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PURIFICATION AND METHYLATION ANALYSIS OF CELL WALL MATERIAL FROM *SOLANUM TUBEROSUM*

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Key Word Index—*Solanum tuberosum*; Solanaceae; potatoes; cell wall material; extraction and purification; methylation analysis.

Abstract—Cell wall material (CWM) of potatoes was prepared by sequentially extracting the wet ball-milled tissue with 1% aq. Na deoxycholate, PhOH–HOAc–H_2O and 90% (v/v) aq. DMSO. The purity of the CWM (e.g. absence of residual starch) was established by carbohydrate analysis using different acid hydrolysis conditions and by methylation studies. The partially methylated alditol acetates from the $CHCl_3$–MeOH soluble fraction (S) of the methylated CWM were separated into 15 main peaks by GLC. Fourteen of these peaks were carbohydrate derivatives and the identity of most of these was established by MS. Reduction of the hydrolysate of S with $NaBD_4$ was used to identify the carbohydrate derivatives present in peaks 7 and 11 above. The occurrence of 4-linked galacturonosyl residues in the methylated polymers was established after reduction of S with $LiAlH_4$ and $LiAlD_4$. The main glycosidic linkages present in the non-cellulosic polysaccharides of the wall in descending order of concentration are: 4-linked galactose, 4-linked galacturonic acid, 5-linked arabinose and 4,6-linked glucose. The major branch points are those through 0–6 of glucose and 0–4 of rhamnose. Arabinose, galactose and xylose residues constituted the non-reducing ends. Graded acid hydrolysis of the CWM made it possible to assess the relative strengths of some of the glycosidic linkages. The general structural features of the CWM are discussed in the light of these results.

INTRODUCTION

Most investigations on the texture of vegetables have centred on the cell wall itself, which is the obvious subject to study in as much as it is the cell wall which serves to give rigidity to the plant tissue. It is the changes in the amounts and properties of the cell wall constituents which influence any major changes in texture that occur during maturation, ripening and cooking of edible plant tissues [1]. Work on the texture of potatoes [2] and the possible importance of 'fibre' in vegetables [3] had led us to investigate the cell wall polymers of potatoes. This vegetable was chosen because little is known about its cell wall polymers and because it is a staple component of the diet and presumably contributes much 'fibre' to the diet. The high starch content has made the preparation of cell wall material from potatoes very difficult, but Hoff and Castro [4] succeeded in broadly defining the major groups of polysaccharides of the walls.

We have developed an improved method for preparing appreciable quantities of cell wall material (CWM) from potatoes. With this method contamination with intracellular constituents (including starch) was minimal. Following the work on white mustard [5] and cultured sycamore cells [6] we have surveyed the wall polysaccharides by methylation analysis and partial acid hydrolysis studies. This paper reports the major structural features of the CWM.

RESULTS

The cell wall material of potatoes was prepared in two stages. In the first stage, the fresh ball-milled tissue was sequentially extracted with 1% aq. Na deoxycholate (SDC) and PhOH–HOAc–H_2O (PAW; 2:1:1, w/v/v) as described before [7]. The residue thus obtained (Preparation 1) contained large quantities of starch. This is reflected in the very high value obtained for glucose on hydrolysis with 2 N trifluoroacetic acid (TFA) for 2 hr (see column 1, Table 1). From 50 g fresh tissue, the following figures were obtained for the starch solubilized by SDC, PAW and starch in the residue: SDC (381 mg), PAW (697 mg) and residue (6.4 g). The second stage was concerned with the removal of starch from Preparation 1.

Because the validity of the ball-milling procedure was queried (possible degradation of cell wall polymers), checks were carried out with potato starch. Aqueous suspensions of starch were ball-milled at 2° for differing periods (16, 32 and 48 hr) and the ball-milled starch was isolated as a freeze dried solid. Viscosity measurements of the starch in DMSO showed that there was no detectable degradation of the starch even after the 48 hr ball-milling period. These results suggest that the cell wall polymers are unlikely to be degraded under the conditions used in this study.

Removal of starch from the SDC–PAW-insoluble residue (Preparation 1)

While the bulk of the residual starch could readily be removed by digestion with α-amylase, alternative starch solubilizing reagents were tried because the enzymic method is time consuming and expensive. Of the methods tried, destarching with 90% aq. DMSO proved to be the most promising. Ultrasonic pretreatment of the suspension greatly facilitated the subsequent solubilization of

Table 1. Comparison of the neutral sugars of the cell wall preparations at various stages of purification by the different hydrolytic procedures

Neutral sugars	Relative monosaccharide composition				
	TFA hydrolysis			H_2SO_4 hydrolysis	Saeman hydrolysis
	Prepn 1	Prepn 2	Prepn 3	Prepn 3	Prepn 3
Rhamnose	0.7	4.5	6.2	7	3.4
Arabinose		9.3	15.8	15	12.1
Xylose		3	4.3	5	4.1
Mannose	—	t	t	t	t
Galactose	2.1	44.2	69.2	68.4	37.3
Glucose	97.2	22.9	4.5	4.6	43

The hydrolysis conditions and the details of the preparations are given in the text. The sugar compositions were computed as mole % of the total neutral sugars. Cellulose is not hydrolysed by TFA and H_2SO_4 hydrolysis. t—trace.

