Glycoprotein of the Wall of Sycamore Tissue-Culture Cells

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1. A glycoprotein containing a large amount of hydroxyproline is present in the cell walls of sycamore callus cells. This protein is insoluble and remained in the α -cellulose when a mild separation procedure was used to obtain the polysaccharide fractions of the wall. The glycoprotein contained a high proportion of arabinose and galactose. 2. Soluble glycopeptides were prepared from the α -cellulose fraction when peptide bonds were broken by hydrazinolysis. The soluble material was fractionated by gel filtration and one glycopeptide was further purified by electrophoresis; it had a composition of 10% hydroxyproline, 35% arabinose and 55% galactose, and each hydroxyproline residue carried a glycosyl radical so that the oligosaccharides on the glycopeptide had an average degree of polymerization of 9. 3. The extraction of the glycopeptides was achieved without cleavage of glycosyl bonds, so that the glycoprotein cannot act as a covalent cross-link between the major polysaccharides of the wall. 4. The wall protein approximates in conformation to polyhydroxyproline and therefore it probably has similar physicochemical properties to polyhydroxyproline. This is discussed in relation to the function of the glycoprotein and its effect on the physical and chemical nature of the wall.

The discovery that hydroxyproline was an important component of the cell-wall protein of plants, although being present only in trace amounts in the cytoplasm (Lamport & Northcote, 1960; Dougall & Shimbayashi, 1960), provided a means of identifying and studying the protein component of the wall. Great interest has been shown in the possible morphogenetic role of the wall protein, and several reviews have dealt with this subject (Newcomb, 1963; Lamport, 1965, 1970).

The existence of specific proteins containing hydroxyproline as components of the cell wall has been doubted by Steward on the basis of radioautographic studies on carrot phloem explants (Steward, Israel & Salpeter, 1967; Israel, Salpeter & Steward, 1968), although the results of similar experiments by Sadava & Chrispeels (1969), who used plasmolysis to separate the radioactivity of the wall from that of the cytoplasm, do not agree with these negative findings. Further evidence that the proteins of isolated walls are not contaminants of cytoplasmic origin comes from painstaking fractionation of the proteins of the leaves of bean (Jennings & Watt, 1967; Clarke & Ellinger, 1967; Jennings, Pusztai, Syngé & Watt, 1968; Pusztai & Watt, 1969). These workers found some hydroxyproline-

rich protein still remaining in their cell-wall residues, in addition to a range of cytoplasmic proteins containing hydroxyproline.

Lamport (1965, 1967*b*, 1969*a*) has shown that the wall protein hydroxyproline residues carry carbohydrate units attached at the 4-hydroxyl group, and the details of the synthesis of the glycoprotein have been studied (Chrispeels, 1969, 1970*a,b*; Doerschug & Chrispeels, 1970; Sadava & Chrispeels, 1971).

Methods so far applied to the wall protein have not shown clearly the nature of the attached carbohydrate units or their association with the major polysaccharides of the wall. As these questions are crucial to current speculations on the morphogenetic role of the wall protein, the present work has been undertaken in an attempt to elucidate them.

MATERIALS AND METHODS

Chemicals. 4-Hydroxy-L-proline (puriss. grade) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., and hydrazine (purity greater than 95%) from Eastman Kodak Co., Rochester, N.Y., U.S.A.

Radiochemicals. L-[1-¹⁴C]Arabinose (52 μ Ci/mmol) and L-[U-³H]proline (578 mCi/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Tissues. Cultures of sycamore (*Acer pseudoplatanus*) were grown, both as solid callus and in suspension, as described by Stoddart, Barrett & Northcote (1967).

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Tissue in suspension was harvested by centrifugation and washed with water.

Radioactive labelling of tissue. L-[1-¹⁴C]Arabinose (20 μ Ci) and L-[U-³H]proline (200 μ Ci) were placed in a culture bottle containing 150 ml of rapidly growing callus suspension. After 40 h the cells were harvested and washed with water.

Wall preparation. Tissues were suspended in water and the cells were broken by sonication with a Soniprobe Converter (Dawe Instruments, London W.3, U.K.) for 5 min at 4.2 A. Separated walls were gathered by centrifugation and washed ten times with water.

