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INSOLUBILIZATION OF HYDROXYPROLINE-RICH CELL WALL GLYCOPROTEIN IN AERATED CARROT ROOT SLICES

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Summary: The hydroxyproline-rich glycoprotein of plant cell walls is secreted from the cytoplasm as a soluble monomer which slowly becomes insolubilized. A tyrosine derivative, isodityrosine, is formed in the cell wall during this insolubilization and could serve as a protein-protein crosslink. Glycoprotein insolubilization is inhibited by peroxidase inhibitors and free radical scavengers, the most effective of which is L-ascorbate. These data support a hypothesis that the hydroxyproline-rich cell wall glycoprotein forms a covalently crosslinked wall network under the control of an extracellular peroxidase/ascorbate oxidase system.

Cells of higher plants secrete a variety of substances which form a cell wall matrix embedding oriented cellulose microfibrils. The biophysical properties of this matrix probably play a role in controlling cell growth and development. Hydroxyproline-rich glycoproteins are an important element of the cell wall matrix, comprising as much as 18% of the wall's mass (1). The characterization of this wall component has been difficult because little hydroxyproline can be solubilized from most cell walls without proteolysis.

Aerated carrot root slices synthesize large amounts of cell wall HRGP*, some of which can be extracted from the wall with solutions of high ionic strength (2). The major soluble HRGP has been purified and characterized (3,4). It closely resembles bacterial agglutinins isolated from tobacco callus and potato tubers (5,6). The chemical composition of the carrot HRGP is also similar to that of cell wall glycopeptides isolated from partially hydrolyzed walls of tomato suspension culture cells (7) and nonextractable cell wall proteins deposited during cessation of growth in both pea and bean stems (8,9). The composition is clearly different from that of hydroxyproline-rich arabinogalactan proteins (10,11). We have used aerated carrot root discs to inves-

*Abbreviations: HRGP, hydroxyproline-rich glycoprotein

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tigate the relationship between the soluble HRGP and the nonextractable wall HRGP. Our results support the hypothesis that the cell wall HRGP is secreted as a soluble monomer which becomes insolubilized in the wall by the formation of isodityrosine crosslinks (12,13).

MATERIALS AND METHODS

Large tap roots of Daucus carota were obtained from a local merchant and stored at 4°C until use. Sterile discs (7 mm diameter by 1.5 mm thick) were washed extensively with sterile water and aerated at 25°C for at least 30 h to induce synthesis of the cell wall HRGP (14)(less than 5 g tissue in 50 ml water in each 500 ml erlenmeyer flask shaken at 120 cycles per min). Tissue discs were labeled, 2 discs/ml, in 50 mM K-phosphate buffer, pH 6.5, homogenized in 50 mM K-phosphate buffer (pH 6) containing 1 mM carrier amino acid and 0.1 mM phenylmethylsulfonylfluoride (Sigma) using a ground glass tissue grinder, and centrifuged at 1000 x g for 3 min. This cell wall pellet was washed extensively with cold 1 mM carrier amino acid (about 1 l/g fresh weight). Soluble wall proteins were extracted sequentially with 0.5 M $CaCl_2$ at 4°C (50 ml/2 discs) and 5 M guanidine thiocyanate at 50°C (Fluka AG)(10 ml/ 2 discs). At least 80% of the extractable wall proteins labeled with proline or tyrosine migrated on isopycnic CsCl gradients as the peak with buoyant density 1.44 g/ml which has been identified as the HRGP (3). Peptides of extractable HRGP and extracted cell walls were prepared following deglycosylation with 0.1 N HCl (100°C, 30 min) by hydrolyzing with trypsin or chymotrypsin (Sigma) 1 mg/ml in 60 mM $(NH_4)_2CO_3$ buffer (pH 9) at 37°C for 48 h. Peptides were separated on microcrystalline cellulose plates (Eastman) using electrophoresis (pH 10.9, 50 V-h/cm) and chromatography (butanol/acetic acid/H_2O, 12/3/5) and visualized by autoradiography on Kodak X-OMAT R film. Cell walls were lyophilized before hydrolysis with 6 N HCl (Pierce) under N_2 for 24 h at 120°C. Hydrolysates were analyzed by paper electrophoresis (Whatmann 3 MM) and by ion-exchange chromatography using L-tyrosine (Sigma) and di-and tritryosine, synthesized by the method of Gross and Sizer (15), as internal standards. The diluted acid hydrolysates (pH 2 to 3) were applied to cellulose phosphate (Sigma) equilibrated with 0.2 M acetic acid in long glass columns (1 cm diameter by 80 cm long), and eluted with a step to 0.5 M NaCl in 0.2 M acetic acid (16). Electrophoresis buffers were: 4.35% acetic acid/1.3% formic acid, pH 1.9; 2.5% pyridine/1.2% acetic acid, pH 5.9; and 2.5% triethylamine/carbonate, pH 10.9.