of starch. The DMSO-insoluble residue gave a negative reaction with I_2/KI, showing 'complete' removal of starch

As a further check, two batches of wall material were prepared, one using the α-amylase method (Preparation 2) and the other using the 90% aq. DMSO method (Preparation 3). The neutral sugars liberated from both preparations on TFA hydrolysis were determined and these are shown in Table 1 (columns 2 and 3). TFA hydrolysis does not hydrolyse the β (1 → 4) links of α-cellulose and only partially hydrolyses the corresponding links in the hemicelluloses. The glucose detected in the hydrolysate of Preparation 3 was shown to arise from the hemicelluloses and not from residual starch by fractionation with aq. inorganic solvents. No glucose was detected in either the hot water- or oxalate-soluble polysaccharides, the presence of which would have shown residual starch in Preparation 3 (Ring and Selvendran, unpublished results). These results showed that while there was some residual starch (amylopectin?) in Preparation 2, Preparation 3 was free of starch. The residual starch in Preparation 2 could be extracted with 90% aq. DMSO.

Because it is possible that some of the very acid labile sugar linkages (e.g. arabinofuranoside residues) of the cell wall polymers may have been hydrolysed by the conditions of acid extraction used for the preparation of the wall material, the following experiments were carried out. Wall preparations were obtained from potatoes by two methods. The first by sequential extraction of the fresh ball-milled tissue with 1% SDC, 90% aq. DMSO and absolute alcohol and the second as described for Preparation 3. Both preparations were treated with 50% aq. HOAc at 1–2° for 1 hr (conditions simulating treatment with PAW) and the sugars present in the extract and residue were determined. The results showed no evidence of hydrolysis of the sugar–sugar linkages from either preparation under these conditions.

Analysis of the DMSO-soluble polymers

Although the analysis of the DMSO-soluble polymers (DMSO-S) was not the main purpose of this investigation, some experiments were carried out to confirm that starch was the major polysaccharide solubilized by DMSO and to extend previous findings on potato starch. The DMSO-S were isolated as a freeze dried solid after dialysis and the neutral sugars obtained on TFA hydrolysis were determined. Glucose accounted for 98% of the neutral sugar fraction. The other sugars were present in very small amounts and therefore could not be identified and estimated. Methylation analysis of DMSO-S gave 2,3,4,6-tetra-*O*-methyl, 2,3,6-tri-*O*-methyl and 2,3-di-*O*-methyl-glucose in the ratio 1:31:1 respectively, as shown by analysis of alditol acetates by GC–MS. Therefore DMSO-S must contain 3% terminal glucose, 93% (1 → 4) and 3% (1 → 4,6) glucosidic linkages. These glycosidic linkages are typical of starch.

The bulk of the starch present in DMSO-S could be removed by treatment with α-amylase. TFA hydrolysis of the residue obtained after the third stage of amylosis of DMSO-S gave mainly glucose (97%), which accounted for *ca* 3% of the glucose released on hydrolysis of DMSO-S. Methylation analysis of the amylase treated DMSO-S showed that it contained 22% terminal-glucose, 74% (1 → 4) and 4% (1 → 4,6) glucosidic linkages. These glycosidic linkages are suggestive of the occurrence of limit-dextrins. From the combined evidence given above, it is proposed that *ca* 98% of the polysaccharides present in DMSO-S is composed of starch. The evidence, however, does not exclude the presence of very small amounts of other polysaccharides (notably glucans).

Composition of the cell wall material (Preparation 3)

In the following studies Preparation 3 which is referred to as the cell wall material (CWM) was used. A large part of the dry matter of the CWM could be considered as polysaccharides, wall proteins (Kjeldahl nitrogen × 6.25) and ash. The percentage composition was : carbohydrate (84), protein (2), ash (2) and unaccounted for material (12). Some of the material unaccounted for could be 'lignin-like material', 'residual water', sugar destroyed during acid hydrolysis (expecially xylose), or not measured because of incomplete hydrolysis by the methods used. Gordon *et al.* [8] have made similar observations with mesophyll cell-walls prepared from grasses. To test the effectiveness of the three hydrolytic procedures (see Experimental), the CWM was hydrolysed by all 3 procedures and the neutral sugar composition was determined by GLC. From the results (see Table 1, columns 3, 4 and 5) it is clear that Saeman-hydrolysis is the most effective. However, TFA- and H_2SO_4-hydrolysis may be used to hydrolyse preferentially the bulk of the neutral sugars of the non-cellulosic polysaccharides of the wall. The galacturonic acid residues of the CWM were only partially hydrolysed by all 3 procedures. However, pretreatment of the CWM with pectinase followed by H_2SO_4-hydrolysis

resulted in the release of the bulk of the galacturonic acid residues. TFA- and Saeman-hydrolysis of the CWM liberated only 50 and 15% respectively, of the uronic acid obtained by the 'pectinase–H_2SO_4 procedure'. The uronic acid content of the CWM obtained by the 'pectinase–H_2SO_4 procedure' was 20% of the total carbohydrate (including cellulose). This figure is in broad agreement with that obtained by methylation analysis of the $CHCl_3$–MeOH soluble fraction of the methylated CWM.