Fractionation (Scheme 1). Wall preparations were treated with 0.5% EDTA (disodium salt) in 0.05 M-sodium phosphate buffer, pH 6.8, at 100°C for 1 h (Stoddart *et al.* 1967). Pectic substances (fraction 1), were precipitated from the extract by addition of 4 vol. of ethanol. The material remaining after extraction was further treated with NaOH (175 g/l) in boric acid (40 g/l) at room temperature for 10 h (Timell, 1960). The residue was mainly cellulose (fraction 3). Hemicelluloses (fraction 2) were precipitated from the extract by addition of 4 vol. of ethanol-acetic acid (10:1, v/v).

Drying of cell walls and fractions. Samples were freeze-dried at 0.01 mmHg and then stored over KOH pellets at 0.5 mmHg.

Hydrazinolysis. By using the method of Yosizawa, Sato & Schmid (1966), the material (10 mg) was sealed in a glass tube and heated at 100°C for 10 h with 0.5 ml of hydrazine containing 1.5% (w/v) hydrazinium sulphate. Hydrazine was removed by vacuum desiccation over conc.

H₂SO₄, the material was extracted with water and the solution fractionated on Sephadex G-25.

Paper electrophoresis. This was carried out on Whatman no. 1 paper at 5 kV for 30 min in a buffer (98% formic acid-acetic acid-water, 1:4:45, by vol., pH 2; or acetic acid-pyridine-water, 13:100:900, by vol., pH 6.5).

Amino acid analyses. Samples (10 mg) were treated, in sealed tubes which were not evacuated, with 0.5 ml of 6 M-HCl at 110°C for 18 h. Amino acid analyses were performed on a Technicon AutoAnalyzer or Beckman model 120C Amino Acid Analyzer (Moore, Spackman & Stein, 1958).

Determination of hydroxyproline. The method used was that of Leach (1960). Samples were purified by paper electrophoresis (pH 2). Amino acid marker strips were stained with the reagent of Kolor & Roberts (1957).

Hydrolysis of polysaccharides. Samples (up to 20 mg) were hydrolysed by dissolving the material in 72% (w/w) H₂SO₄, diluting to 3% (w/v) H₂SO₄ and autoclaving at 120°C and 15 lb/in² for 1 h (Saeman, Moore, Mitchell & Millett, 1954) and the solution was neutralized with Amberlite IR-4B resin (carbonate form). Sugars were converted into their trimethylsilyl ethers as described by Ludlow, Harris & Wolf (1966) and separated on a Pye series 104 gas chromatograph (Harris & Northcote, 1970).

Paper chromatography. Sugars were separated by descending chromatography on Whatman no. 1 paper, with ethyl acetate-pyridine-water (8:2:1, by vol.) as the solvent. Marker strips were stained with the aniline hydrogen phthalate reagent of Wilson (1959).

Determination of carbohydrate. Total carbohydrate was determined colorimetrically by using the tryptophan-H₂SO₄ method (Dische, 1962). Galactose was used as a standard.

Alkaline hydrolysis. Samples were sealed in Pyrex tubes with 0.5 ml of saturated Ba(OH)₂ solution (approx. 0.22 M) and then heated to 105°C for 6 h (Lampert, 1969a). The solution was neutralized with 0.5 M-H₂SO₄, centrifuged to remove the precipitate of BaSO₄, evaporated to dryness and then dissolved in 50 μ l of water and subjected to electrophoresis at pH 2 for 1.5 h at 5 kV.

Scintillation counting. Paper strips were placed in counting vials with 0.5 ml of toluene fluor (Stoddart & Northcote, 1967); aqueous samples were placed in counting vials with 10 vol. of Triton-toluene fluor (Patterson & Greene, 1965). The vials were placed in 20 ml bottles and counted simultaneously for ¹⁴C and ³H radioactivity in either a Packard model 3375 Tri-Carb liquid-scintillation spectrometer or a Philips model A liquid-scintillation Analyzer.

RESULTS

Composition of the wall preparation. The preparation of cell walls was examined under the light-microscope to ensure that cytoplasmic contaminants had been removed.

The amino acid composition of the wall protein of the cells of the solid callus of sycamore is shown in Table 1. From the recovery of amino acids from a wall sample, the protein content was calculated at 5.6% (w/w). The hydroxyproline content of the protein is very high (18.1 mol/100 mol of amino

Scheme 1. Fractionation of wall polysaccharides.

