Phytochemistry, 1975, Vol. 14, pp. 2169-2174. Pergamon Press. Printed in England.

CELL WALL GLYCOPROTEINS AND POLYSACCHARIDES OF MATURE RUNNER BEANS

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(*Received* 23 *January* 1975)

Key Word Index—*Phaseolus coccineus*; Leguminosae; runner beans; cell wall; sodium chlorite treatment; delignification; cell wall proteins; hydroxyproline; galactose; arabinose.

Abstract—A novel use of chlorite–HOAc treatment (delignification procedure) for the isolation of hydroxyproline (HP) rich "glycoproteins" from the depectinated cell wall material of mature runner beans is described. This procedure can be used for the isolation of wall proteins even from heavily lignified tissues. Its main disadvantage is that some of the constituent amino acids are either destroyed or modified; the nature of these changes was studied using gelatine, lysozyme and "cytoplasmic proteins" of mature beans. The main amino acids to be affected were tyrosine, cystine, methionine and lysine. The chlorite–HOAc solubilized proteins were separated by PhOH–H_2O fractionation into two distinct "glycoprotein fractions". The major fraction (isolated from the aqueous layer) contained most of the HP of the solubilized proteins. The sugars obtained on hydrolysis of both "glycoproteins" were galactose, arabinose, glucose, xylose, rhamnose and uronic acid. Most of the proteins remaining in the holocellulose could readily be extracted with cold alkali and were relatively poor in HP.

INTRODUCTION

The isolation of cell wall proteins from plant tissues is difficult, because they are embedded in the wall matrix and are strongly bonded to the wall polymers, possibly by covalent linkages [1]. Heath [2] and Monro *et al.* [3] found that the HP-containing glycoproteins could not be extracted from wall preparations with solvents known to disrupt hydrogen bonds. Lamport used drastic enzymes [4] and hydrolysis with $Ba(OH)_2$ solution [5] to extract much of the glycoproteins from the wall material of suspension cultures of tomato. Heath and Northcote [6] employed hydrazinolysis to obtain soluble glycopeptides rich in HP from the "α-cellulose" fraction of the wall of sycamore tissue culture cells. In this paper, the use of warm Na chlorite–HOAc to solubilize HP rich glycoproteins from the wall material of runner beans and thus separate it from a (wall) protein relatively poor in HP is described.

RESULTS AND DISCUSSION

The results of fractionation of the wall material of mature beans is shown in Table 1. Some of the arabinose and galactose detected in the hydrolysates of the pectic substances, hemicelluloses and α-cellulose could have arisen from the (wall) glycoproteins associated with them. It appears that some of the $-CH_2OH$ groups of the constituent glucose molecules of α-cellulose of parenchyma are susceptible to oxidation with alkali after chlorite–HOAc treatment. In contrast the corresponding groups in the α-cellulose of lignified tissues from beans (e.g. "strings" and "parchment layer") are not readily oxidizable (R. R. Selvendran, unpublished results). This would account for the presence of an appreciable amount of uronic acid (probably glucuronic acid) in the hydrolysates of the α-cellulose fraction from mature beans. Similar results were obtained with the α-cellulose fraction of lignified (stem and root

Table 1. Percentage composition of the cell wall material of mature runner beans

Constituent	Composition % (w/w)	Sugars present in hydrolsates
Cell wall material	(2·7)	
1. Pectic substances	24·8 (0·6)	GalA (73·6), Gal (12·7), Ara (6·7), Man (2·5), Glc (2·1), Xyl (1·4), Rha (1·0)
2. $NaClO_2$–HOAc soluble polymers*	3·0 (10·2)	Ara (35·2), Gal (32·2), uronic acid (14·0), Glc (10·3), Xyl (6·3), Rha (2·0)
2a. Polymer from H_2O layer of 2	2·0 (7·6)	Gal (41·9), Ara (23·6), uronic acid (18·1), Glc (9·8), Rha (4·1), Xyl (2·5)
2b. Polymer from PhOH layer of 2	0·7 (8·8)	Gal (62·6), Ara (19·5), Xyl (10·5), Glc (3·7), uronic acid (2·6), Rha (1·1)
$NaClO_2$–HOAc soluble compounds†	15·5	
3. Hemicellulose A	21·5 (5·1)	Xyl (40·8), Ara (23·6), Glc (17·9), Gal (12·2), Man (4·3), Rha (1·2)
Hemicellulose B	1·2	Glc (39·2), Xyl (26·8), Man (14·4), Gal (10·7), Rha (5·3), uronic acid (2·0), Ara (1·6)
4. α-Cellulose	34·0 (0·8)	Glc (66·5), uronic acid (11·4), Gal (11·3), Xyl (5·9), Ara (4·0), Rha (2·9)

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 have been corrected for coprecipitated hexametaphosphate.