Methylation analysis of CWM

CWM (50 mg) was methylated and separated into $CHCl_3$–MeOH-soluble (S, *ca* 60%) and insoluble (I) fractions. In this study, no attempt was made to separate S into acidic and neutral components. The IR spectrum of S showed very weak OH absorption indicating that the methylation of this fraction was complete. The GLC separation of partially methylated alditol acetates obtained from S showed 15 peaks. Fourteen of these peaks (all except peak 2) were shown to contain carbohydrate derivatives by GC–MS. (1) 2,3,5-tri-*O*-Me arabinose (3) 2,3,4-tri-*O*-Me xylose (4) 3,5-di-*O*-Me arabinose (5) 3,4-di-*O*-Me rhamnose (6) 2,3-di-*O*-Me arabinose (7) 2,3- and 3,4-di-*O*-Me xylose and 2,3,4,6-tetra-*O*-Me galactose (8) 3-*O*-Me rhamnose (9) 2-*O*-Me arabinose (10) unknown (11) at max. 2,3,6-tri-*O*-Me galactose (12) unknown (13) 2,6-di-*O*-Me galactose (14-1) 3,6-di-*O*-Me galactose (14-2) 4,6-di-*O*-Me galactose (15-1) 2,3-di-*O*-Me glucose (15-2) 2,3-di-*O*-Me galactose. Continuous scanning of peaks 7 and 11 suggested that they contained more than a single derivative. The identity of most of the carbohydrate derivatives in these peaks was established by GC–MS as described in the text. The RR_t of the peaks on OV-225 (run isothermally) are only given below. The pertinent ions in the MS of the peaks given below are followed by their relative intensities within brackets.

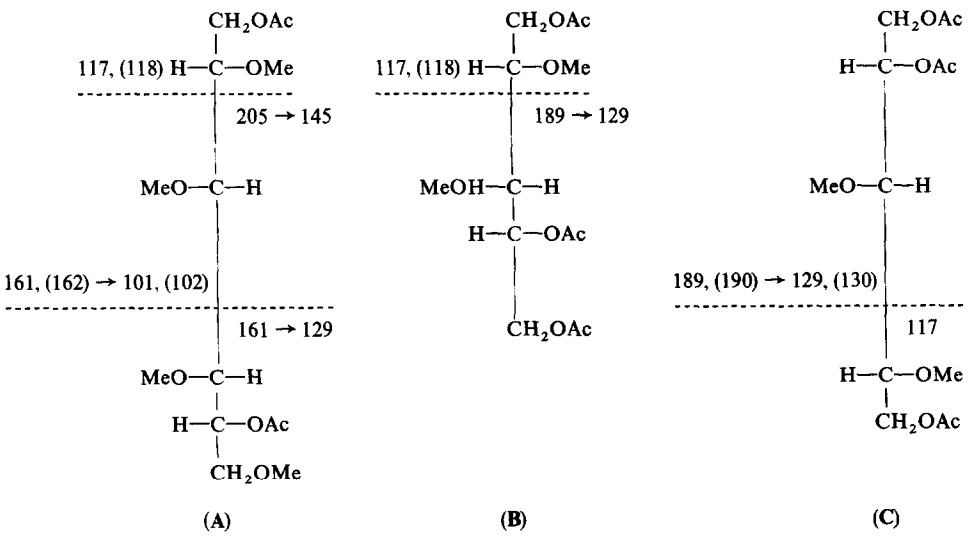
Peak 7. The RR_t of this peak (1.19, at maximum) corresponded with that 2,3,4,6-tetra-*O*-Me galactose (**A**), and 2,3- (**B**) and 3,4- (**C**) di-*O*-Me xylose derivatives. The detection of the above derivatives was achieved by selected ion monitoring of the continuously scanned MS data over the peak obtained from the derivatives of the hydrolysate of S after reduction with $NaBH_4$ and $NaBD_4$ respectively. An estimate of the relative proportions of the derivatives was obtained from the pertinent peaks in the mass spectrogram. Some pertinent fragments in the MS of these derivatives and their deuterated analogues which enabled their identification are shown in the formulae below. The mass numbers within brackets refer to the the fragments obtained after reduction of the methylated sugars with $NaBD_4$.

Before deuteration peak 7 gave ions at *m/e* 43 (100), 45 (26), 71 (10), 87 (33), 101 (46), 117 (62), 129 (49), 145 (28), 161 (17), 189 (5) and 205 (8), corresponding to all the primary and many of the secondary fragments. After deuteration it gave ions at *m/e* 43 (100), 45 (25), 71 (9), 72 (7), 87 (17), 88 (10), 101 (17), 102 (32), 117 (16), 118 (36), 129 (35), 130 (21), 145 (30), 161 (11.5), 162 (9.3), 189 (3.2), 190 (3.1) and 205 (11). The main distinguishing ions are 205 (**A**), 189 (**B**) and 190 (**C**). Application of the principles for the fragmentation of partially methylated alditol acetates supports the structures **A**, **B** and **C** above.

Peak 11. (Max. RR_t 2.25) gave ions at *m/e* 43 (100), 45 (66), 87 (63), 101 (79), 113 (93), 117 (97), 129 (35), 161 (17), 189 (1) and 233 (65) corresponding to all the primary and many of the secondary fragments expected from 2,3,6-tri-*O*-Me galactose or glucose derivative. The bulk of this peak must be the galactose derivative. This can be inferred from the TFA-hydrolysis studies of the whole CWM and from the RR_t of the glucose derivative. The glucose derivative leaves the column on the tail of the galactose derivative and has an RR_t of 2.33. When a small amount of the glucose derivative is present, a slight inflexion on the tail of peak 11 at the expected R_t and corresponding MS might indicate its presence.