Table 1. *Amino acid composition of a hydrolysate of the cell walls of sycamore solid callus*

Amino acid	Content (mol/100 mol of amino acids)
Hydroxyproline	18.1
Aspartic acid	7.7
Threonine	4.2
Serine	9.6
Glutamic acid	7.8
Proline	8.1
Glycine	6.4
Alanine	5.7
Valine	6.9
$\frac{1}{2}$ -Cystine	0.4
Methionine	0.2
Isoleucine	3.2
Leucine	5.2
Tyrosine	2.4
Phenylalanine	2.5
Lysine	7.1
Histidine	1.9
Arginine	2.6

acid). The neutral sugar composition of the isolated walls is shown in Table 2.

Products of fractionation. The treatments used (Scheme 1) were chosen so as to cause the minimum damage to proteins. Fraction 1 consisted chiefly of pectic substances; fraction 2 contained mainly hemicelluloses; fraction 3 had α -cellulose as its principal constituent. Table 3 shows the size and hydroxyproline content of each of the three fractions, and clearly most of the hydroxyproline (95%) was in fraction 3. Table 4 shows that fraction 3 has a higher proportion of hydroxyproline than the whole wall although the total protein content remains the same. Tables 5 and 6 show the amino acid and sugar composition of fraction 3. Besides α -cellulose the fraction contained arabinan and galactan but no uronic acids.

Hydrazinolysis. The selectivity of this hydrolytic procedure was tested by hydrazinolysis of Whatman cellulose powder (Standard grade). One sample was subjected to the standard hydrazinolysis as described above, and a control sample was sealed in a glass tube with 0.5 ml of glass-distilled water and heated at 100°C for 10 h. The aqueous extract of the hydrazine-treated sample and the water from the water-treated sample were each separated on a Sephadex G-25 column (1.2 cm $\times$ 25 cm) into high- and low-molecular-weight fractions, which were then assayed for carbohydrate. The results are shown in Table 7. Very little carbohydrate was extracted by the hydrazine, which therefore could not have been splitting glycosidic bonds. Hence any carbohydrate extracted by hydrazinolysis from cell wall material (which has

Table 2. *Neutral sugar composition of the cell walls of sycamore solid callus*

Neutral sugar	Content (mol/100 mol of neutral sugars)
Arabinose	22
Xylose	9
Mannose	1
Galactose	21
Glucose	47
(Uronic acids were not determined)	

Table 3. *Products of fractionation (Scheme 1) of the cell walls of sycamore solid callus*

Fraction no.	Type	% (w/w) of total recovered material	
		Dry weight	Hydroxyproline
1	EDTA soluble	28	3
2	Cold alkali soluble	7	2
3	Alkali insoluble	65	95

Table 4. *Comparison of the hydroxyproline and protein contents of fraction 3 and whole wall*

	Fraction 3	Whole wall
Hydroxyproline (μ g/mg dry wt.)	18.8	12.3
Hydroxyproline (mol/100 mol of amino acids)	28.6	18.1
Protein (mg/100 mg dry wt.)	5.5	5.6

already been subjected to aqueous extraction) was probably released by proteolysis or by solution in the hydrazine.

When fraction 3 was subjected to hydrazinolysis, about 22% (w/w) of the carbohydrate was extracted. The solution was separated into high- and low-molecular-weight fractions by chromatography on a Sephadex G-25 column (1.2 cm $\times$ 25 cm). Table 8 shows a comparison of the amino acid analyses of the three fractions.

It is evident that more than half of the protein had been extracted into the high-molecular-weight fraction, which was particularly rich in hydroxyproline. Table 9 shows that the neutral sugars of the high-molecular-weight fraction were principally arabinose and galactose. Lampert (1967a) found that arabinose and galactose were the principal sugars of glycopeptide material extracted from tomato cell walls by enzymic digestion.

Radioactively labelled material. Suspension callus

tissue was incubated with L-[U-³H]proline and L-[1-¹⁴C]arabinose, and the walls were isolated. The three fractions (Scheme 1) were prepared from the walls, and fraction 3 was treated with hydrazine. The aqueous extract was then chromatographed on a Sephadex G-25 column (1.6 cm × 80 cm), fractions (1.4 ml) were collected and samples (0.2 ml) were taken for scintillation counting. Four peaks of radioactive material were eluted from the column (Fig. 1). The first peak was mainly carbohydrate of high molecular weight (it contained only ¹⁴C), the second and third peaks were subsequently found to be glycopeptide material (they contained both ¹⁴C and ³H) and the final peak was low-molecular-weight material and its radioactivity was due to the presence of ³H only. It probably contained oligopeptides produced as a result of the treatment with hydrazine.

