Phytochemistry, Vol 23, No. 6, pp 1233–1239, 1984
Printed in Great Britain

0031–9422/84 $3 00 + 0 00

ISOLATION OF EXTENSIN PRECURSORS BY DIRECT ELUTION OF INTACT TOMATO CELL SUSPENSION CULTURES

JAMES J. SMITH, E. PATRICK MULDOON and DEREK T. A. LAMPORT

MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824, U.S.A.

(*Revised received* 18 *November* 1983)

Key Word Index—*Lycopersicon esculentum*; Solanaceae; tomato, extensin precursor; cell suspension cultures, peroxidase; cell wall; hydroxyproline rich glycoprotein.

Abstract—Dilute salt solutions eluted peroxidase and hydroxyproline-rich glycoproteins (HRGP's) very rapidly (60% within 10 s) from the surface of intact tomato cells grown in suspension culture. Further purification of the HRGPs based on (a) their solubility in 10% trichloroacetic acid and (b) chromatography on carboxymethyl cellulose, gave two components (P1 and P2) rich in serine, tyrosine, lysine and arabinosylated hydroxyproline. The sum of the hydroxyproline arabinoside profiles of P1 and P2 approximated that of the wall. P1, unlike P2, was histidine-rich and also contained proline. Significantly, isodityrosine (IDT) was absent from P1 and P2 but present in cell wall hydrolysates where the Hyp:IDT molar ratio was *ca* 15:1. In cells 4 days after subculture, ^{3}H-proline pulse-chase data indicated turnover of P1 and P2 presumably resulting from covalent attachment to the wall as neither P1 nor P2 appeared in the growth medium. At day four the cell mean generation time (MGT) was 4.6 days, the cell hydroxyproline content was 0.7% (w/w), the half lives of P1 and P2 were both *ca* 12 hr, and the combined $CaCl_2$ elutable P1 and P2 precursor pools contained *ca* 400 μg Hyp/g cells (dry weight). Calculated from the MGT and Hyp content, the cell demand was 44 μg Hyp/g cells (dry weight)/hr. The precursor pool size was therefore sufficient for 9 hours growth. However the pool turnover calculated from half life and pool size was 5.6%/hr or 22.4 μg Hyp/g cells (dry weight)/hr. Thus the supply of P1 and P2 precursors met > 50% of the cell wall demand. Corroborative experiments showed that after depletion of the P1 and P2 pools by salt elution, washed cells resuspended in growth medium repleted the precursor pools at a rate corresponding to a synthesis of 43 μg Hyp/g cells (dry weight)/hr, or 98% of the demand. These data allow us to make the following suggestions: P1 and P2 represent monomeric extensin precursor subunits. Salt elution of P1 and P2 indicates their ionic binding by pectic carboxyl groups. The rapidity of elution indicates a high diffusivity of these extended rodlike macromolecules through the cell wall. This may imply a preferred orientation for P1 and P2 perpendicular rather than parallel to the plane of the wall. The lack of IDT in P1 and P2 implies that IDT forms *in muro*, possibly via peroxidase. We speculate that some of these IDT residues may crosslink an extensin precursor 'weft' around a cellulose microfibrillar 'warp'. Such formation of heteromultimeric extensin interpenetrated by microfibrils would create a mechanically coupled extensin-cellulose network.

INTRODUCTION

Extensin is a hydroxyproline-rich glycoprotein (HRGP) component firmly bound to the primary cell wall [1]. Characteristically extensin is insoluble and also rich in serine, valine, tyrosine and lysine, and thus highly basic. Glycosylation involves short oligoarabinosides attached *O*-glycosidically to most of the hydroxyproline residues while some of the serine residues are *O*-galactosylated. The polypeptide backbone has repeating sequences containing the pentapeptide Ser–Hyp–Hyp–Hyp–Hyp. The difficulties of further characterizing this insoluble glycoprotein led to the search for a soluble precursor of extensin. However the isolation of such a precursor from the cytoplasm was not straightforward [2]. An early report [3] of a putative extensin precursor from carrot cell walls gave kinetics and composition difficult to reconcile either with cell wall growth rates or known wall amino acid composition. Furthermore, it was not possible to detect a putative extensin precursor in the salt-soluble fraction of cell walls prepared from *Acer pseudoplatanus* suspension cultures [4]. In recent reinvestigations of the carrot system [5, 6], elution of cell wall preparations yielded a single salt-soluble extensin-like polypeptide with some precursor characteristics. Thus extensin precursors secreted into the wall are soluble until covalently attached. Recent identification of the new crosslinked amino acid isodityrosine in cell wall hydrolysates [7] and in extensin tryptic peptides [8, 9] supports an earlier speculation that extensin network formation involves phenolic cross-linkages [10]. This led us to reinvestigate the possible isolation of soluble extensin precursors by a direct elution technique developed earlier [11].