The rising part of the shoulder of peak 11 gave ions at *m/e* 43 (100), 45 (31), 87 (29), 101 (32), 113 (47), 117 (79), 129 (19), 161 (9), 189 (1.4) and 233 (16). The 233/161 ion ratio of peak 11 at maximum is 3.8 as compared with 1.8 at the shoulder. This shows that the shoulder contains other derivatives besides 2,3,6-tri-*O*-Me galactose derivative. 2,4,6-Tri-*O*-Me galactose and 2,3,6-tri-*O*-Me mannose have the same RR_t values as the shoulder and would give rise to the fragments found in it; however, the data do not permit their positive identification. GLC on ECNSS-M did not improve the resolution.

Peak 10. (RR_t, 2.07) gave ions at *m/e* 43 (100), 45 (27),



99 (28), 101 (27), 113 (38), 117 (67), 143 (4), 157 (3), 159 (2), 161 (6), 173 (12), 189 (1.3) and 233 (14), showing that it contains a methylated sugar derivative, but its identity could not be determined.

Peak 12. (RR_t, 2.5) gave ions at *m/e* 43 (100), 115 (15), 117 (9), 127 (7), 145 (9), 175 (2) and 187 (6), showing that it contains a methylated sugar derivative, but its identity could not be determined.

Galacturonic acid residues in CWM

The galacturonosyl residues in S could not be analysed by the GLC procedure employed, as they do not form sufficiently volatile, partially methylated galacturonic acid acetate derivatives. Evidence for the linkage composition and an estimate of the galacturonosyl residues in the CWM was therefore obtained from an analysis of S after reduction with $LiAlH_4$. Formolysis and acetylation of the reduced product gave rise to a large peak corresponding to 2,3-di-*O*-Me-1,4,5,6-tetra-*O*-acetyl-galactitol. This compound can be distinguished from the corresponding glucitol derivative (which overlaps with it on the chromatographic system used) and also from (1 → 4,6)-linked galactose by performing the reduction with $LiAlD_4$ instead of the hydride. It was found that the cleavage products of S, after carboxyl reduction, gave rise to increased amounts of mono- and di-*O*-Me derivatives of rhamnose.

A summary of the identifiable neutral sugar derivatives obtained from S, their relative mole per cent composition and the mode of linkage of the sugar residues are shown in Table 2. The proportions of the neutral sugars in the TFA-hydrolysate of the CWM are shown in the last column for comparison. It should be borne in mind that during methylation analysis there may be selective losses. If there should be such losses, the structures established may not relate exactly to the structures in the CWM. The configuration of the sugar residues was not determined, but by analogy with related studies, the galactose, glucose and xylose were assumed to be the D-form and the arabinose and rhamnose the L-form. The relatively low figure for galactose residues in S could be due to incomplete solubilization of 'methylated galactans' by $CHCl_3$–MeOH from methylated CWM and because the total contribution of galactose derivatives from the shoulder of peak 11 is not included. The 2,3-di-*O*-Me glucose content of S is relatively high and this suggests that the glucose residues of the non-cellulosic polysaccharides of the CWM are incompletely hydrolysed by either TFA or H_2SO_4 hydrolysis. It is clear from the results that it is impossible to obtain a balance between end groups (10.9%) and branch points (21.4%). This may be attributable in part to under methylation and also to the occurrence of wall glycoproteins. Nevertheless, certain major structural features of the wall material can be recognised. The major branch points are those through *O*-6 of D-glucose and *O*-4 of rhamnose residues. The modes of linkage of the galactose residues were established by the isolation of several Me ethers, but it would be premature to discuss their position in the structure. However, it is clear that long chains of (1 → 4)-linked galactose residues exist. Detection of 2,3,5-tri-*O*-Me-L-arabinose, 2,3,4-tri-*O*-Me-D-xylose and 2,3,4,6-tetra-*O*-Me-D-galactose, indicated that L-arabinose, D-xylose and D-galactose groups constituted the non-reducing ends. The presence of 2,3,5-tri- and 2,5-di-*O*-Me arabinose indicates that some of the arabinose residues are in in the furanose form. Confirmatory evidence for this was obtained from partial acid hydrolysis studies. None of the polymers contained glucose in terminal positions. The interior part of the polysaccharides was made up of D-

Table 2. Sugar linkages in the non-cellulosic polysaccharides of potato cell wall

