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The Isolation and Partial Characterization of Hydroxyproline-Rich Glycopeptides Obtained by Enzymic Degradation of Primary Cell Walls*

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With the Technical Assistance of Leon Clark

ABSTRACT: Enzymic degradation of cell walls isolated from suspension cultures of tomato released glycopeptides rich in hydroxyproline. Five glycopeptides accounted for about 20% of the total hydroxyproline, with approximate compositions as follows: (1) $Ara_{25}Gal_6Hyp_{10}Ser_3Tyr$, (2) $Ara_{14}Gal_3Hyp_{10}Ser_3Lys_2Thr,Val$, (3) $Ara_{20}Gal_4Hyp_9Ser_3Lys,Tyr$, (4) $Ara_{18}Gal_4Hyp_9Ser_3Tyr$, and (5) $Ara_{16}Gal_2Hyp_9Ser_3Lys_3Val,Tyr$. The composition and chemical properties of the glycopeptides indicated that the sugar amino acid linkage was a glycosidic link involving the hydroxyl group of hydroxyproline. This was confirmed by alkaline hydrolysis of the glycopeptides and subsequent isolation of hydroxyproline *O*-arabinosides.

In his classical work Heyn (1940) showed that the plant growth hormone auxin increases the plasticity of the primary cell wall and that plasticity is necessary for growth by cell extension.

The chemical basis for these changes in cell wall plasticity is unknown. Recently however I speculated (Lamport, 1965) that these changes might involve the hydroxyproline-rich cell wall protein, provisionally named "extensin," if it acted as a variable cross-link between wall polysaccharides. Two predictions followed: first the existence of a covalent linkage between extensin and the polysaccharides of the wall, and second the existence of *labile* cross-links directly affecting plasticity.

The data presented here verify the first prediction, after suitable enzymic degradation of cell walls we have been able to isolate hydroxproline-rich glycopeptides and identify the carbohydrate–protein linkage.

Our experimental approach involved a search for enzymes which would release hydroxyproline-rich material from isolated primary cell walls, the suitability of any degradation procedure being determined by the amount of peptide-bound hydroxyproline released and by its heterogeneity as judged initially from gel filtration on Sephadex columns. When choosing which cell walls to degrade we aimed for those richest in hydroxyproline and which also released the largest proportion of that hydroxyproline. We used cell suspension cultures because their use ensured the isolation of fairly pure preparations of primary cell walls rich in hydroxyproline.

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Materials

Enzymes were bought from commercial sources as follows. Lot numbers are given when the enzyme is suspected to be a crude mixture.

Cellulase was purchased from Worthington (lot nos. 6533 and 6329), General Biochemicals (lot no. 53985), Fisher (lot no. 741278) and Calbiochem (lot no. 44532).

Meicelase P (a cellulase) was purchased from Meiji Seika Kaisha Ltd. (lot no. CEB008).

Hemicellulase was purchased from General Biochemicals (lot no. 51693).

Pectinase was purchased from General Biochemicals (lot no. 54123).

Takadiastase was purchased from Parke Davis and Co. and from the International Chemical & Nuclear Corp.

Chitinase was purchased from Nutritional Biochemicals (lot no. 8044).

Pronase and *nagarse* (subtilisin) were purchased from Calbiochem.

Chymotrypsin, *collagenase*, *elastase*, *papain*, *pepsin*, and *trypsin* were purchased from the Worthington Biochemical Corp. Sephadex G-25 and G-50 were used in the fine bead form and bought from Pharmacia Fine Chemicals, Inc. Aminex 50W-X2 and Dowex 1-X2 (AG 1-X2 – 400 mesh) (were bought from Bio-Rad Laboratories. L-[1-^{14}C]Arabinose (specific activity 10 mCi/mmole) was bought from Calbiochem.

Tritiated L-proline (generally labeled; specific activity 403 mCi/mmole) was bought from Schwarz BioResearch, Inc. Sodium hypobromite was prepared as a stock solution by adding 3.2 ml of Br_2 to 500 ml of ice-cold 5% NaOH. This stock solution was allowed to remain 1 week at 4° before preparation of the dilute solution as needed; 17.5 ml of the stock solution was mixed with 82.5 ml of cold 5% NaOH and stored at 4° for 12 hr before use.

Methods

Cell Culture. We grew cells of tomato (*Lycopersicon esculentum* Mill. Var. Bonny Best), sycamore (*Acer pseudoplatanus* L.), potato (*Solanum tuberosum* L.), and flax (*Linum perenne* L.) as previously described (Lamport, 1964). Cell cultures of tobacco (*Nicotiana tabacum* L.) were a gift from Dr. P. Filner, of this Laboratory.

Isolation of primary cell walls was as described previously (Lamport, 1965).

Removal of Pectic Substances from the Walls. To reduce the possibility of carbohydrate interfering with the hydroxyproline assay, we generally boiled walls overnight in distilled water and washed them two or three times with cold water before enzymic degradation.

Enzymic Degradation. Walls (4–5 mg of dry weight/ml) were incubated at 37° overnight with the appropriate enzyme in a Radiometer pH-Stat.