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

† Value for $NaClO_2$–HOAc soluble compounds was obtained from the difference in weights of the hexametaphosphate extracted residue before and after the delignification step.

wood) and non-lignified (stem and root bark) tissues of the tea plant [7]. Preliminary experiments with the separated fractions (6N HCl hydrolysis followed by PC of the hydrolysates), indicated that the bulk of the HP-rich "glycoproteins" were solubilized during the delignification step along with the lignin. From the delignification liquor, after dialysis and concentration, a polymer rich in HP was obtained by precipitation with alcohol. Acid hydrolysis (2N H_2SO_4 for 12 hr) of the polymer gave arabinose, galactose, uronic acid, glucose and xylose. However, the ratio of arabinose and galactose to the uronic acid in the polymer was considerably higher than the corresponding ratio in the pectic substances. These results suggested that the chlorite–HOAc treatment of the depectinated wall material solubilised the HP-rich wall protein in addition to lignin. In this connection it is of interest to note that Monro *et al.* [8] reported the solubilization of an appreciable quantity of "arabinogalactan" from the depectinated material of lupin hypocotyl cell walls during the delignification procedure. It is possible that in their studies too some of the wall glycoproteins (carrying arabinose and galactose residues) were solubilized. These findings underline the need for caution when using chlorite–HOAc for delignification. However, the delignification procedure appeared to offer a convenient method for isolating (modified) wall proteins.

Because of the results reported by Alexander *et al.* [9,10] and Saito *et al.* [11], it was anticipated that treatment of wall proteins with oxidizing agents would result in the oxidation and subsequent modification of some of the constituent amino acids (and carbohydrate residues). It was thought desirable as a preliminary to establish the nature of some of these changes when known proteins such as gelatine, lysozyme and AIR of mature beans (containing coprecipitated cytoplasmic proteins) were treated with chlorite–HOAc for 4 hr at 70°. Trials with gelatine and lysozyme showed that they could be completely dissolved and almost quantitatively recovered (>90%) after the treatment. As can be seen from the results (Table 2), the above treatment brought about the following major changes in the constituent amino acids: (1) most of the tyrosine was destroyed; (2) methionine was oxidized to its sulphoxide and cystine was oxidized to cysteic acid; (3) most of the lysine was converted to α-aminoadipic acid.

Table 2. Amino acid composition (mol/100 mol of amino acids) of "proteins" treated with chlorite–HOAc for 4 hr at 70°

Amino acid	Protein preparations (1)	(1a)	(2)	(2a)	(3)	(3a)*
Hydroxyproline	8·6	8·0	0	0	3·2	0·6
Aspartic acid	4·7	4·7	17·9	24·4	11·2	11·2
Threonine	1·5	1·6	5·4	7·3	5·2	5·4
Serine	3·6	3·5	8·3	8·2	7·3	6·9
Glutamic acid	7·4	7·7	4·2	6·3	11·0	11·6
Proline	12·6	12·5	1·8	1·4	6·3	4·4
Glycine	36·4	36·0	10·4	10·0	8·1	8·6
Alanine	10·6	11·0	10·2	10·6	9·1	9·6
½ Cystine†	0·05	(0·05)	5·0	(2·7)	0·3	t
Valine	1·7	2·0	4·5	3·6	5·8	8·2
Methionine†	0·15	(0·6)	1·2	t	0·4	(1·4)
Isoleucine	0·8	0·9	4·4	4·1	3·6	5·7
Leucine	2·3	2·3	6·8	5·2	9·0	10·3
Tyrosine	0·3	0	2·4	0	2·8	0
Phenylalanine	1·2	1·1	2·4	3·1	3·8	4·5
Lysine†	2·6	1·2(1·6)	5·1	0·5(4·6)	7·1	1·9(5·8)
Histidine	0·4	0·3	0·7	0	1·9	0·3
Arginine	4·6	4·7	8·8	6·9	3·7	3·6

The preparations used were: (1) gelatine; (1a) treated gelatine; (2) lysozyme; (2a) treated lysozyme; (3) alcohol-insoluble residue of mature beans; (3a) treated AIR.