Radiochemicals were from New England Nuclear and had the following specific activities: L-[^{14}C(U)]histidine, 340 mCi/mmol; L-[^{14}C(U)]lysine, 331 mCi/mmol; L-[2,3-^{3}H]proline, 16.9 Ci/mmol; L-[^{14}C(U)]proline, 274 mCi/mmol; and L-[^{14}C(U)]tyrosine, 487 mCi/mmol.

RESULTS AND DISCUSSION:

Insolubilization of the cell wall HRGP. Changes in solubility of the extractable carrot HRGP were studied with pulse-chase experiments. Immediately following a 20 min pulse-labeling with proline, 73% of the labeled wall HRGP was solubilized by sequential extraction with 0.5 M $CaCl_2$ and 5 M guanidine thiocyanate. This pool of soluble HRGP slowly became nonextractable during a 24 h chase experiment (Fig. 1). No radioactivity appeared in the incubation medium, indicating that the carrot HRGP was not released through the cell wall as has been reported for arabinogalactan proteins (11,17). The experiments of

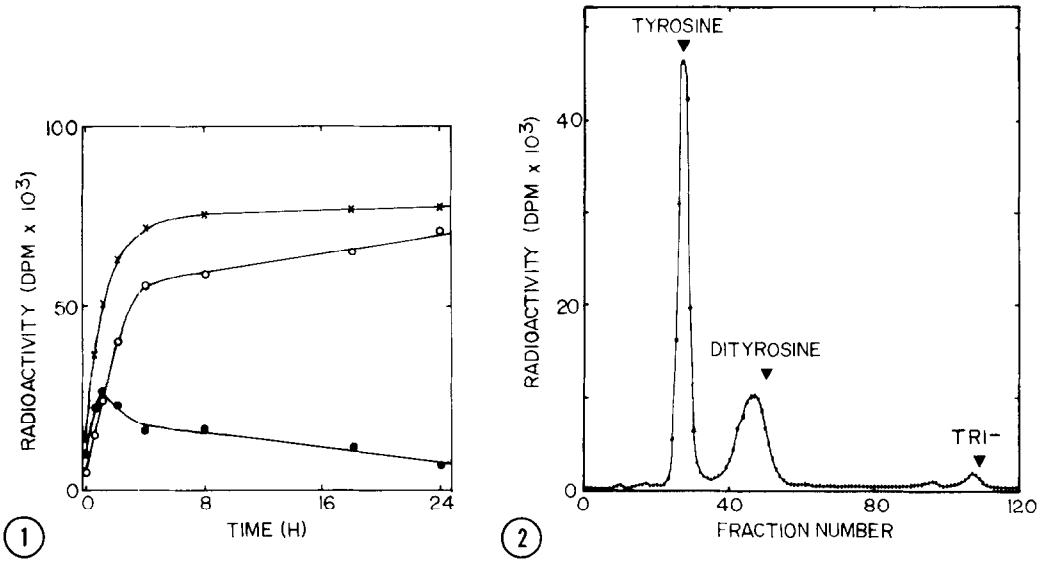

Figure 1. Insolubilization of the cell wall HRGP.
Aerated root discs were pulse labeled for 20 min with [2,3-^{3}H]proline, 5 μCi/ disc, and chased with 1 mM proline. At each time point cell walls were prepared and extracted with 0.5 M $CaCl_2$ at 4°C and 5 M guanidine thiocyanate at 50°C. The residual wall was hydrolyzed and aliquots of the soluble and insoluble wall proteins were counted, corrected for quenching, and normalized to the total tritium uptake of the disc. Extractable wall radioactivity (●) (>80% HRGP (3)), nonextractable wall radioactivity (○), and total cell wall radioactivity (X). These data represent the results of two experiments, each of which had duplicate samples at each time point.