Here we report the isolation of two monomeric extensin precursor polypeptides, designated P1 and P2, by direct elution of the cell surface of intact tomato cell suspension cultures during rapid growth. Judging from pulse-chase kinetics and chemical composition these polypeptides fulfil the criteria required for precursor status although the unexpectedly large pool with its concomitant slow turnover was unexpected and may have delayed recognition of the true status of this material in other plants.

Abbreviations: CMC, carboxymethyl cellulose; Hyp, hydroxyproline; HA_n, hydroxyproline with *n* arabinose residues; HRGP, hydroxyproline-rich glycoprotein; IDT, isodityrosine; MGT, mean generation time; PCV, packed cell volume.

RESULTS

HRGP and peroxidase elution from the cell surface

Elution of cells packed into a small column (8 mm × 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 $CaCl_2$ (Fig. 1). Cell columns eluted with linear gradients of $LaCl_3$ or $AlCl_3$ and NaCl released hydroxyproline-rich material at 10 mM $LaCl_3$ or $AlCl_3$ and 150 mM NaCl respectively.

For subsequent bulk elutions we used 50 mM $CaCl_2$. This did not plasmolyse the cells, nor did it drastically decrease viability, judging from both microscopic and macroscopic observation of cells grown in the presence of 50 mM $CaCl_2$, in which the yield was 15% PCV compared with 24% PCV in a control culture, after 19 days growth from an initial inoculum of 5% PCV. However, cells did not grow in 100 mM $CaCl_2$. The yield of crude (unfractionated) HRGP obtained by $CaCl_2$ elution ranged from 0–0.7 mg Hyp/g cells dry weight, depending on the growth phase (Fig. 4). Elution of HRGP from the cells was complete two minutes after $CaCl_2$ addition (Fig. 2). A separate experiment using rapid sampling gave 60% elution of the total salt-soluble HRGP within 10 seconds (Fig. 2, inset).

On a weight basis the actual amount of peroxidase eluted was much less than HRGP. For example at day seven (Fig. 4) the HRGP yield was 2.3 mg/g cells dry weight, while the peroxidase yield was 28 μg/g cells dry weight, using horseradish peroxidase as a standard.

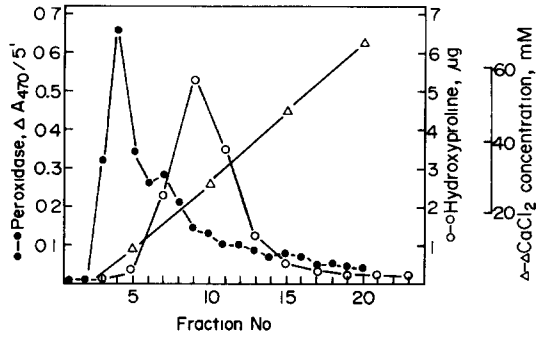

Fig. 1. Elution of peroxidase and hydroxyproline rich glycoprotein from a column of intact tomato cells. Peroxidase activity (ΔA_{470}/5 min) (●); hydroxyproline (μg) (○); $CaCl_2$ (mM) (△).

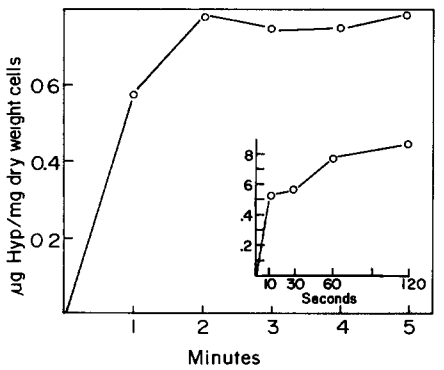

Fig. 2. Elution of HRGP with 50 mM $CaCl_2$ as a function of elution time. Samples not TCA precipitated.

Further fractionation of salt-eluted HRGP

Treatment with 10% (w/v) trichloroacetic acid. Overnight precipitation with 10% w/v TCA at 4° followed by centrifugation removed some contaminating proteins from bulk eluates judging by amino acid analyses and the size of the CMC void peak of columns run before and after TCA precipitation (data not shown). Soluble HRGP remained in the TCA-supernatant consistent with the highly glycosylated and basic character of extensin. Peroxidase activity however remained exclusively in the TCA-precipitate when assayed after dialysis, and accounted for all the original salt-eluted peroxidase activity.

Cation exchange chromatography of HRGP on carboxymethyl cellulose (CM-52). After dialysis of the TCA-soluble HRGP and chromatography on carboxymethyl cellulose in 30 mM pH 7.8 NaPi buffer with a linearly increasing NaCl gradient, we obtained two major HRGP fractions designated P1 and P2 (Fig. 3). P1 eluted at 0.3 M NaCl while P2 eluted at 0.5 M NaCl. The CMC void peak contained a relatively small amount of hydroxyproline and gave a positive reaction with Yariv antigen [12] indicating the presence of arabinogalactan protein.

