleavage was followed under unbuffered conditions at room temperature in a pH Stat using a Corning model 135 pH/ion meter interfaced to a Radiometer Automatic Burette Unit.

B. HPLC Peptide Mapping

Tryptic peptide maps were obtained via reverse phase HPLC of tryptic digests on either DuPont Zorbax ODS (4.6 x 250 mm) or Hamilton PRP-1 (4.1 x 150 mm) columns 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) CH_3CN (aq). For resolution of dP1 tryptides, the gradient began at 100% A and 0% B. B

was then increased from 0 to 50% in 100 min (0.5%/min). For dP2 tryptide resolution, the starting conditions were the same, but solvent B was increased from 0 to 60% in 60 min (1%/min). Absorbancy was monitored at 273 nm using a Spectra-Physics Model 770 Spectrophotometric Detector. Fractions for analyses were collected manually, allowing for the 200 ul dead volume between the detector and "fraction collector".

C. Sephadex G-25 Gel Filtration

Freeze dried tryptides (1-10 mg in 0.5 ml 0.1 M HOAc) were injected onto two 1.25 x 100 cm columns (in series) of Sephadex G-25-80 (fine) and eluted with 0.1 M HOAc at 10 ml/hr using a Syringe pump (Harvard Apparatus Co. Model 2201). Fractions (2 ml) were collected and their absorbances read at 230 (or 280) nm.

D. Automated Edman Degradation

Samples (5-200 nmoles) of HPLC-purified peptides were sequenced [5 mg polybrene added as a carrier (Klapper et al., 1978) without precycling) using a Beckman 890C Spinning Cup Sequencer plus cold trap (0.1M quadrol program; Beckman Program #101078), in conjunction with Sequemat P-6 Autoconversion of anilinothiazolinone derivatives to the corresponding phenylthiohydantoins (PTH). After Sequemat conversion in methanolic HCl [12.5% acetyl chloride (v/v) in methanol] for 6 min at 65 C, the

PTH derivatives were dissolved in 100 uL pH 4.4 Zorbax buffer [0.006 M NaAc, pH 4.4, in 42% (v/v) CH_3CN (aq)] containing 10 nmoles PTH-norleucine as internal standard. They were then chromatographed on DuPont Zorbax ODS (4.6 x 250 mm) isocratically eluted at 0.5 ml/min with pH 4.4 Zorbax buffer or pH 5.0 Zorbax buffer [0.005 M NaAc, pH 5.0, in 50% (v/v) CH_3CN (aq)] for PTH-histidine identification. The eluate was monitored at 269 nm using an ISCO Model 1840 Absorbance Detector and the peak areas were calculated using an SP4100 computing integrator.

In the later stages of this work, I used an IBM cyano column (4.5 x 250 mm) with the SP8000 liquid chromatograph to separate PTH-amino acids. For these separations I used the following ternary solvent program at a flow rate of 0.5 ml/min:-

Solvent A: 0.015M NaAc (pH 5.8)
Solvent B: CH_3CN
Solvent C: MeOH

Time	%A	%B	%C
0	85	15	0
10	58	30	12
14	67	15	18
30	50	25	25
40	40	30	30
44	85	15	0

Detection was at 254 nm using the SP770 Model Spectrophotometric Detector and data capture was via the IBM 9001 computer running CAPS software. This column and solvent program gave a better separation of all PTH-amino acids and allowed identification of histidine and arginine without using a separate solvent system (Hunkapiller &

Hood, 1983).

PTH-serine and PTH-threonine were sometimes identified by the presence of distinctive peaks resulting from their degradation during conversion (Edman, 1970) but most often these degradation peaks merely verified identification.

RESULTS

I. Elution Of Hydroxyproline-rich Glycoprotein From The Cell Surface And Its Subsequent Fractionation

A. Columns Of Intact Cells And Bulk Elutions

Elution of cells packed into a small column (8 x 100 mm) with a linear gradient of unbuffered $CaCl_2$ (0-100 mM, pH 6) released cell wall peroxidase at 10 mM $CaCl_2$ and HRGP at 30 mM $CaCl_2$ (Figure 1). Cell columns eluted with linear gradients of $LaCl_3$ and NaCl released hydroxyproline-rich material at 10 mM $LaCl_3$ and 300 mM NaCl respectively (Figure 2).

I initially used 50 mM $CaCl_2$ for bulk elutions (see Figure 3 for flow chart). Fifty mM $CaCl_2$ did not plasmolyse the cells or drastically decrease viability (based on microscopic and macroscopic observation). Furthermore, when cells were grown in 50 mM $CaCl_2$, the cell yield was 15% PCV versus 24% PCV in the control (19 days growth, initial inoculum 5% PCV). Cells did not grow in 100 mM $CaCl_2$.

Hydroxyproline elution from the cells was complete two minutes after $CaCl_2$ addition (Figure 4). In a separate experiment (using rapid sampling) 60% of the total

Figure 1. Profiles of peroxidase and hydroxyproline-rich glycoprotein eluted from a column of intact suspension cultured tomato cells.

Cell column (8 x 100 mm) eluted with a linear gradient of 0 to 100 mM unbuffered $CaCl_2$.

Closed circles: peroxidase activity.

Open circles: hydroxyproline.

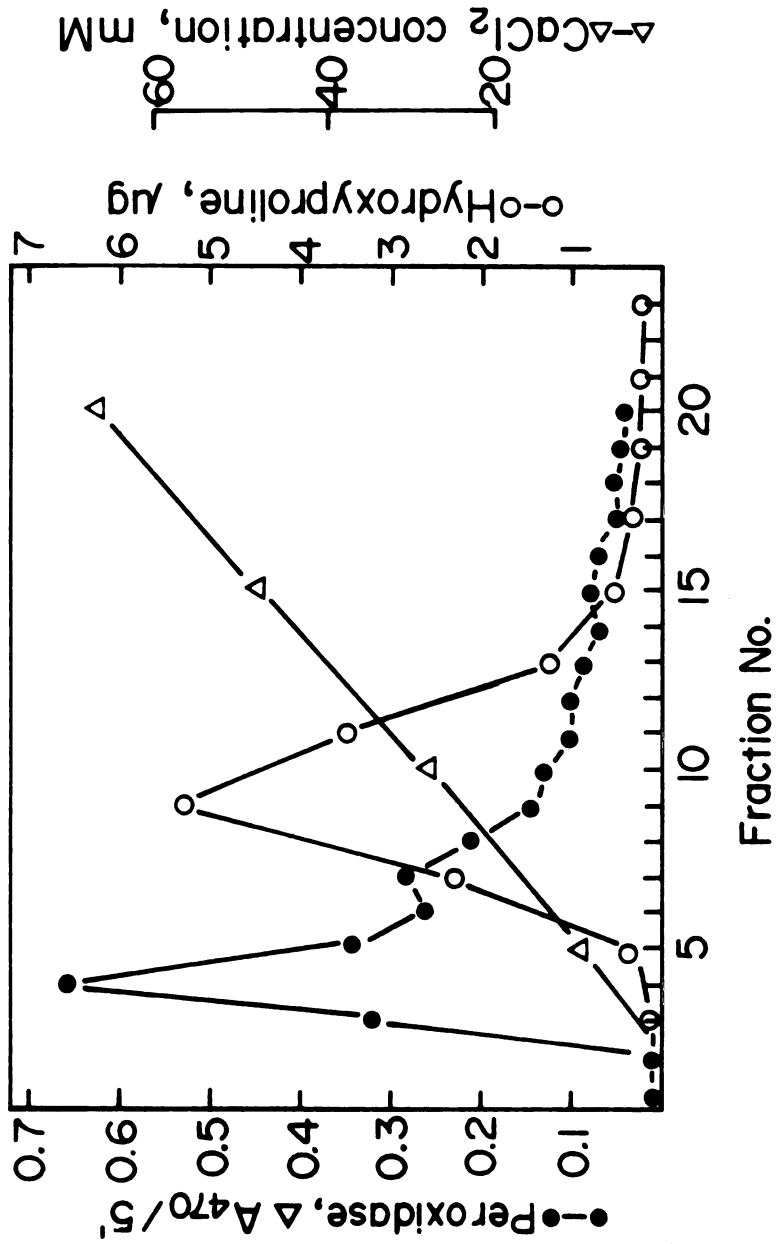

●–● Peroxidase, Δ A470/5'
o–o Hydroxyproline, µg
Δ–Δ CaCl2 concentration, mM
Fraction No.
0.1
0.2
0.3
0.4
0.5
0.6
0.7
1
2
3
4
5
6
7
20
40
60
5
10
15
20

Figure 1

Figure 2. Hydroxyproline-rich glycoprotein eluted from cell columns with Na^{+} and La^{+++}.

Dashed line indicates gradient for both NaCl and $LaCl_3$. An elution profile from cell columns eluted with $AlCl_3$ was similar to that of $LaCl_3$ (peak at 10 mM). Column dimensions as in Figure 1.

Open circles: NaCl.

Closed circles: $LaCl_3$.

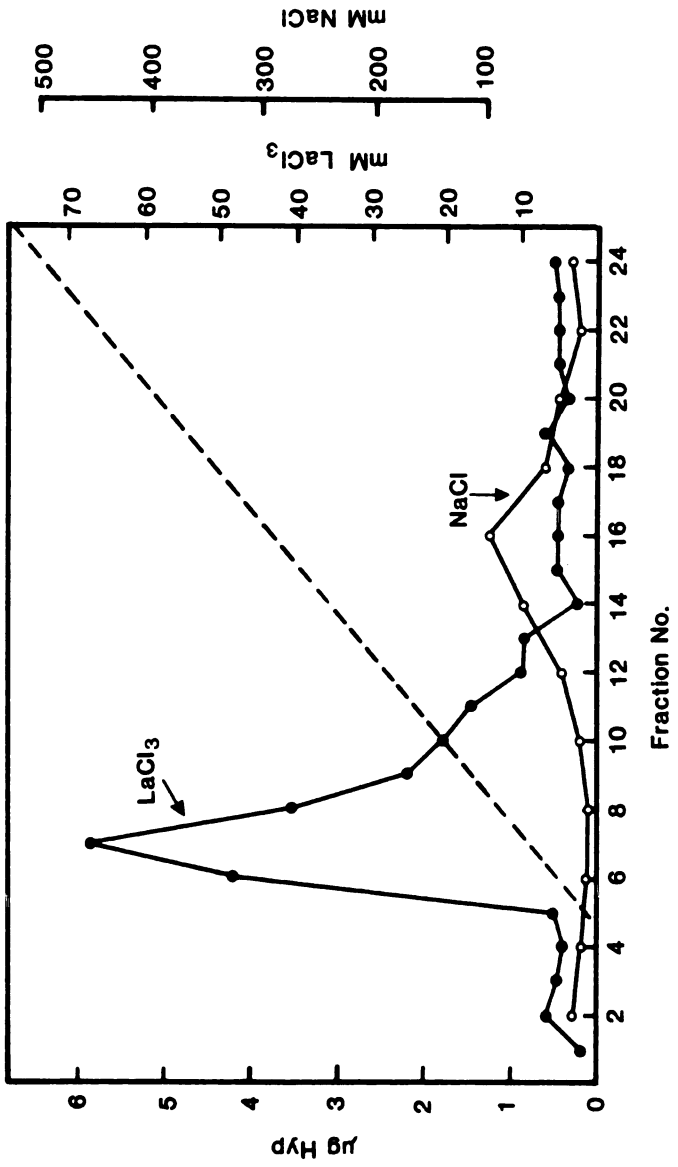

mM NaCl
500
400
300
200
100
mM LaCl3
70
60
50
40
30
20
10
µg Hyp
6
5
4
3
2
1
0
LaCl3
NaCl
2
4
6
8
10
12
14
16
18
20
22
24
Fraction No.

Figure 2

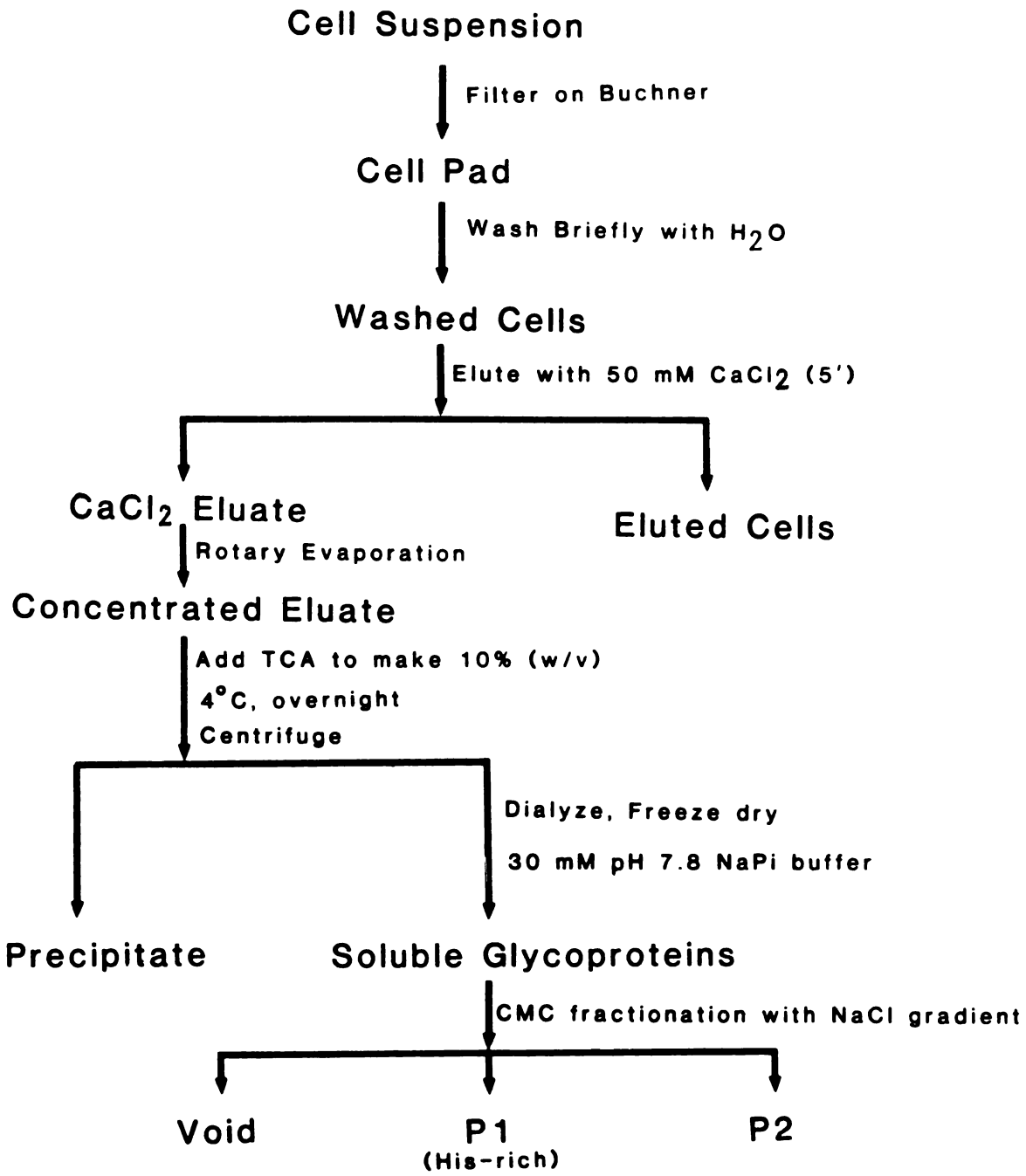


Figure 3. Flow chart for bulk elution and purification of hydroxyproline-rich glycoprotein from tomato suspension cultures.

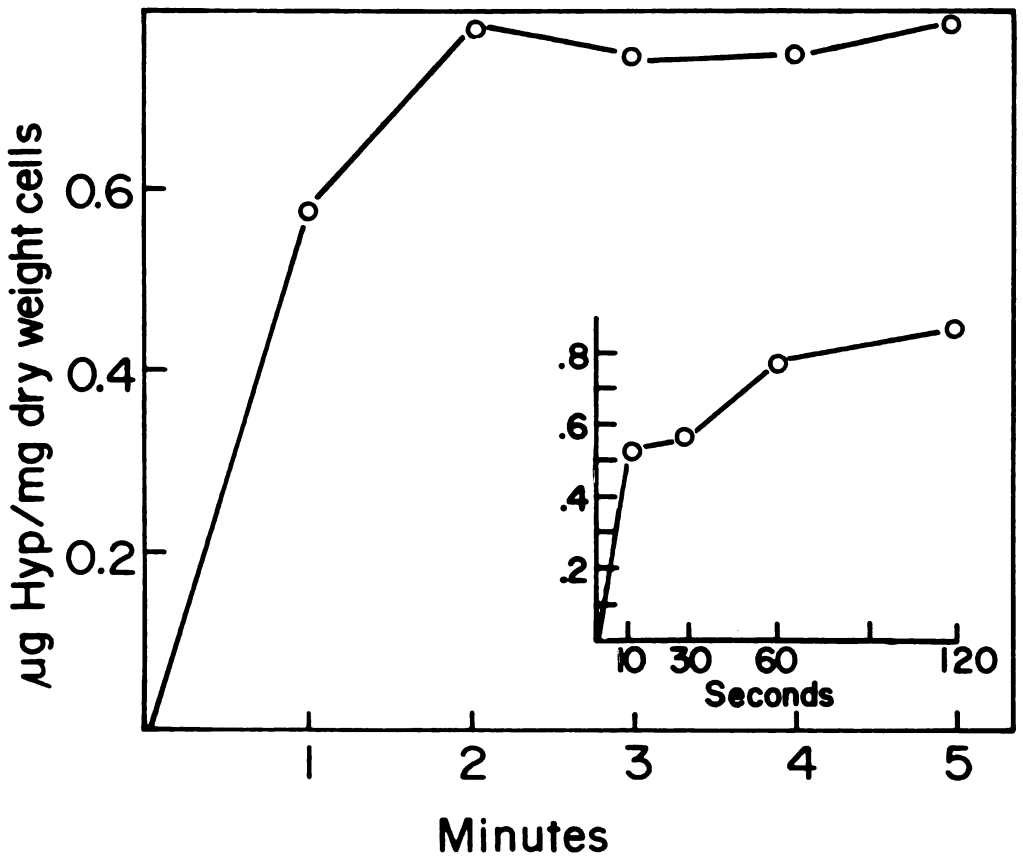


Figure 4. Elution of hydroxyproline-rich glycoprotein with 50 mM $CaCl_2$ as a function of time.

The cell pad was bathed in eluting salt for various times and then assayed for hydroxyproline. Samples were not TCA precipitated.

Inset: separate experiment showing the amount of HRGP present after very short elution periods.

salt-soluble HRGP eluted within 10 seconds (Figure 4, inset). The yield of hydroxyproline obtained by $CaCl_2$ elution ranged from 0-0.7 mg Hyp/g cells dry weight, depending on the growth phase (Figure 5).

B. Treatment With Trichloroacetic Acid

Overnight precipitation with 10% (w/v) TCA at 4 C followed by centrifugation removed contaminating protein from crude eluates; amino acid analyses showed significant hydroxyproline enrichment after TCA precipitation (Table 1). This was consistent with the highly glycosylated and basic character of extensin.

Peroxidase activity remained exclusively in the TCA-precipitate as determined by assays after dialysis. On a weight basis the actual amount of peroxidase eluted was much less than HRGP. For example, at day seven (Figure 5) the HRGP yield was 2.3 mg/g cells dry weight, while the peroxidase yield was 28 ug/g cells dry weight, using horseradish peroxidase as a standard.