* Only the protein amino acids of the insoluble residue (holocellulose) were determined. The bulk of the coprecipitated cytoplasmic proteins remain in the "holocellulose", although most of the hydroxyproline rich proteins would have been solubilized. The hydroxylysine content of untreated gelatine was 0·5 mol/100 mol of amino acids.

† 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-aminoadipic acid (lysine). t—trace.

It appeared that some ornithine was produced from arginine (especially in the alkali treated samples) and this made the estimation of lysine difficult, because these two acids overlapped in the chromatographic system used. Incomplete oxidation of lysine is not surprising in view of the stereochemical restrictions which must exist in the 3-dimensional network of cross-linked polypeptide chains. No significant loss of HP and proline was noticed in the case of gelatine. The marked decrease in the HP and proline level of treated AIR was due to the fact that some of the wall proteins have been solubilized (see footnote to Table 2). Aspartic acid showed negligible change in the case of gelatine and AIR, and its increase in the treated lysozyme is not readily explicable. The other amino acids were not significantly affected within the limits of the experimental error of analysis. The effect of chlorite treatment on the peptide links is uncertain. Hydrolytic fission of peptide links, followed by oxidation of the amino acids so produced may occur. From this study, all that seems certain is that these oxidative processes produce HP-rich wall "glycoprotein" fragments and that this degradation is accompanied by an increase in acidic groups, mainly in the side chains of some of the amino acid residues. While many additional studies are necessary to clarify the mode of oxidation (and release of wall proteins), it seems clear that, despite the obvious disadvantages, the above treatment may prove useful for isolation of wall proteins (especially from lignified tissues).

Table 3 shows the amino acid composition of the proteins associated with the various groups of polysaccharide fractions. The amino acid composition of the cell wall material has been reported before [12]. By using milder extraction procedures it is possible to show that the HP-rich "glycoprotein" is actually associated with the α-cellulose fraction of parenchyma and that it can be readily liberated by treatment with chlorite–HOAc [13]. The chlorite–HOAc solubilized proteins were separated by PhOH–H_2O fractionation to give a HP-rich "glycoprotein X" (soluble in H_2O) and a "glycoprotein Y" which contained relatively less HP (soluble in PhOH). Table 3 also shows that amino acid composition of these "glycoproteins". It is of course possible that these "glycoproteins" are fragments of a macromolecu-

Table 3. Amino acid composition (mol/100 mol of amino acids) of proteins from the cell wall material of mature runner beans

Amino acid	Protein preparations* (1)	(2)	(2a)	(2b)	(3)
Hydroxyproline	6·0	24·7	33·9	9·5	4·1
Aspartic acid	9·2	8·7	7·7	9·8	10·9
Threonine	3·7	2·8	2·5	3·5	4·5
Serine	14·9	10·4	11·4	6·4	9·4
Glutamic acid	9·0	7·2	6·1	9·8	10·3
Proline	5·0	9·8	8·6	10·2	6·9
Glycine	7·1	6·0	4·5	9·3	9·0
Alanine	6·9	4·1	2·4	5·9	8·8
½ Cystine†	t	(0·5)	t	t	t
Valine	4·7	5·4	5·0	8·9	6·2
Methionine†	t	t	(0·3)	t	(0·8)
Isoleucine	2·7	1·7	1·1	3·0	4·0
Leucine	4·8	3·5	1·7	6·2	8·3
Tyrosine	2·0	0·2	0·3	0·2	0·4
Phenylalanine	2·2	1·2	0·7	2·9	3·5
Lysine†	15·5	0·8(10·3)	1·0(10·9)	1·2(9·6)	3·0(6·4)
Histidine	3·6	1·1	1·1	0·7	0·7
Arginine	2·4	1·7	0·9	2·9	2·5

