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Cell Surfaces in Plant-Microorganism Interactions

I. A STRUCTURAL INVESTIGATION OF CELL WALL HYDROXYPROLINE-RICH GLYCOPROTEINS WHICH ACCUMULATE IN FUNGUS-INFECTED PLANTS[1]

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ABSTRACT

Infection of muskmelon *Cucumis melo* seedlings by the fungus *Colletotrichum lagenarium* causes a 10-fold increase in the amount of cell wall hydroxyproline-rich glycoprotein. Evidence for this increase was provided by studying two specific markers of this glycoprotein, namely hydroxyproline and glycosylated serine. The lability of the *O*-glycosidic linkage of wall-bound glycosylated serine in the presence of hydrazine, was used to determine the amount of serine which is glycosylated.

A large increase in the hydroxyproline content of infected plants is shown, but the ratios of glycosylated serine to hydroxyproline are similar in healthy and infected plants. As far as these markers are concerned, the hydroxyproline-rich glycoproteins secreted into the wall as a result of the disease are similar to those of healthy plants. In addition, the extent of glycosylation of the wall serine, in both healthy and infected plants, decreases as the plant ages.

Serine- and hydroxyproline-rich (glyco)peptides were also isolated after trypsinolysis of the wall. These (glyco)peptides include the galactosyl-containing pentapeptide, serine-hydroxyproline$_4$. This pentapeptide is characteristic of cell wall protein.

Hydroxyproline is an integral component of plant cell wall macromolecules (2, 7, 25, 26, 31, 32). It can account for up to 20% of the wall amino acid residues in suspension cultured cells and is found in a hydroxyproline-rich glycoprotein (HRGP)[2] that has been called "extensin" (19, 20). Most of the hydroxyproline residues of this glycoprotein have *O*-glycosidically attached oligosaccharides consisting of from one to four L-arabinofuranosyl residues (1, 20). The serine residues frequently have a single galactosyl residue *O*-glycosidically bound to them (24). The glycosylated hydroxyprolyl and seryl residues are often found in the sequence -Ser-Hyp$_4$- which occurs with a high frequency in wall peptides (23).

Some aspects of the biosynthesis (4, 5, 27) and secretion (6) of this glycoprotein have also been studied.

The present paper partially characterizes the macromolecules responsible for the 10-fold increase in hydroxyproline which occurs in the cell walls of fungal infected melon plants (10, 28). This increase was formerly shown to be coincident with an increase in hydroxyproline arabinosides (12), to be dependent on protein synthesis (11) and paralleled by a concomitant enrichment of the wall in proteins (10, 28). These findings suggested that the synthesis of HRGP is enhanced in diseased plants. The possibility that these molecules could be unique was raised by their higher extent of hydroxyproline glycosylation than in healthy plants (12).

For glycosylation of a protein to occur, the glycosyl transferases must recognize a sequence of amino acids (34). The experiments reported below were designed to determine whether the observed increased glycosylation of hydroxyproline might reflect a modification in the sequence of the amino acids at the glycosylation sites. Analyses were undertaken of the amino acid residues known to be in the vicinity of the hydroxyproline arabinosides of the wall. Hydroxyproline, serine, and particularly the ratios of glycosylated serine to total hydroxyproline were thus determined during the course of the disease and during natural aging. Hydroxyproline-rich (glyco)peptides were also isolated, with the aim of determining their amino acid sequence.

MATERIALS AND METHODS

PLANT MATERIAL

Melon seedlings (*Cucumis melo*, variety Cantaloup charentais) were grown in a greenhouse, sprayed at the two-leaf stage with a suspension of *Colletotrichum lagenarium* conidia and harvested 1 to 7 days later. Details of the whole procedure have been given in a previous paper (9). The disease symptoms appeared 3 to 4 days after the seedlings had been sprayed with the pathogen; numerous necroses then developed on the leaves and stems, leading to death of the seedlings about 4 days later. Healthy plants were simultaneously obtained when controls were necessary. Resistant varieties of *C. melo* to this pathogen are not available at the present time.

The plant material was used either directly or after storage at −20 C. All extractions were performed on the whole stem and petioles, or parts of them: hypocotyl, or epicotyl + petioles.