The ratio of P1 and P2 changed as a function of culture age as did the total amount of TCA-soluble P1 and P2 (Fig. 4). The P1 yield rose and fell a day or two ahead of P2. On subculture total soluble HRGP fell rapidly, was

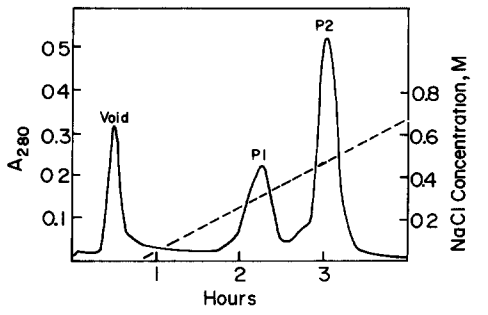

Fig. 3. Elution of P1 and P2 from carboxymethyl cellulose. 10 mg crude precursor preparation from a seven day old culture injected. Details in Experimental.

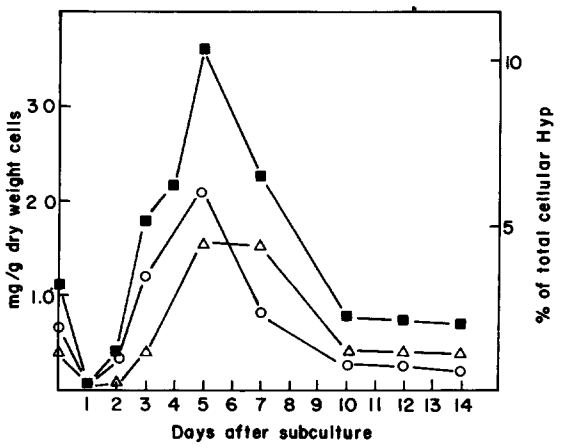

Fig 4. Yields of P1 (○), P2 (△), and total P (■) as a function of culture age. Cell is taken to be 0.7% hydroxyproline by weight.

minimal at day 2 then rose to peak at day 5, subsequently declining.

Apparent MW and purity via SDS-PAGE. The size of extensin precursors before incorporation into the wall matrix is crucial to hypotheses concerning mechanisms of cell wall assembly and growth. 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 (Fig. 5), although 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 and IDT) indicated that deglycosylated P1 and P2 were monomeric. Virtual absence of other bands apart from a very faint band, possibly attributable to a trace dimer component, suggested that P1 and P2 were highly purified.

The following sections show that the composition and kinetic behaviour of HRGP fractions P1 and P2 were consistent with their role as precursors to covalently wall-bound extensin.

Criteria for precursor status of HRGP P1 and P2

We set out to answer two questions: did the putative precursor look chemically like extensin and did it behave kinetically like a precursor? This involved amino acid analyses, determination of hydroxyproline arabinoside profiles, and two kinds of kinetic experiments to determine approximate rates of pool influx and efflux for comparison with wall demand calculated from growth rates.

Composition: 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 and contained *ca* 5 mol % proline. This distinguished P1 from both P2 and the 'bulk' of covalently bound extensin. Both P1 and P2 were also rich in tyrosine but lacked the crosslinked isodityrosine (IDT), while covalently bound extensin contained IDT but less tyrosine *per se* than P1 and P2.

Composition: hydroxyproline arabinoside profiles. Oligoarabinoside substituents of the hydroxyproline hydroxyl groups represent the major carbohydrate com-