The materials of the second and third peaks were divided into five separate samples (A–E) as shown in Fig. 1, dried by evaporation under reduced pressure, taken up in glass-distilled water (50 μl) and run on paper electrophoresis (pH 2) with glu-

cose as a marker for neutral material and a mixture of amino acids as a set of reference markers.

The electrophoretograms were cut into strips (1 cm long × 4 cm wide) for scintillation counting (Fig. 2). They showed two major components, corresponding to the second and third peaks obtained from the Sephadex column. The higher-molecular-weight component, which gave a sharp peak on the electrophoretogram (Fig. 2B) and which could be easily purified, was investigated further. The purified material eluted from the electrophoretogram was designated component B.

Investigation of component B. The experiment illustrated by Fig. 2 showed that component B was neutral at pH 2. On electrophoresis at pH 6.5 component B was again found to run as a single sharp band with the neutral material and it remained as a single band at the origin of a paper chromatogram (in pyridine–ethyl acetate–water, 8:2:1, by vol.).

A sample of component B was sealed in a glass tube with 0.1 M-hydrochloric acid (1 ml) and heated at 100°C for 1 h, conditions which will split all the furanose bonds but not all the pyranoside or the peptide bonds (Lamport, 1969b). The treated material was dried by evaporation under reduced pressure followed by desiccation *in vacuo* over potassium hydroxide pellets, and on electrophoresis at pH 2 it ran as a single neutral band. Chromatography of the treated material on a Sephadex G-25 column (1.6 cm × 80 cm) showed that both ¹⁴C and ³H radioactivity occurred in the completely retarded fractions, with elution volumes significantly greater than that for untreated component B (Fig.

Table 5. *Amino acid composition of fraction 3 (Scheme 1)*

Amino acid	Content (mol/100 mol of amino acids)
Hydroxyproline	28.6
Aspartic acid	5.3
Threonine	3.2
Serine	8.8
Glutamic acid	6.5
Proline	9.3
Glycine	4.1
Alanine	4.1
Valine	7.7
Methionine	0.3
Isoleucine	2.5
Leucine	4.0
Tyrosine	2.5
Phenylalanine	1.9
Lysine	7.7
Histidine	2.1
Arginine	1.4

Table 6. *Sugar composition of fraction 3 (Scheme 1)*

Sugar	Content (mol/100 mol of sugars)
Arabinose	26
Xylose	8
Mannose	1
Galactose	18
Glucose	47
Uronic acids	0

Table 7. *Extraction of carbohydrate from Whatman cellulose by hydrazine*

Samples were treated with water or hydrazine as described in the text, separated into high- and low-molecular-weight fractions on Sephadex G-25, and the fractions assayed for carbohydrate.

Treatment	Sample weight (mg)	Fraction	Carbohydrate (μg)	Extracted material (%)
Water	11	High-mol.wt.	39	0.35
		Low-mol.wt.	35	0.30
Hydrazine	14	High-mol.wt.	76	0.55
		Low-mol.wt.	59	0.40

Table 8. *Distribution of amino acids after hydrazinolysis of fraction 3*

Amino acid	Recovery of amino acid (% in each fraction)		
	High-mol.wt.	Low-mol.wt.	Residue
Hydroxyproline	78	6	17
Aspartic acid	30	62	8
Threonine	54	37	9
Serine	60	34	5
Glutamic acid	42	37	20
Proline	50	21	28
Glycine	51	40	9
Alanine	59	30	10
Valine	55	17	28
Isoleucine	55	25	21
Leucine	56	30	13
Tyrosine	32	42	27
Phenylalanine	Trace	79	21
Lysine	63	7	29
Histidine	36	32	32
Arginine*	69	19	12
Total amino acids	57	24	19

* Measured as ornithine after hydrazinolysis.

Table 9. *Sugar composition of high-molecular-weight material from aqueous extract of fraction 3 after hydrazinolysis*

Sugar	Content (mol/100 mol of sugars)
Arabinose	42
Xylose	6
Mannose	0
Galactose	40
Glucose	12
Uronic acids	0

3; cf. Fig. 1), but the peaks of ^{14}C and ^3H radioactivity were not quite coincident. A paper chromatogram of the acid-treated component B separated the ^{14}C and ^3H radioactivity (Fig. 4). The material containing ^{14}C corresponded in position to arabinose (R_{Glc} 1.6), and when eluted from this region and hydrolysed (Saeman *et al.* 1954) and re-chromatographed its position was unchanged. The material containing ^3H ran faster, with R_{Glc} 2.2. The ^3H -labelled material was eluted from the chromatogram with water and the eluate was divided into two parts. When one part was hydrolysed with 6M-hydrochloric acid and analysed by electrophoresis (pH 2) the ^3H label was nearly all found in hydroxyproline. The other portion was rechromatographed after removal of sugars by hydrolysis with sulphuric acid (Saeman *et al.* 1954). The position on the chromatogram of the ^3H -containing material was the same as that previously found (R_{Glc} 2.2).

