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CELL WALL GLYCOPROTEINS AND POLYSACCHARIDES OF PARENCHYMA OF *PHASEOLUS COCCINEUS*

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Abstract—Fractionation of the cell wall material of parenchyma of mature runner beans with and without chlorite–HOAc treatment, clearly showed that at least two main types of wall proteins were present. One relatively rich in hydroxyproline (HP) associated with α′-cellulose, from which most (90%) of it could be readily liberated by chlorite–HOAc treatment and the other relatively poor in HP associated with hemicellulose A. The chlorite HOAc solubilized "glycoprotein" contained a high proportion of arabinose and galactose. It was purified by PhOH–H_2O fractionation and the molar ratios of HP, arabinose, galactose, xylose, rhamnose, glucose and uronic acid in the purified glycoprotein ("glycoprotein X") were 1:2·6:2·4:0·2:0·2:0·1:0·3. The principal amino acids of glycoprotein X were HP (43·5 mol%), serine and proline which together comprised 66 mol% of the total. These results suggest that the HP-rich wall glycoprotein is associated with cellulose microfibrils and approximates in conformation to polyhydroxyproline carrying arabinose and galactose oligosaccharide side chains.

INTRODUCTION

The occurrence of hydroxyproline (HP) rich glycoproteins in the cell walls of higher plants is now well established [1–6]. These glycoproteins have been generally regarded as structural [5], although some of them may (also) be enzymes [7,8]. However, the exact role of these glycoproteins in the formation of crosslinks between the well known polysaccharides is far from being established. The presence of HP-containing proteins in the cytoplasm of some plant tissues has been reported [9–11] and they may contaminate the isolated wall preparation by an unspecific adsorption unless powerful protein solubilising reagents are used in the preparation of the wall material [12]. These adsorptive effects may give misleading information on the association of the (wall) proteins with the major polysaccharides of the wall. Because of these difficulties, most of the detailed work on cell wall proteins has been carried out with relatively simple tissues, such as cell suspension cultures [1,3,4]. Although such information may be useful, it cannot be used with any certainty to make generalisations on wall protein-polysaccharide association of higher plants because culture cells and cells of ordinary plants differ in many ways. Therefore, there is still need for methods which permit the isolation and characterization of the glycoproteins or glycoprotein–polysaccharide complexes from cell walls of higher plants in a state as near as possible to the native walls.

In the preceding paper [6], the occurrence and isolation of HP-containing proteins from the cell wall material of mature runner beans was described. The evidence presented there gave no clear indication as to the association of these proteins with the wall polysaccharides. In the present

Table 1. Amino acid composition (mol/100 mol of amino acids) of proteins from parenchyma of mature beans

	SDC soluble	SDC/PAW soluble	SDC/PAW insoluble
Hydroxyproline	—	3·1	9·9
Aspartic acid	23·2	8·9	8·9
Threonine	4·4	5·0	4·4
Serine	7·6	7·0	9·1
Glutamic acid	10·7	8·7	9·0
Proline	4·1	7·6	6·7
Glycine	7·5	9·5	8·5
Alanine	7·0	8·2	7·5
½ Cystine	—	0·3	0·2
Valine	6·0	8·6	4·5
Methionine	0·7	1·1	0·8
Isoleucine	4·2	4·8	2·8
Leucine	7·3	8·7	7·3
Tyrosine	1·6	3·6	3·0
Phenylalanine	3·3	4·5	3·4
Lysine	7·2	5·6	8·4
Histidine	1·0	1·0	2·0
Arginine	4·2	3·7	3·6

investigation, parenchyma cell wall from mature runner beans was used for chemical fractionation studies. The aim was four-fold: to confirm the presence of HP-rich proteins in the cell wall; to establish the existence of more than one type of wall glycoprotein; to gain information about the association of the wall glycoproteins with the various groups of wall polysaccharides; and to isolate the wall glycoproteins (fragments?) for characterization.