Cellulase Preparation. A stock solution was prepared by mixing the enzyme powder with distilled water (50 mg/ml) to form a suspension. This was shaken gently overnight at 4° and then centrifuged for 30 min at 15,000 rpm. The supernatant was used as the enzyme source ("50 mg/ml of cellulase") and stored as 1-ml samples at −10°.

Fractionation of Enzymic Digest. After enzymic treatment we centrifuged the wall suspension at 15,000 rpm and evaporated the supernatant to dryness in a rotary evaporator (with the heating bath at 45–50°), finally taking up the dry material in 0.5 ml of 0.1 M acetic acid. We carried out the initial fractionation on a Sephadex G-25 (fine bead form) column (120 × 1 cm) equilibrated with 0.1 M acetic acid. Fractions of 1–2 ml were collected. After gel filtration we pooled appropriate fractions, dried them from the frozen state, and usually further fractionated them on an Aminex column (20 × 1 cm) H^+ form using a pyridine–formate gradient. Again appropriate fractions were pooled and refractionated on a Dowex 1-X2 column (60 × 1 cm) using the pyridine–*N*-ethylmorpholine–α-picoline buffer system described by Schroeder and Robberson (1965). Figure 1 summarizes the fractionation procedures.

Hydrolysis of Peptide Fractions. A. HCl (6 N) HYDROLYSIS. For accurate amino acid detection and estimation we mixed 10–50 nmoles of peptide with 200 μl of redistilled constant-boiling HCl in a small glass tube which was evacuated, then sealed, and heated for 18 hr at 105°. We removed the HCl rapidly at 40°. If a peptide contained tyrosine we generally added 5 μl of 0.1 M phenol before hydrolysis, to reduce tyrosine degradation according to the method of Sanger and Thompson (1963). Acid hydrolysis was always used when estimating the hydroxyproline content of a glycopeptide. Control experiments indicated that 20% of the serine was destroyed during acid hydrolysis.

B. H_2SO_4 (1 N) HYDROLYSIS. Prior to chromatographic identification of sugars, 10–50 nmoles of glycopeptide was mixed with 500 μl of 1 N H_2SO_4 in a small glass tube which was then sealed and autoclaved at 15 psi and 121° for 1 hr. The hydrolysate was passed through an AG 1-X8 ion-exchange column (2 × 1 cm) (acetate form) to remove sulfate ions as described by Ray (1963).

C. NaOH (2.5 N) HYDROLYSIS. For a rapid approximate estimation of hydroxyproline in column eluates we hydrolyzed peptide samples with 1 ml of 2.5 N NaOH in unstoppered glass test tubes (18 × 150 mm) and heated for 3.5 hr in a 90° water bath (Hirs *et al.*, 1956). After hydrolysis we neutralized by adding *a slight excess* of 2.5 N HCl to each test tube, and then estimated hydroxyproline using a salt-insensitive method (Kivirikko, 1963).

D. $Ba(OH)_2$ (0.44 N) HYDROLYSIS. To obtain partial alkaline hydrolysis, the glycopeptides were heated at 105° with 0.44 N $Ba(OH)_2$ in a sealed tube for 6 hr. The hydrolysate was neutralized with H_2SO_4, centrifuged, and evaporated to a small volume.

Amino Acid Estimation. A. ON PAPER. After 6 N acid hydrolysis and removal of acid as described above we separated the amino acids in two dimensions and developed the paper by the method of Heilmann *et al.*, (1957). The first dimension was electrophoresis on Whatman No. 4 paper (45 × 10.5 in.) 7 kV for 70 min using a pH 1.9 buffer (8.7% formic–2.5% acetic in water, v/v). In the second dimension we chromatographed the paper 18 hr, using the epiphase prepared from a 1:1 mixture

TABLE I: Percentage Distribution of Hydroxyproline-Rich Material in Sephadex G-25 Fractions Obtained by Enzymic Degradation of Various Cell Walls.[a]

	Cellulase				Pronase			
	% Wt Lost from Wall	% Wall Hyp Released	% of Total Wall Hyp in G-25 Peak I	% of Total Wall Hyp in G-25 Peak II	% Wt Lost from Wall	% Wall Hyp Released	% of Total Wall Hyp in G-25 Peak I	% of Total Wall Hyp in G-25 Peak II
Tomato	55	70	50	20	17	41	26	15
Tobacco	66	75	46.5	28.5	21	50	29	21
Potato	28	5.5	2.6	2.9	7	7	4.3	2.7
Sycamore	42	25			0[b]	16		
Linum	50	25	14.3	10.7	0	26		

[a] Boiled walls, *i.e.*, with pectic substances removed. [b] The weight of the walls actually increased after incubation, apparently due to adsorption of pronase.

of *t*-amyl alcohol and buffer. The buffer was 5% pyridine–0.6% *N*-ethylmorpholine (v/v), adjusted to pH 8.2 with acetic acid.

B. AUTOMATIC AMINO ACID ANALYSIS. We used a modified Technicon Autoanalyser where the colorimeter was replaced by a Gilford Model 300 spectrophotometer plus flow-through cell. The spectrophotometer output was fed to a Sargent Model TR recorder. Elution and development details were according to standard Technicon procedures, and allowed the estimation of 5–100 nmoles (±5%) of a single amino acid. For hydroxyproline and proline the sensitivity was about one-tenth of that for the other amino acids.