CELL WALL PREPARATION

After plasmolysis of the tissues in 1 M sucrose for 2 h and subsequent homogenization, a cell wall extract was made by successive low speed centrifugation at 500*g* of the homogenates and resuspensions of the pellet in phosphate buffer, Triton X-100,

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[2] Abbreviations: HRGP: hydroxyproline-rich glycoprotein; -Ser-Hyp$_4$-: serine-hydroxyproline-; ATZ: phenylthiazolinone derivative; PTH: phenylthiohydantoin derivative; *N-O*-BSA: *N-O*-bis(trimethylsilyl)acetamide; TMS derivatives: trimethylsilyl derivatives; He: healthy; I: infected; AGP: arabinogalactan proteins.

sodium salts, distilled H_2O, and acetone as described previously (12).

CELL WALL SERINE AND HYDROXYPROLINE DETERMINATIONS

The hydroxyproline and total serine as well as the amino acid content of the wall were determined after hydrolysis of the cell wall with 6 N HCl (2.5:1, w/v) for 18 h at 100 C, in a sealed tube. We verified that no condensation of sugars and amino acids occurred because their presence would have subsequently affected the amino acids determinations. To remove the HCl, the hydrolysates were evaporated to dryness, then put in a desiccator under vacuum, in the presence of a NaOH pellet for 24 h. Values for serine were obtained by ion exchange chromatography in the course of routine amino acid analysis (33) which allowed the assay of 50 to 500 nmol (±5%) of a single amino acid. Hydroxyproline was assessed colorimetrically according to the method of Kivirikko and Liesmaa (17) which allowed from 5 to 100 nmol (±1%) of this amino acid to be measured.

HYDRAZINOLYSIS

Glycosylated serine residues in the wall have been shown to be destroyed in the presence of hydrazine, according to a presumed β-elimination process (24). The difference between the total serine content of the wall and the number of serine residues remaining after hydrazinolysis thus provides the amount of glycosylated serine.

After preliminary experiments to obtain optimal conditions, the cell walls were treated with hydrazine in the proportions of 10 mg of wall/1 ml of N_2H_4OH (24%, Merck), at 110 C in a sealed tube. The tube was opened after 18 h, and its content quickly dried under a stream of compressed air at 50 C, followed by storage under vacuum over anhydrous $CaCO_3$, overnight. The conversion of the obtained amino acids hydrazides into the corresponding amino acids was brought about by submitting, in the same tube, the dried residue to a 6 N HCl hydrolysis, under the conditions given above. Amino acid levels were determined according to Spackman *et al.* (33). The threonine content of the wall was similarly investigated after HCl hydrolysis, with or without prior hydrazinolysis, since threonine residues in glycoproteins behave in the same way as serine, when glycosylated.

TRYPSINOLYSIS AND HYDROXYPROLINE-RICH (GLYCO)PEPTIDES RECOVERY

An extraction of hydroxyproline-rich (glyco)peptides was carried out on infected cell walls, according to the procedure of Lamport *et al.* (24). This involved, after a preliminary step of stripping (*i.e.* deglycosylation at pH 1.0 of the hydroxyproline residues), a digestion of the wall glycoproteins by trypsin (TPCK, Worthington) at pH 8.5. The (glyco)peptides obtained were then isolated by successive gel filtration on Sephadex G-25, and ion exchange chromatography on Aminex AG 50-W-X2, 200 to 325 mesh (Bio-Rad) and Aminex 5 (Bio-Rad), both resins being equilibrated in a pyridine-acetate buffer at pH 2.7 (see Figs. 2–4).

The column effluents were monitored for peptidylhydroxyproline detection according to Lamport and Miller (25) and, occasionally, for an additional ninhydrin amino acid detection. The fractions corresponding to each of the hydroxyproline-positive peaks recovered after Aminex 5 were pooled, evaporated to dryness, and taken up into 2 ml of H_2O.