It seems therefore that acid treatment (pH 1) splits the neutral glycopeptide material B into arabinose and possibly other unlabelled sugars, and a neutral peptide or peptide hydrazide containing hydroxyproline.

Lamport (1967b) isolated hydroxyproline arabinosides from intact sycamore cell walls by hydrolysis with saturated barium hydroxide. A sample of fraction 3 material (10mg) was hydrolysed by barium hydroxide and subjected to electrophoresis. Spots staining for hydroxyproline and sugars were found with R_{Hyp} values similar to those recorded by Lamport (1967b). A sample of component B was then treated with barium hydroxide. The distribution of radioactivity on an electrophoretogram of this hydrolysate is shown in Fig. 5. Neutral material, a small amount of an acidic material and at least five components that ran towards the cathode were detected (Fig. 5). Probably the neutral material was arabinose and the acidic material resulted from the breakdown of sugars. The positively charged components correspond in position with spots detected in the hydrolysate of fraction 3 material. There was no free hydroxyproline in the hydrolysate of component B and from this it may be deduced that all the hydroxyproline residues of this material must carry carbohydrate (Chrispeels, 1970b).

Chemical analysis of glycopeptide material. To identify the amino acid and sugar residues present in component B, 40mg of unlabelled fraction 3 was hydrazinolysed together with 4mg of radioactively labelled material to provide a radioactive marker.

Fig. 1. Separation of glycopeptide fractions from isolated, radioactively labelled cell walls. Suspension callus tissue was incubated with L-[U- ^3H]proline and L-[1- ^{14}C]arabinose and the walls were isolated. Alkali-soluble polysaccharides were removed and the residue was treated with hydrazine. The aqueous extract of the treated material was chromatographed on a Sephadex G-25 column, and the ^3H (○) and ^{14}C (●) radioactivity of each fraction of the eluate was assayed. Five samples (A-E) were prepared from the fractions.

Fig. 2. Electrophoresis of isolated glycopeptide fractions. The five samples (A-E) (Fig. 1) were each subjected to electrophoresis at pH 2, and the electrophoretograms were assayed for ^3H (—) and ^{14}C (---) radioactivity.

Fig. 3. Gel filtration of acid-treated glycopeptide B. The material of the major peak of sample B (Fig. 2) was eluted from the electrophoretogram. The eluate was treated with 0.1 M-HCl, then chromatographed on a Sephadex G-25 column. The ^3H (○) and ^{14}C (●) radioactivity of each fraction was assayed.

Fig. 4. Paper chromatography of acid-treated glycopeptide B. The material of the major peak of sample B (Fig. 2) was eluted from the electrophoretogram. The eluate was treated with 0.1 M-HCl, then chromatographed on paper with ethyl acetate-pyridine-water (8:2:1, by vol.) as solvent. The chromatogram was assayed for ^3H (○) and ^{14}C (●) radioactivity.

By means of this marker the component B was separated by gel filtration and electrophoresis in the normal manner. The material was eluted from the electrophoretogram with water, and the eluate was divided into two parts. One was analysed for amino acids and the other for sugars. A separate sample eluted from an equivalent piece of electrophoresis paper was analysed for any soluble carbohydrate present in the paper. The glycopeptide contained only three components (Table 10).

DISCUSSION

Wall protein resists many non-degradative extraction procedures to render it soluble. Removal of alkali-soluble polysaccharide by gentle procedures yields insoluble α -cellulose and wall protein, plus

some associated arabinan-galactan which would, on harsher treatment, be extracted with the hemicellulose material.

As a degradative procedure is required to obtain the protein material, it is necessary to select one of high specificity if the products are to be useful as guides to the nature of wall protein. Several proteolytic enzymes have been tried with little success (Olson, 1964; Lamport, 1965, 1969a). A crude cellulase preparation was used successfully by Lamport (1969a), but this preparation also had proteolytic activity, which was said to be of great importance for the extraction of the glycopeptides. Hence the specificity of the enzyme preparation was too ill-defined for conclusions to be made about the wall protein structure and its possible combination with wall polysaccharides from the nature

Fig. 5. Electrophoresis of alkaline hydrolysate of glycopeptide B. The material of the major peak of sample B (Fig. 2) was eluted from the electrophoretogram. The eluate was treated with saturated Ba(OH)₂, and the neutralized hydrolysate was subjected to electrophoresis at pH 2. The electrophoretogram was assayed for ³H (○) and ¹⁴C (●) radioactivity.