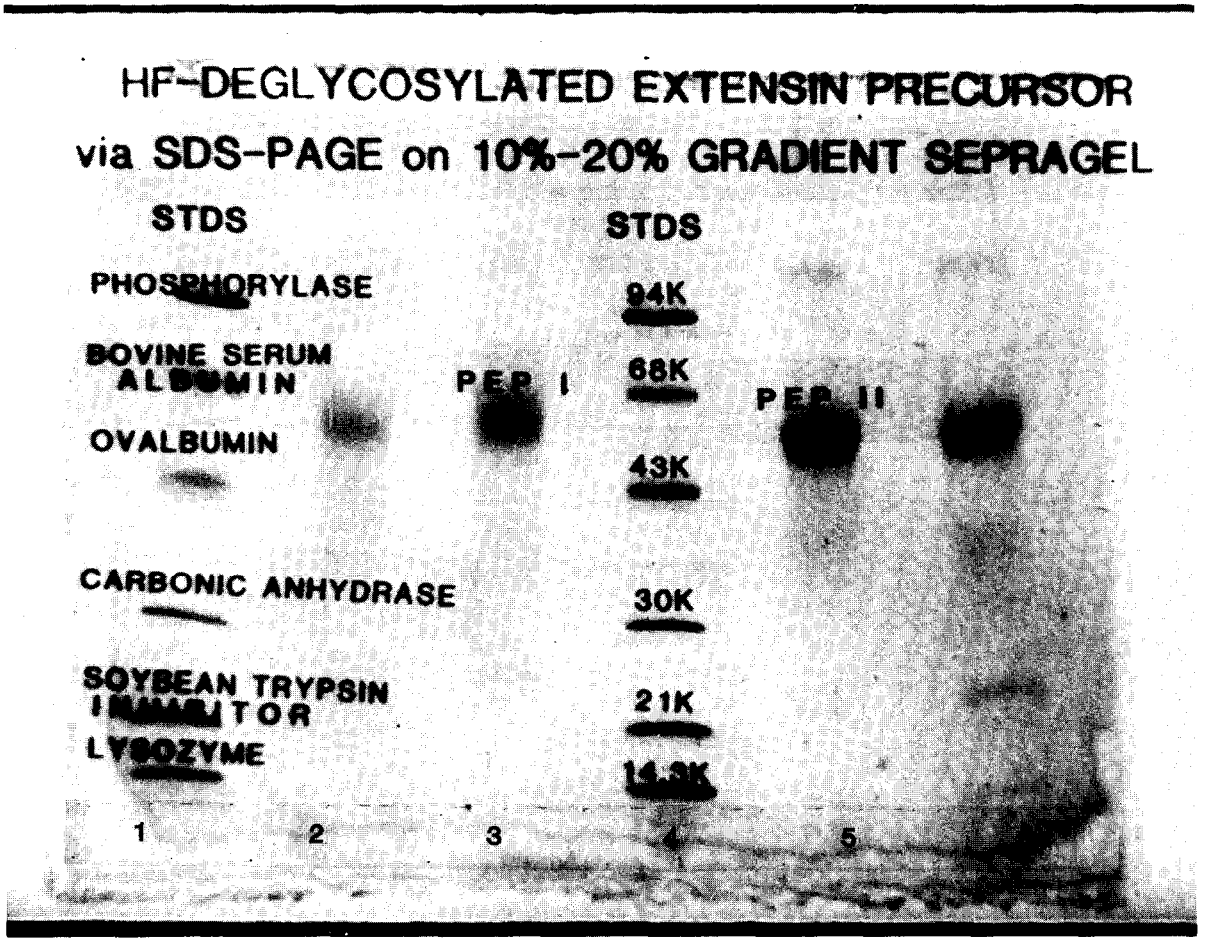

Fig. 5. SDS-PAGE of HF deglycosylated P1 and P2, Lane 1, molecular weight markers. Lane 2, 50 μg P1. Lane 3, 100 μg P1. Lane 4, molecular weight markers. Lane 5, 50 μg P2. Lane 6, 100 μg P2. All weights before deglycosylation. PEPI = P1, PEPII = P2. Details in Experimental.

Table 1 Amino acid compositions* of extensin precursors P1, P2 and HF-deglycosylated tomato cell walls

AA	P1	P2	Tomato cell wall†
Hyp	33.5	41 8	28 5
Asp	1.8	0.7	4.0
Thr	7.2	1 0	4.6
Ser	9.5	12.1	14 2
Glu	1.9	0.3	2 8
Pro	8 3	0.8	3 9
Gly	1 6	0.3	3.3
Ala	2.0	0.5	3 2
Cys	0	0	0
Val	5.0	5 1	7.0
Met	0	0	0.3
Ile	0.9	0.9	1.8
Leu	0.8	0.2	2.5
Tyr	8 9	14.9	6 3
Phe	0 6	0.2	1 3
His	7 1	1.0	2.7
Lys	10 1	20 1	10 5
IDT	0	0	1.9
Arg	0 7	0 1	1.2
Total	100.0	100.0	100.0

*Expressed as mol %

†Cell walls were prepared by sonic disruption as described previously [11], 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 Experimental

ponent 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 [13] although minor variations may occur as a function of growth rate [14]. The hydroxyproline arabinoside profile is therefore a critical test of a possible extensin precursor.

Hydroxyproline tetraarabinosides (HA_4) and triarabinosides (HA_3) predominated in P1 and P2 which in this respect were therefore similar to covalently bound extensin of the wall (Table 2). 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.

Table 2 Hydroxyproline arabinoside profiles of P1, P2 and tomato cell walls

	P1	P2	P1 + P2	Tomato cell wall†
Hyp*	11 7	7 3	9.5	5.3
Hyp-Ara	9.3	5.9	7.6	9.9
Hyp-Ara_2	7 6	8 2	7.9	9.1
Hyp-Ara_3	33.2	24.2	28.7	27.5
Hyp-Ara_4	38 1	54 4	46 3	48.3
Total	100 0	100.0	100 0	100.0

*Expressed as % of total Hyp

†SDS-boiled.

However the hydroxyproline arabinoside profile of P1 plus P2 closely approximated that of the cell wall (Table 2).