RESULTS

Composition of the wall material

To afford a comparison of wall proteins with other cellular proteins, particularly with those likely to contaminate the wall preparation, the amino acid composition of the proteins solubilized by SDC, PAW from SDC-extracted material and that of the final preparation are given in Table 1. The relatively high value of HP in the final preparation and the resistance of the residual proteins to pronase action shows that the wall preparation is reasonably "clean".

Products of fractionation of wall material

The results of fractionation of the wall material by Methods 1 and 2 are shown in Tables 2 and 3. Tables 4 and 5 show the amino acid composition of the proteins associated with the various groups of polysaccharides isolated by Methods 1 and 2 and that of the glycoproteins solubilized by the chlorite–HOAc treatment. From these results the following conclusions may be drawn.

(1) Very few proteins are associated with the pectic substances and the associated proteins are relatively poor in HP. It seems likely that these proteins are essentially the same as those that are associated with hemi-cellulose A. (2) Most (90%) of the HP-rich glycoprotein associated with α′-cellulose (Method 2) could be liberated by treatment with chlorite–HOAc. However, mild acid

Table 2. Products of fractionation by Method 1 of the cell wall material of parenchyma

Constituent	% (w/w) Composition	% (w/w) Sugars present in hydrolysates
Cell wall material	(4·8)	
1. Pectic substances	34·0 (0·7)	GalA (75·4), Gal (15·2), Ara (6·7), Gl (1·2), Xyl (1·2), Rha (0·2)
2. $NaClO_2$–HOAc soluble polymers*	8·0 (7·6)	Ara (49·2), Gal (44·9), uronic acid (2·3), Glc (1·4), Rha (1·4), Xyl (0·8)
2a. Polymer from H_2O layer of 2 after PhOH–H_2O fractionation	6·0 (8·3)	Gal (45·3), Ara (41·3), uronic acid (6·2), Rha (3·2), Xyl (2·8), Glc (1·3)
3. Hemicellulose A	16·0 (10·5)	Gal (39·4), Glc (23·7), uronic acid (18·9), Ara (10·8), Xyl (9·3)
Hemicellulose B	3·4	Glc (43·9), Xyl (21·5), Gal (15·7), Man (9·8) Fuc? (4·1), Ara (4·0), Rha (1·1)
4. α-Cellulose	33·5 (0·3)	Glc (77·6), uronic acid (11·8), Gal (6·4), Ara (2·9), Xyl (1·3)

The figures in parentheses are the percentage protein content of the polysaccharide fractions. For each fraction, the sugars liberated on hydrolysis are listed in descending order of concentration and their percentage composition are given within parentheses.

The pectic substances has been corrected for coprecipitated hexametaphosphate.

* The chlorite–HOAc soluble polymers may contain coprecipitated NaOAc and it was purified by PhOH–H_2O fractionation.

Table 3. Products of fractionation by Method 2 of the cell wall material of parenchyma

Constituent	% (w/w) Composition	% (w/w) Sugars present in hydrolysates
1. Pectic substances	32·0 (0·8)	See Table 2
2. Hemicellulose A′	17·2 (11·2)	Gal (42·4), Xyl (19·7), Glc (17·6), Ara (13·0), uronic acid (3·9), Fuc? (1·8), Rha (1·1), Man (0·5)
Hemicellulose B′	4·6	Glc (47·5), Xyl (22·1), Gal (15·8), Ara (9·0), Rha (5·6)
3. α′-Cellulose	47·4 (3·4)	Glc (81·1), Gal (8·5), Ara (5·6), Man (2·0), Rha (1·3), uronic acid (1·2), Xyl (0·6)
4. $NaClO_2$–HOAc treated α′-Cellulose	35·9 (0·35)	Glc (87·3), Gal (4·9), Ara (2·8), uronic acid (2·1), Man (1·4), Xyl (0·8), Rha (0·7)
$NaClO_2$–HOAc soluble polymers from α′-Cellulose*	9·1 (6·4)	Ara (46·6), Gal (37·4), Glc (10·2), uronic acid (3·9), Rha (1·3), Xyl (0·5)

The figures in parantheses are the percentage protein content of the polysaccharide fractions. For each fraction, the sugars liberated on hydrolysis are listed in descending order of concentration and their percentage composition are given within parentheses.