Elution of intact cells with 25 mM $AlCl_3$ gave higher TCA-soluble crude precursor yields than 50 mM $CaCl_2$. Large batches of tomato cell suspension (15L, 0.6 kg. wet weight, 30 g dry weight) after 7 days growth on M6E medium consistently yielded about 200 mg TCA-soluble crude precursor upon $AlCl_3$ elution while $CaCl_2$ elution yielded about 120 mg crude. I subsequently changed over to 25 mM

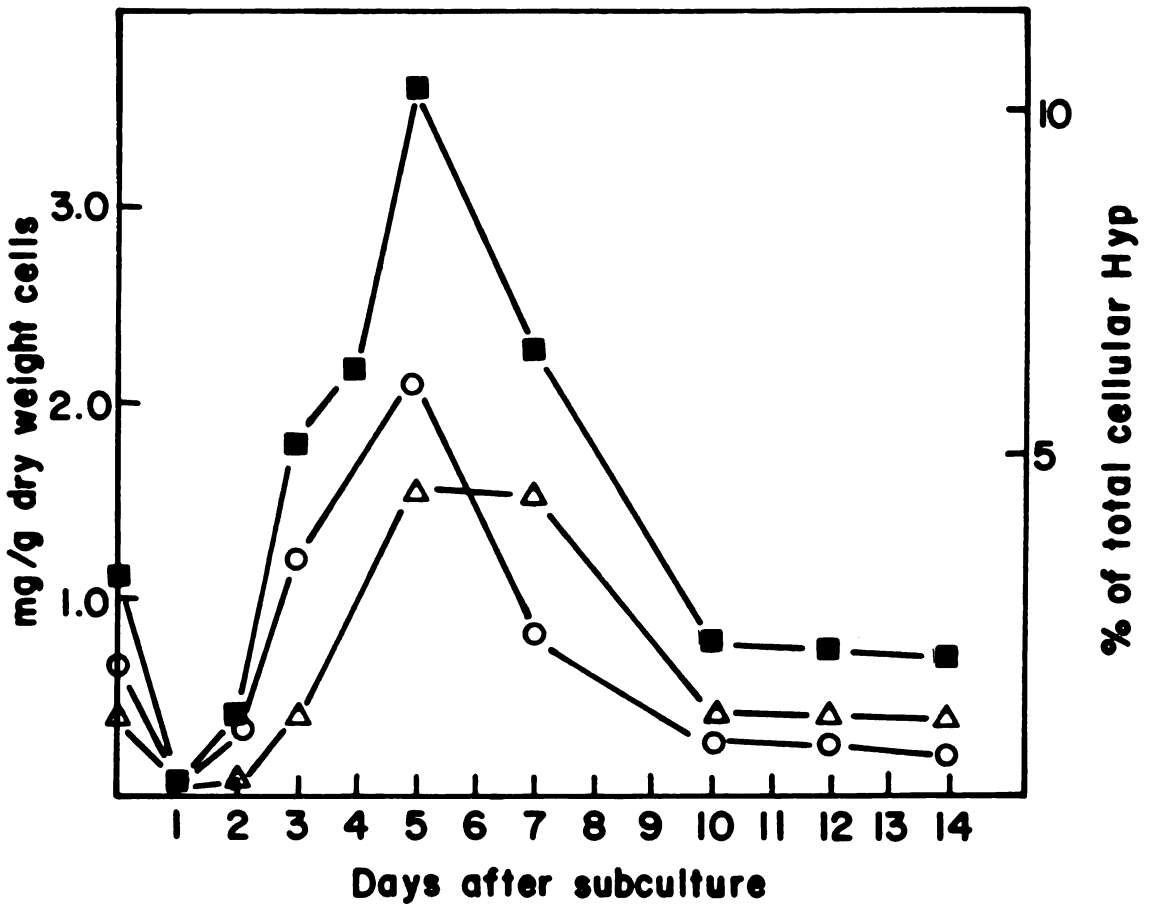


Figure 5. Yields of P1, P2, and P1 + P2 as a function of culture age.

Yield also expressed as the % of total cellular hydroxyproline eluted assuming the cell is 0.7% hydroxyproline by weight.

Open circles: P1.

Open triangles: P2.

Closed squares: sum of P1 and P2.

Table 1. Amino Acid Compositions* of Crude Eluates Before and After TCA Precipitation, the TCA Precipitate, the CM-52 Void Peak, P1, P2, and Isolated Cell Walls

A.A.	Crude (before TCA)	Crude (after TCA)	TCA ppt.	Void	P1	P2	Isolated cell walls**
Hyp	21.4	35.3	1.5	3.3	33.5	41.8	28.5
Asp	5.9	2.6	11.8	7.6	1.8	0.7	4.0
Thr	5.0	3.4	6.6	5.0	7.2	1.0	4.6
Ser	10.5	10.6	9.6	21.0	9.5	12.1	14.2
Glu	3.7	2.2	6.9	14.5	1.9	0.3	2.8
Pro	5.3	4.0	6.1	2.3	8.3	0.8	3.9
Gly	4.2	2.8	10.0	16.5	1.6	0.3	3.3
Ala	2.6	1.2	6.1	8.9	2.0	0.5	3.2
Val	6.0	6.7	5.5	3.7	5.0	5.1	7.0
CysA	0.7	0.3	1.5	1.0	0.0	0.0	0.0
Met	0.0	0.0	0.0	0.0	0.0	0.0	0.3
Ile	3.1	1.8	4.6	2.4	0.9	0.9	1.8
Leu	4.4	1.4	9.1	3.4	0.8	0.2	2.5
Tyr	7.5	9.0	3.2	2.0	8.9	14.9	6.3
Phe	2.7	0.0	4.7	2.4	0.6	0.2	1.3
His	2.8	2.7	1.9	1.6	7.1	1.0	2.7
Lys	11.3	14.3	6.6	3.2	10.1	20.1	10.5
Arg	2.1	0.9	3.6	1.2	0.7	0.1	1.2

*- Expressed as mole%.

**- Cell walls were prepared by sonic disruption as described by Lamport (1965), followed by boiling in 1% (w/v) SDS for 3 hr to remove contaminants. The clean walls were then deglycosylated and hydrolyzed for 24 hr as described in Materials and Methods.

$AlCl_3$ for bulk elutions.

C. Cation exchange chromatography of HRGP on carboxymethyl cellulose (CM-52)

After dialysis of the TCA-soluble crude HRGP, chromatography on carboxymethyl cellulose (in 30 mM NaPi buffer (pH 7.8) with a linearly increasing NaCl gradient) yielded two major HRGP fractions designated P1 and P2 (Figure 6). P1 eluted at 0.3 M NaCl while P2 eluted at 0.5 M NaCl. The P1:P2 ratio changed with culture age as did the total amount of TCA-soluble P1 and P2 (Figure 5). The P1 yield increased and decreased a day or two ahead of P2. After subculture total soluble HRGP decreased rapidly, was minimal at day 1, then rose to peak at day 5, subsequently falling.

To determine whether salt-elutable HRGP arose from a rapid secretion process or was simply washed from the cell surface, cells were eluted in the cold room using rinse water and eluent at 4 C. This gave a CMC profile identical to CMC profiles obtained from elutions done at 20 C (Figure 7). In addition, sonic disruption of cells for 1 min did not have any effect on P1 and P2 yield (Figure 8). From these results I concluded that P1 and P2 were being released from the cell surface (probably via ionic displacement), and not being rapidly secreted (see discussion).

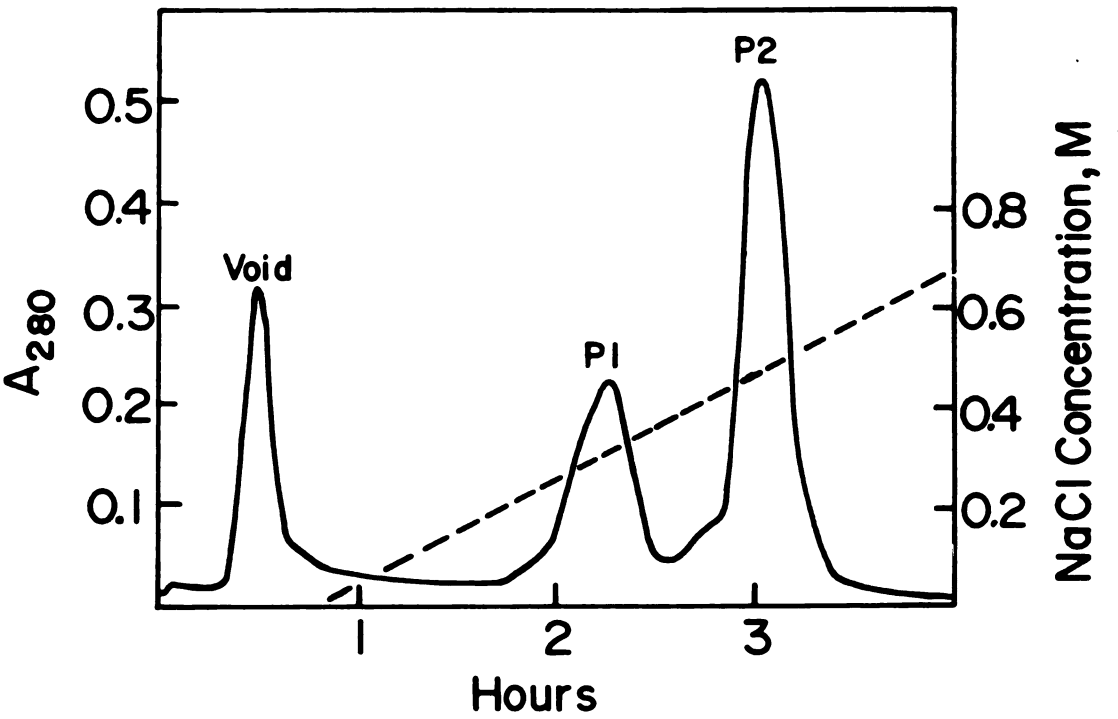


Figure 6. Separation of TCA-precipitated crude HRGP on carboxymethyl cellulose (CM-52), giving P1 and P2.

Crude precursor (10 mg) in 1 ml 30 mM NaPi buffer (pH 7.8) was applied to an 8 x 100 mm carboxymethyl cellulose column (Whatman CM-52) equilibrated with buffer. P1 and P2 were eluted with a 0-1.0 M NaCl gradient (in buffer) at a flow rate of 10 ml/hr.

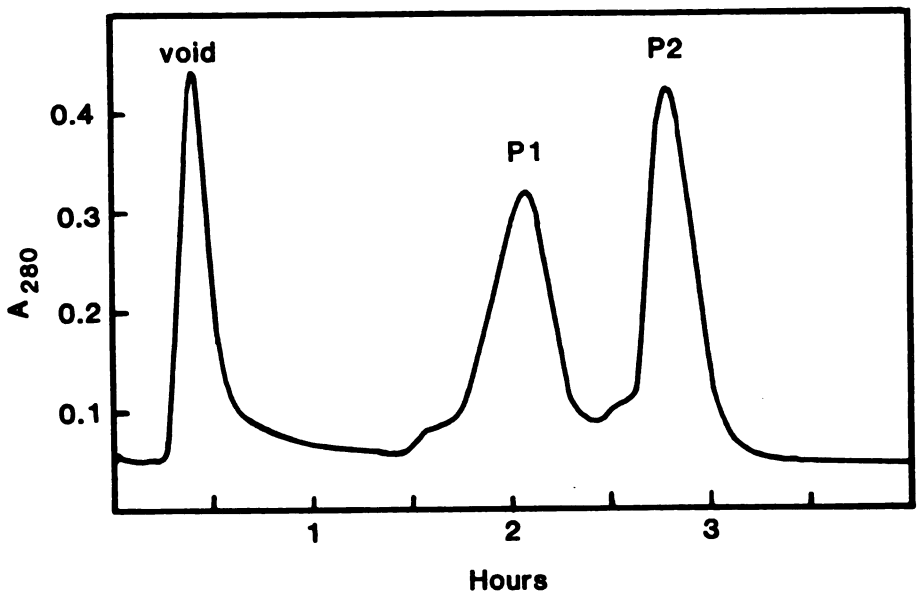


Figure 7. Profile of the separation of P1 and P2 on carboxymethyl cellulose after elution from cells at 4 C.

Figure 8. Comparison of the elution of P1 and P2 from whole cells with their elution from isolated cell walls.

Profile (a): 100 ml cell suspension (6 day culture) chilled in ice water for 2 min (with constant swirling), then eluted with $CaCl_2$ as usual.

Profile (b): 100 ml cell suspension chilled in ice water for 1 min, sonicated with a needle probe at a setting of 35 (on ice) for 1 min, then eluted with $CaCl_2$ as usual. Sonication broke a minimum of 50% of the cells (estimated by visual examination under the microscope).

Profile (c): medium decanted from 100 ml cell suspension, cells suspended in 100 ml ice water for 1 min, followed by sonication and elution as in the middle curve. This treatment ensured that P1 and P2 did not arise from the medium by adhesion after cell breakage.

Elution from carboxymethyl cellulose columns was as described in Figure 6.

Note: y-axis is in relative A280 units; all three profiles begin at zero absorbance.

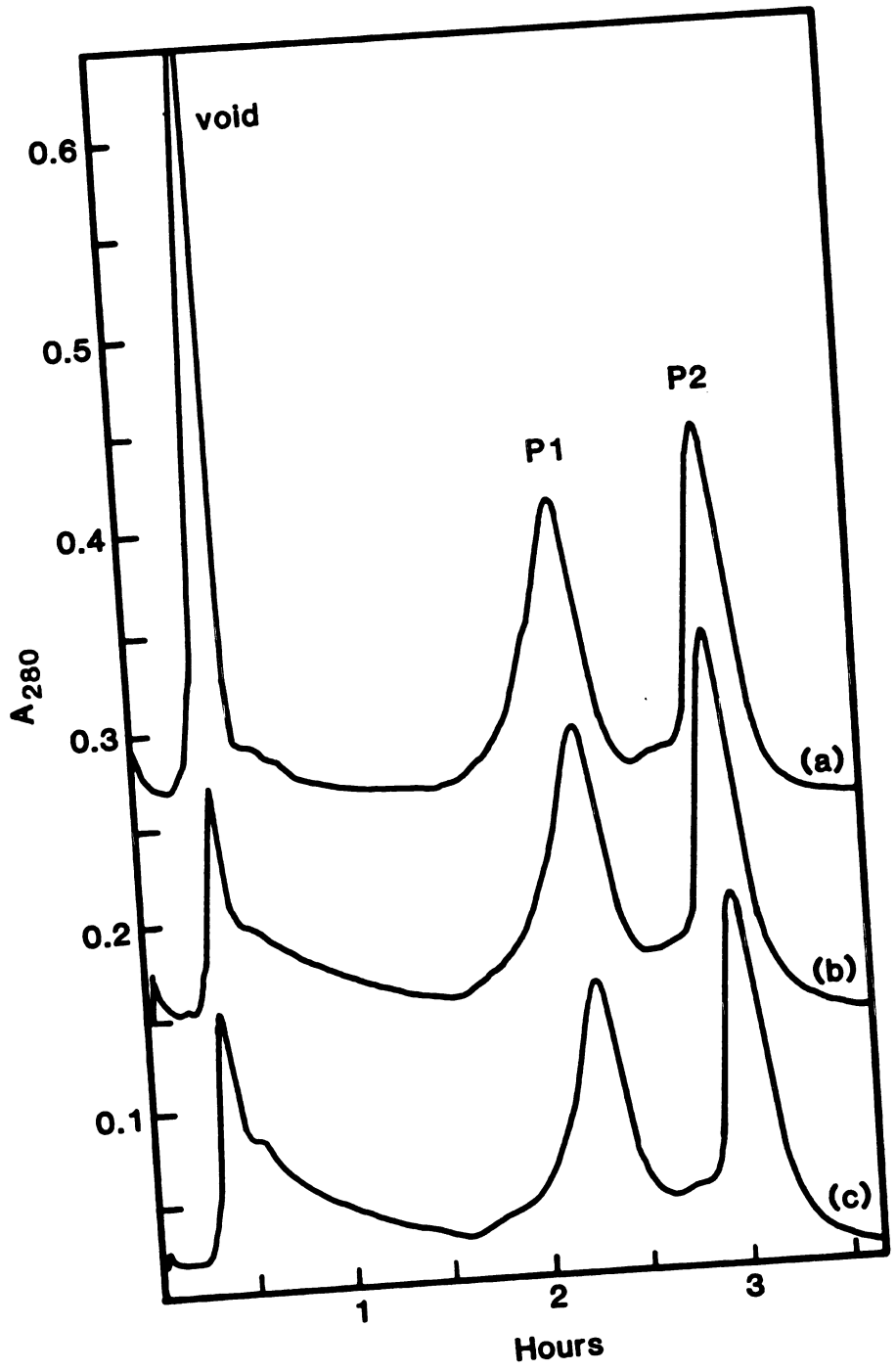


Figure 8

Another possibility was that P1 and P2 were secreted into the medium and some of these secreted molecules attached themselves to the cell wall via ionic bonds. However, there was no P1 or P2 in the growth medium (Figure 9) making this explanation unlikely.

TCA precipitation greatly reduced the CMC void peak and also removed some proteins retarded by CMC (Figure 10). The CMC void peak (after TCA) contained a relatively small amount of hydroxyproline and gave a positive reaction with Yariv antigen (Jermyn & Yeow, 1975) indicating the presence of arabinogalactan protein (AGP). Void peaks were also enriched in amino acids common to the hydroxyproline-poor glycoproteins (Tables 1 & 2). Brysk and Chrispeels (1972) 'anomalous' composition of the TCA-soluble HCMM from carrot may have been due to contamination by these proteins; Van Holst and Varner (1984) sometimes obtained similar compositions from their preparations. I did not further characterize the TCA-precipitable proteins which were retarded on CMC.

Although $AlCl_3$ gave higher TCA-soluble crude yields than $CaCl_2$, final yields of purified precursors were not significantly different; both eluting salts routinely gave 20-30 mg pure P1 and P2 (6 day culture, 30 g cells DW). The excess crude eluted by $AlCl_3$ voided the CMC column. $AlCl_3$ may remove P1 and P2 in a state of wall incorporation in which they are associated with HPGP's.

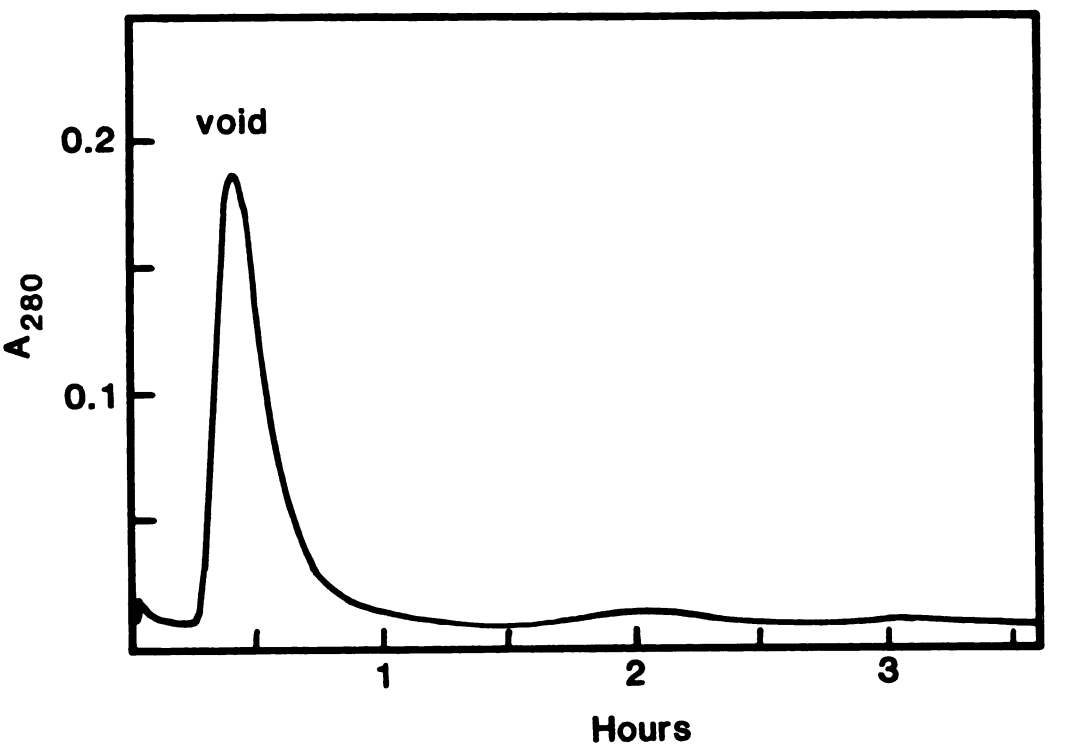


Figure 9. Profile of the TCA-soluble fraction of the culture medium eluted from carboxymethyl cellulose.

Filtered culture medium (200 ml) from a 10 day culture was treated with TCA. The soluble fraction was dialyzed, freeze-dried and 10 mg were applied to a carboxymethyl cellulose column. Elution from the CMC column was as described in Figure 6.