^{3}H-Proline pulse-chase experiments. Tomato cell suspension cultures (4–6 days after subculture *ca* 10% PCV) incubated in their own growth medium (650 ml) containing 75 μCi ^{3}H-proline incorporated ^{3}H-proline into P1 and P2 within *ca* 15 min in short-term labelling experiments (Fig. 6). 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 *ca* 12 hr (Fig. 7). There was no labelled or unlabelled P1 or P2 in the incubation or growth medium before or after pulse-chase labelling (data not shown). The kinetic data therefore reflect *in muro* events. CMC profiles of P1 and P2 were constant during the course of these pulse-chase experiments except for a slight decrease in P1 relative to P2 at the 24 hr time point indicating that the experiments were performed under almost steady state conditions. The data therefore show 'turnover' in the strict sense of that term [15].

Restoration kinetics of the in muro *extensin precursor pool following initial pool depletion.* Depletion of the *in muro* extensin precursor pool by simple salt elution followed by water washing and reincubation of eluted cells in growth medium, allowed us to corroborate 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 8 shows that rapidly growing

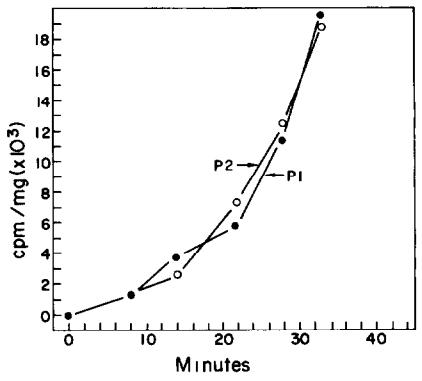

Fig. 6. Short term pulse labelling of P1 and P2 Curves coincide at $t = 0$ min and $t = 8$ min. Details in Experimental.

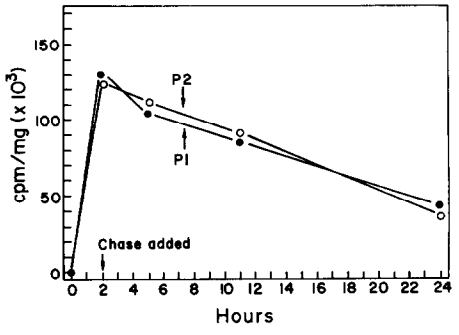

Fig. 7. Pulse-chase labelling of P1 and P2. Details in Experimental.

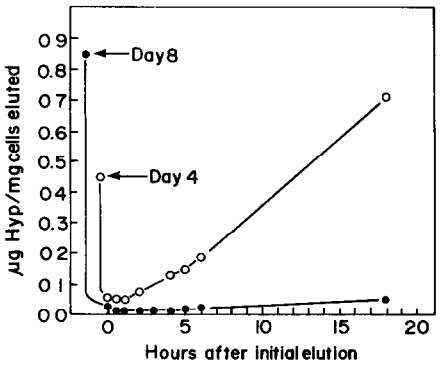

Fig. 8. Restoration of total precursor pools in an 8 day old culture (●) and a 4 day old culture (○). Arrows indicate initial levels of elutable hydroxyproline. Details in Experimental.

cells restored the precursor pool to its initial level within 12 hr with an apparent precursor synthesis rate of 43 μg Hyp/g cells (dry weight)/hr. Clearly this was a minimum rate not accounting for endogenous depletion of the pool by transfer of precursor material to covalently wall-bound extensin during the experimental time course. However the 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.

DISCUSSION

Our quest for precursor extensin was delayed by preconceptions of a small rapidly turning over extensin precursor pool, and the adequacy of the sycamore cell suspension system. Neither was correct. It turns out that direct elution of extensin precursors was, for unknown reasons, highly variable from sycamore cells (data not shown), yet highly reproducible from rapidly growing tomato suspension cultures. Furthermore during rapid growth this elutable pool constitutes a surprisingly high proportion (up to *ca* 10%, Fig. 4) of total wall bound hydroxyproline. Not surprisingly such a large pool turned over slowly and in this respect paralleled the slow turnover reported for HRGP elutable from the cell walls of aged carrot root explants [3, 6]. A relationship between precursor pool size and growth rate is also consistent with the observation that, in mung bean hypocotyls the amount of easily 'extractable extensin' decreased as the growth rate decreased [16].

A prime criterion of precursor status is precursor 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. As the cells contain 7 mg Hyp/g dry weight, a 0.625% increase translates into a cell wall demand of 44 μg Hyp/g cells/hr.

The pulse-chase data (Fig. 7) were obtained from a culture containing an elutable precursor pool of 400 μg Hyp/g cells. With a half-life of 12 hr (corresponds to a 5.6% pool efflux/hr), this pool could provide 22.4 μg Hyp/g cells (dry weight)/hr to the growing cells or approximately 50% of the apparent steady state requirements. On the other hand, restoration kinetics (Fig. 8) showed pool repletion occurring at a minimum rate of 43 μg Hyp/g cells/hr, or 98% of the requirements.