Parent sugar	*O*-Methyl ether	Relative mole % of ether residues in non-reduced S	Mode of linkage	Relative mole % of parent sugar calc from ether ratio	Relative mole % of parent sugar by direct analysis
Arabinose	2,3,5-tri	4.1	L-Ara f-(1 → *		
	3,5-di	2.8	→ 2)-L-Ara f-(1 →		
	2,3-di	12.3	→ 5)-L-Ara f-(1 →	20	15.8
	2-	0.8	→ 3,5)-L-Ara f-(1 →		
Xylose	2,3,4-tri	1.6	D-Xyl p-(1 → *		
	2,3-di	1.9	→ 4)-D-Xyl p-(1 →		
	3,4-di	1.9	→ 2)-D-Xyl p-(1 →	5.4	4.3
Rhamnose	3,4-di	2.1 (3.2†)	→ 2)-L-Rha p-(1 →		
	3-	2.4 (3.8†)	→ 2,4)-L-Rha p-(1 →	4.5 (7†)	6.2
Galactose	2,3,4,6-tetra	5.2	D-Gal p-(1 → *		
	2,3,6-tri	40.3	→ 4)-D-Gal p-(1 →		
	4,6-di	1	→ 2,3)-D-Gal p-(1 →		
	3,6-di	1.6	→ 2,4)-D-Gal p-(1 →	50.3	69.2
	2,6-di	1.2	→ 3,4)-D-Gal p-(1 →		
	2,3-di	1	→ 4,6)-D-Gal p-(1 →		
Unknown hexose (Gal?)	-tri‡	7.8		7.8	
Glucose	2,3-di	12	→ 4,6)-D-Glu p-(1 →	12	4.5

The relative yields of the products of methylation analysis of the $CHCl_3$–MeOH soluble fraction (S) of methylated potato cell walls are expressed as relative mole % of the recovered neutral sugar ethers. Although the amount of the galacturonosyl linkages was estimated from $LiAlD_4$ reduction results as described in the text, the corresponding value was not included when computing the figures in column 3 for clarity. The amount of 1 → 4 linked galacturonosyl linkages was 27–30% of the total sugars in S. The values for the results of direct analysis (column 5), before methylation, are from Table 1 column 3. *End groups. †Rhamnose residues in reduced S. ‡Shoulder of peak 11—not fully characterized.

xylose, L-rhamnose, D-glucose, L-arabinose, D-galactose and D-galacturonic acid residues of which the last two constituted the major portion. The galacturonic acid residues were (1 → 4)-linked and comprised 27–30 mole % of the total carbohydrate content of S.

The presence of appreciable quantities of 2,3-di-*O*-Me glucose, 2,3-, 3,4- and 2,3,4-tri-*O*-Me xylose suggested the occurrence of hemicelluloses containing the corresponding glycosidic linkages. Subsequently, these linkages were shown to be present in the alkali-soluble hemicelluloses of the CWM (Ring and Selvendran, unpublished results).

Graded acid hydrolysis of CWM

The amount of neutral sugars obtained after various acid hydrolysis conditions is shown in Scheme 1. The occurrence of oligosaccharides in the 'various fractions' (diffusates from the different acid treatments—see Experimental) was apparent from the increased values of galactose and other sugars obtained on hydrolysis of the diffusates with 2 N TFA for 2 hr. Whilst hydrolysis of the CWM with N/100 TFA for 1 hr produced negligible amounts of free sugars (including arabinose), further hydrolysis of the residue (CWM-1) with N/10 TFA produced appreciable amounts of free arabinose and galactose and oligosaccharides containing mainly galactose. Hydrolysis of the residue (CWM-2) with N/2 TFA for 1 hr released mainly galactose and smaller amounts of glucose, arabinose, xylose and rhamnose and oligosaccharides containing mainly galactose. Hydrolysis of CWM-3 with 2N TFA for 2 hr liberated mainly galactose and some glucose, rhamnose, arabinose and xylose. Because cellulose is not hydrolysed under the above conditions, the glucose and xylose must have arisen mainly from the hemicelluloses. It is evident from the above data that most (*ca* 93 %) of the bonds linking the arabinose residues are very acid labile. The removal of arabinose is followed by a slow release of galactose, glucose, xylose and rhamnose.

The preferential release of arabinose and some arabinose containing oligosaccharides suggests that residues of this sugar are probably present in the furanose form in the exterior of the chains of the molecular structure [9]. The arabinose residues in CWM-3 (*ca* 7 % of total arabinose residues) are relatively stable suggesting that they are probably involved in a different type of linkage (β-linked?) or are not readily accessible for hydrolysis (cf. results of methylation analysis). While these results are of limited significance, they nevertheless give some insight into the relative strengths of the various glycosidic linkages.

CWM
↓ 0.01 N TFA, 100°, 1 hr
→ Ara (3.1) and traces of other sugars
[Ara (9.2), Gal (1.9) and traces of other sugars]
Degraded CWM-1
↓ 0.1 N TFA, 100°, 1 hr
→ Ara (50) Gal (18.6), Rha (1.5) and Xyl (1.4)
[Ara (48.5, Gal (46.2), Rha (3.5), Xyl (2.2) and Glc (1.6)]
Degraded CWM-2
↓ 0.5 N TFA, 100°, 1 hr
→ Gal (74.6), Glc (6.2), Ara (6.1), Xyl (6) and Rha (1.5)
[Gal (93.4), Glc (5.9), Ara (5.8), Xyl (5.8) and Rha (4.1)]
Degraded CWM-3
↓ 2 N TFA, 100°, 2 hr
→ Gal (32.6), Glc (5.8), Rha (5.1), Ara (4.6) and Xyl (0.8)
Degraded CWM-4
↓ Saeman Hydrolysis
Glc (184.6)

Scheme 1. Showing graded acid hydrolysis of CWM. The sugar values are expressed as μg/mg CWM. The values within double brackets are those obtained on furtner hydrolysis with 2 N TFA at 100° for 2 hr. For details of Saeman hydrolysis see Experimental.