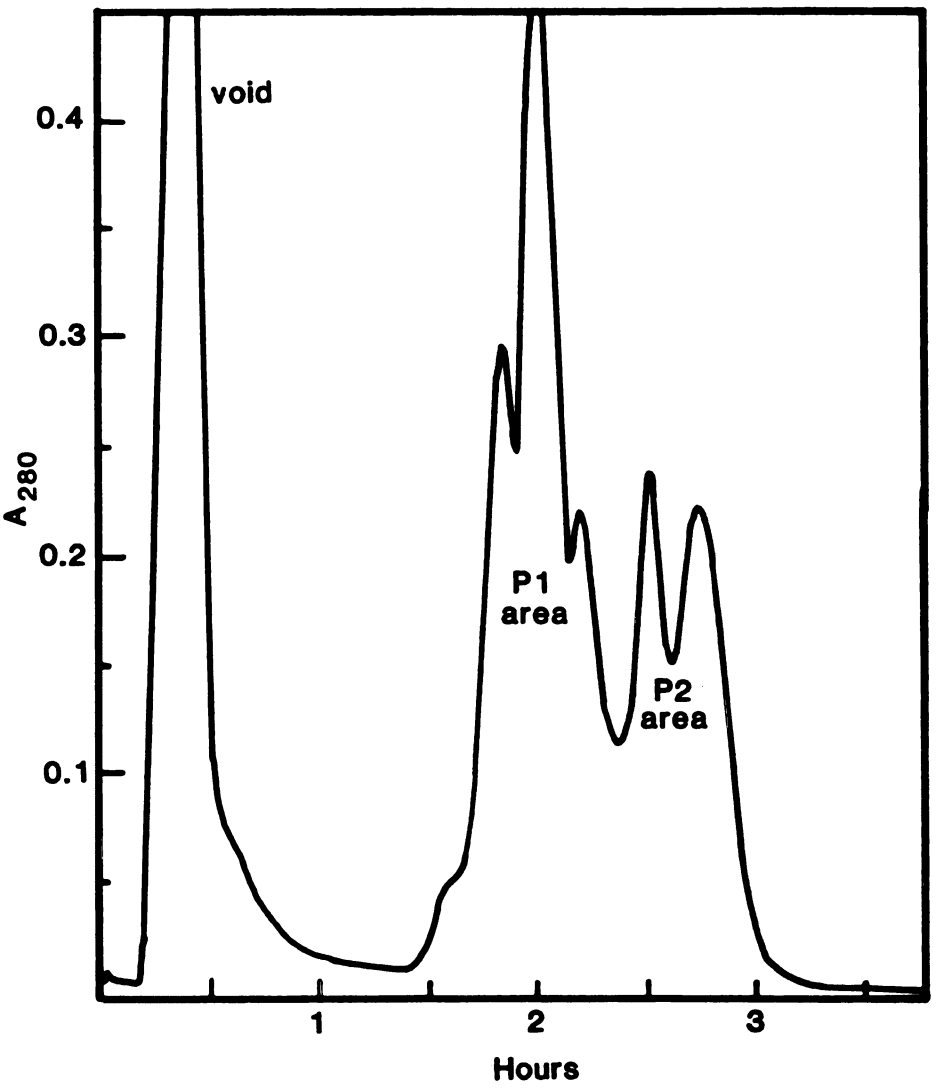


Figure 10. Profile of crude $CaCl_2$ eluate before TCA precipitation as separated on carboxymethyl cellulose.

Crude eluate from one 650 ml culture (day 5). The contaminating peaks which appeared in the P1 and P2 areas were not further characterized.

Table 2. Amino Acid Compositions of Various Hydroxyproline-containing Glycoproteins

A.A.	Carrot extensin (a)	Tobacco Agglutinins (b)	Potato (c)	Potato Lectin (d)	AGP (e)	HPGP (f)
Hyp	45.5	38.0	41.7	20.5	14.8	2.0
Asp	0.3	5.5	0.7	5.0	5.6	7.0
Thr	1.2	5.6	3.2	5.7	6.6	5.0
Ser	14.0	8.9	9.4	12.6	10.0	7.0
Glu	0.3	0.4	1.1	6.9	4.9	10.0
Pro	0.9	8.4	9.2	6.9	5.7	7.0
Gly	0.4	0.4	1.1	12.2	6.8	11.0
Ala	0.4	1.2	0.9	4.1	22.6	11.0
Val	5.9	5.2	3.8	0.4	5.2	6.0
Cys	0.0	0.2	0.1	10.6	1.0	0.0
Met	0.0	0.2	ND	0.4	1.6	1.0
Ile	0.3	0.3	0.3	1.6	1.3	5.0
Leu	0.3	0.8	0.2	1.2	5.1	7.0
Tyr	11.0	12.2	6.2	3.3	1.2	4.0
Phe	0.0	ND	0.1	0.2	1.5	4.0
His	11.8	5.0	5.1	0.0	0.6	1.0
Lys	6.5	12.4	15.9	3.7	3.3	7.0
Arg	0.0	ND	0.2	1.3	2.2	4.0
Trp	1.2	ND	1.9	3.3	0.0	1.0

ND- not detected
(a) from Van Holst and Varner (1984)
(b) from Mellon and Helgeson (1982)
(c) from Leach et al. (1982)
(d) from Allen et al. (1978)
(e) from Anderson et al. (1977)
(f) from Brown and Kimmins (1978)

Jim Willard recently surveyed several weak cation exchangers (carboxymethyl cellulose (DE-52), BioRex 70, CM-Sepharose, and CM-Trisacryl). He was able to optimize precursor separation conditions by changing from CMC to BioRex 70 (100-200 mesh) which was eluted with a shallow salt gradient superimposed on a decreasing pH gradient (described in Materials and Methods). This resolved P1 into two components (P1a and P1b; Figure 11) of almost identical amino acid composition (Table 3) but distinguished by the slightly higher histidine and lysine content of P1b (consistent with its later elution from the cation exchanger). P2 remained a single peak of unchanged composition (Table 1). Subsequently P1 is used as an inclusive term when describing features common to both P1a and P1b.

D. Molecular Weight And Purity

1. Sepharose CL-6B and DuPont GF-250 gel filtration

The size of extensin precursors before incorporation into the wall is crucial to hypotheses concerning mechanisms of cell wall assembly and growth. Gel filtration of each precursor (P1 and P2) on Sepharose CL-6B gave single retarded symmetrical peaks at approx. 1.5 V_o (Figure 12). HF-deglycosylated material interacted strongly with the CL-6B gel; I could not elute deglycosylated P1 or P2

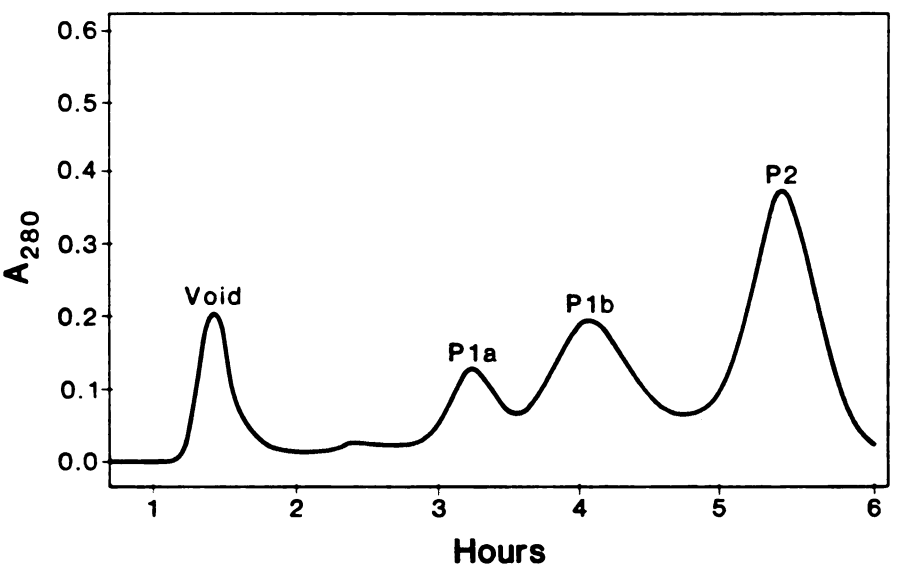


Figure 11. BioRex 70 cation exchange chromatographic separation of P1a, P1b, and P2.

A crude precursor preparation (120 mg) from a 5 day culture (15% packed cell volume, 28 g cells DW) was applied to a 1.5 x 90 cm column of BioRex 70. The sample was dissolved in 12 ml of 30 mM NaPi buffer (pH 7.6). The column was developed with a linear gradient of 0 to 1 M NaCl (in buffer).

Table 3. Amino Acid Compositions* of Pla and Plb

A.A.	Pla	Plb
Hyp	32.7 +/- 3.4	30.9 +/- 4.9
Asp	1.4 +/- 0.5	1.9 +/- 0.6
Thr	6.2 +/- 0.5	6.5 +/- 0.5
Ser	9.8 +/- 1.0	9.5 +/- 0.9
Glu	1.5 +/- 0.6	1.6 +/- 0.4
Pro	9.6 +/- 2.3	10.2 +/- 1.2
Gly	1.7 +/- 1.1	2.0 +/- 0.6
Ala	2.9 +/- 0.5	1.9 +/- 0.7
Val	8.3 +/- 0.8	7.1 +/- 0.8
Ile	1.0 +/- 0.2	1.1 +/- 0.2
Leu	1.0 +/- 0.3	0.8 +/- 0.5
Tyr	7.7 +/- 1.0	8.0 +/- 1.1
Phe	0.0 +/- 0.0	0.0 +/- 0.0
His	6.1 +/- 0.7	7.1 +/- 1.1
Lys	9.5 +/- 1.3	10.3 +/- 0.9
Arg	0.7 +/- 0.3	1.2 +/- 0.5

* Expressed as mole% +/- s.d. (10 preparations)

Figure 12. Sepharose CL-6B gel filtration of glycosylated P1 and P2.

P1 or P2 (5 mg in 500 ul 1 M NaCl) were injected onto a 100 x 1.25 cm column of Sepharose CL-6B. The columns were developed isocratically with 1 M NaCl at a flow rate of 13 ml/hr and 65-70 2 ml fractions were collected.

Top curve: P1.

Bottom curve: P2.

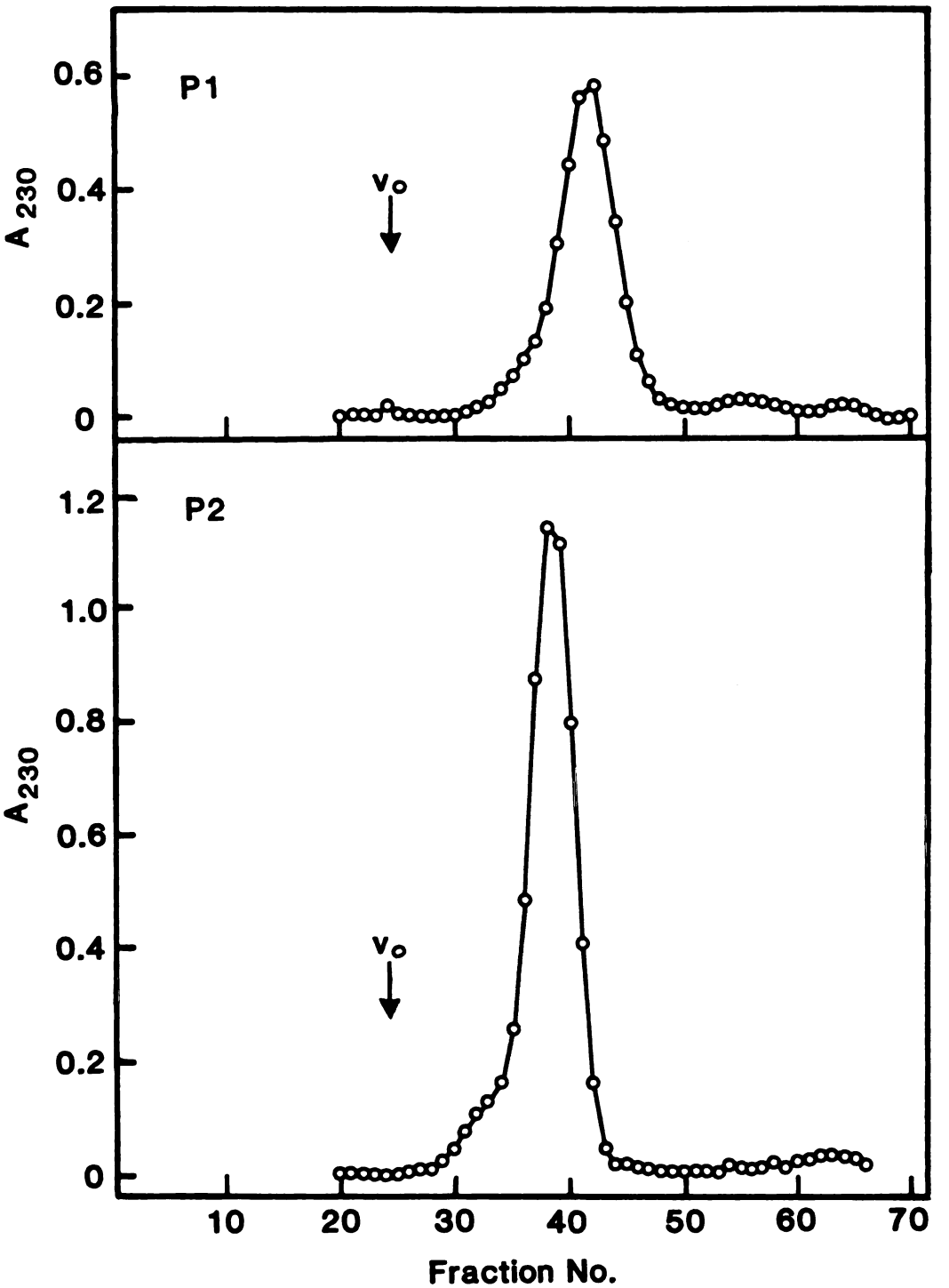

P1
V_o
A_{230}
0.6
0.4
0.2
0
P2
V_o
A_{230}
1.2
1.0
0.8
0.6
0.4
0.2
0
10
20
30
40
50
60
70
Fraction No.

Figure 12

from CL-6B. Treatment of P1 and P2 with succinic anhydride puts negative charges on all positively charged lysine epsilon-amino groups. When Jim Willard did this, dP1 and dP2 eluted from the CL-6B column but still interacted with it (not shown).

After gel filtration via HPLC on Dupont GF-250, the intact precursors eluted at V_o (Figure 19). Upon HF-deglycosylation, P1a and P1b still eluted at V_o while P2 remained adsorbed to the column. Succinylation of dP2 overcame this adsorption; succinylated dP2 eluted at V_o (not shown).

2. SDS-PAGE

Glycosylated P1 and P2 hardly migrated on SDS gel electrophoresis and stained poorly with Coomassie Blue (despite their high lysine content). However, after deglycosylation in anhydrous hydrogen fluoride, P1 and P2 migrated with apparent molecular weights of 55 and 53.5 kDa respectively and stained well with Coomassie blue (Figure 13; cf. Stuart & Varner, 1980). Excessive destaining readily decolorized the bands, presumably due to elution of the basic protein from the gel by the acidic destaining solution. The lack of crosslink amino acids (i.e. cystine) indicated that deglycosylated P1 and P2 were monomeric. Virtual absence of other bands apart from a very faint band, possibly attributible to a trace dimer component, suggested that P1 and P2 were highly purified.

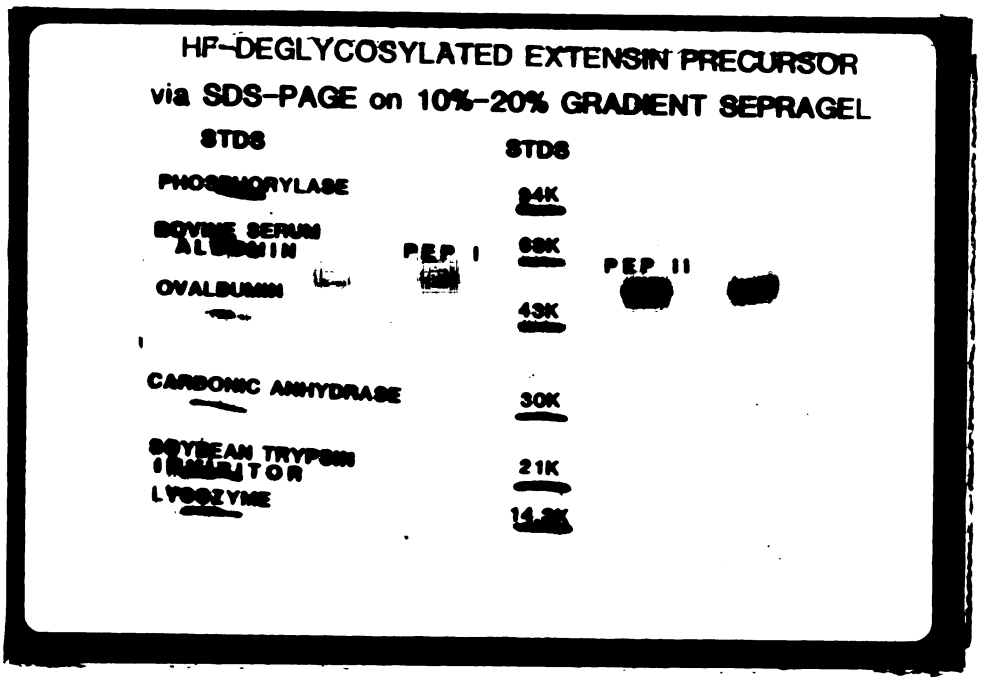


Figure 13. SDS-PAGE of HF-deglycosylated P1 and P2.

From left to right: Lane 1, molecular weight markers; Lane 2, 50 ug P1; Lane 3, 100 ug P1; Lane 4, molecular weight markers; Lane 5, 50 ug P2; Lane 6, 100 ug P2.

Sample weights given are weights before deglycosylation. PEP I = P1, PEP II = P2.

II. Criteria For Precursor Status Of HRGP's P1 and P2

I set out to answer two questions: did the putative precursors look chemically like extensin and did they behave kinetically like precursors? The resolution of these questions involved amino acid analyses, sugar analyses, determination of hydroxyproline arabinoside profiles, and kinetic experiments to determine approximate rates of pool influx and efflux for comparison with wall demand calculated from growth rates.

A. Composition

1. Amino acid analyses of P1, P2, and deglycosylated cell wall preparations

Amino acid analyses showed that, like covalently bound extensin, P1 and P2 are rich in hydroxyproline, serine, lysine, and valine (Table 1). However P1 was also histidine-rich, contained 8.3 mol% proline and 7.2 mol% threonine. This distinguished P1 from both P2 and the 'bulk' of covalently bound extensin.

P1 and P2 were also rich in tyrosine and P1 lacked the crosslinked isodityrosine (IDT); however P2 contained IDT (Figure 14) and gave a Tyr:IDT molar ratio of about 8:1 (or Hyp:IDT of 20:1). Covalently bound extensin contained more IDT than could be accounted for by P2 alone (Hyp:IDT of 15:1) and also contained less tyrosine per se than P1 and

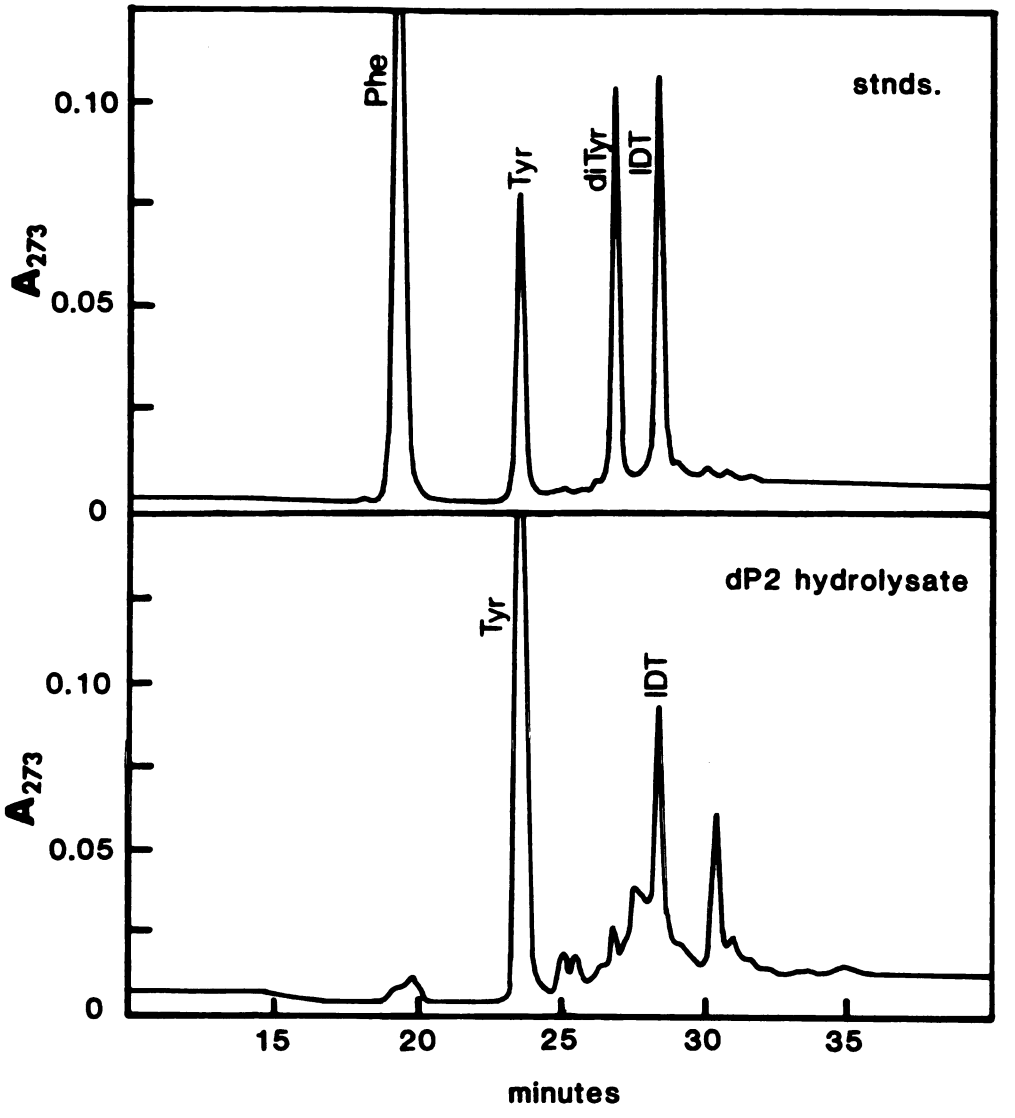


Figure 14. Quantitation of isodityrosine in deglycosylated P2: HPLC separation of a dP2 acid hydrolysate on Hamilton PRP-1.