* The preparations used were: (1) Pectic substances; (2) Chlorite–HOAc soluble polymer; (2a) polymer from H_2O layer of 2; (2b) polymer from PhOH layer of 2; (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-aminoadipic acid (lysine). t—trace.

lar protein. From these results it is clear that the bulk of the HP-rich proteins are solubilized by chlorite–HOAc and the proteins associated with hemicellulose A are relatively poor in HP. It would appear that the protein moiety of the holocellulose is "bound" to it by a bond as sensitive to alkali as an ester, because dilute alkali solubilised it. The small protein residue remaining in the α-cellulose is unlikely to have been held by electrostatic forces or hydrogen bonds. The "glycoprotein X" (which may be contaminated with some pectic substances) contains HP, galactose, arabinose, uronic acid, glucose, rhamnose and xylose residues in the molar ratio 7·7:14·0:9·5:5·6:3·3:1·5:1·0, whereas the "glycoprotein Y" contains the above compounds in the molar ratio 4·0:54·0:20·2:2·1:3·2:1·0:10·9. From these results it is clear that the galactose and arabinose are well in excess of the acidic sugar, showing that these sugars are presumably linked to the proteins and are not liberated from pectic substances which may be present as contaminants. Moreover, the pectic substances are not readily solubilized by PhOH from aqueous solutions, clearly showing that the "glycoprotein Y" has sugar residues linked to it. It could also be inferred from the work of Pusztai [14] that the protein remaining in the aqueous layer "glycoprotein X") is heavily glycosidated. Future work might show that the acidic sugar found in the polymers may be due to either the partial oxidation of galactose residues or the presence of small amounts of pectic acid in the preparation. However, by the techniques used so far this distinction is not possible.

These results show that treatment of the depectinated wall material with chlorite–HOAc enables the separation of two major types of wall proteins with some degradation. Further, because the starting material is virtually free from cytoplasmic proteins, these results suggest that more than one protein is located in the cell wall as suggested by some workers [15–19]. It is likely that some of these proteins are wall bound enzymes. The ease with which the bulk of the HP-rich "glycoproteins" are solubilized by the above treatment and thus enabling the isolation of suitable modified wall protein fragments for characterization is surprising and has not been reported before. The possibility of bonding between the wall glycoproteins and polysaccharides is a much debated issue. Earlier, Heath and Northcote [6] came to the conclusion that the HP-rich glycoprotein of sycamore tissue culture cells is closely associated

with α-cellulose possibly through hydrogen bonding. In the next paper [13] the occurrence of such association in parenchyma of mature beans is reported.

EXPERIMENTAL

Chemicals. Gelatine (BDH Ltd.) was dialysed and lyophilized. Lysozyme (muramidase) from egg white was purchased from the Sigma. All evaporations were carried out in a rotary film evaporator under red pres at 50°.

Plant material. Mature runner beans (cv. Streamline) used in this investigation were collected from plants grown in experimental plots near the laboratory.

Isolation and fractionation of cell wall material. Cell wall material of beans was prepared by sequential treatment of the fresh ball milled tissue with 1% sodium deoxycholate and PhOH–HOAc–H_2O (PAW) (2:1:1) as described before [12]. To minimize irreversible "artificial" binding of the wall proteins to other wall polymers, the wall preparation after PAW extraction was washed thrice with distilled water and used directly for fractionation studies (i.e. treatment with alcohol was omitted). Wall material was extracted 2× with 2% hexametaphosphate at 95° for 2 hr, followed by washing with H_2O. Residual material was then treated with sodium chlorite–HOAc essentially as described by Wise *et al.* [20]. Depectinated material (5 g) was suspended in 200 ml of dist. H_2O in a loosely-stoppered 250 ml round-bottomed flask. HOAc (0·5 ml) and sodium chlorite (1·25 g) were added and the flask was immersed up to the neck in a water bath at 70°. Same amounts of HOAc and chlorite were added at hourly intervals. After 4 hr extraction (depectinated material–chlorite–HOAc, 1:1:0·4, the contents of the flask were centrifuged and residue (holocellulose) washed 6 × with 3 bed vol. cold dist. H_2O on the centrifuge. Holocellulose was then extracted with 1 and 4 N KOH for 2 hr each to remove the hemicelluloses. In all these treatments the sample solvent ratio was 1:40. Residue was then washed with dist. H_2O 4 × and finally with absolute alcohol and Et_2O. The final residue was air dried till most Et_2O had evaporated and then dried over Si gel in a vacuum dessicator. The polymers were isolated from the extracts by methods described before [12,21]. Gelatine, lysozyme and AIR of mature beans were treated with chlorite–HOAc as described for the depectinated material.