Sugar Chromatography and Electrophoresis. For routine sugar separation we used an ethyl acetate–pyridine–water (8:2:1, v/v) single-phase solvent (Timell, 1960). We detected sugars by use of the aniline phthalate dip (Wilson, 1959) or the silver nitrate reagent (Trevelyan *et al.*, 1950). Sugar identification was checked by electrophoresis in 50 mM borate buffer (Frahn and Mills, 1959).

Arabinose Estimation. We used the ferric chloride orcinol method for pentoses (Dische, 1962).

Galactose Estimation. We estimated galactose enzymically, using Galactostat, a preparation of galactose oxidase obtained from the Worthington Biochemical Corp.

NH_2-Terminal Identification and Estimation. We identified the NH_2-terminal amino acids of the glycopeptides by the dansylation technique (Gray and Hartley, 1963). When possible we checked the identification of the NH_2 terminus by a subtractive method in which the peptide composition is determined before and after cyanoethylation with acrylonitrile (Fletcher, 1966). The cyanoethylation method allowed direct estimation of the amount of a given amino acid which existed as NH_2 terminal, excluding lysine.

Radioactivity Measurements. Radioactivity of samples taken from column eluates was monitored in a three-channel Beckman Model 1650 liquid scintillation spectrometer. The composition of the scintillation fluid was: napthalene, 200 g/l.; phenylbiphenylyloxadiazole-1,3,4, 9 g/l.; 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, 250 mg/l.; and 1,4-dioxane to make 1 l.

Sodium borohydride reduction of glycopeptides was by the method described by Edstrom and Heath (1965). We fractionated the reaction mixture on a G-25 Sephadex column (120 × 1 cm).

Performic acid oxidation was by the method of Schram *et al.*, (1954).

Attempted reaction of glycopeptide with [^{14}C]potassium cyanide was by the method of Moyer and Isbell (1958).

Attempted Alkaline β Elimination of Serine. We mixed 250 μl of glycopeptide with 250 μl of 1 N NaOH in an ice bath and evacuated the tube for 10 min at the water pump. After 18 hr at 2–4° we neutralized by adding 250 μl of 1 N HCl and finally adjusted the pH to 6.5, and dried the material from the frozen state. Then we added 0.5 ml (5 mg) of insulin to act as a marker and fractionated the mixture on a G-25 Sephadex column.

Attempted Detection of Hexosamine. Approximately 20 nmoles of each glycopeptide was hydrolyzed with 6 N HCl in a sealed tube at 105° for 8 hr. After removal of HCl in a vacuum desiccator we separated the hydrolysates electrophoretically at pH 1.9 using 0.5 μg of glucosamine as a marker. We developed the paper with the alkaline silver nitrate reagent.

Results and Conclusions

Isolation of Hydroxyproline-Rich Glycopeptides from Tomato and Sycamore Cell Suspensions. Sycamore walls were first boiled under reflux overnight to remove pectic substances and were then treated with either trypsin, chymotrypsin, pronase, subtilisin, pepsin, elastase, or collagenase. The solubilized material was fractionated on Sephadex G-25 or G-50, and fractions were assayed for hydroxyproline. Despite earlier promising results (Lamport, 1965), proteases released only small amounts of hydroxyproline-rich material from sycamore walls (Table I).

As resistance to *proteases* might result from the large amount of hydroxyproline in the molecule and/or the presence of carbohydrate, we examined the ability

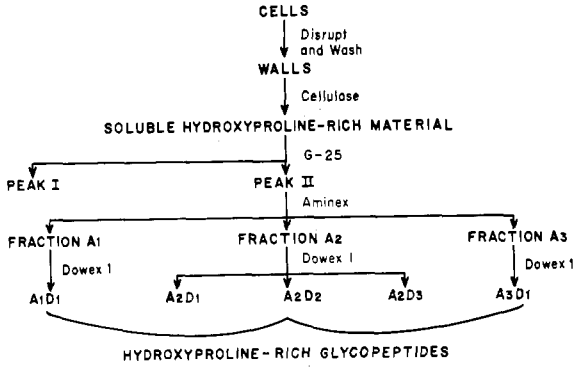

FIGURE 1: Summarized protocol for the isolation of hydroxyproline-rich glycopeptides from tomato cell walls.

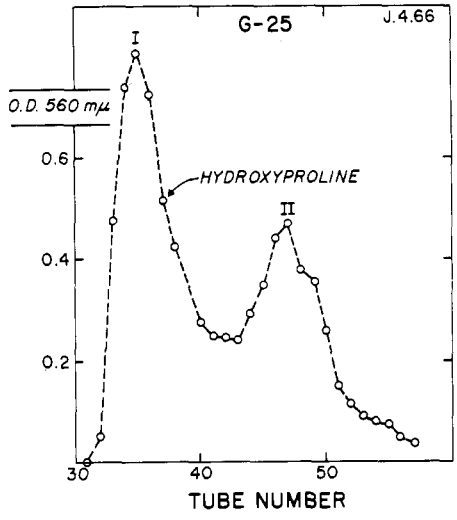

FIGURE 2: Tomato walls incubated with crude cellulase; gel filtration of the hydroxyproline-rich material released. Previously boiled tomato walls (470 mg dry weight) were incubated with 94 mg of Worthington cellulase at pH 4.5 and 27 overnight. The soluble material was fractionated on a Sephadex G-25 column (120 × 1 cm). The fraction volume was *ca.* 1 ml of which 20 μl was used for the hydroxyproline assay. With the same assay conditions 10 μg of polyhydroxyproline gave an optical density of 0.360. The G-25 column (120 cm long) used throughout these experiments had an exclusion volume of 34.5 ml and a retention volume of 70.5 ml. The code in the upper right-hand corner of each graph indicates the approximate date of the experiment. Thus J-4-66 for example signifies that this was experiment J in April 1966.