Hydrolysis and HPLC conditions as described in Methods. No peaks eluted before 15 min.

Top: Standards; 5 ug phenylalanine, 2.5 ug each of tyrosine, dityrosine, and isodityrosine.

Bottom: 100 ug dP2 after acid hydrolysis.

P2 (Table 1).

2. Sugar analysis of P1 and P2

Quantitative sugar analysis showed that both P1 and P2 contain 90 mole% arabinose, 6-7 mole% galactose and 2 mole% glucose (<1% mannose, xylose, and rhamnose; Table 4). The Ara:Hyp molar ratio was 2.58:1 for P1 and 2.96:1 for P2. HF-deglycosylation of P1 and P2 followed by dialysis removed >98% of the sugar and gave a weight loss of approximately 60%.

3. Hydroxyproline arabinoside profiles

Oligoarabinoside substituents of the hydroxyproline hydroxyl groups represent the major carbohydrate component of extensin. The relative amounts of these oligoarabinosides, as determined from the chromatographic profile of alkaline hydrolysates, seem to be relatively constant for a given species (Lamport & Miller, 1971), although minor variations may occur as a function of growth rate (Klis & Eeltink, 1979). Hydroxyproline arabinoside profiles are therefore a critical test for possible extensin precursors.

Hydroxyproline tetraarabinosides (HA_4) and triarabinosides (HA_3) predominated in P1 and P2 which in this respect were similar to the covalently bound extensin of the wall (Table 5). There were some minor differences. In P1, HA_4 was only slightly greater than HA_3, while in P2, HA_4 was more than twice that of HA_3. The average

Table 4. Sugar Compositions of P1 and P2

Sugar*	P1	P2
Rhamnose	0.1	0.2
Fucose	0.0	0.1
Arabinose	89.9	90.2
Xylose	0.4	0.2
Mannose	0.6	0.9
Galactose	6.8	6.2
Glucose	2.2	2.2
Ara:Hyp	2.58:1	2.96:1
wt% sugar**	47.0	51.6

* expressed as molar percentages
** % of glycoprotein assayed as sugar

Table 5. Hydroxyproline-arabinoside profiles of P1, P2 and tomato cell walls*

	P1	P2	P1 + P2 (avg.)	Tomato cell wall**
Hyp*	11.7	7.3	9.5	5.3
Hyp-Ara	9.3	5.9	7.6	9.9
Hyp-Ara2	7.6	8.2	7.9	9.1
Hyp-Ara3	33.2	24.2	28.7	27.5
Hyp-Ara4	38.1	54.4	46.3	48.3
Total	100.0	100.0	100.0	100.0

* Expressed as % of total Hyp
** % of glycoprotein assayed as sugar

hydroxyproline arabinoside profile of P1 and P2 closely approximated that of the cell wall (Table 5).

B. Kinetics

1. Pulse-chase experiments with ^{3}H-proline

Tomato cell suspension cultures (4-6 days after subculture approx. 10% PCV) incubated in their own growth medium (650 ml containing 75 uCi ^{3}H-proline) incorporated ^{3}H-proline into P1 and P2 within about 15 min in short-term pulse labelling experiments (Figure 15). In longer term (24 hr) pulse-chase experiments the labelling pattern of P1 and P2 (i.e. specific activity and total counts) decayed exponentially with a half-life of about 12 hr (Figures 16 & 17).

2. Restoration kinetics of the *in muro* extensin precursor pool following initial pool depletion

In an experiment conceived by DTA Lamport and carried out by Pat Muldoon, the *in muro* extensin precursor pool was depleted by simple salt elution followed by water washing. Reincubation of the eluted cells in the growth medium allowed corroboration of the pulse-chase half-life data by direct measurement of the rate at which precursors P1 and P2 reappeared in the wall after initial depletion. Figure 18 shows that rapidly growing cells restored the precursor pool to its initial level within 12 hr, with an apparent precursor synthesis rate of 43 ug Hyp/g cells (dry

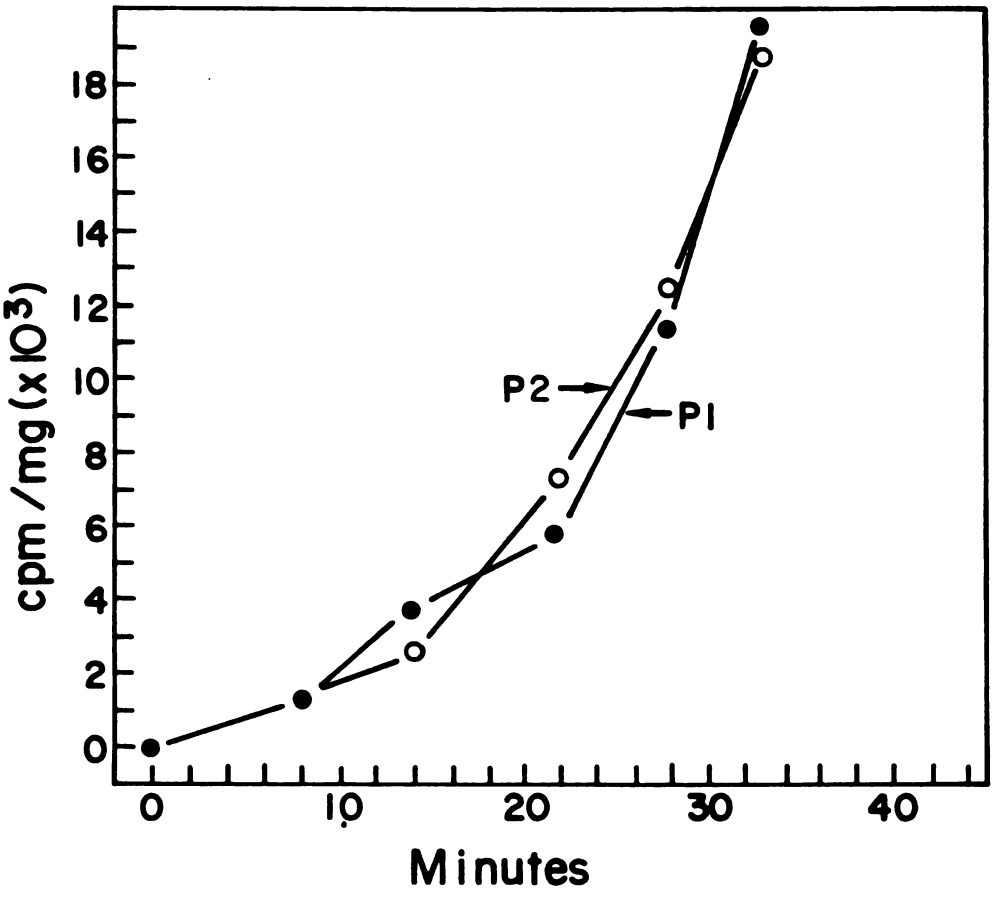


Figure 15. Short-term pulse labelling of P1 and P2.

Carrier-free L-[5-^{3}H]-proline (40 uCi) in 1 ml H_2O were added by syringe to a 1 liter flask containing 650 ml of a 6 day-old cell suspension. Aliquots of 100 ml of the cell suspension were taken at various times and crude extensin was isolated. P1 and P2 were separated via carboxymethyl cellulose and the tritium content of each fraction was measured.

Curves coincide at t = 0 min and t = 8 min.

Closed circles: P1.

Open circles: P2.

Figure 16. Pulse-chase kinetics of P1 and P2: Incorporation and turnover of ^{3}H-proline.

CMC profiles taken as a function of time during the pulse-chase experiment.

Carrier-free L-[U-^{3}H]-proline (75 uCi) in 1 ml H_2O were added by syringe to a 1 liter flask containing 650 ml of a 4 day-old cell suspension. After 2 hours, 1 g of unlabelled proline was added as chase. Aliquots of 50 ml of the cell suspension were taken at various times and crude extensin was isolated. P1 and P2 were separated via carboxymethyl cellulose and the tritium content of each fraction was measured.

Solid lines: A280nm.

Closed circles: counts in each CMC fraction.

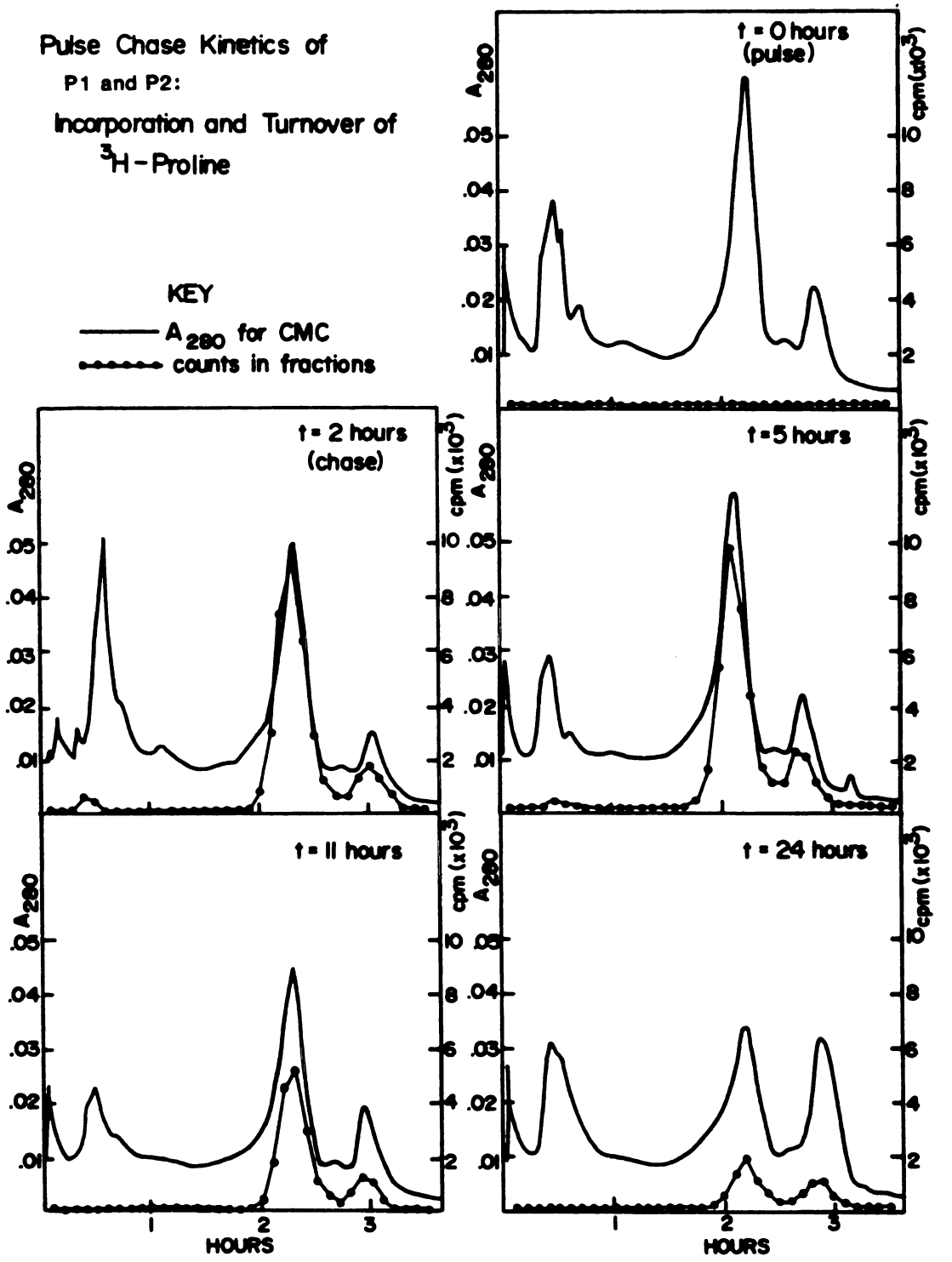

Pulse Chase Kinetics of
P1 and P2:
Incorporation and Turnover of
^{3}H-Proline
KEY
A_{280} for CMC
counts in fractions
t = 0 hours (pulse)
t = 2 hours (chase)
t = 5 hours
t = 11 hours
t = 24 hours
A_{280}
cpm (x10^{-3})
HOURS

Figure 16

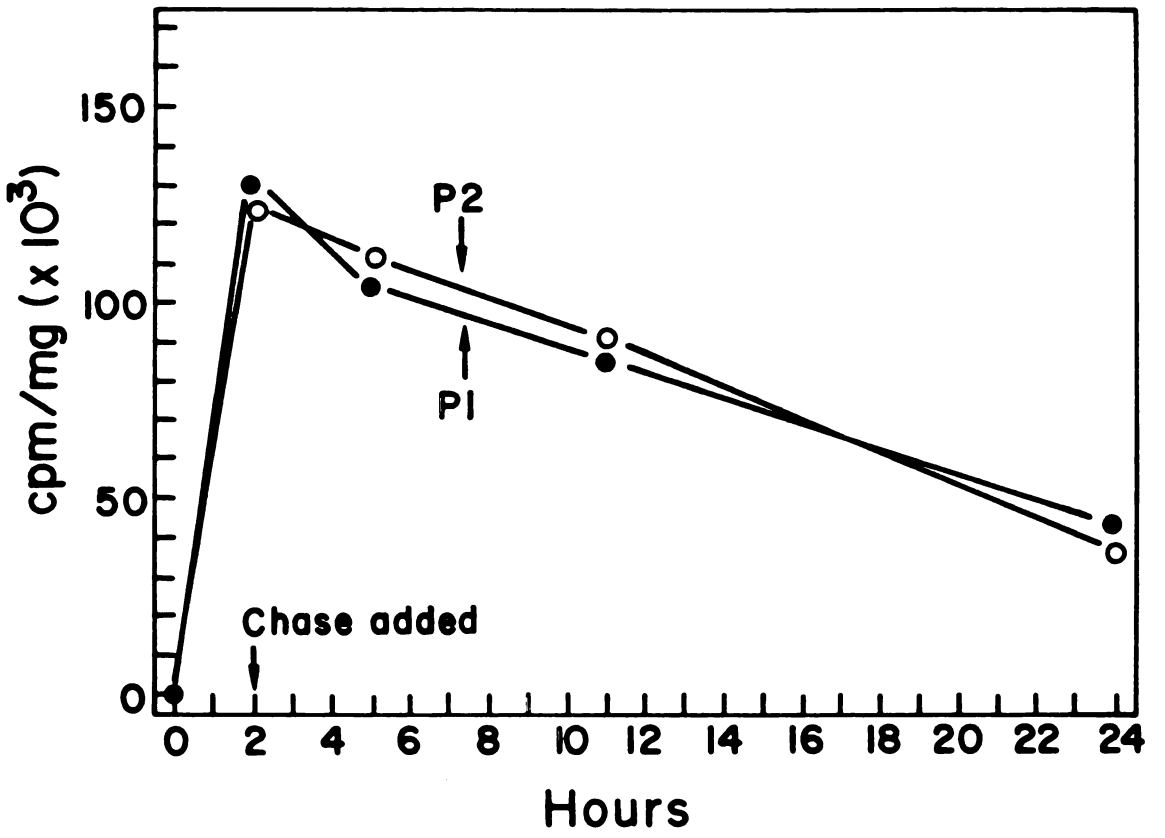


Figure 17. Pulse-chase kinetics of P1 and P2: Specific activities of P1 and P2 as a function of time.

See legend for Figure 16.

Closed circles: P1.

Open circles: P2.

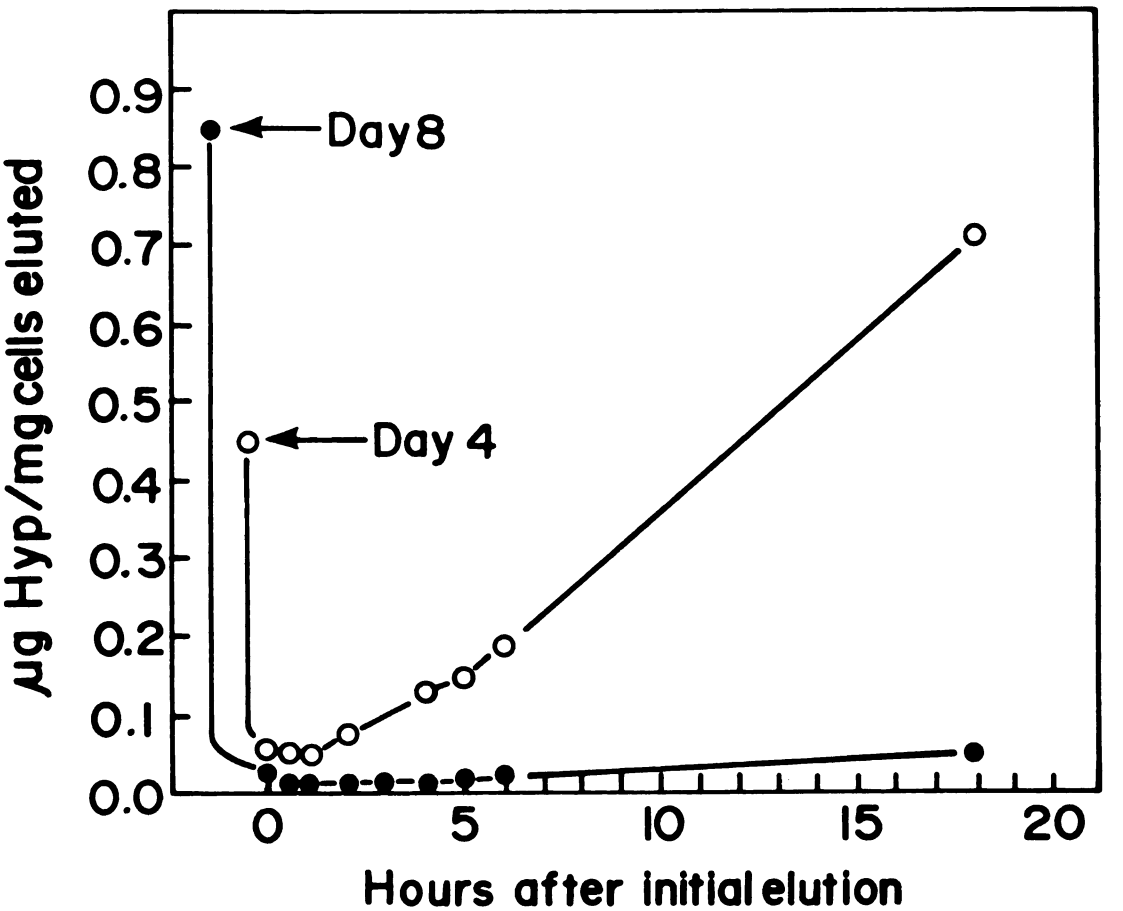


Figure 18. Restoration kinetics of P1 and P2 pools after an initial depletion.

Aliquots of 650 ml of cell cultures of the age indicated were filtered, washed briefly with water, and the cells subsequently treated with 50 ml of $CaCl_2$. The cells were then resuspended in their original growth media and at the times indicated 50 ml aliquots of the depleted cells were assayed for salt-elutable hydroxyproline.

Arrows indicate initial levels of elutable hydroxyproline.

Open circles: Pool restoration in a four day culture.

Closed circles: Pool restoration in an eight day culture.

weight)/hr. This was a minimum rate which did not account for endogenous depletion of the pool by transfer of precursor material to covalently wall-bound extensin during the experimental time course. This repletion 'overshoot' in 4 day cells indicated a precursor secretion rate much greater than the attachment rate at that stage of growth, while the reverse occurred by day 8, where the attachment rate probably exceeded the secretion rate.

From the chemical analyses and kinetic data presented above, I concluded that P1 and P2 were indeed bona fide precursors of the covalently bound extensin matrix (see discussion). The next sections describe the results of experiments that were designed to determine the primary sequences (and more specifically the "crosslink domains") of polypeptides P1 and P2.