DISCUSSION

An ealier paper [7] emphasized the importance of preventing coprecipitation of cytoplasmic compounds when isolating and purifying cell wall material from plant tissues. This was achieved by using as solvents simple substances which have a high affinity for the cytoplasmic molecules. Both SDC and PAW extract the cytoplasmic compounds efficiently, dissociating without hydrolysing them. In addition, the tissue was ball-milled after blending in an Ultra Turrax to ensure thorough maceration. This produced a homogenous product which was very rich in starch and could readily be separated by centrifugation. Experiments with potato starch showed that the cell wall polysaccharides are not likely to be degraded under the ball-milling conditions used in this study.

Enzymic removal of starch from the SDC–PAW insoluble residue is not only expensive and time consuming but is suitable for preparing only small quantities of the wall material. Water sonication of the residue followed by miracloth filtration (sieving) is a useful alternative method [4], however, this method suffers from the disadvantage that the final preparation contains a small quantity of residual starch which obscures the significance of amyloid type 'xyloglucans'. Another means of obtaining starch-free CWM became possible from the finding that aq. DMSO solubilized starch [10]. In the course of our work, the use of aq. DMSO for destarching crude potato wall preparation was described by Mullen and Bateman [11]. However, these workers have not undertaken any carbohydrate analysis to show the effectiveness of complete solubilization and the relative merits of the procedure. Our results show that treatment of the SDC–PAW residue with 90 % aq. DMSO under the standard extraction conditions removed the residual starch quantitatively without solubilizing any cell wall polysaccharides. It should be noted that in order to facilitate the solubilization of starch by aq. DMSO, pretreatment of the suspension in an ultrasonicator is necessary.

Although methylation of polysaccharides containing a high proportion of uronic acid residues is often difficult to achieve, and may be complicated by some degradation, there was sufficient indication of the nature of the acidic and neutral polysaccharide complexes present in the the non-cellulosic polysaccharides of the wall. A further investigation will be necessary to distinguish between the acidic and neutral polysaccharides. In studies of this nature, the presence of pectinic acid (pectic acid in which an appreciable proportion of the galacturonic acid residues are esterified) may cause complications when the CWM is treated with the strong base methylsulphinylmethyl sodium in DMSO. During the formation of the polyalkoxide ions, the uronic esters will be in contact with the base for several hr and the esterified residues may undergo β-elimination, yielding unsaurated residues [12]. This would invariably lead to some depolymerization of pectinic acid and thus to an underestimation of galacturonic acid residues.

The neutral sugar compositions calculated from the partially methylated alditol acetates were, apart from rhamnose, galactose and glucose in 'non-reduced S', in good agreement with the proportions of the sugars in the 'non-cellulosic polysaccharides' before methylation. The possible reasons for the discrepancies in the values of galactose and glucose have been mentioned before. Rhamnose was probably under-estimated in non-reduced-S, as it would be incompletely released under the conditions of acid hydrolysis used. Talmadge *et al.* [6] have made similar observations with their work on cell walls of suspension cultures of sycamore. The higher proportions of the various rhamnose ethers in reduced S implies that contiguous rhamnose residues occur in the chain. Thus by converting galacturonosyl residues to galactosyl residues, the yields of rhamnose derivatives are increased. This would be the expected result if the relatively acid resistant galacturonosyl-(1 → 2)-rhamnose linkages occurred in the molecules. Then the glycosidic linkage obtained on reduction between galactose and rhamnose would not have the acid resistant properties of the aldobiouronic acid linkage. Such linkages are a characteristic feature of pectic substances from plant tissues [13 and references therein].

It is clear that the non-cellulosic polysaccharides contain mainly backbones of (1 → 4)-linked D-galactopyranose units and (1 → 4)-linked galacturonan chains which are interspersed with (1 → 2)-linked rhamnosyl residues which carry substituents on C-4. Also end groups of D-galactose, L-arabinose and D-xylose occur but no D-glucose end groups are present. The occurrence of hemicelluloses containing → 4,6)-D-Glup-1(→ residues could be inferred from these results. Because the CWM is rich in polysaccharides containing (1 → 4)-linked galactose residues, it is possible that the cold water soluble galactan of potatoes described by Wood and Siddiqui [14] is a cell wall constituent.

The bulk of the $CHCl_3$–MeOH insoluble fraction of the methylated CWM is accounted for by (1 → 4)-linked glucose. The occurrence of substantial amounts of partially methylated glucose in this fraction indicates that the cellulose is not fully methylated. It also contains significant amounts of (1 → 4)-linked galactose and small amounts of (1 → 5)-linked arabinose and traces of terminal arabinose, xylose and galactose. These residues probably arise from incompletely removed 'pectic substances' and hemicelluloses.

A full assessment of the structural significance of the various sugar residues and cell wall polysaccharides can only be made in the light of detailed characterization of neutral and acidic oligosaccharides formed on partial hydrolysis of the cell walls by both chemical [12, 13] and enzymic methods [15]. Our results, however, provide the first evidence for the overall structural features of potato cell walls.