(GLYCO)PEPTIDE ANALYSIS

Amino Acid Composition. An aliquot of the above pooled fractions corresponding to about 200 nmol of hydroxyproline was hydrolyzed by 6 N HCl (~400 µl) at 105 C for 18 h in a microvial (Precision Sampling Corp.) under N_2. The hydrolysates were evaporated to dryness under a stream of N_2, and taken up into 100 µl of a pH 2.75 citrate buffer. Amino acid analyses were then performed on aliquots containing about 100 nmol of hydroxyproline, with an amino acid analyzer described by Lamport (21) suitable for the determination of small amounts of these compounds.

Determination of N-Terminal Amino Acid. This was accomplished by a subtractive method using the fixation of acrylonitrile on the free NH_2-terminus of the peptide, according to Fletcher (14). The amount of peptide used corresponded to 20 µg of hydroxyproline.

Edman Degradation. The sequential degradation of peptides via Edman and Begg (8) was achieved by initially coupling the peptides with 4-sulfophenylisothiocyanate followed by 5% phenylisothiocyanate using 300 nmol of peptides, in a Beckman automatic sequencer (model 890 C). The amino acids were recovered separately under the form of ATZ derivatives, converted into PTH amino acids, and taken up into ethyl acetate (25 µl) according to the Beckman recommendations; aliquots (1 µl) were then silylated by *N-O*-BSA (2.5 µl) for their identification on a gas chromatograph equipped for amino acid analysis.

Sugar Analysis. An amount of (glyco)peptide material, assumed to contain about 10 µg of sugar, was hydrolyzed with 200 µl of trifluoroacetic acid (15%, v/v) for 2 h at 121 C, in a sealed tube. Inositol (10 µg) was used as an internal standard. The hydrolysate was evaporated to dryness under N_2 and put under vacuum in a desiccator at 60 C, over P_2O_5. The TMS derivatives of the sugar residues were then directly obtained by adding 25 µl of TMS, of which 2 µl were injected into the column of a gas chromatograph equipped for sugar analysis.

Electrophoresis. The peptide material (corresponding to 20 nmol of hydroxyproline) and a lysine standard (12.5 nmol) were run simultaneously on Whatman 4, according to Lamport (19).

RESULTS AND DISCUSSION

Hydrazinolysis of Cell Wall and Standard Proteins. A study of the glycosylated seryl residues of the wall was first undertaken by calculating the number of seryl residues undergoing β-elimination in the presence of alkali. The recovery of the serine after hydrazinolysis was shown to decrease with time (Fig. 1) and to become constant after 14 to 16 h at 100 C, regardless of the amount of hydrazine used for a given quantity of cell wall. It was assumed that during this period of time, the labile seryl residues, *i.e.* glycosylated residues, were destroyed; therefore, in later experiments, hydrazinolyses of the cell walls were carried out for 18 h. Under these conditions, the experimental error was ±7% for the estimation of N_2H_4-labile serine. This base was preferred to the mild alkali solutions usually retained for β-elimination because of the previously demonstrated failure of such solutions to remove

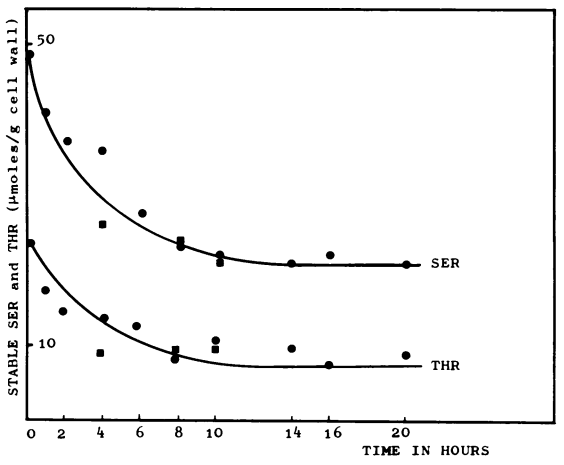

FIG. 1. Recovery of wall serine and threonine after hydrazinolysis as a function of time in the presence of 1 ml (●) or 2 ml (■) of hydrazine per 10 mg cell wall.

the galactosyl residues linked to serine in extensin (22) whether this glycoprotein was entrapped in the cell wall or extracted as large (glyco)peptides.