Isolation of HP-rich glycoproteins from chlorite–HOAc extracts. The supernatant obtained after chlorite–HOAc treatment was filtered and dialysed, against 15 vol dist. H_2O, for 6 hr at room temp. Dialysed extract was concentrated and poured into 5 vol. of EtOH containing 1% HOAc. After 24 hr, the pp obtained by centrifugation was dissolved in H_2O and reprecipitated from EtOH. The pp was then washed with absolute alcohol followed by Et_2O, and dried over Si gel in a vacuum desiccator. One gram (dry) wall material gave about 30 mg crude "wall protein".

Extraction of "wall protein" with PhOH–H_2O. The "wall protein" was extracted with PhOH–H_2O essentially according to the procedure of Westphal and Jann [22]. Powdered protein (100 mg) was placed in a centrifuge tube and gently dispersed in warm dist. H_2O (15 ml). Solid phenol (15 g) was added to the colloidal soln and the mixture quickly warmed to about 70° on a H_2O bath. The contents of the tube were mixed with a glass rod and rapidly cooled to room temp. by immersing the tube in a cold H_2O bath, when the 2 phases separated (centrifugation helped). The aq. layer was siphoned off and the procedure repeated with further additions of H_2O and the aq. phases were pooled. The 2 phases were evaporated to dryness and the residues were taken up in minimum vol. H_2O and the proteins were precipitated by the addition of EtOH containing 1% HOAc. Resulting precipitates were washed with EtOH and then ether, and dried over silica gel in a vacuum desiccator. The "glycoproteins" isolated from the H_2O and PhOH layers are referred to as glycoproteins X and Y respectively.

Amino acids of protein hydrolysates. The proteins (and polysaccharide fractions having associated proteins) were hydrolysed with 6 N HCl for 24 hr and the liberated amino acids were determined by the methods described before [12]. The presence of α-amino adipic acid in the chlorite treated proteins was inferred from its position on the ion-exchange chromatogram and was confirmed by GLC by the method of March [23]. Amino acid analyses of proteins associated with polysaccharides yielded a number of (minor) unidentified ninhydrin-positive peaks, which are characteristic of this type of hydrolysates. These are due to condensation products formed from amino acids, sugars and uronic acids. These characteristic peaks were present in the hydrolysates of the wall material and polysaccharide fractions, but were diminished in quantity in the chlorite–HOAc soluble polymer and were completely absent in the PhOH soluble glycoprotein-Y. It should be noted that in the results given in Tables 2 and 3, allowance was not made for loss due to hydrolysis in the presence of carbohydrates.

Sugars present in polysaccharides and glycoproteins. The polysaccharide fractions were hydrolysed with 2 N H_2SO_4 for 12 hr and the liberated sugars were isolated by methods described before [7,21]. In the case of the pectic substances, it was found that pretreatment with (Sigma) prectinase made it more soluble in dil. H_2SO_4. "Glycoproteins X and Y" isolated from the chlorite soluble polymer were hydrolysed with 3% HNO_3 (v/v) containing 0·05% (w/v) urea at 100°C for 3 hr and the sugars were isolated from the hydrolysates after removal of HNO_3 by repeated evaporation with H_2O. The isolated sugars were determined by ion exchange chromatography, using borate buffers by the newly developed method of Davies *et al.* [24].

Total nitrogen and "protein". These were determined as described before [12].

Acknowledgements—The authors thank Professor R. L. M. Synge, FRS and Dr. F. A. Isherwood for helpful discussions. They also thank Mr. J. F. March for the identification of α-amino adipid acid by GLC and Miss N. M. Gilmour and Mrs. V. E. Newby for their technical assistance.

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