The unexpected presence of two extensin precursor polypeptides (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 (Fig. 6) labelling experiments, and disappeared from P1 and P2 at essentially equal rates in longer term (Fig. 7) labelling experiments. Therefore we conclude that there is no precursor–product relationship between P1 and P2 which also show significant differences in amino acid composition (Table 1) and hydroxyproline arabinoside profiles (Table 2). P1 contains considerably more histidine and proline than P2, while the hydroxyproline arabinoside profiles indicate that P2 is somewhat more highly glycosylated than P1. These data reinforce the conclusion that P1 and P2 are separate precursors, as do the similar MW's of HF-deglycosylated P1 and P2 via SDS-PAGE (Fig. 5).

We must now consider how the compositions of P1 and P2 compare with firmly bound extensin. Significantly both P1 and P2 lacked isodityrosine crosslinks (or cystine crosslinks) but were tyrosine-rich; cell wall amino acid analyses of HF deglycosylated cell walls, precleaned by boiling with SDS, showed less tyrosine than the precursors (Table 1) but the Hyp/IDT ratio was *ca* 15:1. Thus excluding these exceptions, the combined P1 and P2 amino acid compositions strongly resembled that of the wall (Table 1). A simple average of P1 and P2 hydroxyproline arabinoside profiles also corresponded closely to that of the wall (Table 2), suggesting that the P1:P2 stoichiometry is 1:1 in multimeric extensin. However those data do not account for the molar ratios of elutable P1 and P2 which change characteristically during growth (Fig. 4), and for which we currently have no simple explanation.

From these kinetic and compositional data we conclude that both P1 and P2 are monomeric subunit precursors which become crosslinked *in muro* to form a heteromultimeric extensin network. Peroxidatically formed IDT is a prime candidate for the intermolecular crosslink.

Isolation of these extensin precursors by elution from the cell 'surface' raises many questions We address three as follows:

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*?

1. The hydroxyproline-rich 'agglutinins' of tobacco and potato described recently [17, 18] match the amino acid, sugar composition and molecular weights (after HF deglycosylation) of tomato P1 and are therefore similar proteins although from different species. The possible structural and agglutination roles are not mutually exclusive as 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 [19]. Virulent bacteria may escape attachment or agglutination by secretion of a neutral capsule. The hydroxyproline-rich

potato lectin [20] is also rich in cystine and is therefore not directly related to extensin. The arabinogalactan proteins (AGPs) are freely soluble, highly acidic, alanine-rich, and contain arabinogalactan polysaccharide attached via hydroxyproline [1]. Therefore AGP's are also quite different from extensin.

2. A recent report proposed that cations control polysaccharide secretion at the plasma membrane of sycamore cell suspensions [21]. "The steady-state rate of secretion of all the polymers was increased *within seconds* of adding various electrolytes and polyelectrolytes to the growth medium" (our italics). As 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 we suggest that our 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!) Thus elution occurred with increasing efficacy in the order $Na^+ < Ca^{2+} < La^{3+}$, Al^{3+}, suggesting simple cationic 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 but not temperature dependent. Release was sixty percent complete within 10 seconds and total release occurred within two minutes of the $CaCl_2$ additions. We calculated that the 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 an MGT of 4.6 days. Our kinetics show that the large precursor pool had a half life of *ca* 12 hr. Quantitative secretion of such a pool within seconds is quite unlikely, a view confirmed by our demonstration that the pool was also quantitatively eluted from cell wall preparations (data not shown) and therefore pre-exists within the cell wall.

3. Our final question concerns localization and orientation of extensin precursors. The elution experiments demonstrate that a highly basic rodlike [22] glycoprotein of about 100 kDa is 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. [23]); and the rapid facile quantitative elution (even when newly synthesized, Fig. 6) of a rodlike molecule whose length suffices to span the width of the primary cell wall (*ca* 100 nm) indicates a preferred orientation perpendicular to the plane of the cell wall rather like needles stuck in a pin cushion. Other arrangements would sterically hinder movement of the precursors and significantly increase resistance to their diffusion or mass transfer, according to current estimates of cell wall porosity [24], especially those based on the complete exclusion of a 67 kDa globular protein (3.5 nm radius) from primary cell walls [25]. The possible arrangement of two interpenetrating cell wall polymer systems, periclinal cellulose, and anticlinal extensin, is a basis for the recently proposed cellulose 'warp' extensin 'weft' model [8] where peroxidase is the hypothetical crosslinking enzyme *in muro*.

Coincidentally there is a recent report of analogous interpenetrated polymer systems in Wharton's jelly (from umbilical cord) where an insoluble network is based on glycoprotein fibrils (13 nm dia.) interpenetrating a fibrillar (39 nm average dia.) collagen network [26]. In the primary cell wall, mechanical coupling between cellulose microfibrils and the extensin network may lead to new insights into the old problem of cell extension.