III. Precursor Trypsinization, HPLC Peptide Mapping, And Edman Degradation

A. Trypsin Digestion Of Glycosylated P1a, P1b, and P2

Incubation with trypsin (24 h) did not cleave glycosylated P1a, as judged by GF-250 HPLC gel filtration (Figure 19). P1b was slightly less resistant to trypsin, degrading within 24 h to give a minor peak at 1.1 V_o in addition to the main GF-250 peak at V_o. However, glycosylated P2 gave a single retarded peak at V_e(salt) on

Figure 19. DuPont GF-250 HPLC gel filtration chromatography of glycosylated P1a, P1b and P2 before and after 24 hr. tryptic digestion.

Each precursor sample [200 ug in 0.2 M NaPi buffer (pH 7.0) containing 0.005% NaN_3] was injected onto a 9.4 x 250 mm column of DuPont GF-250 and eluted with the sample buffer using an LDC Model I Constametric pump. V_e(salt) = 14 ml. Absorbance was monitored at 254 nm.

The observed difference in trypsin digestion of P1a and P1b requires corroboration.

Top row: Before digestion.

Bottom row: After digestion.

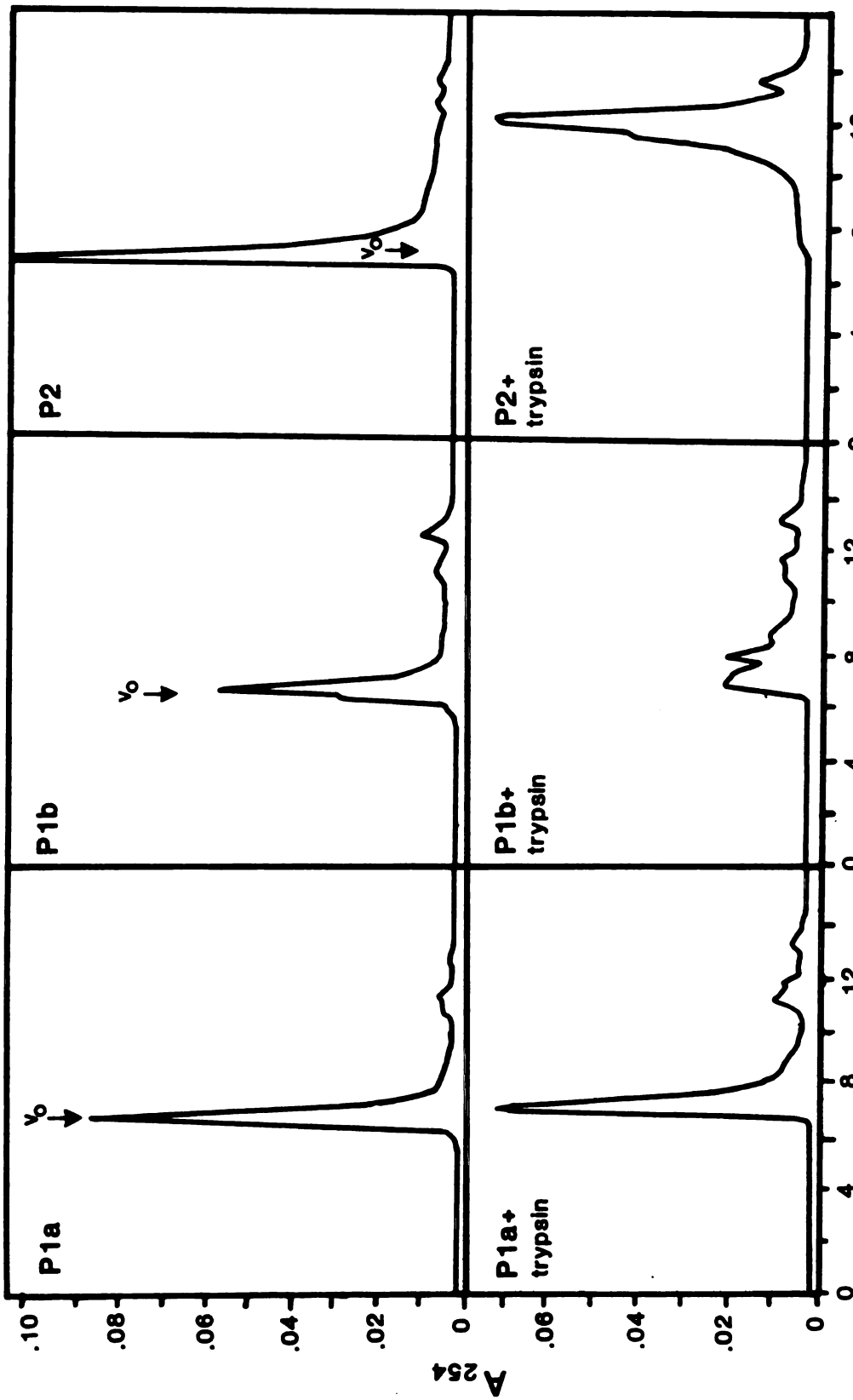

P1a
P1b
P2
P1a+ trypsin
P1b+ trypsin
P2+ trypsin
V_o
A_{254}
.10
.08
.06
.04
.02
0
0
4
8
12
Ve (ml)

Figure 19

GF-250 after 24 h incubation with trypsin, indicating extensive degradation.

B. Tryptic Peptide Maps of dP1a and dP1b, and Primary Structure of the Major Tryptides

Trypsin rapidly cleaved HF-deglycosylated P1 (dP1). In a pH-stat the reaction was 50% complete within 20 min and virtually complete by 2 hr (Figure 20; 85% theoretical cleavage, excluding Lys-Pro, Table 6). Sephadex G-25 gel filtration resolved the complete tryptic digest into 2 retarded peaks (S1 and S2; Figure 21), with two minor shoulders on the high molecular weight side of S1.

Figure 22 shows a typical peptide map obtained by fractionation of the complete tryptic digest via gradient-elution reverse-phase HPLC on Dupont Zorbax ODS. Zorbax ODS resolved 20 tryptides while Hamilton PRP-1 improved the resolution of the more hydrophobic peptides, resolving 28 tryptides (Figure 23). The P1a and P1b maps were slightly different, but both had two major peptides, H5 and H20, which together accounted for 44% of the total absorbance at 273nm and 33% of the total recoverable peptide weight (Table 7; 70% peptide recovery from PRP-1). Amino acid analysis (Table 7) and sequencing via automated Edman degradation (Table 8) showed that H5 and H20 were decapeptide and hexadecapeptide respectively (in accord with their G-25 elution behavior: H5 in peak S2 and H20 in

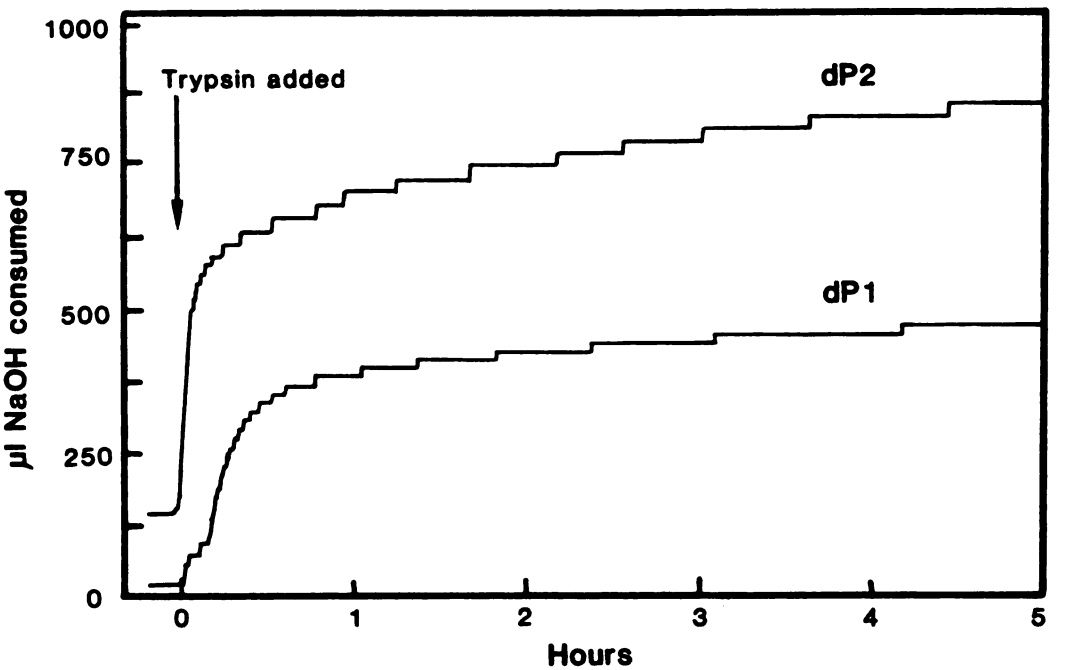


Figure 20. Time course of P1 and P2 tryptic cleavage (after HF-deglycosylation) as measured with the pH-Stat.

HF-deglycosylated P1 (7.3 mg) and P2 (6.5 mg), both dissolved in 2 ml H_2O containing 40 ul 0.5 M $CaCl_2$, were digested with TPCK-trypsin (substrate:enzyme = 100:1) for five hours. Base (0.1 N NaOH) was added to the reaction mixture throughout the time course to maintain the pH at the starting point of 8.0. NaOH deliveries, recorded at t = 20 min, 2 hr, and 5 hr, were corrected for base consumed during trypsin addition and used to calculate the extent of cleavage (see Table 6).

Table 6. P1 and P2 Tryptic Cleavage (after HF-deglycosylation) via the pH-Stat

	(a) Amt. digested (mg)	(b) Total Peptide bonds (umoles)	(c) % of total cleaved			(d) # cleaved per molecule			(e) # cleavable per molecule	(f) % of theoretical % cleavage		
			20'	2h	5h	20'	2h	5h		20'	2h	5h
dP1	7.3	55	4.5	6.8	7.7	14	20	23	24	56	85	96
dP2	6.5	49	9.2	11.7	14.3	28	35	43	54	51	65	79

(a) HF-deglycosylated precursors dP1 and dP2 prepared as described in methods.

(b) Calculated from $\frac{\text{total peptide ug}}{\text{average residue weight}}$ using 133 as the average residue weight.

(c) Calculated from pH-stat delivery of NaOH umoles equivalent to umoles peptide bond cleaved, and expressed as a percentage of the total peptide umoles in column (b).

(d) Best estimate of molecular size is approximately 40 kDa, an interpolation based on: our SDS-PAGE data (Figure 13) which showed rodlike dP1 and dP2 have apparent MW's of 55 kDa and 53.5 kDa respectively (overestimates), CsCl gradient centrifugation of a similar carrot HRGP (Stuart and Varner, 1980) giving 35 kDa, and the cDNA sequence (Chen and Varner, 1985b) of a carrot HRGP giving MW = 35 kDa. Taking the average residue weight (derived from the composition) of 133 gives 300 residues/molecule for both P1 and P2, allowing calculation of the number of bonds cleaved/molecule from percent cleaved (column c).

(e) P1 is 10 mole% lysine and thus would have 30 bonds available for tryptic cleavage, except for 4 uncleavable Lys-Pro bonds. Peptide compositions suggest more as peptides H21-H28 contain more than 1 lysine; Table 7a). Therefore we corrected for 6 trypsin-resistant lysyl bonds. Edman degradation showed that P2 (20 mole% lysine) also contained trypsin-resistant lysyl bonds (lysine surrounded by IDT in H11 and H12). If Tyr:IDT = 8:1, there are 6 such bonds in P2.

(f) Expresses column (d) as a percentage of column (e).

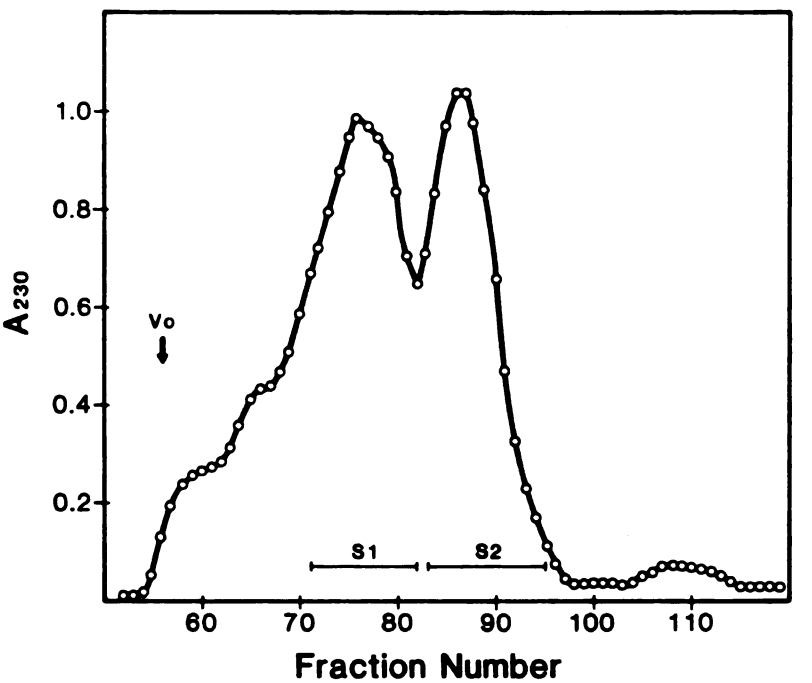


Figure 21. Sephadex G-25 gel filtration of HF-deglycosylated P1 after a 24 hr. tryptic digestion.

Freeze dried HF-deglycosylated P1 tryptides (9 mg) dissolved in 0.5 ml 0.1 M HOAc were injected onto two 1.5 x 100 cm columns (in tandem) of Sephadex G-25 and eluted with the same solvent. Fractions (120) of 2 ml were collected. Peptides H1 to H15 (except for H3) are in S2 and peptides H16 to H28 are in S1.

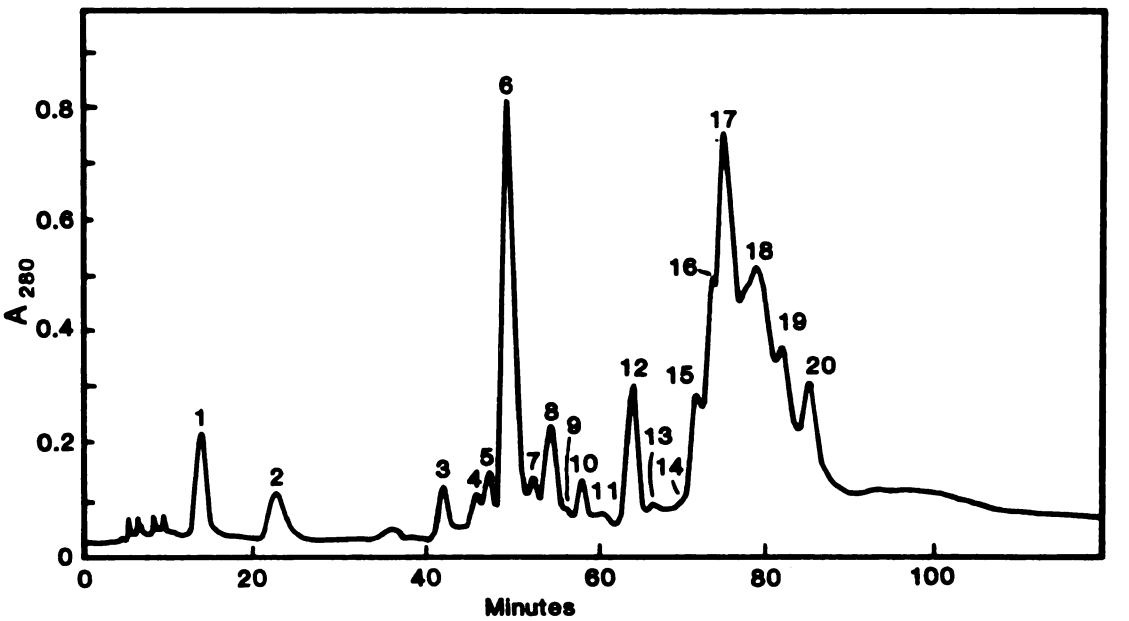


Figure 22. Zorbax ODS HPLC peptide map of dP1 after complete tryptic digestion (24 hr).

A trypsin digest (500 ug dP1 tryptides in 50 ul) was injected onto a 4.6 x 250 mm DuPont Zorbax ODS HPLC column. The tryptides were eluted with a programmed gradient consisting of solutions of 0.13% heptafluorobutyric acid (HFBA) and 0.13% HFBA in 80% aqueous acetonitrile. Peaks 1 and 2 appear in this tracing, done at 280 nm, but do not appear in 230 nm tracings. Amino acid analyses showed that peaks 1 and 2 did not contain amino acids. Peaks 1 and 2 are probably sugar degradation products seen here because of incomplete dialysis; their relative heights changed while the relative heights of the peptide peaks did not.

Figure 23. Hamilton PRP-1 HPLC peptide maps of HF-deglycosylated P1a and P1b.

Conditions the same as in Figure 22 except I used a 4.1 x 150 mm Hamilton PRP-1 HPLC column.

a) 10 ul of a trypsin digest containing 100 ug dP1a tryptides

b) as (a) except 10 ul reaction mix contained 100 ug dP1b tryptides

No peptides eluted before 40 min although the void contained minor amounts of serine and glycine.

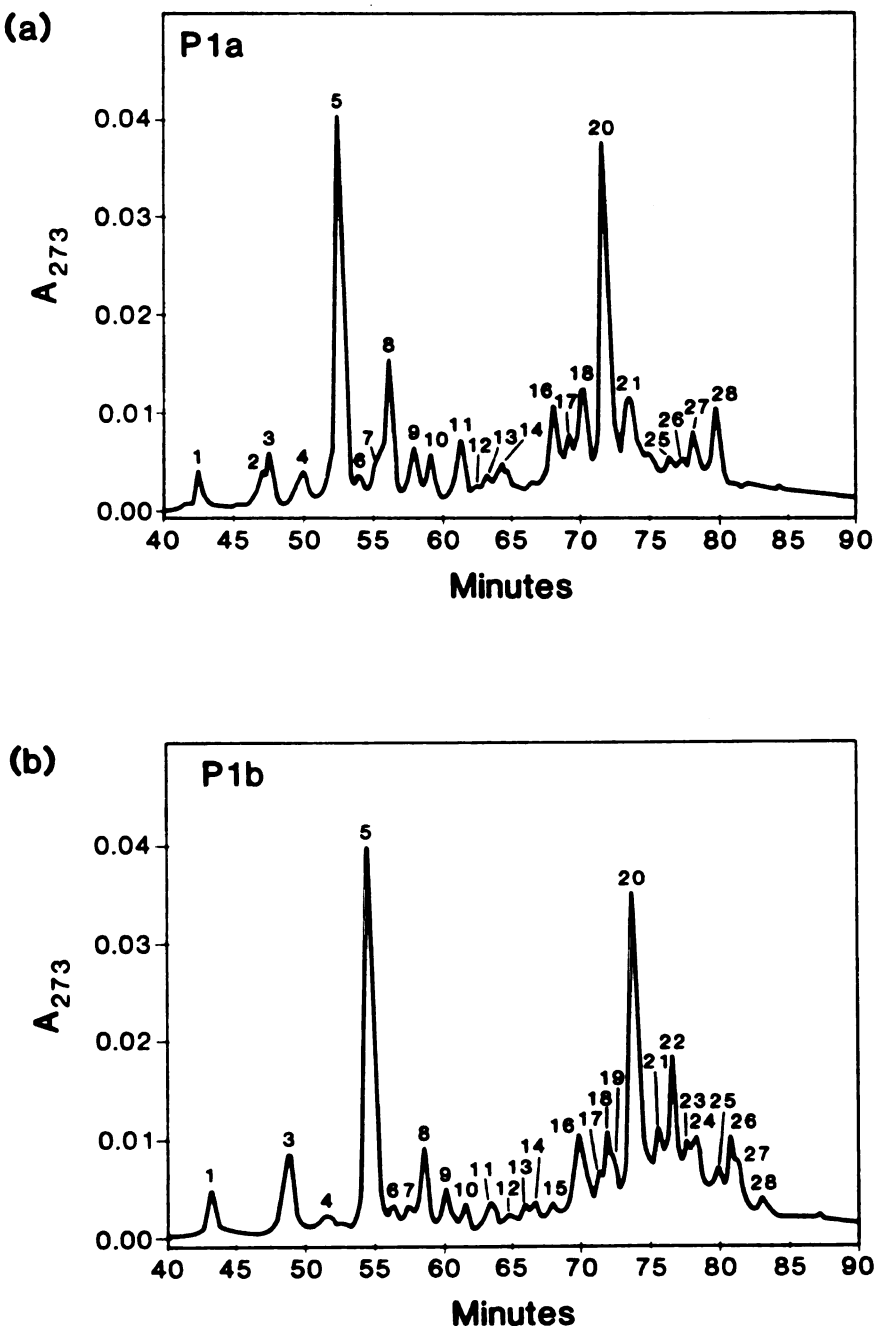

(a)
P1a
A_{273}
0.04
0.03
0.02
0.01
0.00
40 45 50 55 60 65 70 75 80 85 90
Minutes
(b)
P1b
A_{273}
0.04
0.03
0.02
0.01
0.00
40 45 50 55 60 65 70 75 80 85 90
Minutes

Figure 23

Table 7. Amino Acid Compositions* of Major P1 Tryptic Peptides

A.A.	H3	H5	H8	H9	H10	H11	H16	H20	H20/C1	H20/C2 before cyanoethylation	H20/C2 after cyanoethylation	H28
Hyp	10.0 (10)	5.0 (5)	5.0 (5)	5.0 (5)	5.0 (5)	4.8 (5)	5.0 (5)	4.3 (5)	4.1 (4)	1.0 (1)	1.1 (1)	4.2 (4)
Asp												
Thr	1.2 (1)	1.1 (1)	1.1 (1)	1.1 (1)	0.5	0.8 (1)	1.0 (1)	1.0 (1)		0.5 (1)	0.6 (1)	0.8 (1)
Ser	3.2 (3)	1.3 (1)	1.1 (1)	1.3 (1)	1.7 (2)	1.5 (2)	1.4 (1)	1.3 (1)	1.0 (1)			1.1 (1)
Glu												
Pro				1.5 (2)	2.2 (2)	0.8 (1)	2.6 (3)	2.0 (2)	1.1 (1)	1.1 (1)	0.9 (1)	2.1 (2)
Gly												
Ala												
Val	1.2 (1)	1.0 (1)		1.0 (1)	1.3 (1)	0.8 (1)	1.8 (2)	1.6 (2)	0.9 (1)	0.6 (1)	1.0 (1)	1.2 (1)
Ile			0.8 (1)									
Leu				0.8 (1)		0.6 (1)						
Tyr	1.4 (2)	0.5 (1)	1.2 (1)	1.3 (1)	1.4 (1)	1.0 (1)	1.9 (2)	1.4 (2)	0.8 (1)	0.6 (1)	0.5 (1)	1.9 (2)
Phe												
His	1.0 (1)					0.6 (1)	2.3 (2)	1.4 (1)	0.4	1.0 (1)	0.1	2.7 (3)
Lys	2.0 (2)	1.1 (1)	1.0 (1)	2.0 (2)	1.6 (2)	1.0 (1)	3.1 (3)	1.7 (2)	1.0 (1)	0.3		1.0 (1)
Arg												
% (w/w)**		16.6						15.7				
%A273		21.2						21.8				

*expressed as molar ratios

**% of total weight recovered from PRP-1 HPLC column.

Note: Although we were unable to completely separate peptides H16-H19 and H21-H27 in high enough yield for compositions of pure peptides, we did determine compositions across these areas of the HPLC maps. Most of the histidine and proline of P1 is in these areas. H1-H8, which size in the decamer range on G-25 (S2; except H3) contain no proline or histidine (except H3). H16-H28 are all in the G-25 S1 fraction.