The pectic substances have been corrected for coprecipitated hexametaphosphate.

* The chlorite–HOAc soluble polymers from α′-Cellulose may contain coprecipitated NaOAc.

treatment (dil. HOAc, pH 2·9, 4 hr at 70°) did not solubilize the HP-containing polymer. (3) The carbohydrate composition of the chlorite–HOAc solubilized polymer from the depectinated material (Method 1) is comparable with that solubilized from α′-cellulose (Method 2), a characteristic feature being the relatively high levels of arabinose and galactose. The presence of an appreciable quantity of glucose in the hydrolysate of the polymer solubilised from α′-cellulose suggests that

Table 4. Amino acid composition (mol/100 mol of amino acids) of proteins from the cell wall material of parenchyma of mature runner beans fractionated by Method 1

	Protein preparations* (1)	(2)	(2a)	(3)
Hydroxyproline	1·2	32·1	43·5	2·3
Aspartic acid	9·4	7·0	5·2	10·2
Threonine	3·6	3·0	2·7	4·8
Serine	13·7	14·7	16·4	8·4
Glutamic acid	11·4	6·1	4·6	11·0
Proline	6·1	6·9	6·0	5·1
Glycine	12·7	5·8	3·1	9·2
Alanine	7·1	3·3	1·7	9·5
½ Cystine†	t	(0·3)	t	0·2
Valine	4·3	3·0	2·9	4·9
Methionine†	t	(1·3)	t	0·7
Isoleucine	3·0	0·8	0·5	3·7
Leucine	5·8	2·0	0·6	10·2
Tyrosine	1·7	0·2	0·5	0·2
Phenylalanine	2·6	1·0	0·3	4·4
Lysine†	11·0	1·8(8·0)	1·6(8·5)	2·2(6·1)
Histidine	2·4	1·2	1·2	1·2
Arginine	3·9	1·3	0·5	4·5

* The preparations used were: (1) pectic substances; (2) chlorite–HOAc soluble polymer; (2a) polymer from H_2O layer of 2 after PhOH–H_2O fractionation; (3) hemicellulose A.

† The values within parentheses are the concentrations of the modified amino acids of the parent. These would be cysteic acid (cystine), methionine sulphoxide (methionine) and α-amino adipic acid (lysine). t—trace.

Table 5. Amino acid composition (mol/100 mol of amino acids) of proteins from the cell wall material of parenchyma of mature runner beans fractionated by Method 2

	Protein preparations* (1)	(2)	(2a)	(3)
Hydroxyproline	3·2	17·8	25·8	7·2
Aspartic acid	11·2	7·7	8·7	9·3
Threonine	4·7	3·7	3·2	4·8
Serine	8·0	12·3	11·4	7·6
Glutamic acid	11·2	7·2	9·5	7·7
Proline	5·6	7·5	7·6	4·5
Glycine	8·9	6·7	7·0	9·6
Alanine	8·7	7·0	4·0	9·6
½ Cystine†	(0·3)	t	t	t
Valine	4·7	3·8	5·1	6·1
Methionine†	(0·7)	t	(0·4)	t
Isoleucine	3·2	1·7	1·7	5·5
Leucine	9·1	5·7	3·5	11·1
Tyrosine	2·7	2·9	—	0·3
Phenylalanine	3·7	3·0	0·1	5·9
Lysine†	7·5	8·2	2·1(8·1)	2·3(4·0)
Histidine	1·6	1·9	—	0·5
Arginine	4·3	2·0	1·2	2·9

* The preparations used were: (1) hemicellulose A′; (2) α′-cellulose; (2a) polymer solubilized from α′-cellulose by chlorite–HOAc; (3) chlorite–HOAc treated α′-cellulose (residual proteins).