EXPERIMENTAL

Suspension cultures. We grew tomato cell suspension cultures (derived from a callus culture of the variety 'Bonnie Best' donated to us by Dr. H. Murakishi in 1967) in 1 l flasks containing 580 ml M6E medium shaken at 120 rpm on a gyrotory shaker at 27° under subdued fluorescent lighting, and subcultured, except where noted, every 7 days to an initial packed cell volume of 1–5%

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); $FeCl_3$ $6H_2O$ (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_2O)

Cell columns We prepared small (8 mm i.d. × 100 mm) cell columns by pouring 10–20 ml of the appropriate cell suspension into the column, allowing the cells to settle and then washing briefly with *ca* 10 ml distilled water, followed by elution with a 0–100 mM $CaCl_2$ gradient (total volume 50 ml) at a flow rate of 15–20 ml/hr, and collecting 2 ml fractions We dialysed each fraction in a multiple sample microdialysis apparatus (Bethesda Research Laboratories) before taking aliquots for hydroxyproline assays.

Assay of peroxidase. The peroxidase assay involved spectrophotometric determination of tetraguaiacol formation from guaiacol monitored at 470 nm [27] The assay mixture contained 8 mM guaiacol in 10 mM pH 6.1 NaPi buffer (2.0 ml), 5 μl 3% H_2O_2, and 50 μl aliquots taken from the cell column fractions before dialysis. Peroxidase activity is expressed as ΔA_{470} after 5 min reaction.

Assay of hydroxyproline. We determined the hydroxyproline content by Kivirikko's method [28] involving acid hydrolysis (6 N HCl, 110°, 18 hr) followed by alkaline hypobromite oxidation and coupling with acidic Ehrlich's reagent.

Isolation of crude precursors. We prepared the crude precursors from appropriate cultures (0 to 14 days post-subculture) by rapid filtration of the cultures (650 ml) on a large coarsely-sintered funnel followed by a brief water wash and then gentle agitation of the cell pad in 50 mM $CaCl_2$ for 5 min before final suction. Addition of TCA to a final concentration of 10% w/v in the eluate yielded a precipitate after 18 hr at 4°. Centrifugation of the TCA treated eluate (at 9000 rpm, 1 hr) yielded a hydroxyproline-poor pellet (discarded) and a hydroxyproline-rich supernate which was dialysed 48 hr at 4° and then freeze dried The yield of crude precursor was highly dependent on the growth stage (Fig. 4)

Carboxymethyl cellulose ion exchange chromatography. We dissolved crude precursors in 30 mM pH 7.8 NaPi buffer (10 mg/ml), applied a maximum of 15 mg to a Whatman CM-52 carboxymethyl cellulose column (8 mm i.d. × 100 mm) equilibrated with 30 mM NaPi buffer (pH 7.8), and then eluted with a 0–1.0 M NaCl gradient (in buffer) at a flow rate of 10 ml/hr, monitoring the $A_{280\,nm}$ using an ISCO UV monitor and chart recorder.

Amino acid analysis We performed amino acid analysis using a modified Dionex system fitted with a 16 cm DC5A microcolumn eluted with Dionex Hi Phi Buffers A and B (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 we monitored at 570 nm and 440 nm for accurate estimation of Hyp and Pro. The most accurate estimations of

IDT (involving accurate determination of a ninhydrin response factor [9]) were obtained by additional amino acid analyses with buffer C elution alone, which improved the Lys/IDT resolution.

Hydroxyproline arabinoside profiles. We obtained hydroxyproline arabinoside profiles after alkaline hydrolysis of appropriate samples followed by careful neutralization and separation of the arabinosides on Technicon Chromobeads C eluted with a pH gradient and monitored as previously described [13].

HF *deglycosylation.* We deglycosylated *ca* 5 mg precursor material in a micro apparatus containing *ca* 2 ml anhydrous HF and 10% (v/v) anhydrous MeOH for 1 hr at 0° as described previously [29]. After removal of the HF we immediately dissolved the deglycosylated material in 1 ml H_2O and removed 50 and 100 μl aliquots for SDS-PAGE.

Gel electrophoresis We applied deglycosylated extensin precursors in 25 μl sample buffer (Trizma base, 0.01 M, SDS, 1%; EDTA, 0 001 M, beta-mercaptoethanol, 5%) to the 'sepracomb' of commercially prepared Sepra-Gels (Separation Science Inc.), using the 10–20% acrylamide gradient preparation. We ran the gels in Tris–Gly buffer (Trizma base, 0.025 M; glycine, 0.192 M; SDS, 0.1%) for 3 hr at constant power (15 watts) with bromophenol blue tracking dye

Short term pulse labelling. We added 40 μCi carrier-free L-[5-^{3}H]proline (Amersham, 21 Ci/mmol) in 1 ml H_2O to a 1 l flask containing 650 ml cell suspension (6 day old *ca* 10% PCV). At various times after the pulse (see Fig. 5), we took 100 ml aliquots, prepared and separated crude extensin precursor via CMC as described above, and then monitored the tritium content of the fractions in aquasol or ACS (1 ml in 10 ml) using a Beckman LS 133 or LS 7500 scintillation counter