The effect of hydrazinolysis on the other amino acids of the wall (Table I) showed that threonine was the only one to be affected in a similar way, and to the same extent as serine (Fig. 1), suggesting that some of the threonyl residues are glycosylated as well. We also checked that the above conditions for hydrazinolysis did not significantly affect the recovery of most of the amino acids of a known protein, BSA (Table I). The effect of hydrazinolysis on aspartic acid, lysine, and arginine recoveries was independent of the glycosylation of the proteins under investigation; the observed losses could be explained by side reactions.

Relationships between Hydrazino-labile Serine (Threonine) and Hydroxyproline Contents of Wall. The data reported in this section were obtained in two or more independent experiments. The wall N_2H_4-labile serine and threonine were analyzed in the stems of infected seedlings from day 1 to day 7 after inoculation and also in their healthy counterparts, and compared to the corresponding amounts of hydroxyproline. Similar comparisons from the total serine content of the wall were not made since serine may be expected in all of the wall proteins, while glycosylated seryl residues are specifically found in hydroxyproline-rich peptides.

The cell wall hydroxyproline content of the whole stems of healthy plants within a given batch remained almost constant (on a wall dry weight basis) in the 7-day period under investigation, while a decrease of N_2H_4-labile serine (threonine) was seen as the seedlings aged (Table II). As a result of the disease, hydroxyproline accumulated in the cell wall, as already described (28). Addition of the corresponding glycoproteins caused the absolute amount of glycosylated serine (threonine) to increase as well; however, this increase was far smaller than the hydroxyproline increase. Within experimental error, the N_2H_4-labile serine (threonine) to hydroxyproline ratios were of the same magnitude in healthy and infected plants of the same age.

These ratios, however, varied from 1 to 0.2, due to the decreasing extent of glycosylation of serine (threonine) which depended not on the state (healthy or infected) but on the age of the plant. The fact that low values characterized mature cell walls as opposed to high values for younger ones was strengthened by the data obtained from different parts of the stems. In the oldest part (hypocotyls) of stems harvested from 13- to 27-day-old healthy plants, the ratios decreased continuously with age (Table III). In the youngest parts (epicotyl + petioles), which always showed a lower hydroxyproline content (Table IV), the ratios were still high and, once again, a similar discrimination of the ratios was seen between the mature and young organs of infected stems. It should, however, be noted that the effects of infection tended to mimic those of age on cell walls, particularly when one considers young organs. These data apparently contrast with a previous report (18) that such ratios remained constant varying around 0.2 during elongation in etiolated pea stems; the use of etiolated melon stems at two stages of elongation (Table V) in fact showed that low values were also obtained, whereas higher ones were found in control plants grown under usual lighting conditions. It thus seems that at least in melon cell walls, whenever the hydroxyproline content increased, whether as a result of infection or growth in darkness, the glycosylated serine to hydroxyproline ratios decreased in a manner similar to the decreases found in mature healthy organs *versus* young organs (*i.e.* hypocotyls *versus* epicotyls and petioles). This suggests that the regulation of the hydroxyproline content of the wall, in the three reported instances, was dependent on the regulation of the same kind of hydroxyproline-rich material.

Hydroxyproline-containing arabinogalactan proteins (AGP) in which the arabinogalactan and peptide moieties are linked through galactose and hydroxyproline have been reported in wheat endosperm (13), in numerous other seeds (16), and in the extracellular medium of cultured plant cells (15, 30). A hydroxyproline-poor material has also been identified in the cytoplasm of virus-infected plants (3). The similarities of the glycosylated serine (threonine) to hydroxyproline ratios in healthy and infected plants, at a given stage, rule out the possibility that the hydroxyproline which accumulates in diseased melons is present in glycoproteins related to the AGPs or the hydroxyproline-poor molecules. Major

Table I. *Amino Acid Composition of the Wall and of Bovine Serum Albumin With or Without Prior Hydrazinolysis*