Figure 2. Cellulose phosphate chromatography of the cell wall tyrosine derivative.
Cell walls were prepared from carrot discs labeled with [^{14}C(U)]-tyrosine for 24 h, and hydrolyzed with 6 N HCl at 120°C for 24 h. The hydrolysate was diluted to 0.06 N HCl, mixed with standards (15), adjusted to pH 3 with KOH, and applied to a phosphocellulose column equillibrated with 0.2 M acetic acid. The column was eluted with 0.5 M NaCl in 0.2 M acetic acid. Aliquots of each fraction were counted. Standards (marked with arrows) were detected by measuring the absorbance at 280 nm.

Brysk and Chrispeels (18), while comparable, were too short to demonstrate this insolubilization. Incorporation of the soluble HRGP into the insoluble wall matrix was further demonstrated by electrophoretic analysis of tryptic and chymotryptic peptides prepared from both the soluble and the insoluble wall HRGP. Both of the wall fractions contained several highly basic hydroxyproline rich peptides which all had similar chromatographic behavior and pIs greater than pH 11 (not shown).

Formation and characterization of a putative crosslinking amino acid. The cause of cell wall HRGP insolubility has long been a subject of speculation. No

covalent bonds have been found linking the HRGP to wall carbohydrates. Purified carrot HRGP contained large amounts of tyrosine, lysine and histidine (3), all of which serve as protein-protein crosslinks in animal cells; and Lamport reported the presence of a tyrosine derivative in nonextractable hydroxyproline-rich cell wall glycopeptides (7). A tyrosine derivative was labeled in carrot cell walls during the period in which the HRGP was being insolubilized. This derivative, labeled with [^{14}C]tyrosine, separated from tyrosine during phosphocellulose chromatography, and eluted slightly faster than authentic dityrosine (Fig. 2). The derivative was absent in hydrolysates of cytoplasmic proteins and the salt-extractable HRGP, was not produced when [^{14}C]tyrosine-labeled HRGP was hydrolyzed with large amounts of unlabeled cell wall material, and was not labeled *in vivo* with either [^{14}C]histidine or [^{14}C]lysine (data not shown).

The elution of this tyrosine derivative from phosphocellulose indicates the presence of two amino groups. During high-voltage electrophoresis at pH 1.9, the derivative comigrated with tyrosine, indicating that the compound contained one amino group for each tyrosine mass unit. The derivative had a low mobility at pH 5.9, indicating the presence of equal numbers of amino and carboxyl groups. At pH 10.9, the mobility of the derivative was about 0.4 relative to tyrosine, indicating that at least one of the phenolic hydroxyls was blocked. Ultraviolet absorption spectra of the cell wall tyrosine derivative were obtained under both acid and alkaline conditions. The shift in absorption maximum at pH 13 indicates that at least one tyrosine phenolic hydroxyl could be ionized (Table I).

The absorption maxima of the derivative were different from the maxima of either tyrosine or dityrosine, but were virtually identical to those of isodityrosine, a cell wall tyrosine derivative recently characterized from potato callus (13). Likewise, the absorption maxima of isodityrosine at pH 1 and pH 13 were similar to the absorption extrema of the difference spectrum (pH 13/pH 1) reported for a hydroxyproline-rich cell wall glycopeptide isolated from tomato cell walls containing an "unidentified tyrosine derivative" (7). Based on the similarity in UV absoption and electrophoretic mobility, we conclude

Table I. Ultraviolet Absorption Characteristics of Tyrosine and Tyrosine Derivatives.