† The values within parentheses are the concentrations of the modified amino acids of the parent. t = trace.

some glucans are also extracted. It appears that the $-CH_2OH$ groups of the galactose residues of the glycoproteins are not readily oxidizable by chlorite–HOAc. (4) The HP-rich glycoprotein solubilised by chlorite–HOAc from the depectinated material (Method 1) is probably the one associated with α′-cellulose (Method 2). (5) Ph OH–H_2O fractionation of the polymer solubilised by chlorite–HOAc (Method 1) resulted in the removal of a protein (fragment?) relatively poor in HP into the PhOH layer, leaving the heavily glycosidated protein in the aq. layer. This protein was found to be very rich in HP (43·5 mol%). (6) The protein content (and amino acid composition) of the hemicellulose A isolated by both methods is not significantly different and the associated proteins are relatively poor in HP. It is tentatively concluded from this that the protein represents material closely associated with hemicellulose A. (7) Chlorite–HOAc treatment of the depectinated material (Method 1) appears to render the holocellulose susceptible to partial oxidation with alkali. This would account for the production of an appreciable quantity of uronic acid (probably glucuronic acid) on hydrolysis of the α-cellulose (Method 1). (8) Some of the galactose and arabinose detected in the hydrolysates of the hemicellulose A, α-cellulose (Method 1) and Chlorite–HOAc treated α′-cellulose (Method 2) could have arisen from the wall glycoproteins associated with them. These results taken together suggest that polysaccharide fractionations of plant tissues after delignification may be only of limited meaning in terms of the *in vivo* matrix polymers.

DISCUSSION

From the limited data on the wall polysaccharide-protein association of cell suspension cultures, it is difficult to make any generalizations about the nature of these associations and their interactions in higher plant tissues [5]. This problem has been further complicated by the lack of suitable methods for the isolation of wall material free of cytoplasmic proteins and the resistance of the wall proteins to non-degradative procedures to render it soluble. In this study, the first difficulty was overcome by using a relatively simple, homogenous higher plant tissue, namely parenchyma, from mature beans. This tissue is convenient to handle and the wall material can be fractionated into the various groups of polysaccharides with and without subjecting it to the delignification step, because it is free of lignin. In this respect it is similar to cell suspension cultures. The second difficulty was overcome by using powerful protein solubilizing reagents to remove cytoplasmic proteins and other contaminants giving relatively "clean" wall preparations [12]. Preliminary experiments showed that by slightly modifying the standard fractionation procedure, illuminating results about wall polysaccharide-protein association could be obtained. Further, the chlorite–HOAc treatment proved to be a convenient method for isolating hydroxyproline-rich wall glycoproteins (fragments?). No doubt some of the constituent amino acids of proteins are oxidized by this reagent but the nature of these oxidations was studied using known proteins [6]. By using these probes fairly definite conclusions about the type of wall polysaccharide-protein associations have been obtained.

It has been shown in this study that the HP-proteins of the wall material of parenchyma can be separated into (at least) two distinct fractions on the basis of their solubility (in inorganic solvents). The purity of these fractions is difficult to assess and some cross-contamination may occur, but their difference in HP level suggests that the fractions are distinct. The glycoprotein relatively rich in HP is associated with α′-cellulose and the one relatively poor in HP is associated with hemicellulose A. Because very few proteins are associated with the pectic substances it is difficult to draw any postive conclusions about them. Chlorite–HOAc treatment of α′-cellulose either destroyed one type of cross-linkage or hydrogen bond by oxidation, or cleaved the peptide chain, liberating most of the HP-rich glycoproteins. These results suggest a potential use of chlorite treatment for isolation of (modified) wall glycoproteins. Alkali treatment of the holocellulose affected another type of cross-linkage in a similar manner, solubilizing proteins relatively poor in HP. It is possible that some of these proteins are enzymes very firmly bound to the wall. These results strongly support the view that more than one protein is located in the wall. Further, they are in agreement with those from sycamore sus-

pension culture cell walls, in which insolubility of HP-rich proteins in alkali has been reported [4]. However, they are in contrast to those from lupin hypocotyls [13], in which almost complete solubility of the HP-rich proteins by strong alkali was reported. This difference in alkali solubility may be due to either the tissue used, or the difference in the stage of maturity of the tissue. In the present study parenchyma from mature beans was used, compared with the immature tissues from lupin hypocotyls.