EXPERIMENTAL

Chemicals. $LiAlD_4$ and $NaBD_4$ were purchased from Fluka, Switzerland; α-Amylase (from hog pancreas) and pectinase were purchased from Boehringer Corp. (London) Ltd., and Sigma Chemical Co. Ltd., respectively. DMSO and NaH were obtained from BDH. The DMSO was redistilled to remove any contaminating dibutylphthalate and kept dry over a molecular sieve before use. The disaccharides and polysaccharides used in the synthesis of partially methylated alditol acetate standards were obtained from the following sources: gentiobiose, araban and galactan from Koch–Light Laboratories Ltd; cellobiose, amylose and amylopectin from BDH; xylans from lignified tissues of mature runner bean pods as described in ref. [16].

Potatoes. (Var. Desiree) were obtained from plants grown in experimental plots near the laboratory. From a batch of mature potatoes harvested 10 September 1975, those between 100 and 150 g were selected and stored for 3–4 months at 7° before analysis. The composition of the cell wall material did not change appreciably during this period.

Preparation of SDC–PAW-insoluble residue (Prepn 1). Prepn 1 of potatoes was obtained by sequential treatment of the fresh ball-milled tissue with 1% aq. Na deoxycholate (SDC) and PhOH–HOAc–H_2O (PAW) (2:1:1, w/v/v) as described before [7]. Slight modifications were introduced to minimize contamination with cytoplasmic compounds. A 50 g sample of frozen powder was blended with 100 ml of 1% SDC containing 5 mM $Na_2S_2O_5$ for 5 min. The triturated material was centrifuged and washed with 0.5% SDC containing 3 mM $Na_2S_2O_5$. The resulting product was quantitatively transferred with 100 ml of 0.5% SDC containing 3 mm $Na_2S_2O_5$ to a Pascall ball-milling apparatus. Optimal cell disruption was obtained by ball-milling at 50 rev/min for 16 hr. The rest of the procedure was as described earlier [7].

Isolation of macromolecules from SDC and PAW extracts. Macromolecules present in the dialysed SDC extract were isolated by pptn with EtOH and the bulk of the contaminating starch was removed by prolonged treatment with α-amylase. The product thus obtained dissolved easily in H_2O and on acid hydrolysis yielded, in addition to glucose (from residual starch) and galactose (from galactan), small and differing amounts of arabinose (*ca* 1–4%). Calculations based on the galactose content showed that the soluble galactan would account for *ca* 0.06% by wt of fr. tissue. The PAW extracts on dialysis against several changes of H_2O gave a flocculent white ppt. in the tubing; this ppt. contained mainly deoxycholic acid. The supernatant on freeze drying yielded a white solid which was mainly starch.

Removal of starch from Prepn 1. (1) *Digestion with α-amylase.* Starch was removed by treatment with α-amylase twice at 30° for 36 hr using toluene as bactericide [7]. After the first 36 hr treatment, the digest was centrifuged and the residue treated with a further quantity of α-amylase for another 36 hr. The final prepn (Prepn 2) gave a pale brown colour with I_2/KI showing incomplete removal of starch (amylopectin?). This prepn can be freed of residual starch by extraction with 90% aq. DMSO. (2) *Extraction with 90% (v/v) aq. DMSO* The wet residue (~15 ml) was blended in 150 ml of 90% aq. DMSO in a Waring blender before sonication. Conditions of sonication depended on the instrument used. With the Bransonic, the wall suspension was placed in a 250 ml round bottomed flask and sonicated (max. output) for 10 min. The temp. was not allowed to rise above 30°. The mixture was then stirred at room temp. for 16 hr. Centrifugation of the thick 'emulsion' at 15°

for 10 min at 12000 *g* yielded an opalescent aq. organic phase which contained starch which was removed. The residue was then dispersed in 50 ml of aq. DMSO, ultra-sonicated for 1 hr and centrifuged as before. The organic layers were combined dialysed at room temp. against frequent changes of H_2O and freeze dried to isolate the DMSO-S which was mainly composed of starch. The insoluble residue was washed with H_2O (× 5) and freeze dried. This resulted in complete removal of starch as shown by negative reaction with I_2/KI and carbohydrate analysis, so that only cell wall polysaccharides remained (Prepn 3). 100 g fr. tissue gave 1–1.2 g (dry) wall prepn containing *ca* 2% wall protein. In the following studies Prepn 3 was used unless otherwise stated.

Methylation analysis of cell walls. Hakomori methylation. Methylation of CWM (25–50 mg) was carried out by the method of ref. [17] as described in ref. [18], except that the inert atmosphere in the reaction flask was maintained by flushing with dry argon instead of N_2. Argon being a denser gas effectively prevented possible back diffusion of air. Following methylation of the walls, 1 vol. of $CHCl_3$–MeOH (1:1) was added to the reaction mixture and the product was filtered on a sinter and washed with $CHCl_3$–MeOH. The insoluble material (I) consisted mainly of incompletely methylated cellulose, some residual 'pectic substances', hemicelluloses and wall glycoproteins. This fraction was not analysed in detail. The methylated non-cellulosic polysaccharides present in the filtrate were isolated by dialysis against several changes of 50% aq. EtOH and evapd to dryness. The resulting product (S) was dissolved in 5 ml of $CHCl_3$–MeOH (1:1). IR absorption spectra of a 10% soln of S in CCl_4 was recorded in the 4000–1600 cm^{-1} region to confirm the 'complete' substitution of all free OHs in S.