	A_{max} (pH 1)	A_{max} (pH 13)
Cell wall derivative	274 nm	298 nm
L-Tyrosine	275	293
Dityrosine	284	317
Isodityrosine[1]	273	298
	A_{max}(pH1/pH13)	A_{max}(pH13/pH1)
Cell wall glycopeptide[2]	275	297

UV absorption spectra of the compounds were obtained by scanning the aborbance from 250 nm to 330 nm at both pH 1 and pH 13 in 1 cm pathlength quartz cuvettes with a Gilford DB spectrophotomenter. The carrot cell wall tyrosine-derivative was isolated from acid hydrolyszates of unlabeled cell walls by phosphocellulose chromatography. Dityrosine, prepared by the method of Gross and Sizer (17), and L-tyrosine (Sigma) were also purified by phosphocellulose chromatography.

[1]from (13)

[2]from (7)

that isodityrosine is formed in carrot cell walls during HRGP insolubilization, supporting a role for this amino acid in HRGP crosslinking (13).

Inhibition of crosslinking, in vivo. Biphenyl ether linkages, similar to that found in isodityrosine (13), are formed in plant cell walls during the peroxidase-catalyzed polymerization of lignin. Extracellular peroxidases also catalyze the formation of structural tyrosine-tyrosine crosslinks in extracellular matrices of several animal systems (16,19,20,21,22,23). Peroxidase inhibitors and free radical scavengers were tested for their ability to inhibit HRGP insolubilization. Carrot discs were pulse-labeled with radioactive proline and chased in the presence of the inhibitors. During the 2 h chase period, about 50% of the initially soluble HRGP was insolubilized in control discs (chased with buffer and proline alone). Iron chelators, which act as noncompetitive peroxidase inhibitors and scavengers of oxygen radicals, inhibited HRGP insolubilization by at least 70% (Table II). Competitive peroxidase inhibitors (glycine ethyl ester and phloroglucinol) inhibited HRGP insolubilization poorly, probably because structural similarities with the natural substrate are required for competitive inhibitory activity. Mannitol, a scavenger of hydroxyl radicals, was without effect. The antioxidants

Table II. Inhibitors of Cell Wall Glycoprotein Crosslinking, in vivo.

Inhibitor	% Insolubilization
Potassium phosphate (pH 6.5), 50 mM	100%
n-Propyl Gallate, 2 mM	21
Potassium Cyanide, 2 mM	32
Phloroglucinol, 25 µM	81
Glycine Ethyl Ester, 250 mM	78
Mannitol, 100 mM	98
Butylated Hydroxyanisole, 1 mM	40
Butylated Hydroxytoluene, 1 mM	50
3,4,5-Trichlorophenol, 0.2 mM	45
L-Ascorbic Acid, 4 mM	7

Root discs were pulse-labeled for 20 min with [2,3-^{3}H]proline and chased for 2 h with 1 mM proline in 50 mM K-phosphate buffer and the inhibitors. Cell walls were prepared and sequentially extracted as described in the materials and methods. About 50% of the soluble wall glycoprotein was insolubilized during this 2 h period in discs chased with proline and buffer only. These results are the average of at least four independent cell wall preparations.

(butylated hydroxyanisole, butylated hydroxytoluene, 3,4,5-trichlorophenol and ascorbic acid), which scavenge superoxide anion and organic radicals, all inhibited crosslinking by at least 50%. Ascorbate was the most effective inhibitor of the compounds tested, which may have physiological significance since plant cell walls contain an ascorbate oxidase with unknown function (1,24). It has recently been reported that ascorbate inhibits crosslinking of elastin in smooth muscle cultures (25). Our results with antioxidants/radical scavengers are in agreement with those of Fry (13), who reported that strong reductant (millimolar dithiothreitol) inhibited both the insolubilization of [^{14}C]tyrosine labeled wall proteins and the formation of isodityrosine in cultured potato cells. The control of HRGP network formation in plant cell walls should provide a challenging and important problem for future work.

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