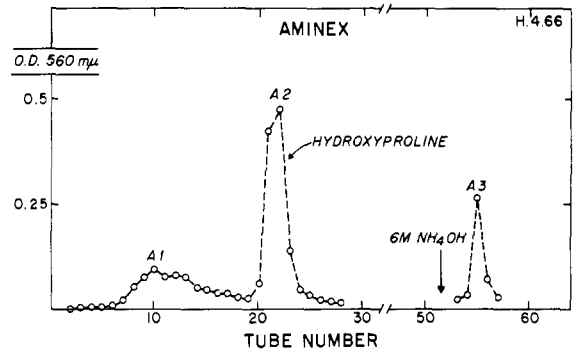

FIGURE 3: Further Aminex fractionation of cellulase-released G-25 peak II tomato hydroxyproline-rich material. Peak II from the G-25 fractionation was fractionated further on an Aminex column (H^+ form) eluted with a pyridine formate gradient (100 ml of 0.2 M pH 3.3 buffer in mixing chamber and 100 ml of 0.4 M pH 5.3 in the reservoir).

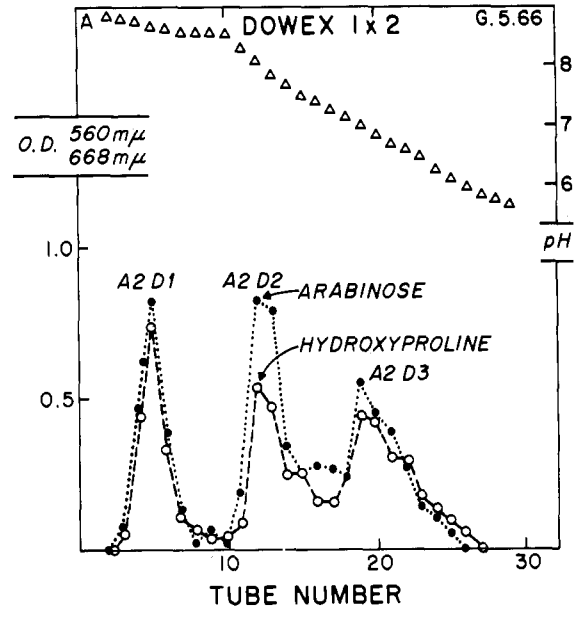

FIGURE 4: Further fractionation of Aminex fraction 2 (tomato Cel G-25 II A2) on Dowex 1-X2. Fraction A2 was fractionated further on a Dowex 1-X2 column (30 × 1 cm) using the buffer system described by Schroeder and Robberson (1965). The glycopeptide peaks are designated as follows: Cel G-25 II A2D1, Cel G-25 II A2D2, and Cel G-25 II A2D3 (abbreviated as A2D1, A2D2, and A2D3 in the tables).

of *carbohydrases* to release hydroxyproline-rich material from sycamore walls, bearing in mind that the commercially available preparations are relatively crude compared with most of the proteases available. Pectinase, hemicellulase, chitinase, takadiastase, and Meicelase released only negligible amounts of hydroxyproline-rich material from sycamore walls, but certain crude cellulases (notably Worthington, GBI, and Fisher) released significant amounts of hydroxyproline-rich material which could be fractionated reproducibly on Sephadex G-50. Cellulase released only 10–25% of the sycamore wall hydroxyproline, but 70–75% of the hydroxyproline from tomato or tobacco walls (Table I). Therefore we used tomato walls for most of the work described here.

Two-thirds of the soluble hydroxyproline-rich material released by the action of cellulase on tomato walls was not retarded on Sephadex G-25 (Figure 1) but gave a reproducible elution pattern when fractionated on Sephadex G-50. The remaining third appeared as a single retarded peak (peak II on Sephadex G-25) (Figure 2). This G-25 elution pattern was similar in several different species examined.

TABLE II: Composition of Tomato Glycopeptides Purified from the Sephadex G-25 Peak II Fraction.