Amino Acid[1,2]	Wall		Bovine Serum Albumin	
	N_2H_4 + HCl	HCl	N_2H_4 + HCl	HCl
Asp	9.2	12.2	7.4	10.1
Thr	3.1	5.2	3.7	4.9
Ser	6.4	11.9	3.9	4.5
Glu	9.5	9.3	17.2	15.0
Pro	6.2	6.3	6.1	5.3
Gly	15.8	15.2	5.5	5.7
Ala	9.3	8.3	10.2	8.3
Val	7.4	7.2	5.9	5.3
Ile	3.4	3.8	1.8	1.9
Leu	7.0	6.9	12.0	11.9
Tyr	4.2	3.0	3.3	3.4
Phe	4.1	3.8	4.4	4.6
Lys	11.2	6.2	15.5	10.3
His	1.4	1.6	2.7	3.0
Arg		3.5		4.0

[1] Amino acids, expressed in μmol/100 μmol total amino acids, are the mean of two determinations.

[2] Hyp not determined in the course of routine amino acid analysis.

Table II. *Hydroxyproline and N_2H_4-labile Serine and Threonine, in the Cell Walls of Infected Stems in the Course of the Disease, and in Healthy Controls*

Amino Acid[1]	Days After Inoculation[2]				
	1	6	3	5	7
Hyp					
I[3]	6.6	42.7	13.0	53.4	76.3
He[3]	5.7	6.9	10.7	11.2	10.4
N_2H_4-labile Ser					
I	6.7	10.5	10.6	13.3	15.8
He	7.0	2.4	7.3	3.7	2.3
N_2H_4-labile Thr					
I	6.1	7.6	6.0	10.3	9.4
He	7.3	4.1	8.4	4.8	2.8
Labile Ser/Hyp					
I	1.0	0.2	0.8	0.2	0.2
He	1.2	0.3	0.7	0.3	0.2
Labile Thr/Hyp					
I	0.9	0.2	0.5	0.2	0.1
He	1.3	0.6	0.8	0.4	0.3

[1] Hyp, Ser, and Thr expressed as μmol/g cell wall.

[2] Data obtained from two different batches of seedlings.

[3] I, He = cell walls from infected and healthy plants.

Table III. *Hydroxyproline and N_2H_4-labile Serine and Threonine, in the Cell Walls of Healthy Hypocotyls Harvested at Different Times of Culturing of Healthy Seedlings*

Amino Acid[1]	Days of Culture		
	13	21	27
Hyp	9.2	7.6	7.2
N_2H_4-labile Ser	12.8	6.9	3.5
N_2H_4-labile Thr	15.4	6.2	4.5
Labile Ser/Hyp	1.4	0.9	0.5
Labile Thr/Hyp	1.7	0.8	0.6

[1] μmol/g cell wall.

Table IV. *Hydroxyproline and N_2H_4-labile Serine and Threonine, in the Cell Walls of the Oldest (Hypocotyl) and Youngest (Epicotyl + Petioles) Parts of Stems from Infected and Healthy Seedlings*

Amino Acid[1]	Hypocotyl Cell Walls[2]	Epicotyl + Petioles Cell Walls[2]
Hyp		
I	82.4	67.9
He	11.8	5.2
N_2H_4-labile Ser		
I	20.1	33.5
He	2.9	4.3
N_2H_4-labile Thr		
I	11.4	11.4
He	8.8	5.4
Labile Ser/Hyp		
I	0.2	0.5
He	0.2	0.8
Labile Thr/Hyp		
I	0.1	0.2
He	0.7	1.0

[1] μmol/g cell wall.

[2] Organs harvested 7 days after inoculation and the corresponding controls.

Table V. *Hydroxyproline, and N_2H_4-labile Serine and Threonine, in the Cell Walls of Stems of Dark-grown and Normally Grown Melon Seedlings*

Amino Acid[1]	Days of Culture	
	20	27
Hyp		
Etiolated	17.6	19.1
Control	7.6	7.3
N_2H_4-labile Ser		
Etiolated	3.5	3.1
Control	4.0	3.0
N_2H_4-labile Thr		
Etiolated	2.2	4.1
Control	3.8	4.2
Labile Ser/Hyp		
Etiolated	0.2	0.2
Control	0.5	0.4
Labile Thr/Hyp		
Etiolated	0.1	0.2
Control	0.5	0.5

[1] μmol/g cell wall.

discrepancies would be expected, in that case, between the ratios obtained from both kinds of plants, due to a great dilution, in infected plants, of seryl, glycosylated seryl, and hydroxyprolyl residues, by residues from a new kind of glycoprotein. From the above data, it was thus concluded that the disease-induced HRGP has a peptide backbone characteristic of the wall HRGP of healthy plants. A further characterization of the HRGP was undertaken from infected plants, since their level is much higher in these plants.