Table 7a. Amino Acid Compositions* Across the P1/H16-H28 Area of the HPLC Peptide Map

A.A.	H16,17	H18,19	H20	H21,22	H23-25	H26-28
Hyp	33.7	33.9	30.6	26.9	31.9	26.3
Asp	0.5	0.3	0.0	0.8	1.8	1.4
Thr	3.2	6.0	4.9	7.0	5.4	4.9
Ser	8.1	7.4	6.5	6.7	8.3	7.0
Glu	0.3	0.8	0.0	2.1	0.8	0.9
Pro	15.6	13.7	14.5	15.2	14.0	13.3
Gly	1.6	1.6	0.9	0.8	0.5	0.7
Ala	1.4	2.7	2.7	1.4	0.9	1.0
Val	7.9	9.0	9.8	6.1	6.7	7.3
Cys	0.0	0.0	0.0	0.0	0.0	0.0
Met	0.0	0.0	0.0	0.0	0.0	0.0
Ile	0.3	0.0	0.4	0.8	1.2	0.8
Leu	0.0	0.0	0.0	0.0	0.2	0.0
Tyr	8.1	7.4	10.7	10.0	8.0	11.8
Phe	0.0	0.0	0.0	0.0	0.0	0.0
His	8.3	9.6	8.5	12.1	11.8	16.8
Lys	10.7	7.7	10.5	9.8	7.8	6.3
Arg	0.3	0.0	0.0	0.3	0.8	1.7

* Expressed as mole%.

Table 8. Amino Acid Sequences of Major P1 Tryptic Peptides.

P1/H5 (50 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Ser	47.6
2	Hyp	50.4
3	Hyp	73.3
4	Hyp	63.7
5	Hyp	68.4
6	Thr	46.3 (O 29.3)(c)
7	Hyp	39.0
8	Val	54.2 (O 13.5)
9	Tyr	38.1 (V 36.9)
10	Lys	40.6 (Y 18.3)

P1/H8 (50 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Ser	4.0
2	Hyp	8.0
3	Hyp	22.4
4	Hyp	20.4
5	Hyp	22.4
6	Thr	2.6 (O 6.0)
7	Hyp	13.4
8	Ile	17.0 (O 6.6)
9	Tyr	13.6 (I 4.4)
10	Lys	7.4
11	---	

P1/H20 (20 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Ser(a)	
2	Hyp	25.0
3	Hyp	25.5
4	Hyp	27.0
5	Hyp	45.5
6	Val	74.5 (O 31.5)
7	Lys	33.0 (V 25.5)
8	Pro	57.5 (K 14.0)
9	Tyr	15.0 (P 13.0)
10	---	(Y 17.0)
11	Pro	35.5 (Y 9.5)
12	Thr(a)	(P 24.0)
13	Hyp	16.5
14	Val	27.5 (O 13.5)
15	Tyr	26.5 (V 29.5)
16	Lys	6.0 (Y 14.5)

P1a/H20 (10 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Ser(a)	----
2	Hyp	35.0
3	Hyp	59.0
4	Hyp	45.0
5	Hyp	42.0
6	Val	49.0 (O 16.0)
7	Lys	29.0
8	Pro	54.0
9	Tyr	51.0
10	---	----
11	Pro	39.0
12	Thr(a)	----
13	Hyp	10.0
14	Val	33.0
15	Tyr	30.0
16	Lys	7.0
17	---	

P1b/H20 (10 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Ser(a)	
2	Hyp	19.0
3	Hyp	100.0
4	Hyp	62.0
5	Hyp	41.0
6	Val	74.0 (O 25.0)
7	Lys	5.0
8	Pro	30.0
9	Tyr	52.0 (P 32.0)
10	His(b)	20.0
11	Pro	65.0
12	Thr(a)	
13	Hyp	27.0
14	Val	28.0
15	Tyr	26.0
16	---	

P1/H20/C1 (50 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Ser	4.0
2	Hyp	12.0
3	Hyp	3.4
4	Hyp	11.6
5	Hyp	9.0
6	Val	10.8 (O 2.6)
7	Lys	7.8 (V 2.4)
8	Pro	8.0 (K 0.6)
9	Tyr	2.8 (P 1.0)
10	---	(Y 1.8)

P1/H20/C2 (12 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	---	---
2	His(b)	81.0
3	Pro	50.0
4	Thr	21.0 (P 10.0)
5	Hyp	28.0
6	Val	75.0 (O 38.0)
7	Tyr	18.0 (V 5.0)
8	---	(Y 8.0)

NOTES

(a) Qualitatively identified by the presence of unique degradation peaks from serine and threonine.

(b) Quantitated using pH 5.5 buffer.

(c) Other amino acids indicated by the standard single letter abbreviations (O=Hyp).

S1) with sequences as follows:-

H5- H2N-Ser-Hyp-Hyp-Hyp-Hyp-Thr-Hyp-Val-Tyr-Lys-COOH, and

H20- H2N-Ser-Hyp-Hyp-Hyp-Hyp-Val-Lys-Pro-Tyr-His-Pro-Thr-Hyp-Val-Tyr-Lys-COOH; [note: trypsin did not cleave Lys-Pro (McBride & Harrington, 1967)]. Verification of H20 via chymotryptic cleavage yielded the two predicted peptides C1 and C2 (Tables 7 & 8) with sequences as follows:-

C1- H2N-Ser-Hyp-Hyp-Hyp-Hyp-Val-Lys-Pro-Tyr-COOH and

C2- H2N-His-Pro-Thr-Hyp-Val-Tyr-COOH.

Edman degradation showed that P1a/H20 and P1b/H20 were identical (Table 8).

C. Tryptic Peptide Map of dP2, and Primary Structure of the Major Tryptides

Trypsin also rapidly cleaved HF-deglycosylated P2 (dP2) and cleaved 65% of the theoretically cleavable bonds in 2 hrs (1/2IDT-Lys-1/2IDT-Lys not cleaved; see Table 10) in the pH-stat (Table 6). Gel filtration of the dP2 tryptic digest on Sephadex G-25 gave two major retarded fractions (S1 and S2; Figure 24) but no large fragments and little trypsin resistant core. The first peak (S1) had shoulders on both the higher molecular weight side (S1a) and the lower molecular weight side (S1c). The highly retarded second peak (S2) was chromatographically homogeneous on Zorbax ODS, contained equimolar tyrosine and lysine (Table 10), and was identified as Tyr-Lys by

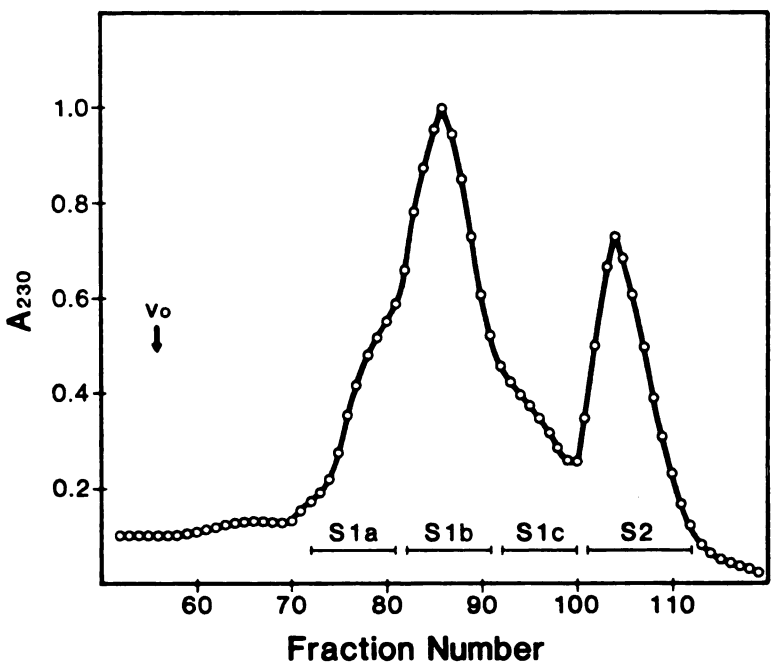


Figure 24. Sephadex G-25 gel filtration of HF-deglycosylated P2 after a 24 hr. tryptic digestion.

Injected 5 mg freeze dried dP2 tryptides and subjected them to the same conditions given in Figure 21. S1a contained peptide H2, S1 contained H4, H5(6), and H9, S1c contained H1, and S2 contained H3 (see Figure 26).

cochromatography with authentic tyrosyl-lysine and by Edman degradation (Table 11). Estimation of Tyr-Lys in tryptic digests after HPLC (using authentic Tyr-Lys as a standard) showed that [Lys]-Tyr-Lys occurred about 15 times in P2 (Table 9; assumes a polypeptide of 40 kDa, see Table 6).

Fractionation of the complete dP2 tryptic digest on Zorbax ODS or Hamilton PRP-1 resolved 12 tryptides (Figures 25 & 26). The two major peptides (H3 and H4) accounted for 60% of the total absorbance at 273 nm and 55% of the total recoverable peptides by weight (Table 10) while a third (H11) accounted for 15% of the total absorbance and 10% of the total weight. The remaining minor peptides accounted for 25% of the absorbance and 35% of the weight, but this is probably a high estimate due to inclusion of major peptides from overlap of pooled fractions.

Amino acid analysis (Table 10) and Edman degradation (Table 11) showed that H3, H4, and H11 were dipeptide, octapeptide and decapeptide respectively (corroborated by G-25 elution data, Figure 24) with sequences as follows:-

H3- H2N-Tyr-Lys-COOH,

H4- H2N-Ser-Hyp-Hyp-Hyp-Hyp-Val-Tyr-Lys-COOH, and

H11- H2N-Ser-Hyp-Hyp-Hyp-Hyp-Val-1/2IDT-Lys-1/2IDT-Lys-COOH.

The minor tryptides H5 (or H6; Zorbax did not resolve H5 & H6 (Figure 25), PRP-1 did (Figure 26)) and H12 are isoleucine for valine substitution analogs of H4 and H11 respectively (Tables 9 & 10). The proportion of tryptide

Table 9. Quantitation of Tyr-Lys in a dP2 Tryptic Digest.

Hyp digested (ug)	ug protein digested (a)	nmoles protein (b)	nmoles Tyr-Lys (c)	#Tyr-Lys / molecule
500	1350	34	566	16.6

(a) ug protein = ug Hyp/0.37
(b) Assumes MW = 40 kDa
(c) Assayed via HPLC (Zorbax ODS) vs. standard Tyr-Lys

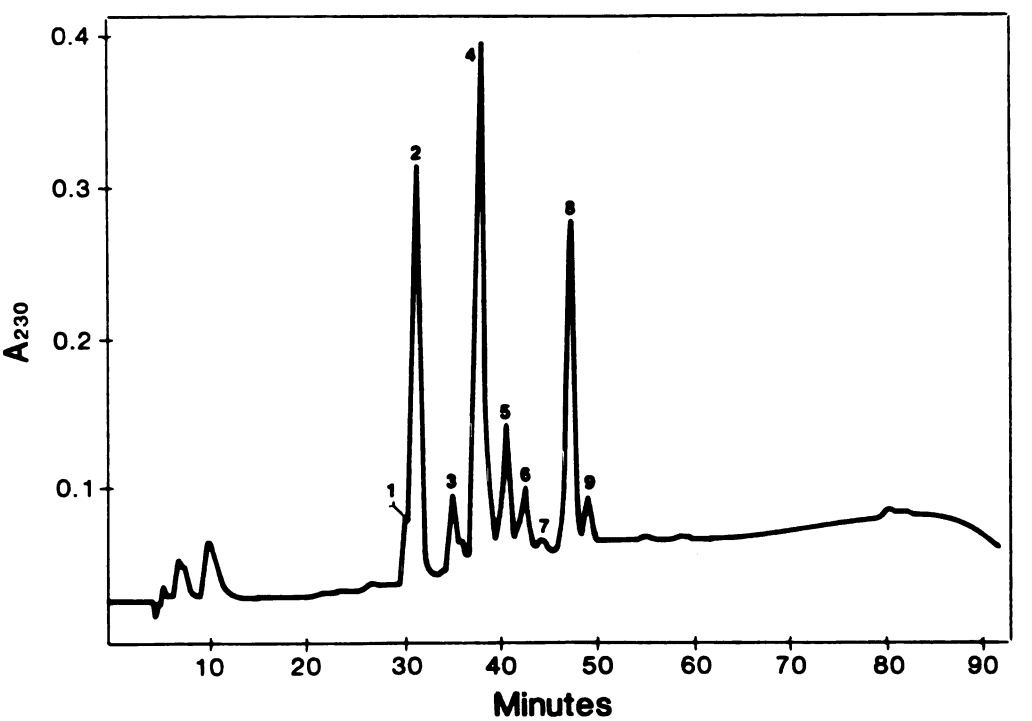


Figure 25. Zorbax ODS HPLC peptide map of dP2 after complete tryptic digestion (24 hr).

Forty ul of a trypsin digest containing 400 ug dP2 tryptides injected. Conditions as described in Figure 22. Note absence of peaks after peak 9.

Figure 26. Hamilton PRP-1 HPLC peptide maps of dP2 90 min and 12 hrs after initiation of digestion.

See legends for Figures 22 and 23.

(a) A tryptic digest (10 ul containing 100 ug dP2 tryptides) after 90 min reaction time. Note dominance of H9 peak.

(b) A tryptic digest (10 ul containing 50 ug dP2 tryptides) after 12 hrs reaction. All peaks beyond H12 disappear after 24 hr reaction (data not shown). Note reduction of H9 peak.

Maps in (a) and (b) are from different batches.

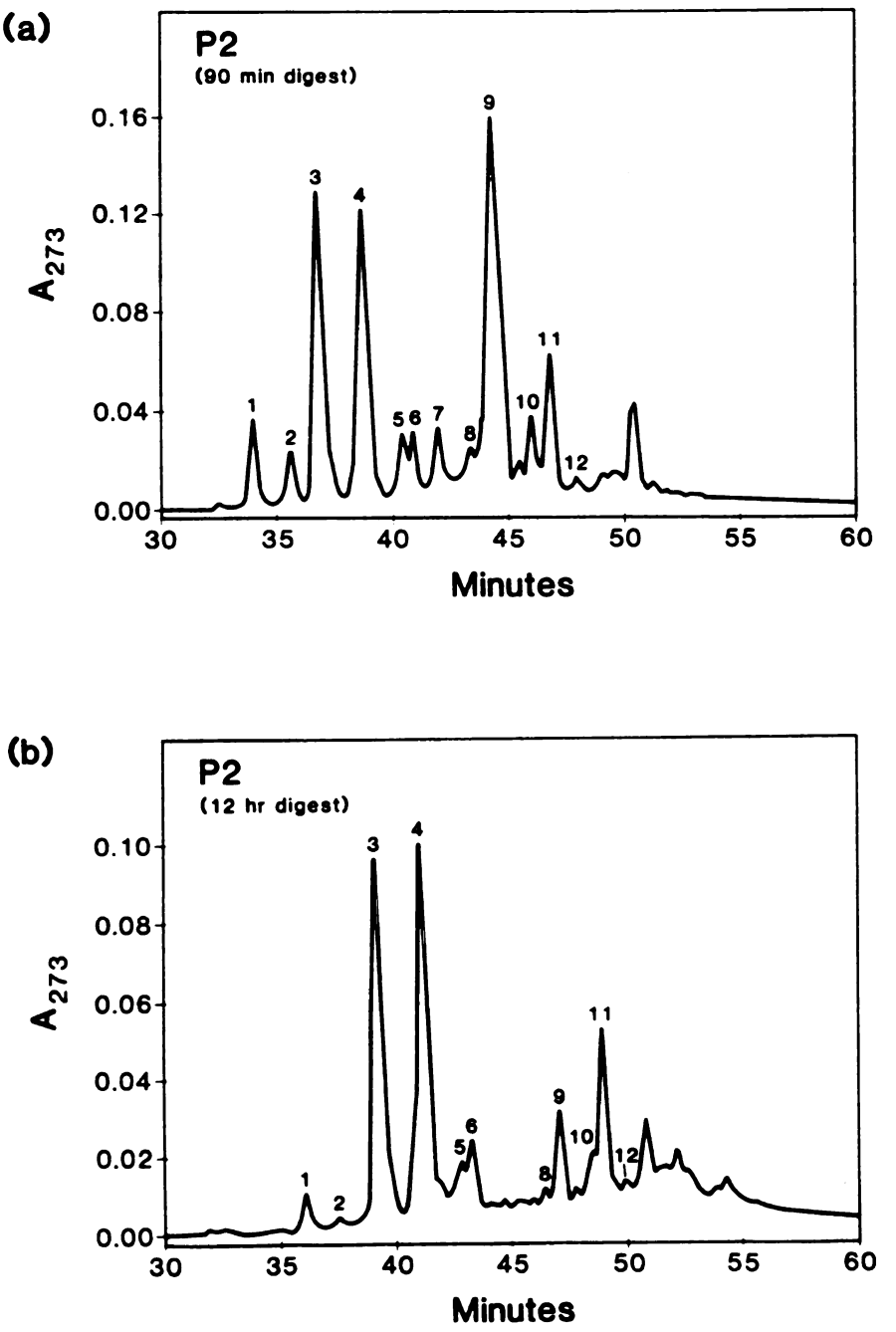

(a)
P2
(90 min digest)
0.16
0.12
0.08
0.04
0.00
A_{273}
1
2
3
4
5
6
7
8
9
10
11
12
30
35
40
45
50
55
60
Minutes
(b)
P2
(12 hr digest)
0.10
0.08
0.06
0.04
0.02
0.00
A_{273}
1
2
3
4
5
6
8
9
10
11
12
30
35
40
45
50
55
60
Minutes

Figure 26

Table 10. Amino Acid Compositions of P2 Tryptic Peptides - Molar Ratios

A.A.	H1	H2	H3	H4	H5(6)	H9	H11*	H12*
Hyp		4.2 (4)		3.9 (4)	3.6 (4)	4.1 (4)	3.6 (4)	3.0 (4)
Asp								
Thr								
Ser		1.0 (1)		1.1 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.0 (1)
Glu								
Pro								
Gly								
Ala								
Val		0.9 (1)		0.9 (1)	0.2	0.7 (1)	0.9 (1)	0.6
Ile					0.7 (1)			0.4 (1)
Leu								
Tyr			0.7 (1)	1.0 (1)	0.5 (1)	1.2 (2)		
Phe								
His								
Lys	1.0 (1)	1.3 (1)	1.0 (1)	1.0 (1)	1.0 (1)	1.6 (2)	1.7 (2)	2.2 (2)
Arg								
% (w/w)**			15.0	39.4			(9.6)	
%A273	5.4	6.5	29.9	29.8	7.1	1.0	14.3	1.8

*Also contain IDT as determined by (1) cochromatography with S_2A_{11} (Epstein and Lamport, 1984), (2) Edman degradation of H11 & H12 (Table11), & (3) UV spectra characteristic of IDT (Epstein and Lamport, 1984).
** % of total weight recovered from PRP-1 HPLC column.