Glycopeptide	Number of Residues[c]				
	A1D1[e]	A2D1	A2D2	A2D3	A3D1
Hyp	10.4 (10)	10.2 (10)	8.9 (9)	8.9 (9)	9 (9)
Ser	3 (3)	2.4 (3)	3.0 (3)	3 (3)	2.6 (3)
Lys		1.6 (2)	1 (1)		3 (3)
Thr		0.7 (1)			
Val		1 (1)			1 (1)
Tyr	0.6 (1)	0.2	0.5 (1)	0.4 (1)	0.2 (1)
Tyr[d] (from ultraviolet spectrum)	2.5	0	2.5	2.7	4.5
Ara	25	14	20	18	16
Gal	6	3	4	4	2
N Terminus	Ser[a]	Lys[b]	Lys[b]	Ser[a]	Lys[b]
Molecular weight	5826	4281	4857	4465	4360

[a] Estimated by subtractive cyanoethylation. [b] Identified by dansylation. [c] Acid hydrolysates of four glycopeptides gave two small peaks of unknown identity. These unknowns were absent from A2D1. [d] Using $\epsilon_{295 m\mu}$ 2.33 × 10^3. [e] In a control experiment we took free amino acids and sugars in the same molar proportions as found in glycopeptide A1D1 and subjected them to hydrolysis conditions. Hydroxyproline was unchanged, serine decreased 19%, and tyrosine decreased 61%. No unknowns were observed.

Further separation of G-25 II on Aminex yielded three major peptide fractions designated A1, A2, and A3 (Figure 3). Each of these fractions also yielded arabinose and galactose after acid hydrolysis followed by paper chromatography. A control experiment in which the crude cellulase preparation was taken through the same separation procedure showed that although carbohydrate was present in the enzyme preparation, its elution behavior was quite different from the hydroxyproline-rich compounds. Experiments both with unlabeled and labeled material gave chromatograms showing an excellent correspondence between the hydroxyproline and arabinose peaks, indicating that we were dealing with hydroxyproline-rich glycopeptides.

Using modifications of the volatile buffer systems (Schroeder and Robberson, 1965) we fractionated each Aminex peak on Dowex 1-X2. Fractions A1 and A3 gave only one major glycopeptide on Dowex 1, whereas fraction A2 yielded several glycopeptides which have varied considerably in relative proportions from run to run (Figure 4). Figure 1 summarizes the experimental procedures involved in the isolation of the glycopeptides. Table II shows the sugar and amino acid composition of the five major glycopeptides. For comparison Table III shows the amino acid composition of the cell wall.

Table I shows that the purified glycopeptides from tomato walls account for about one-third of the hydroxyproline released by enzymic degradation (*i.e.*, about 20% of the *total* wall hydroxyproline). As to the other two-thirds, namely G-25 peak I, it was, as mentioned above, possible to fractionate this peak further by gel filtration on Sephadex G-50. A Sephadex

TABLE III: Amino Acid Composition of Cell Walls Isolated from Tomato Cells (var. "Bonny Best") Grown in Suspension Culture.[a]

	Residues/10^5 g of Protein
Hyp	202
Asp	52
Thr	38
Ser	100
Glu	59
Pro	56
Gly	53
Ala	46
Val	53
Met	11
Ile	33
Leu	58
Tyr	23
Phe	23
Lys	70
His	16
Arg	26

[a] The walls were hydrolyzed with 6 N HCl at 105° for 18 hr in a sealed tube from which air had been removed. The amino acid analysis was obtained by the use of a modified Technicon Autoanalyzer.

TABLE IV: The Tomato Primary Cell Wall: Sugar Molar Ratios and Pentose Content.

Sugar:	Galactose	Glucose	Mannose	Arabinose	Xylose	
Molar Ratio[a]:	4.6	10	2.2	4.2	1.8	
Pentose content of dried cell walls[b]						14%
Arabinose						9.8%
Xylose						4.2%

[a] Determined by paper chromatography (Wilson, 1959). [b] Estimated by the ferric chloride orcinol method (Dische, 1962).

G-50 fractionation of tomato G-25 peak I material labeled with [^{14}C]arabinose and [^{3}H]proline (to discriminate from contaminants in the crude cellulase) showed a remarkably close correspondence between ^{14}C and ^{3}H. As we observed no significant labeling of cell wall sugars after growing tomato cells in the presence of [^{14}C]proline, and as we also found that cells grown in the presence of L-[^{14}C]arabinose showed no significant labeling of the cell wall amino acids we conclude that the ^{3}H- and ^{14}C-labeled material obtained by fractionation on Sephadex consists exclusively of glycopeptides. As there was no evidence of an equilibrium mixture of glycopeptides, peptides, and oligosaccharides we ruled out the possibility that the glycopeptides were artifacts arising during enzymic degradation. The most dramatic conclusion is that enzymic degradation of the wall protein gives *glycopeptides exclusively*.

Experiments with pronase reinforce these conclusions. Pronase released substantial amounts of hydroxyproline-rich material from tomato walls (Table I). Further fractionation of this hydroxyproline-rich material also yielded distinct glycopeptide fractions. On the other hand pronase did not release free sugar or oligosaccharides. Cellulase, of course, always releases sugar monomers from the walls, and is the most efficient enzymic agent yet found for the release of hydroxyproline-rich material. Thus while pronase-treated sycamore walls released insufficient hydroxyproline-rich material for further fractionation, cellulase released enough hydroxyproline-rich material for fractionation and subsequent isolation of a glycopeptide ($Hyp_{11}Ser_4Lys_2ValTyrAra_{31}$). From these results it is clear that cellulase also releases hydroxyproline-rich glycopeptides from sycamore walls.