Serine- and Hydroxyproline-rich (Glyco)peptides of the Wall. A study of the glycoprotein peptides which contained serine and hydroxyproline was carried out using cell walls isolated from infected plants. After deglycosylation of most of the hydroxyproline residues by means of 0.1 N HCl, tryptic digestion allowed the recovery of a soluble material accounting for ~20% of the total wall hydroxyproline. Further trypsinolysis of the remaining cell wall residue, in the same conditions, did not improve this yield, while a subsequent pronase digestion allowed the recovery of only 2% more hydroxyproline. The walls of healthy plants, which released 32% of their hydroxyproline content under tryptic digestion appeared to be somewhat less resistant to proteolysis.

Most of the hydroxyproline-rich material solubilized was present in the form of low mol wt fragments (Fig. 2). This material (S_2), first fractionated by ion exchange chromatography on Aminex 50, gave seven major hydroxyproline-containing peaks (Fig. 3). With regard to their amino acid composition, notably to their hydroxyproline and serine content, the first peaks to be eluted appeared the most complex (Table VI). The sixth (S_2A_6) and the seventh (S_2A_7) being, quantitatively, the most important (50% of the S_2) was subsequently purified on Aminex 5 (Fig. 4). Using this procedure, the hydroxyproline of S_2A_6 and S_2A_7 was recovered under the form of single peaks, $S_2A_6A_3'$ and $S_2A_7A_1'$ respectively, together accounting for 32% of the S_2.

The two corresponding components appeared homogeneous on high voltage electrophoresis at pH 1.9, $S_2A_6A_3'$ showing a R_{Lys} of 0.58 and $S_2A_7A_1'$ a R_{Lys} of 0.66. Their analysis was therefore undertaken (Table VII). The amino acid composition first indicated that the two components corresponded to two peptides, in nearly the same ratios: Hyp~4/Lys~1/Ser~1. The disappearance of serine after cyanoethylation indicated that the peptides contained one serine residue, in the N-terminal position; it followed that $S_2A_6A_3'$ and $S_2A_7A_1'$ contained only six amino acids residues, with lysine being in the C-terminal position, due to the specificity of trypsin. The presence of four consecutive hydroxyprolines in

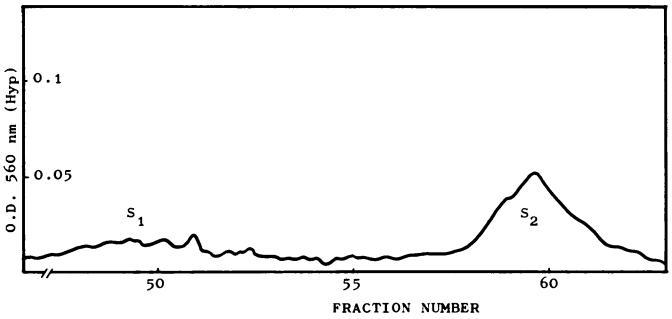

FIG. 2. Gel filtration of the hydroxyproline-rich material released by trypsinolysis of the cell wall. After incubation of stripped cell walls (7 g) with trypsin (42 mg) at pH 8.2 for 18 h, the suspension was filtered and washed; the filtrates were evaporated to dryness, taken up into 25 ml of H_2O, and fractionated on a Sephadex G-25 column (100 × 1 cm) with 0.5 N acetic acid (designated as step S). The fraction volume was 19 ml, of which 200 μl were assayed for peptidylhydroxyproline.