Table 11. Amino Acid Sequences of P2 Tryptic Peptides

P2/H1 (50 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Unk A	101.4*
2	Lys	52.4 (U 21.2)(a)
3	---	(K 14.4,U 17.8)

P2/H2 (200 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Ser	21.9
2	Hyp	22.8
3	Hyp	40.6
4	Hyp	20.0
5	Hyp	20.4
6	Val	17.4 (O 6.9)
7	Unk A	8.9*(V 6.1,O 3.3)
8	Lys	1.1 (U 1.5,V 0.6)
9	Unk A	4.4*(K 2.7,V 1.2)

P2/H5(6) (120 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Ser	19.5
2	Hyp	37.9
3	Hyp	53.9
4	Hyp	45.9
5	Hyp	39.8
6	Ile	22.4 (O 15.7)
7	Tyr	26.9 (I 7.6,O 11.5)
8	Lys	10.6 (Y 5.9,I 5.0)
9	---	(K 4.1,Y 7.2)

P2/H12 (10 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Ser	<10.0
2	Hyp	21.0
3	Hyp	38.0
4	Hyp	39.0
5	Hyp	46.0
6	Ile	32.0 (O 15.0)
7	---	(I 6.0)
8	Lys	17.0
9	Unk B	6.0*
10	Lys	14.0

P2/H3 (64.5 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Tyr	75.1
2	Lys	31.3 (Y < 1%)
3	---	(K 11.9)

P2/H4 (240 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Ser	10.5
2	Hyp	75.4
3	Hyp	49.5
4	Hyp	27.8
5	Hyp	57.8
6	Val	44.8 (O 8.8)
7	Tyr	45.0 (V 14.0)
8	Lys	8.4 (Y 3.8)
9	---	

P2/H11 (60 nmoles in cup)

Cycle	Residue	% Yield of PTH-Amino Acid
1	Ser	39.8
2	Hyp	98.2
3	Hyp	118.0
4	Hyp	68.0
5	Hyp	81.3
6	Val	64.0 (O 21.7)
7	---	(V 17.7,O 8.7)
8	Lys	17.7 (U 2.2,V 4.7)
9	Unk B	18.2*(K 18.3)

NOTES

(a) other amino acids indicated by the standard single letter abbreviations O=Hyp, U=unknown

* used PTH RF=1 for Unknowns; Unknown A had a PTH retention time of ca. 0.1 relative to Norleu. Unknown B had a PTH retention time of ca. 1.1 relative to Norleu and is probably PTH-IDT.
UNK = Unknown.

H9 decreased drastically during digestion (Figure 26) with a concomitant increase in H3 and H4. H9 has a provisional sequence:

H9- H2N-Tyr-Lys-Ser-Hyp-Hyp-Hyp-Hyp-Val-Tyr-Lys-COOH.

H1 and H2 had compositions similar to H3 and H4 except H1 and H2 contained no tyrosine (Table 10; or dityrosine or isodityrosine despite their absorptivity at 280 nm). Sephadex G-25 gel filtration showed H1 in S1c and H2 in S1a, while H3 and H4 appeared in S2 and S1b respectively (Figure 24). Eliminating light and maintaining a low temperature during the precursor preparation virtually eliminated H1 and H2 from the peptide map although one cold dark prep yielded H1 and H2 (Figure 26b). Edman degradation of H1 and H2 yielded an unknown PTH-amino acid at residue 1 in H1 and residue 7 in H2 (Unknown A, Table 11).

Partial tryptic digests of dP2 (4 min) followed by gel filtration gave a void peak and a retarded fraction (S0) between the void peak and S1a (Figure 27). Complete digestion of S0 gave typical P2 peptide maps except for an enrichment in H1 and H2 (Figure 28).

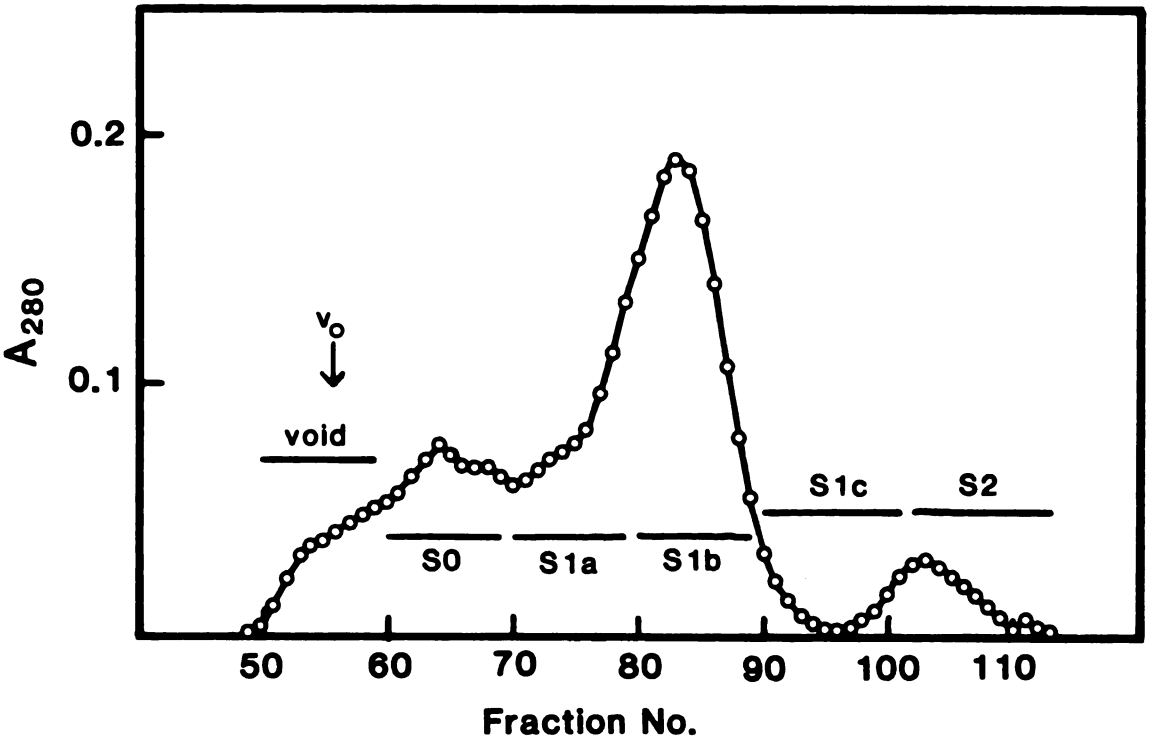


Figure 27. Sephadex G-25 gel filtration of dP2 after a 4 min tryptic digestion.

Trypsin digestion carried out as usual but stopped after 4 min. by adding an equal volume of 0.1 M HOAc and then quickly freezing in liquid N_2. The peptides were freeze dried, dissolved in 0.5 ml HOAc and separated as described in Figure 21. Note the appearance of the S0 peak between the void and S1a (cf. Figure 24).

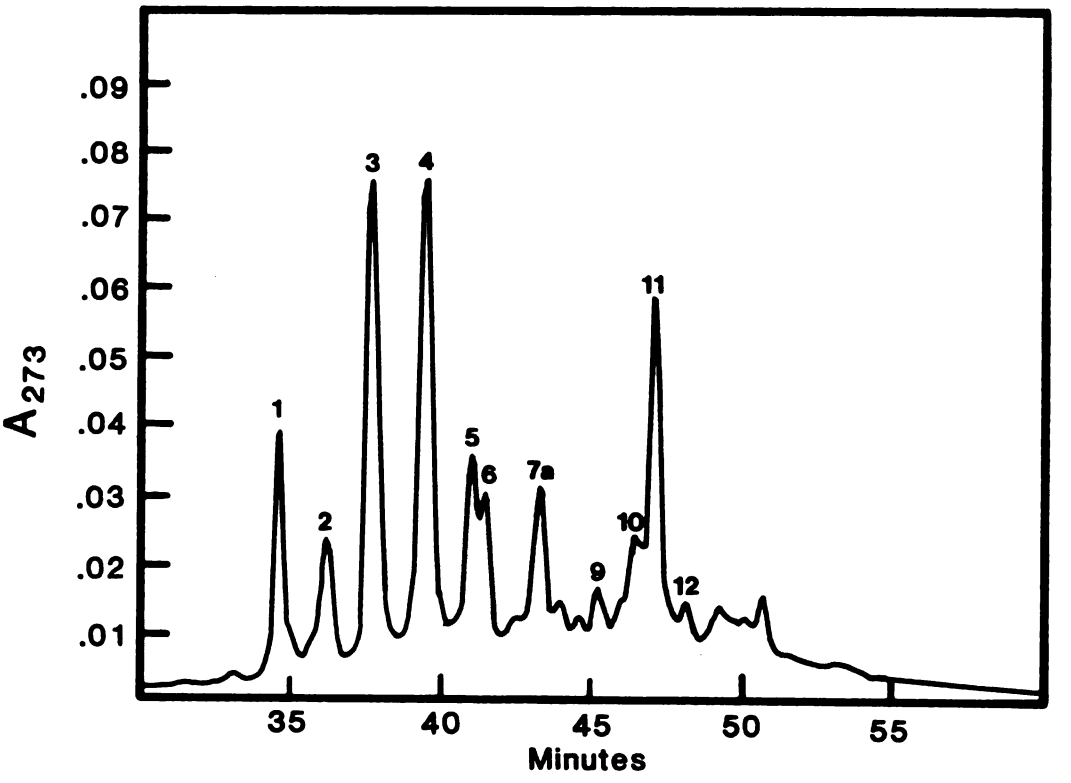


Figure 28. Hamilton PRP-1 HPLC peptide map of dP2/S0 after complete tryptic digestion.

Sephadex G-25 peak S0, from the short-term (4 min.) tryptic digestion, was redigested for 20 hrs. A 100 ug aliquot was then injected onto Hamilton PRP-1. See Figures 22 and 23 for conditions. H1 and H2 tend to be elevated in such digests, H5 is larger than H6, and a new peak (H7a) appears. H7a was not a Tyr-Lys multimer.

DISCUSSION

I. Hydroxyproline-rich Glycoproteins And Extensin Precursors

The quest for precursor extensin was delayed by two preconceptions: 1) that there was a small rapidly turning over extensin precursor pool, and 2) the belief that the sycamore-maple cell suspension system was adequate for precursor studies. Neither was correct. Direct salt-elution of HRGP from sycamore-maple cells gave highly variable results (some Hyp was eluted but not enough to characterize), yet highly reproducible results were obtained from rapidly growing tomato suspension cultures. Furthermore, during rapid growth salt-elutable HRGP constituted a surprisingly high proportion (up to 10%, Figure 5) of total wall-bound hydroxyproline. This large pool turned over slowly (half-life approximately 12 h) and in this respect paralleled the slow turnover reported for HRGP elutable from the cell walls of aged carrot root explants (Brysk & Chrispeels, 1972, Cooper & Varner, 1983). This relationship between tomato HRGP pool size and growth rate is also consistent with the observation that the amount of easily 'extractable extensin' in mung bean

hypocotyls decreased as the growth rate decreased (Bailey & Kauss, 1974).

A. Tomato HRGP's P1 and P2 Are Extensin Precursors

A prime criterion for an HRGP to be an extensin precursor is flux. How well does supply meet demand? During the experimental time-course the tomato cell suspension cultures increased in dry weight at a rate of 0.625%/hr, corresponding to the observed mean generation time of 4.6 days. Since the cells contained 7 mg Hyp/g dry weight, a 0.625% dry weight increase translates into a cell wall demand of 44 ug Hyp/g cells/hr.

The pulse-chase data (Figures 16 & 17) were obtained from a culture containing an elutable HRGP pool of 400 ug Hyp/g cells. With a half-life of 12 hr (corresponding to a 5.6% pool efflux/hr), this pool can provide 22.4 ug Hyp/g cells (dry weight)/hr to the growing cells or approximately 50% of the apparent steady state requirement. On the other hand, restoration kinetics (Figure 18) showed that pool repletion in cells 4 days after subculture occurred at a minimum rate of 43 ug Hyp/g cells/hr, or 98% of the requirement.

As the amounts of P1 and P2 eluted from the tomato cells were constant during the course of the pulse-chase experiments (except for a slight decrease in P1 relative to P2 at the 24 hr time point), the experiments were performed

under almost steady state conditions and the data therefore reflect 'turnover' of the salt-elutable pool in the strict sense of that term (Lamport, 1970).

I concluded that the kinetic data showed incorporation of P1 and P2 into the cell wall because: 1) the medium did not contain P1 or P2 (Figure 9); 2) several workers have already shown that the radioactivity from labelled proline or tyrosine (added to cultures) incorporates into hydroxyproline-containing protein (extensin) of the cell wall (Dougall & Shimbayashi, 1960; Olson, 1964; Chrispeels, 1969); and 3) covalently wall-bound extensin is not catabolized (Chrispeels, 1969).

The unexpected presence of two extensin-like HRGP's (P1 and P2) suggested their possible interconversion by 'processing'. However, ^{3}H-proline appeared in P1 and P2 at approximately equal rates in very short term (Figure 15) labelling experiments, and disappeared from P1 and P2 at essentially equal rates in longer term (Figure 17) labelling experiments. I therefore concluded that there was no precursor-product relationship between P1 and P2. Furthermore, P1 and P2 have significantly different amino acid compositions (Table 1) and hydroxyproline-arabinoside profiles (which indicate that P2 is somewhat more highly glycosylated than P1, Table 5). These data reinforce the conclusion that P1 and P2 are separate precursors, as do the similar molecular weights of P1 and P2 determined by

gel filtration, and HF-deglycosylated P1 and P2 determined by gel electrophoresis (Figure 13).

How do the compositions of P1 and P2 compare with firmly bound extensin? The combined P1 and P2 amino acid compositions strongly resembled that of the wall (Table 1). The cell walls (which were cleaned by boiling with SDS and then HF-deglycosylated) did have two notable differences. First, they contained less tyrosine than P1 and P2, which was expected since the postulated intermolecular extensin crosslinks involve tyrosine residues. Second, the walls contained more Asp, Ser, Glu, Gly, Ala, and Leu than P1 and P2. At present I cannot account for the presence of these other amino acids but they may be due to covalently-bound cell wall enzymes or another non-enzymic protein (or peptide) component of the wall as in the case of the bacterial cell wall peptidoglycan.

Both P1 and P2 contain 50-60% carbohydrate which is 90 mole% arabinose and 6-7 mole% galactose. This is the expected sugar composition for HRGP's that are extensin precursors. A simple average of the hydroxyproline-arabinoside profiles of P1 and P2 closely resembles the hydroxyproline-arabinoside profile of the wall (Table 5). This is also precisely what one would expect from precursors of covalently-bound extensin. In addition, these results suggest a P1:P2 stoichiometry of 1:1 in multimeric extensin, but the data do not account for

the molar ratios of elutable P1 and P2, which change characteristically during growth (Figure 5; see below).

From the kinetic and compositional data I concluded that P1 and P2 are separate, monomeric extensin precursors that become crosslinked to form covalently bound extensin. Therefore, from now on, the terms "P1" and "P2" will be used synonymously with the term "extensin precursor(s)".

B. HRGP Relationships, Elution versus Secretion, And Extensin Precursor Localization

Isolation of the extensin precursors by elution from the cell 'surface' raised many questions. Among them were:

1. What is the relationship of extensin precursors to other HRGP's such as the 'agglutinins', potato lectin, and arabinogalactan proteins?

2. Does 'elution' mean rapid secretion or release from the cell wall by ionic exchange?

3. Do the precursors appear as an actual layer on the outer surface of the cell wall or are they localized and oriented *in muro*?

Answers to these questions are (in order):

1. The hydroxyproline-rich 'agglutinins' of tobacco and potato described recently (Mellon & Helgeson, 1982: Leach et al., 1982) match the amino acid composition, sugar composition and molecular weight (after HF deglycosylation) of tomato P1 (Table 2). The agglutinins are therefore similar proteins although they come from different species.

The possible structural and agglutination roles are not mutually exclusive; one would expect highly positively charged macromolecules to agglutinate negatively charged bacteria. For example, polylysine is frequently used as an adhesive for attaching bacteria to glass or plastic surfaces (Mazia et al., 1975). Virulent bacteria may escape attachment or agglutination by secretion of a neutral capsule. The hydroxyproline-rich potato lectin is also characteristically rich in cystine (Table 2) and is therefore not directly related to extensin. The arabinogalactan proteins (AGP's) are freely soluble, highly acidic, alanine-rich, and contain arabinogalactan polysaccharide attached via hydroxyproline (Lamport & Catt, 1981). Therefore AGP's are also quite different from extensin.

2. An earlier report proposed that cations (specifically Ca^{++}) control polysaccharide secretion at the plasma membrane of sycamore cell suspensions (Morris & Northcote, 1977). "The steady-state rate of secretion of all polymers was increased within seconds of adding various electrolytes and polyelectolytes to the growth medium" (Morris & Northcote, 1977). Since these workers used ^{14}C-arabinose as a marker, their 'polysaccharide' (which contained unspecified amino acids) would almost certainly have included some extensin precursors. Based on the following lines of evidence I propose that elution of

extensin precursors and peroxidase (and the reported 'polysaccharide secretion') represent release of charged molecules by simple ion exchange from a mixed-bed ion exchanger (cell walls!). Elution occurred with increasing efficacy in the order Na^{+} << Ca^{++} < La+++, Al^{+++}, (Figures 1 & 2) suggesting the simple ionic displacement of extensin from pectic carboxyl groups. Chromatography of P1 and P2 on the cation exchanger carboxymethyl cellulose also supports that interpretation. Furthermore, the release was rapid (Figure 4) but not temperature dependent (Figure 7). Release was sixty percent complete within 10 seconds and total release occurred within two minutes after $CaCl_2$ addition (Figure 4). This release rate over 10 seconds would be equivalent to a 'secretion rate' of 0.76 g HRGP/g cells (dry weight)/hr, which is unrealistic for cells with a mean generation time of 4.6 days. The kinetic data show that the large precursor pool had a half-life of about 12 hr. Quantitative secretion of such a pool within seconds is quite unlikely, a view confirmed by my observation that the pool was also quantitatively eluted from cell wall preparations (Figure 8) and therefore pre-exists within the cell wall.

3. The final question concerns localization and orientation of extensin precursors. Electron microscopic observations in three laboratories (Van Holst & Varner, 1984; Stafstrom & Staehelin, 1985; Heckman & Lamport,

unpublished) suggest that salt-elutable HRGP's from carrot and tomato are highly rod-like glycoproteins of approximately 80-100 nm. The elution experiments demonstrate that these 100 kDa glycoproteins are ionically bound to the wall yet highly mobile after ionic exchange. There are two simple deductions: precursor extensin is ionically bound to the major cell wall anionic component, namely pectin (cf. Knee, 1975). In addition, the rapid, facile, quantitative elution (even when newly synthesized, Figure 15) of a rodlike molecule whose length suffices to span the width of the primary cell wall (approximately 100 nm) suggests a preferred orientation perpendicular to the plane of the cell wall (like needles stuck in a pin cushion). If current estimates of cell wall porosity are correct [Carpita et al., 1979; and Tepfer and Taylor, 1981, who noted complete exclusion of a 67 kDa globular protein (3.5 nm radius) from primary cell walls], other spatial orientations would sterically hinder movement of the precursors and significantly increase resistance to their diffusion or mass transfer.

II. Peptides From P1 And P2: Implications For Extensin Structure

A. The Concept Of "Crosslink Domains" In Extensin

Convinced that the salt-elutable HRGP's from tomato cells were indeed extensin precursors, I began experiments to determine the primary structures of the P1 and P2 polypeptides. In this way, I hoped to understand how P1 and P2 are incorporated into the wall matrix. I had a general idea of what kinds of sequences to expect because previous work (Lamport, 1977) had yielded sequences of extensin glycopeptides isolated from tomato cell walls (Lamport, 1977). These peptides had many Ser-Hyp_4 sequences and also contained the "unknown" tyrosine derivative believed to be involved in extensin crosslinkage. However, I had no idea how these peptides might be arranged in monomeric precursors, or which regions of these polypeptides contained amino acid residues that could interact with other cell wall molecules.