Table 10. *Composition of glycopeptide B*

Component	Content [mol/100 mol of components (amino acid plus sugar residues)]
Hydroxyproline	10
Arabinose	35
Galactose	55

of the glycopeptides obtained by use of the cellulase. Hydrazinolysis in the presence of hydrazinium sulphate has been shown here and elsewhere (Yosizawa *et al.* 1966; Aston, Donald & Morgan, 1968) to be an effective and specific technique, degrading only the peptide linkages of the protein and leaving unattacked the carbohydrate side chains.

The glycopeptide (component B) isolated from the wall by hydrazinolysis contained only three components and its composition indicated that the peptide portion consisted of a series of hydroxyproline residues carrying as carbohydrate moieties copolymers of galactose and arabinose. Glycopeptides extracted by cellulase digestion (Lampport, 1969a) showed much lower molar ratios of sugar to hydroxyproline and of galactose to arabinose than did component B. This probably indicates that the extraction by the crude cellulase causes extensive degradation of the carbohydrate portion of the glycopeptides.

Chromatography of the products produced by hydrolysis of component B with 0.1 M-hydrochloric acid separated a peptide that could be re-run with unchanged mobility (*R*_{Glc} 2.2) after further hydrolysis with 3% sulphuric acid, and therefore probably all the carbohydrate units are connected to the peptide via furanoside links.

Alkaline hydrolysis of component B and electrophoresis of the products demonstrated that all the hydroxyproline residues of the glycopeptide were involved in glycoside linkage, probably to arabinose (Lampport, 1967b; Chrispeels, 1970b). No free hydroxyproline was released.

Glycopeptide B has been produced without breakage of carbohydrate linkages and the carbohydrate portions attached to each hydroxyproline are oligosaccharides with an average degree of polymerization of 9 residues. It is clear therefore that at least these glycopeptides are not involved in covalent crosslinks between the major polysaccharides of the cell wall, a role that has frequently been implied (Lampport, 1962, 1965, 1967b, 1969a, 1970). Since 84% of the hydroxyproline residues of the wall can be extracted with hydrazine, it is unlikely that any protein containing hydroxyproline is involved in cross-links of this type.

The amino acid compositions of fraction 3 and component B suggest that the wall glycoprotein may approximate in conformation to polyhydroxyproline. Recently Lampport, Katona & Roerig (1971) have shown that in the glycoprotein of the cell wall of tomato hydroxyproline is present as contiguous units of up to four residues. Proline oligopeptides of as few as three residues have been shown to exist in the conformation of polyproline (form II) in aqueous solution (Rippon, Koenig & Walton, 1970). Poly(4-hydroxy-L-proline) has some noteworthy properties that may be similar to those of wall protein and contribute to the physicochemical nature of the plant cell wall. Its conformation is analogous to that of poly-L-proline form II (Mattice & Mandelkern, 1970), that is an open rigid left-handed helix determined by the steric hindrance of the bond between the α-carbon and the carbonyl carbon atom, this being the only

backbone bond with any degree of free rotation. All the peptide bonds are held in the *trans* configuration. In molecular aggregates of poly(*O*-acetyl-4-hydroxy-L-proline) these helices are folded and stacked antiparallel (Andries & Walton, 1969). Poly(4-hydroxy-L-proline) is much more stable than poly-L-proline II to changes of pH and to high concentrations of aqueous salts (Deveney, Walton & Koenig, 1971; Mattice & Mandelkern, 1970) which makes it unlikely that the wall protein contributes to the changes in the nature of the wall that are brought about by changes in either pH or concentrations of Ca²⁺ and other ions. Like polyproline II, polyhydroxyproline has the same structure in aqueous solution as in the solid state, but unlike it, it shows no alternative solid state structure with *cis* peptide bonds (the poly-L-proline I structure). The glycosylation at the 4-hydroxyl group of the hydroxyprolines should not greatly perturb the conformation of the polymer, as these hydroxyl groups are positioned on the outside of the helix, and widely separated by the *trans* nature of the peptide bonds.

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