The molar ratios of HP, arabinose, galactose, xylose, rhamnose, glucose and uronic acid in the chlorite–HOAc solubilized polymer which was purified by PhOH–H_2O fractionation (glycoprotein X') are 1·2·6:2·4:0·2:0·2:0·1:0·3. Future work might show that the relatively small amounts of uronic acid (xylose, rhamnose and glucose) found in the hydrolysates of the polymer may be due to either the partial oxidation of galactose residues (giving rise to uronic acid), or the presence of small amounts of pectic acid in the preparation. However, by the methods used so far this distinction is not possible. Using these figures and assuming that all the HP is covalently bonded to arabinose and galactose, we can speculate that the average degree of polymerization of the oligosaccharide side-chains of the glycoprotein is 5. This figure has to be regarded as 'minimal' as some of the arabinose- and galactose-containing oligosaccharide side chains may have been hydrolysed by warm dil. HOAc. However it is of interest that a comparable size of oligosaccharide side chain of arabinose and galactose, is found in the HP-containing proteins of sycamore [14] tissue culture cell walls. Lamport using tomato tissue culture cell walls has shown that while the arabinose residues are mainly linked to HP, the galactose residues are linked to serine [15]. These results suggest that the glycoprotein associated with α-cellulose is a polyhydroxyproline polymer, carrying mainly arabinose and galactose side chains which are linked to the cellulose microfibrils by either strong hydrogen bonds as suggested by Northcote or both hydrogen and covalent bonds because 'oxidative degradation' is necessary for their release. Although the present paper deals only with parenchyma of mature beans, the occurrence of the complexes described is probably of general significance in higher plants.

EXPERIMENTAL

Plant material. Mature runner beans (cv. Streamline, av. length 30 cm; width 2 cm) used in this investigation were collected from plants grown in exptl plots near the laboratory. Parenchyma was obtained by scraping the pods (split in half length-wise) up to the parchment layer with a spoon.

Cell wall preparation. Wall material of parenchyma was prepared by sequential extraction of fresh ball milled tissue with 1% SDC and PhOH–HOAc–H_2O (2:1:1, w/v/v) by methods described earlier [12]. To minimize irreversible artificial binding of the wall glycoproteins to other wall polymers, the wall preparation after PAW extraction was washed 3× with dist. H_2O and used directly for fractionation studies (i.e. treatment with alcohol was omitted). 100 g fr. tissue gave 1·1–1·3 g (dry) wall preparation containing about 4·8% wall protein.

Fractionation of wall material. Two methods were used for fractionation. In Method 1 the wall material was fractionated as before [6], by sequential treatments with 2% hexametaphosphate, sodium chlorite–HOAc for 4 hr at 70° (delignification step) and 1 and 4 N KOH for 2 hr each, leaving behind the α-cellulose residue. In Method 2 the chlorite–HOAc treatment was omitted. This gave α'-cellulose which was rich in HP-containing protein, plus some associated arabinan–galactan, most (90%) of which could be liberated by treatment with chlorite–HOAc for 4 hr. at 70°. This is a convenient method for isolation of wall glycoproteins which have been somewhat modified.

Isolation and purification of "glycoproteins" from chlorite–HOAc extracts. Chlorite–HOAc treatment of depectinated material (Method 1) and α'-cellulose (Method 2) solubilized HP-rich glycoproteins. These compounds were isolated from extracts as previously described [6]. 1 g wall material gave about 50 ng (crude) wall glycoproteins. Crude glycoprotein (50 mg from Method 1) was dissolved in warm dist. H_2O (15 ml, 70°) and fractionated with PhOH (15 g) essentially as described before. The bulk of the HP-rich glycoprotein remained in the aq layer and was isolated and analysed. For convenience this will be referred to as "glycoprotein X". The small amount of protein (5–10%) present in the PhOH layer was not analysed.

Amino acid and protein analyses. The protein content of the polysaccharide fractions and glycoproteins and the amino acids liberated from them on hydrolysis with 6 N HCl for 24 hr at 110° were determined by methods described earlier [6,12].

Carbohydrate analyses. The sugars liberated from the polysaccharide fractions and glycoproteins on acid hydrolysis were isolated and analysed by the methods described earlier [6,16].

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