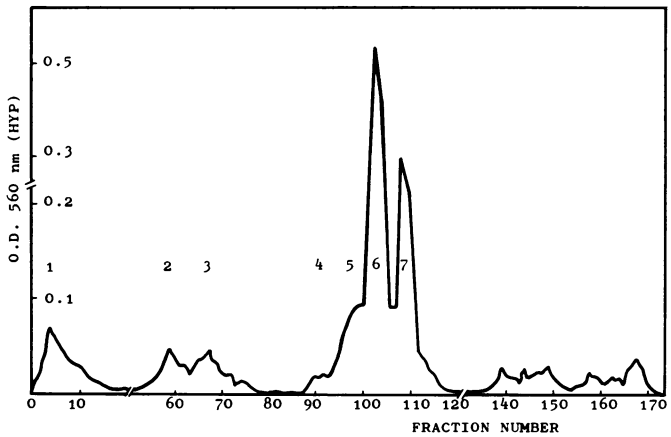

FIG. 3. Fractionation on Aminex AG 50-W-X2 (designated as A) of the Sephadex G-25 peak 2 (=S_2). The fractions corresponding to S_2 were pooled, evaporated to dryness, taken up into 2 ml of a pyridine-acetate buffer at pH 2.7; after fixation onto a column of Aminex AG 50-W-X2, 200 to 325 mesh (100 × 1 cm), equilibrated with the same buffer, the elution was performed at 55 C by a pyridine-acetate gradient using a gradient maker: chambers 1 to 4 each contained 140 ml of 0.07 M pyridine-acetate buffer (pH 2.7); chambers 5 and 6 contained 140 ml of 0.2 M pyridine-acetate buffer (pH 3.1); chambers 7 and 8 contained 140 ml of 2.0 M pyridine-acetate buffer (pH 5.0). Fraction volume was 5 ml of which 500 μl were assayed for peptidylhydroxyproline.

Table VI. *Serine and Hydroxyproline in the Seven Hyp-positive Peaks Obtained by Chromatography on Aminex AG 50-W-X.*

The data are given per lysine residue.

Amino Acid Ratios	Aminex AG 50 (=A) Peaks						
	S_2A_1	A_2	A_3	A_4	A_5	A_6	A_7
Hyp/Lys	9	6	7	2.9	1.2	4	3
Ser/Lys	6	2	2	1.5	1	1	1

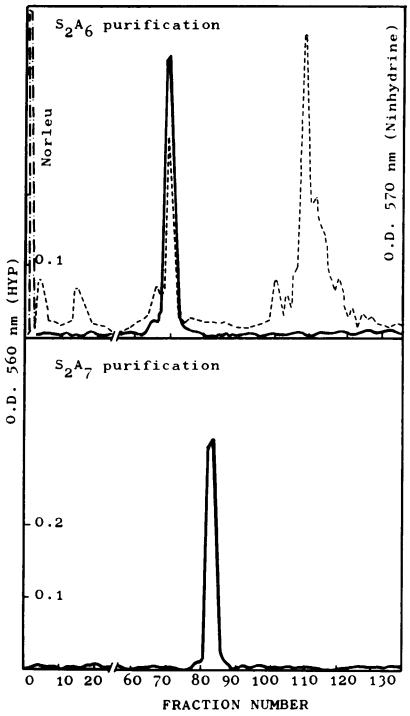

FIG. 4. Further fractionation on Aminex A-5 (designated as A') of the Aminex AG 50-W-X2 peaks 6 and 7, *i.e.* S_2A_6 and S_2A_7. The corresponding fractions were separately pooled, evaporated to dryness, taken up into 1 ml of a pH 2.7 pyridine-acetate buffer and subsequently fractionated on a column of Aminex A-5 (27 × 0.9 cm) kept at 55 C, equilibrated and eluted with the same buffers as above, using only 70 ml per chamber. Three-ml fractions were collected, of which 300 μl were used for peptidyl-hydroxyproline (——) and, in the case of S_2A_6, for an additional amino acid ninhydrin (---) reaction on 300 μl.

Table VII. *Detailed Analysis of the Hydroxyproline-containing Peaks $S_2A_6A_3'$ and $S_2A_7A_1'$ Obtained with Aminex A_5 Chromatography*

Analyses	Aminex A_5 (=A') Peaks[1]	
	$S_2A_6A_3'$	$S_2A_7A_1'$
Amino acid composition	Ser_1 $Hyp_{4.1}$ Lys_1	$Ser_{1.1}$ $Hyp_{3.9}$ Lys_1
C-terminal residue	Lys	Lys
N-terminal residue	Ser	Ser
Sequence (via Edman degradation)	-Hyp-Hyp-Hyp-Hyp	N.D.[2]
Sugar composition	Gal_1	0

[1] All data being the mean of at least two different experiments.