P1 contained histidine, proline and threonine in addition to the serine, hydroxyproline, valine, tyrosine, and lysine found in both P1 and P2 (Table 1). Each of these amino acids have quite different functional groups that could participate in numerous different chemical reactions and interactions.

Hydroxyproline in extensin is arabinosylated. The

arabinose residues (mostly tri- and tetraarabinosides) stabilize the polyproline II helical conformation (Van Holst & Varner, 1984) possibly by wrapping around the helix and hydrogen bonding to the polypeptide backbone (Lamport, 1977). There are probably no other molecules attached to these arabinose residues. Some of the serine residues have a single galactose residue attached to them (the glycosylation state of threonine in extensin is not known), and if this galactose has other sugars attached to it is not known. Serine and threonine must therefore continue to be considered as possible polysaccharide attachment sites (possibly through a ferulic acid ester) because glycosidic and ester linkages are less stable than phenolic ethers. Most treatments which break phenolic ethers also break glycosides and esters, and linkages to galactosyl-serine would go undetected.

The tyrosine-containing regions in P1 and P2 attract the most attention and are the most likely extensin "crosslink domains". Tyrosines can crosslink to form either inter- or intramolecular protein crosslinks via a peroxidase-catalyzed free radical generation and coupling mechanism (similar to lignification). Crosslinked tyrosine, in the form of dityrosine, has been isolated from resilin (Andersen, 1963), while dityrosine and isotrityrosine have been identified in collagen (Fujimoto et al., 1981). Fry (1982) recently identified

isodityrosine (IDT) in acid hydrolysates of suspension-cultured sycamore-maple cell walls. Epstein & Lamport (1984) subsequently sequenced a tryptic peptide containing IDT from tomato extensin (S2A11; Ser-Hyp_4-Val-1/2IDT-Lys-1/2IDT-Lys). IDT crosslinkages form the basis of the recent cell wall model proposed by Lamport (Lamport & Epstein, 1983). In this "warp-weft" model, extensin controls cellulose microfibril slippage by forming intermolecular IDT. This "locks" extensin monomers around the cellulose microfibrils and restricts their movements.

Intermolecularly linked IDT has not been isolated but at least two lines of evidence suggest its existence: one, the Hyp:IDT ratio in isolated cell walls is lower than that observed in dP2 (15:1 vs 20:1) (Smith et al., 1984, Smith et al., 1985) and two, Cooper and Varner (1983) observed that insolubilization of carrot extensin (highly similar to P1 and P2; see below) in carrot root phloem cell walls occurred with an increase in IDT at the expense of tyrosine.

Since tyrosine is considered the primary amino acid involved in extensin crosslinkage, I was particularly interested in determining the amino acid sequences of the tyrosine-containing regions in P1 and P2. This would allow definition of extensin "crosslink domains". In addition, by determining peptide sequences from both P1 and P2, I

would hopefully learn something about how P1 and P2 are related. Finally, I hoped to find out if P1 and P2 (and hence extensin) had fundamental peptide periodicities similar to the Gly-X-Y periodicity observed in collagen.

B. The Highly Periodic Structures Of P1 and P2

Surprisingly, HPLC tryptic peptide maps of HF-deglycosylated precursors dP1a, dP1b and dP2 showed remarkably few major tryptides (these occurred in molar ratios very much greater than one compared to the minor peptides). Since tryptic digestion was complete and the major peptides were small, I concluded that these major peptides were repeating units along the polypeptide backbones of P1a, P1b, and P2. P1a, P1b, and P2 were therefore highly periodic structures.

The P1a map was slightly different from that of P1b; P1a contained H2 but lacked the minor peptides H15,19,22,23, & 24 of P1b. P1a and P1b also had small differences in relative peptide yields (Figure 24 a & b). In spite of these differences, the overall peptide maps of P1a and P1b were remarkably similar. This indicated a periodic structure for both P1a and P1b based on repeats of H5 and H20 which occurred in a 2:1 molar ratio (Tables 6 & 7).

Although I was unable to completely separate P1 peptides H16-H19 and H21-27 in high enough yield for

analysis of the pure peptides, I did determine amino acid compositions across these areas of the HPLC peptide map (Table 7a). Most of the histidine and proline of P1 is in the H16-H28 region. In addition, H16-H28 all appear in the S1 fraction (hexadecamers) when the P1 peptides are separated on Sephadex G-25 (Figure 21). On the other hand, peptides H1-H8, which appear in the G-25/S2 fraction (decamers, Figure 21), contain no proline or histidine (except peptide H3, which is in the S1 fraction and contains proline). Thus, the peptides in the G-25/S1 fraction (except H3) may somehow be related to or derived from peptide H20, while the G-25/S2 peptides may be related to H5.

Tryptide H20 is particularly significant; its sequence is quite unlike any others obtained earlier from tomato (Lamport, 1977) and contains the hexapeptide "insert" Val-Lys-Pro-Tyr-His-Pro. The presence of H5 and the absence of H20 from earlier tryptic digests of wall-bound extensin make H20 the best putative site for intermolecular crosslinkage. Work is currently underway to isolate large histidine- and proline-containing peptides from isolated tomato cell walls. These peptides will then be assayed for crosslinked tyrosine to see if H20 indeed contains an intermolecular crosslink site.

The P2 tryptide map was much simpler than those of P1a and P1b. It consisted essentially of two major

tryptides (H3 and H4) with a third minor but quite noticeable IDT containing tryptide (H11). P2/H11 is identical to the decapeptide S2A11 isolated earlier (Lamport, 1977; Epstein & Lamport, 1984) from wall-bound extensin. Because the octapeptide H4 and the dipeptide H3 were equimolar, and because I was able to isolate the IDT-linked decapeptide H11, Ser-Hyp_4-Val-1/2IDT-Lys-1/2IDT-Lys, I suspect that the entire structure of P2 consists of the repeated decapeptide H11, with occasional isoleucine for valine substitutions (note peptides H5(6) and H12) and varying only in the extent of intramolecular IDT formation. An alternative model consisting of contiguous octapeptides (H4) separated by runs of contiguous dipeptides (H3) is possible, but inconsistent with results obtained from short-term tryptic digests of dP2 (Figures 27 & 28) which gave the IDT-free tryptide H9 (provisionally: Tyr-Lys-Ser-Hyp_4-Val-Tyr-Lys, Figures 26 & 28) but no Tyr-Lys multimers. Generation of H9 also reflects the relative stabilities of peptide bonds to tryptic cleavage; Lys-Ser is more polar and hence more stable than Lys-Tyr (McBride & Harrington, 1967).

C. Molecular Models, Oriented Crosslink Domains, And Peptide Periodicities

Thus, the major tyrosine-containing "crosslink domains" in P1 and P2 are very different.

Val-Lys-Pro-Tyr-His-Pro and Thr-Hyp-Val-Tyr-Lys occur in P1, while Val-Tyr-Lys-Tyr-Lys occurs in P2. To see how these different "crosslink domains" might be oriented and displayed along the polypeptide backbones of P1 and P2, I built CPK molecular models of the major peptides of P1 (H5 & H20) and P2 (H3,H4 and H11). In building the models, I assumed a 100% left-handed polyproline II helical conformation for all peptides (3 residues and 9.4 Angstroms per turn, Figure 29) based on the circular dichroism data of Van Holst and Varner (1984).

Imagine a molecule made up completely of the decapeptide P1/H5. This molecule has dual helical symmetry. Every third residue lies in the same plane and every 30 residues the polypeptide returns to its starting point. Each tyrosine residue is therefore oriented 120 degrees away from the previous tyrosine residue. If we substitute a P1/H20 hexadecapeptide (with its 6 residue insert Val-Lys-Pro-Tyr-His-Pro) for an H5 decapeptide, every third serine residue remains aligned. However, insertion of Val-Lys-Pro-Tyr-His-Pro in the middle of H5 aligns two positively charged lysine residues and a histidine residue within the hexadecapeptide unit. This provides a cluster of oriented positive charge. Furthermore, Val-Lys-Pro-Tyr-His-Pro insertion aligns the two tyrosine residues of P1/H20 in a plane oriented 120 degrees away from the plane of the lysine/histidine cluster

Figure 29. CPK molecular models of tryptic peptides P1/H20, P2/H4+H3, and P2/H11.

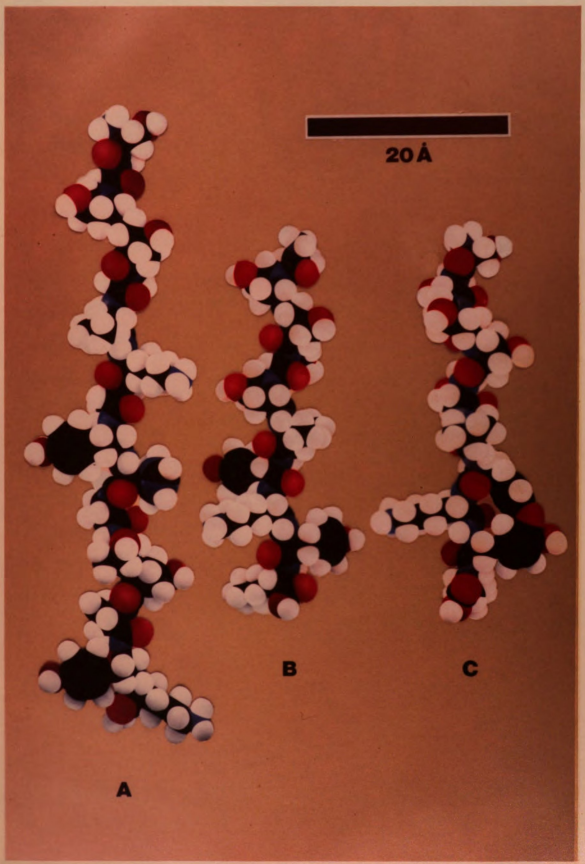
These models show the extended polyproline II helix characteristic of extensin. Peptide P1/H20 is formed by the insertion of the hexapeptide Val-Lys-Pro-Tyr-His-Pro in the middle of peptide P1/H5 (Ser-Hyp4-Thr-Hyp-Val-Tyr-Lys). The proposed decapeptide repeat unit of P2 is formed by attaching P2/H3 (the dipeptide Tyr-Lys) onto the C-terminal end of the octapeptide P2/H4 (Ser-Hyp4-Val-Tyr-Lys). IDT formation in P2/H4+H3 to form P2/H11 does not significantly alter the helix. The hydroxyproline tri- and tetraarabinosides (not shown) form a 'collar' around the Hyp4 regions and stabilize the helix. Note the positions of the lysyl and tyrosyl residues which are oriented differently in the P1 and P2 peptides.

Black = carbon, Red = oxygen, White = hydrogen and Blue = nitrogen.

(A) P1/H20

(B) P2/H4+H3

(C) P2/H11


20 Å
A
B
C

(Figure 29).

I predict that the coplanar positively charged clusters of H20 repeats interact strongly with pectic carboxyl groups (possibly assisting mutual molecular orientation), thereby facilitating crosslinkage reactions of the coplanar tyrosine residues oriented 120 degrees out of that plane. Furthermore, the sequence Val-Lys-Pro-Tyr-His-Pro represents the longest unglycosylated crosslink domain, supporting its suggested role in intermolecular crosslinkage.

The CPK molecular model of the proposed P2 decapeptide repeat unit is similar to the P1/H5 model; three decapeptides bring the molecule back to its original orientation. But in P2, consecutive tyrosine residues are skewed by 120 degrees as are consecutive lysine residues. This allows intramolecular IDT formation but does not allow two coplanar intermolecular crosslinkages involving consecutive tyrosines (as in P1/H20). However, two molecules running in opposite directions (as in double stranded DNA) could crosslink very effectively!

The peptides sequences from P1 and P2 reveal fundamental structural similarities and differences between P1 and P2. They also suggest underlying fundamental periodicities in P1 and P2. All tryptides sequenced that contain hydroxyproline-tetrapeptides have N-terminal Ser. Thus Lys-Ser occurs frequently and most sequences prior to

tryptic cleavage are Lys-peptide-Ser. Therefore the contiguous decamers and hexadecamers of P1 and P2 consist of hydroxyproline tetrapeptides separated by tripeptides which are few both in number and composition (Figure 30). Tyr-Lys-Ser is common to both P1 and P2. Val-Lys-Pro, Tyr-His-Pro and Thr-Hyp-Val are characteristic of P1, while Val-Tyr-Lys is characteristic of P2. Most notably, replacing P1/H5's Thr-Hyp-Val with Val-Tyr-Lys gives the P2 "core" decapeptide).

D. Isodityrosine, The "Warp-Weft" Model, And Extensin Networks

The tetrapeptide and tripeptide periodicities of P1 and P2 combine with the three-fold helical symmetry to emphasize macromolecular orderliness and suggest fine control of crosslink frequency, network topology and ultimately wall rheology. Regularly repeated crosslinks would create an extensin network of defined porosity quite possibly penetrated by cellulose microfibrils (Lamport & Epstein, 1983). Such mechanical coupling of the load-bearing polymers in the growing fabric of the wall could be as important a determinant of cell morphology as the initial direction of cellulose microfibrils (Green & Poethig, 1982).

It is now possible to discuss the role of IDT and crosslink domains in more detail. The presence of IDT,

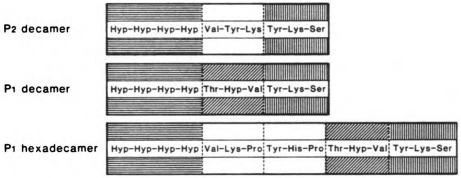


Figure 30. Tri- and tetrapeptide periodicities of P1 and P2.

The P1 and P2 repeat units can be viewed as hydroxyproline tetrapeptides separated by 2 to 4 tripeptides. The P2 decamer differs from the P1 decamer by substitution of Val-Tyr-Lys for Thr-Hyp-Val. This replacement of a potential polysaccharide attachment site with a potential tyrosine crosslink site allows the cell to vary the interaction of extensin with other wall components by differential expression of the genes coding for P1 and P2.

originally identified in cell wall hydrolysates (Fry, 1982) and later in tryptic peptides of bound extensin (Epstein & Lamport, 1984), appears to be correlated with progressive insolubilisation of extensin monomers during the incubation of isolated cell walls (Cooper & Varner, 1984). These results, together with the identification here of the potential crosslink sites Val-Lys-Pro-Tyr-His-Pro (hexapeptide domain in tryptide P1/H20), Thr-Hyp-Val-Tyr-Lys (from P1/H5 & H20), and Val-Tyr-Lys-Tyr-Lys (from P2), make IDT the prime intermolecular crosslink candidate for extensin. However, we have thus far only identified intramolecularly linked IDT (which may stiffen the molecular rod and/or assist in crosslink orientation). Until direct evidence is obtained indicting IDT as the intermolecular crosslink in extensin, the possibility remains that tyrosine residues may react to form other crosslinked species, such as ferulic acid/tyrosine crosslinks or dityrosyl ether (formed by dehydration of the phenolic -OH's). Unknown A, found in peptides P2/H1 and P2/H2, may be a crosslinked tyrosine derivative. P2/H1 and P2/H2 both absorb UV light at 273 nm but neither one of them contain tyrosine (Table 10).

Given their different crosslink domains, one would expect P1 and P2 to crosslink to form different extensin networks. An extensin network composed entirely of P1 would have a much lower crosslink density than a P2 network

(based on crosslink potential, assuming that the crosslinking enzyme has a similar Km for tyrosine residues in different crosslink domains). A P1 extensin network, crosslinked only via the first tyrosine in its P1/H20 type crosslink domains, would have an average crosslink separation of 13-17 nm (assumes that P1 has 300 residues in a polyproline II conformation, with six H20 type sequences in the 80-100 nm rod). This is adequate to wrap around a cellulose microfibril (circumference = 20-25 nm). On the other hand, in order for P2 to crosslink around a 20-25 nm cellulose microfibril, the crosslinks would have to be located every third crosslink domain (this 9 nm crosslink separation is a strict minimum, but interestingly, every third crosslink domain is aligned in the same plane!). P1 and P2 crosslinking is still hypothetical. If extensin and cellulose intertwine to form a warp-weft structure remains to be seen; we are not yet sure how extensin interacts with the other cell wall polymers.

Regulation of extensin networks during development is suggested by the existence of at least two extensin precursors (P1 & P2) whose levels depend on growth phase (Figure 5) and culture medium. P2 elutes only from cells grown on M6E medium and even then not until 3-4 days after subculture. P1 may be involved in division while P2 may be involved in elongation (cf. Klis & Eeltink, 1979).

Division is the major cellular activity early in the culture period (when P1 is dominant) while elongation is prevalent at later stages of growth (when P2 is dominant). In bean cultures the hydroxyproline arabinoside profile of the cell wall varies significantly during the growth cycle (Klis & Eeltink, 1979). As each precursor has a characteristic hydroxyproline arabinoside profile (Table 5) the levels of different extensin precursors in bean probably fluctuate during growth as they do in tomato (Figure 5).

The resolution of P1 into P1a and P1b (Figure 11) suggests variants of P1 due to minor peptide differences (Figure 23), rather than the major differences in peptide sequence seen between P1 and P2. Indirect evidence for a third extensin precursor in tomato cells ("P3"), exists in the form of three major extensin tryptides, Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Lys, Ser-Hyp-Hyp-Hyp-Hyp-Ser-Hyp-Ser-Hyp-Hyp-Hyp-Hyp-Tyr-Tyr-Tyr-Lys, and Ser-Hyp-Hyp-Hyp-Hyp-Lys, which were isolated from bound extensin (Lamport, 1977) but did not appear in tryptic peptide maps of either P1 or P2. This "non-appearance" of "P3" in salt eluates, while puzzling, is paralleled by the "non-appearance" of P2 in salt-eluates of cells grown on MET medium.

Genomic probes indicate there may be as many as seven extensin genes in carrot (J. Varner, personal

communication). Differential expression of extensin genes would allow cells to build different primary cell walls as a function of cell age, position in the plant, and environmental conditions. The suppression of P2 on MET-grown cells may be an example of such developmental regulation.

The peptide sequencing strategy has been nicely complemented by genomic cloning and sequencing data from the carrot root phloem disc system (Chen & Varner, 1985a; b). Carrot clone pDC5A1 contains the sequence Ser-Pro-Pro-Pro-Pro-Thr-Pro-Val-Tyr-Lys eight times (the unhydroxylated analog of P1/H5), and the sequence Tyr-Lys-Tyr-Lys eleven times (note relation to P2). There are also notable differences between the sequence derived for carrot extensin (from clone pDC5A1) and the peptide sequences from P1 and P2. The sequence Ser-Pro-Pro-Pro-Pro-Lys appears in carrot extensin while its hydroxylated analog has thus far only been found in tomato cell walls (not in P1 or P2). In addition, no isoleucine for valine substitutions appear in the carrot sequence, while they are rather common in P1 (peptide H8), and P2 (peptides H5(6) and H12). In spite of these differences, the similarity of extensins from two distant plant families (Umbelliferae and Solanaceae), although in related orders (Umbellales and Solanales), is quite remarkable.

We tentatively assume that in tomato cell cultures,

extensin precursors P1 and P2 create a heteromultimeric network, perhaps with lateral braces of "P3". The "non-elution" of "P3" might be attributed to a periclinal (tangential) rather than anticlinal (radial) orientation. On the other hand secretion of a single major extensin precursor by wounded carrot slices (Stuart & Varner, 1980) suggests the possibility of very tightly crosslinked homopolymeric extensin networks induced as a specific response to stress such as mechanical wounding or infection (Esquerre-Tugaye & Lamport, 1979; Bolwell, 1984). The situation is reminiscent of the analogous metazoan matrix, where the hydroxyproline-rich glycoprotein collagen is quite definitely tailored to the tissue (Bornstein & Sage, 1980; Eyre, 1980).

III. The Future

Many unanswered questions remain concerning extensin precursors, extensin networks, and cell walls. Among them:

What are the complete primary structures of P1a, P1b, and P2? Are P1a and P1b separate gene products or do they arise from some post-transcriptional event such as mRNA processing? Is P2 indeed a repeating decapeptide? How are the hydroxyproline-arabinosides distributed within the P1 and P2 polypeptides? How do they interact with the helical polypeptide backbone? Is threonine glycosylated? Do serine and threonine have polysaccharide attached via

galactose? If not, what does the single galactose residue do? Do the histidine residues have any function? Are they able to regulate crosslinkage via their acid-base properties? How does the cell leave certain proline residues unhydroxylated? Is it possible that extensin is not ionically attached to pectin? What kinds of experiments would prove that extensin is ionically bound to pectin? Does acid-growth occur via a pectin-extensin switch? How are the hemicelluloses oriented in the wall? How do they interact with extensin? Is the warp-weft model correct? Can the cell regulate extensin crosslink density? How? Does this control extension growth? Are specific peroxidases involved? How is their synthesis regulated? Does lignin interact with extensin? Is extensin a template for secondary growth?

The list goes on and on. Obviously there is a lot of work to be done before we completely understand how the plant cell wall is built, what role extensin plays in the wall, and exactly what the wall does once it is built.

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