Molecular Size and Composition of the Purified Glycopeptides Obtained by Degradation of Tomato Cell Walls with Cellulase. The purified glycopeptides obtained from tomato walls were retarded on passing through Sephadex G-25.

A comparison of glycopeptide A1D1 with bovine insulin on a Sephadex G-25 column showed an almost identical retardation and hence probably similar molecular weights. The minimum molecular weights of the glycopeptides, judging from their composition, (Table II) vary from 3900 to 5700.

In two of the glycopeptides we checked the molecular weight assignments by estimating the NH_2 termini *via* a subtractive method in which the composition of the peptide was determined before and after cyanoethylation of free amino groups by reaction with acrylonitrile. Two of the glycopeptides lost one-third of the serine after reaction with acrylonitrile showing that these glycopeptides contain three serine residues. These data thus support the minimum molecular weight assigned. The other three glycopeptides contain NH_2-terminal lysine as determined by the dansylation procedure. For these three glycopeptides therefore we did not use the subtractive method to estimate the NH_2 terminus because *all* the lysine disappeared on cyanoethylation. However, the similarities between glycopeptide composition and behavior on gel filtration indicate a similar molecular weight for *all* the glycopeptides listed in Table II.

We based our identification of the sugars mainly on their electrophoretic and paper chromatographic properties but other data are consistent. For example

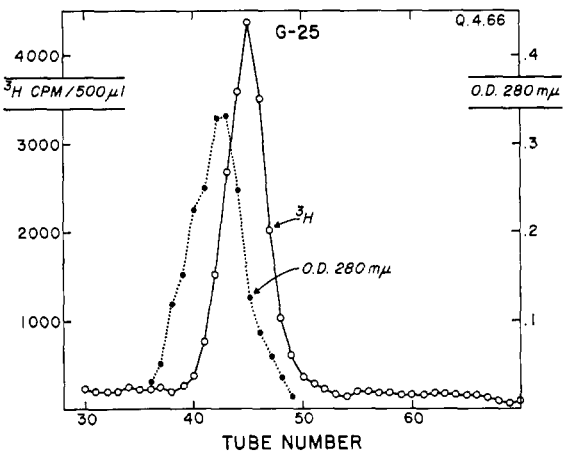

FIGURE 5: The effect of 0.5 N NaOH on tomato glycopeptide Cel G-25 II A1D1. A 250-μl sample of ^{3}H-labeled glycopeptide was mixed with 250 μl of 1 N NaOH (ice cold) and evacuated 10 min at the water pump. After 18 hr at 4°, 250 μl of 1 N HCl was added to the reaction mixture and the pH was finally adjusted to 6.5. The material was then frozen and dried *in vacuo*. Insulin (5 mg in 0.5 ml) was added, and the mixture was then fractionated on a Sephadex G-25 column (120 × 1 cm). The fraction volume was *ca.* 1 ml of which 500 μl was taken for ^{3}H assay in a liquid scintillation counter. The alkaline treatment did *not* alter the retardation of the glycopeptide relative to insulin; a control experiment with untreated glycopeptide showed the same separation of peaks.

TABLE V: Hydroxyproline Arabinoside Molar Ratios in Glycopeptides after Alkaline Hydrolysis.[a]

	Glycopeptide				
	A1D1	A2D2	A2D3	A3D1	Walls
	No. of Hydroxyproline Residues Substituted with				
Ara_4	1	1	2	0	8
Ara_3	2	2	1	1	3
Ara_2	4	4	4	2	4
Ara_1	3	4	0	5	1
	(A) From above Data				
Hyp:Ara	$H_{10}A_{21}$	$H_{11}A_{22}$	H_7A_{19}	H_8A_{12}	$H_{16}A_{50}$
	(B) From Direct Estimation of Hyp and Ara				
Hyp:Ara	$H_{10}A_{25}$	H_9A_{20}	H_9A_{18}	H_9A_{16}	

[a] After hydrolysis of the glycopeptides in $Ba(OH)_2$, the hydroxyproline arabinosides were separated (Lamport, 1967) on an Aminex AG 50W-X2 column (30 × 1 cm) H^+ form eluted with a pH gradient (400 ml of H_2O in mixing chamber and 400 ml of 0.2 N HCl in the reservoir). The arabinosides were detected by automated analysis of the column effluent (methods to be published) from which the molar proportions of the arabinosides were obtained and are rounded to the nearest whole number. The precise arabinoside molar ratios were then calculated using the number of hydroxyproline residues per glycopeptide (Table II) as a guide to the maximum number of arabinosides in any given glycopeptide.

the results with galactose oxidase (galactostat from the Worthington Corp.) also indicate the presence of D-galactose after acid hydrolysis of the glycopeptides. We also based our identification of arabinose on the fact that L-[^{14}C]arabinose was an excellent precursor to the glycopeptides. After acid hydrolysis, chromatography and autoradiography showed ^{14}C label exclusively and matching identically with the arabinose region of the chromatogram. Both galactose and arabinose are well established as major components of primary cell walls (*cf.* Northcote, 1958). Arabinose accounts for about 10% of the dry weight of the tomato walls studied here (Table IV). The partially characterized glycopeptides contain approximately 2 moles of arabinose/mole of hydroxyproline (averaging the data in Table II) and account for one-fifth of the wall hydroxyproline which itself is about 2% of the wall dry weight. Thus the arabinose of these glycopeptides accounts for approximately 1% of the dry weight of the wall. On a similar basis the galactose of the glycopeptides accounts for about 0.2% of the wall dry weight.