Pulse chase. We added 75 μCi carrier-free L-[U-^{3}H]proline (Amersham, 653 mCi/mmol) in 1 ml H_2O after sterilization by millipore filtration (0 2 μ porosity) to a 1 l flask containing 650 ml cell suspension (4 day old *ca* 7% PCV). After 2 hr we added 1 g unlabelled proline as a chase, and took 50 ml aliquots which were eluted with $CaCl_2$; crude extensin precursor was then prepared, separated and monitored as described above

Restoration kinetics. We determined the rate of pool repletion after an initial depletion as follows: First we filtered 650 ml of a 4 or 8 day old culture on a coarsely sintered funnel, washed the cell pad briefly with water, extracted the cells for 5 min with 50 mM $CaCl_2$ (100 mM for 8 day cells; eluates saved for Hyp assay), then briefly washed the cells with water, finally resuspending them in their original growth medium At various times we took 50 ml aliquots of the depleted cells and assayed for the reappearance of salt-elutable Hyp

Acknowledgements—We thank Mr. Nathan Krupp for early technical help, Dr. Lynn Epstein for helpful discussion and Ms. Debby DeGryse for valuable assistance throughout the course of the work. Work supported by U.S. DOE Contract # DE-AC02-76ER-01338

REFERENCES

1. Lamport, D. T. A. and Catt, J W. (1981) in *Encyclopedia of Plant Physiology New Series* (Tanner, W and Loewus, F. A., eds) Vol. 138, pp. 133–165 Springer, Berlin.
2. Dashek, W V. (1970) *Plant Physiol* **46**, 831
3. Brysk, M. M. and Chrispeels, M. J. (1972) *Biochim. Biophys Acta* **257**, 421.
4. Pope, D G. (1977) *Plant Physiol.* **59**, 894
5. Stuart, D. A. and Varner, J E. (1980) *Plant Physiol.* **66**, 787.
6. Cooper, J. B. and Varner, J. E. (1983) *Biochem Biophys. Res Commun.* **112**, 161.
7 Fry, S. C. (1982) *Biochem. J.* **204**, 449.
8. Lamport, D T. A. and Epstein, L (1983) in *Current Topics in Plant Biochemistry and Physiology* (Randall, D D. *et al.*, eds) Vol. 2, p. 73 University of Missouri Columbia.
9. Epstein, L and Lamport, D. T. A. (1984) *Phytochemistry* **23**, 1241
10. Lamport, D T. A (1978) in *Frontiers of Plant Tissue Culture* 1978 (Thorpe, T. A., ed) pp 235—244 International Association for Plant Tissue Culture 1978, Calgary.
11. Lamport, D. T. A. (1965) in *Adv. Bot. Res* (Preston, R D., ed) Vol. 2, pp. 151–218. Academic Press, London.
12. Jermyn, M. A. and Yeow, Y M (1975) *Aust. J. Plant Physiol.* **2**, 501.
13 Lamport, D T. A. and Miller, D. H. (1971) *Plant Physiol* **48**, 454.
14 Klis, F. M. and Eeltink, H (1979) *Planta* **144**, 479.
15. Lamport, D T. A. (1970) *Ann. Rev Plant Physiol.* **21**, 235
16. Bailey, R. W and Kauss, H. (1974) *Planta* **119**, 233
17. Mellon, J. E. and Helgeson, J P (1982) *Plant Physiol.* **70**, 401.
18. Leach, J. E , Cantrell, M A. and Sequeira, L (1982) *Plant Physiol.* **70**, 1353
19. Mazia, D., Schatten, G. and Sale, W. (1975) *J Cell Biol.* **66**, 198.
20. Allen, A. K , Desai, N. N., Neuberger, A and Creeth, J M. (1978) *Biochem. J.* **171**, 665.
21. Morris, M. R and Northcote, D. H. (1977) *Biochem J* **166**, 603.
22. Van Holst, G J. and Varner, J E. (1983) *Plant Physiol.* (in press)
23. Knee, M. (1975) *Phytochemistry* **14**, 2181
24. Carpita, N , Sabularse, D., Montezinos, D and Delmer, D. P. (1979) *Science* **205**, 1144.
25. Tepfer, M. and Taylor, I. E P (1981) *Science* **213**, 761
26. Meyer, F. A., Laver-Rudich, Z and Tanenbaum, R (1983) *Biochim. Biophys. Acta* **755**, 376.
27. Maehly, A. C. and Chance, B. (1954) in *Methods of Biochemical Analysis* (Glick, D , ed.) Vol. 1, pp. 357–424. Interscience, New York.
28. Kivirikko, K I. and Liesmaa, M (1959) *Scand J Clin Lab. Invest* **11**, 128.
29 Sanger, M. P. and Lamport, D T. A. (1983) *Analyt. Biochem.* **128**, 66