[2] N.D., not determined.

$S_2A_6A_3'$ was also established by Edman degradation, the mean yield obtained for each residue being about one-fifth the original amount. This method was not successfully applied to $S_2A_7A_1'$ because of still lower yields in the recovery of the amino acids. The over-all data, however, allowed us to conclude that the two purified peptides exhibited the same sequence: -Ser-Hyp_4-Lys-. The different electrophoretic mobilities resulted from the presence of a single galactosyl residue in $S_2A_6A_3'$ while $S_2A_7A_1'$ was devoid of any glycosyl residue. The linkage of galactose to serine would also explain why PTH-Ser was not observed in $S_2A_6A_3'$ by Edman degradation.

Isolation of hydroxyproline-rich (glyco)peptides thus allowed the recovery of peptides which possess the typical -Ser-Hyp_4- sequence characteristic of extensin, hitherto reported only in cultured plant cells. In the extensin of these cells, with the exception of a few single residues encountered along the polypeptide, hydroxyproline has always been found under the form of such pentapeptides repeated with high frequency. This frequency could not be quantified in infected plants, due to the partial extraction of hydroxyproline by trypsinolysis. The possibility that the nonextracted hydroxyproline enters sequences different from the classical -Ser-Hyp_4- sequences, was not checked. This possibility was, however, very unlikely if one considers the similarities of the peptide backbone of the wall HRGP in healthy and infected plants, and if one assumes that this glycoprotein is identical in healthy plants and cultured plant cells.

CONCLUSION

This paper reports the partial characterization of hydroxyproline-rich material which accumulates in the cell wall of infected plants. This work contributes to a study of the cell wall HRGP of intact plants.

It has been reported (10, 12, 28) that the wall hydroxyproline increase in diseased plants is not due to a greater amount of proline hydroxylation *in situ* but resulted from the addition of HRGP. The question has also been raised whether these glycoproteins represent a variant of the HRGP already present in the wall of healthy plants, with some variations in the sequences of the amino acids, at least at the sites of glycosylation. By studying the amino acids which are glycosylated, we now ascertain that as far as these markers are concerned, the HRGPs of infected and healthy plants have similar peptide backbones. These glycoproteins contain hydroxyprolyly-arabinose and seryl-galactose linkages, and also the -Ser-Hyp_4- pentapeptides which are unique to extensin, the wall HRGP of cultured plant cells. It thus seems that the wall HRGP of intact healthy and infected melon plants are closely related to extensin.

A study of the glycosylation pattern of the wall HRGP also provides some insight into the understanding of this glycoprotein. In contrast to the extent of glycosylation of hydroxyproline which is higher in infected plants (12), the extent of the serine (threonine) glycosylation does not vary significantly upon infection but decreases with age in healthy as well as infected plants. This indicates that the regulation processes involved in the behavior of the two types of carbohydrate to protein linkages are independent, implying a different role for the carbohydrates in each case.

It has been hypothesized that the glycosylated seryl residues of the wall could provide cross-links between sugar polymers and proteins (23). Rather than a stiffening of the wall through an increase of such linkages, one should assume, for instance, that some galactose-containing polymers need this linkage either to be transferred or properly orientated, or both, inside the cell wall before being detached in a subsequent step.

The glycosylated hydroxyprolyl residues of the wall are linked to arabinose chains, the percentage and the length of which are remarkably constant in healthy plants (11). The arabinose residues protect the wall HRGP against proteolysis, since enzymes showing either a broad specificity (pronase) or a narrow one (trypsin) as well as proteases secreted by *C. lagenarium* in culture (29, and unpublished data) are ineffective in extracting hydroxyproline-rich (glyco)peptides without prior deglycosylation of the hydroxyproline residues. Because of the increased glycosylation of these residues in diseased plants, one may ask whether a special meaning can be attributed to the triggering of the wall HRPG synthesis in diseased plants.

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