The Sugar–Amino Acid Linkage. Of all the amino acids, only hydroxyproline and serine are invariably present in each glycopeptide (Table II). The carbohydrate–protein linkage therefore involves hydroxyproline or serine. Weak alkali (0.5 *N* KOH at 4°) which specifically cleaves, by a β-elimination reaction, glycosidic linkages involving the hydroxyl group of serine in peptide linkage (Adams, 1965a,b; Anderson *et al.*, 1965), neither released the protein from intact walls nor degraded glycopeptide A1D1 (Figure 5). A glycosidic link through the hydroxyl group of serine is therefore most unlikely, although the *O*-glycosyl linkage of an NH_2-terminal serine residue would be stable in weak alkali.

As two of the glycopeptides have NH_2-terminal serine in stoichiometric amounts; the linkage is not an NH_2-terminal *N*-glycosidic type. Reduction with sodium borohydride did not degrade glycopeptide A1D1, again judging from gel filtration on Sephadex G-25. This excludes a glycosidic ester type of linkage involving a C-terminal carboxyl group.

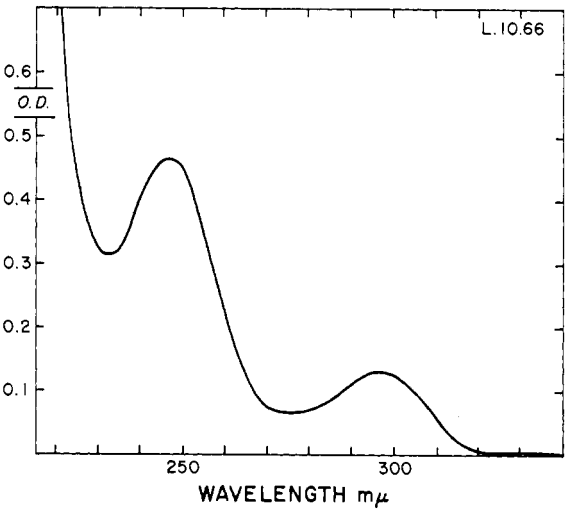

FIGURE 6: Ultraviolet difference spectrum of tomato glycopeptide (Cel G-25 II A1D1). The reference cuvet contained the glycopeptide in 1 ml of 0.1 N HCl. The sample cuvet contained the glycopeptide in 1 ml of 0.1 N NaOH. The cuvet path length was 1 cm and the glycopeptide concentration was calculated from the hydroxyproline content (164 nmoles of Hyp/ml) was 16.4 nmoles/ml.

Figure 6 illustrates the ultraviolet absorption difference spectrum of the glycopeptide A1D1 in acid vs. alkali. The absorption peaks at 295 and 245 mμ in-

dicate that the tyrosine hydroxyl group is free to ionize and does not participate in a covalent linkage.

A sugar–amino acid *ether* linkage is unlikely in view of the fact that we have been unable to detect a sugar reducing end group in the glycopeptide (there was no reaction with [^{14}C]KCN to form the cyanhydrin, and the glycopeptide on paper showed little or no reaction when treated with the alkaline silver nitrate reagent), and because acid hydrolysis degrades the glycopeptide to its monomer constituents. From these data we concluded that the most likely sugar–amino acid linkage is a glycosidic linkage through the hydroxyl group of hydroxyproline.

This was confirmed by partial alkaline hydrolysis of each glycopeptide with 0.44 N $Ba(OH)_2$ followed by separation and identification of hydroxyproline arabinosides similar to those which have recently been obtained by direct alkaline hydrolysis of cell wall preparations (Lamport, 1967).

By electrophoretic and column chromatographic comparison of the alkaline hydrolysis products from the glycopeptides with those obtained directly from cell walls as reported earlier we were able to estimate the approximate number of arabinose residues attached to each hydroxyproline residue in a given glycopeptide (Table V). The total number of arabinose residues obtained in this way for each glycopeptide agrees reasonably well with the number obtained by direct estimation with the exception of glycopeptide A3D1 which was available only in small amounts.

Judging from the very weakly reacting hydroxyproline areas obtained after paper electrophoresis of the alkaline hydrolysates it looks as though there is almost complete glycosylation (say nine out of ten) of the hydroxyprolyl residues in the glycopeptides. There is some doubt about the precise number of hydroxyproline residues in glycopeptide A2D2 as the arabinoside data indicate two more residues than were found by direct estimation of hydroxyproline.

Discussion

Although enzymic methods of degradation have been used to great effect in the analysis of bacterial and fungal cell walls, suprisingly few workers have utilized this powerful approach to study cell walls of higher plants. At the outset there is the problem of deciding whether to purify enzymes or use the available crude preparations. We adopted the latter approach, especially in view of the possibility that release of peptides might require two enzymes, a protease and carbohydrase, as Tipper and Strominger (1966) reported for some bacterial cell walls.