Analysis of methylated non-cellulosic polysaccharides of S. The methylated polysaccharides of S were sequentially hydrolysed with 90% HCO_2H and 0.25 M H_2SO_4 and the resulting partially methylated sugars isolated. They were then reduced with $NaBH_4$ and converted to the corresponding alditol acetates as described in ref. [19]. The mixture of partially methylated alditol acetates were injected directly on to the GLC columns. Because the (1→4)-linked galacturonosyl residues of the wall polymers cannot be detected by the standard methylation analysis, the mode of occurrence of these residues was determined after reduction of S with $LiAlD_4$ (or $LiAlH_4$) [19]. This introduced two D atoms at C-6 on the galactose derivative thus formed, thereby facilitating its identification by MS. In a third expt the mixture of methylated sugars from S was reduced with $NaBD_4$ and analysed as alditol acetates. This produced a mixture containing partially methylated alditol acetates deuterated at C-1. Some pertinent fragments in the MS of the deuterated analogues enabled their identification, e.g. the alditol acetates derived from 2,3,4,6-tetra-*O*-Me galactose (**A**) and 2,3- and 3,4-di-*O*-Me-D-xylose (**B**) and (**C**) respectively (see Results), which, although not separable by the GLC system used in this study, give different MS. GLC was performed using dual columns. Separations were carried out on two glass columns (2.8 m × 2.2 mm). One containing 3% OV 225 on JJ's diatomite CQ and the other 3% ECNSS-M on Gas Chrom Q. The partially methylated alditol acetates were run isothermally at 180° (OV - 225) or 170° (ECNSS-M) to obtain RR_f. For better resolution, temp. programming (150–200° at 0.5°/min) was used. This method was preferred for GC–MS because the alditol acetates eluted as sharper peaks on GLC (especially the later ones). Although the results using both columns were complementary, the OV-225 column was preferred, because with ECNSS-M the phase was not stable and tended to leak out on use resulting in increased background and contamination of the ionization chamber on GC–MS. The mass spectrometer was operated at an inlet temp. of 250°, an ionization potential of 70 eV and an ion source temp. of 220°. The identities of most of the alditol acetates were established from the following data. (a) RR_f by reference to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-Me glucitol on the two different GLC columns. Our results were comparable with those reported in refs [20, 21]. (b) MS recorded at the apex of small peaks and at the rising, apex and descending portions of large peaks. In order to avoid eventual ambiguities when interpreting MS, the methylated sugars were reduced with $NaBD_4$ and continuous monitoring of the peaks was undertaken for more positive identification. The interpretation of these results was as described in refs [19, 21] and refs therein. The MS fragmentation pathways were similar to those described for methylated alditol acetates in [19, 21]. The use of known disaccharides and polysaccharides enabled exact chemical identification to be made from this MS data. (c) The sugar composition of the TFA-hydrolysate of the CWM.

Graded acid hydrolysis of CWM. Graded hydrolysis was carried out with TFA of different strengths as shown in Scheme 1. The results obtained made it possible to assess the relative strengths of some of the glycosidic linkages. The CWM (50 mg) was heated with 0.01 N TFA (10 ml) at 100° for 1 hr and the mixture was cooled and dialysed against 300 ml H_2O for 5 hr. The neutral sugars and oligosaccharides present in the diffusate were analysed directly and after hydrolysis with 2N TFA at 100° for 2 hr. The latter analysis indicated the presence of oligosaccharides in the diffusate. The dialysis residue was then dialysed against a large excess of H_2O and the resulting diffusate was discarded. The contents of the tube (*ca* 25 ml) were then adjusted to 0.1 N acidity with 2 N TFA heated at 100° for 1 hr. The resulting mixture was processed as before. The procedure was repeated with the residue with solns of 0.5 and 2 N acidity. The final residue was freeze dried and subjected to Saeman hydrolysis [22].

Acid hydrolysis of CWM. Three methods were used for hydrolysis of the CWM and the relative merits were assessed. (1) CWM (5 mg) was suspended in 2 N TFA (1 ml) and heated in a sealed tube at 120° for 2 hr (TFA hydrolysis). (2) CWM (5 mg) was suspended in 2 N H_2SO_4 (1 ml) and heated at 100° for 5 hr (H_2SO_4 hydrolysis). (3) CWM (10 mg) was wetted with 72% H_2SO_4 (0.82 g) and left at 25° for 3 hr. It was then diluted with H_2O (5.8 g) to give 2 N H_2SO_4 and heated at 100° for 5 hr (Saeman hydrolysis).

Estimation of sugars. Sugars liberated from the CWM were isolated and analysed as their alditol acetates by GLC as described in refs [19, 23] and by ion exchange chromatography using borate buffers [24]. The alditol acetates were run isothermally at 200°. The estimation of neutral but not acid sugars as alditol acetates was found to be quantitative [25]. The ion exchange method on the other hand gave good recoveries of galacturonic and glucuronic acids but failed to separate them. The uronic acid content of the CWM was determined as follows. The CWM was first treated with pectinase for 15 hr and the resulting mixture was subjected to H_2SO_4 hydrolysis. The sugars thus obtained were determined by ion exchange chromatography and the uronic acid content was computed from these results and those of H_2SO_4- and Saeman hydrolysis of the CWM. Since uronic acids are partially decarboxylated on prolonged exposure to hot acids [26], the effect of H_2SO_4 hydrolysis on the decarboxylation of glucuronic acid was studied. The results showed that less than 6% decarboxylation occurred under the above conditions.

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