Hence it was not suprising to find that a crude cellulase preparation released glycopeptides. Because the cellulase also released free amino acids as well as sugars from suitably labeled walls (unpublished experiments) we conclude that the enzyme is a mixture of proteases and carbohydrases. Precisely which of these activities releases the glycopeptides is not evident from these data. However, pronase also releases hydroxyproline-rich glycopeptides from the cell wall, yet (judging from the lack of sugar monomers released) pronase shows no obvious carbohydrase activity. Thus all or most of the glycopeptides were probably released by the proteolytic activity of the cellulase preparations used. The 30–50% of the tomato wall bound hydroxyproline which is *not* released by cellulase or pronase, is mostly released by treatment with periodic acid (unpublished experiments) and therefore probably represents highly cross-linked material.

We have tacitly assumed throughout this paper that extensin is indeed a protein and that the hydroxyproline-rich glycopeptides represent cleavage products of the macromolecule. However, it is possible to construct several different macromolecular models from the data given here. Perhaps the two models which are most worthwhile considering are backbone side-chain models. Thus model A would consist of a polysaccharide backbone with the peptide side chains (as in the bacteria; *cf.* Strominger, 1965) and model B would consist of a polypeptide backbone with oligosaccharide side chains. Model A is difficult to reconcile with the data obtained from the use of pronase; model B, on the other hand, is quite consistent with all the data, expecially the evidence reviewed recently that extensin *is* a protein (Lamport, 1965). Also supporting this conclusion are data (Holleman, 1967) showing that cycloheximide inhibits the incorporation of [^{14}C]proline into cytoplasmic protein and extensin. Holleman also pointed out that at tracer levels, hydroxyproline is not a *direct* precursor to *protein*-bound hydroxyproline (including extensin), whereas direct incorporation of hydroxyproline into the hydroxyprolyl *peptide* actinomycin I, occurs readily (Katz *et al.*, 1962). The inference is that ribosomes mediate the biosynthesis of extensin.

All available data therefore indicate that extensin is a protein, and, judging from recent work (Crook and Johnston, 1962; Bartnicki-Garcia, 1966; Thompson and Preston, 1967; Punnett and Derrenbacker, 1966; Gotelli and Cleland, 1968) widely distributed throughout the plant kingdom. Probably the plant extensins represent a class of structural proteins much the same way as the collagens constitute a class of structural proteins in animals. An interesting finding in relation to extensin although of unknown significance, arises from the recent demonstration (Shannon *et al.*, 1966) that some horseradish peroxidase isozymes contain hydroxyproline; some do not. All the isozymes are glycoproteins and contain various sugars. However, those isozymes which contain hydroxyproline also contain galactose and arabinose. Those isozymes which have no hydroxyproline have neither galactose nor arabinose. Quite possibly the hydroxyproline-galactose-arabinose components of a peroxidase isozyme represent an extensin subunit which allows the peroxidase to be covalently linked with the cell wall, and this view is supported by current work in which hydroxyproline *O*-arabinosides have been obtained from those peroxidase isozymes which contain hydroxyproline (Liu and Lamport, 1968).

The implication of hydroxyproline in a carbohydrate–protein linkage is novel but entirely consistent, not only with our previous data and our data presented

here, but also with the recent isolation (Boundy *et al.*, 1967) of a hydroxyproline-rich protein polysaccharide complex which was extracted by chemical degradation (hot 15% trichloracetic acid) of *Zea mays* pericarp. These workers noted that an enzyme resistant core of the complex contained hydroxyproline, serine, threonine, and glucosamine. They concluded that: "It is possible that they serve a functional purpose in the carbohydrate–amino acid linkage." From the alkali stability of the protein polysaccharide complex these workers also favored a glycosidic rather than an ester type of carbohydrate–amino acid linkage. If further work confirms the conclusions drawn here then the role of extensin hydroxyproline at the chemical level is clearly to provide carbohydrate–protein links in a non-α-helical polypeptide. Thus the hydroxyproline is far from being a relatively unimportant mere distinguishing feature of the primary cell wall protein.

The role of extensin at the biological level is of course still speculative and must involve a consideration of the glycoprotein *per se*. It is important to note (and relevant to hypotheses dealing with the control of cell extension) that proline and cysteic acid (after oxidation) are absent from the cell wall glycopeptides characterized up to the present time. The present fragmentary glycopeptide data also indicate that tomato extensin contains about 50% protein and at least 50% carbohydrate. Taking as a rough estimate based on the amino acid analysis of tomato walls that 30% of the amino acid residues by weight are hydroxyproline, then a cell wall containing 2% hydroxyproline (dry weight basis) must consist of about 7% protein, or about 14% glycoprotein. Another estimate can be made if we assume that pronase specifically releases extensin from tomato cell walls. The data (Table I) show that pronase released about *half* of the hydroxyproline with a concomitant 17% loss of cell wall dry weight, which indicates that extensin makes up about 22% of the intact (unboiled) tomato cell walls.

These primary cell walls from tomato cultures thus contain nearly as much glycoprotein as α-cellulose.

Extensin is then a major component of these primary cell walls. It may exist as a highly cross-linked macromolecule which with the cross-links under cellular control could provide a chemical basis for changes in cell wall plasticity. Which linkages are labile and *how* auxin might lead to their rupture are questions for future experimentation.

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