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SOLUBLE AND WALL-BOUND GLYCOPROTEINS OF APPLE FRUIT TISSUE

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Abstract—Apple fruit tissue contains small amounts of readily soluble glycoproteins, rich in hydroxyproline; polymethylgalacturonide is not covalently bound to the soluble glycoproteins. Barium hydroxide hydrolysis of apple fruit cell walls liberated glycopeptides containing 4 arabinosyl residues per hydroxyprolyl residue, which were attacked very slowly by α-L-arabinofuranosidase. Hydrazinolysis liberated similar glycopeptides, which were difficult to separate from a polysaccharide containing galactose residues. Protease treatment of walls also released glycopeptides containing hydroxyproline, and a small proportion of these were associated with polyuronide. Polygalacturonase pretreatment of walls led to increased release of hydroxyprolyl residues by protease. Susceptibility of the hydroxyproline containing glycoprotein in the cell wall to attack by protease and arabinosidase did not change during fruit ripening. The amount of an unknown hexosamine associated with the wall was less in ripe than in unripe fruit.

INTRODUCTION

The discovery of wall protein, rich in hydroxyprolyl residues [1,2], which are glycosylated with arabinose [3], led to the suggestion that this glycoprotein was linked to cellulose microfibrils and, therefore, had a structural role [4]. More recently the discovery of a galactosyl serine linkage in wall protein [5], and detailed analysis of sycamore suspension culture cell walls [6,8], has resulted in a model of wall structure, which postulates a rhamnogalacturonan linked through an arabinogalactan to seryl residues in the protein. Association between polyuronide and hydroxyproline containing proteins has been observed in a number of plant tissues [8–11], but it has been suggested that this could result from aggregation of components during preparation [11].

In the present work the association between soluble glycoproteins and polyuronide, and the role of glycoproteins in the wall structure of apple fruit tissue have been further investigated.

RESULTS

Fractionation of tissue

The hydroxyproline content of hydrolysates of the wall fraction of apple fruit tissue was low, and a relatively high proportion of total hydroxyproline was in soluble fractions (Table 1). A small proportion of total protein could be extracted in aqueous media, following acetone extraction (Table 1). More protein could be extracted by disintegrating apple tissue in a suitable aqueous medium, but the amounts of hydroxyprolyl residues extracted were similar (Table 2). Material extracted in M KCl precipitated from solution on dialysis. The precipitate contained uronic acid and small amounts of arabinose and galactose residues. Similar precipitation occurred on freezing and thawing of 5 mM phosphate extracts.

The presence of small amounts of hexosamine in cell wall hydrolysates was surprising. Concentrated hydrolysates, purified on columns of Dowex 50 were examined by TLC on cellulose

Table 1. Composition of protein fractions of acetone extracted apple fruit tissue

Fruit sample	Composition of fractions (μg per g fr. wt) 5 mM Phosphate soluble		M KCl (pH 7·5) soluble		Residue		
	Hydroxy-proline	Amino acid	Hydroxy-proline	Amino acid	Hydroxy-proline	Amino acid	Hexos-amine
Initial	2·71	158	0·39	136	10·2	1090	9·0
Ripened off tree	2·91	274	0·70	394	9·1	509	3·8
Ripened on tree	2·90	230	0·81	383	9·2	424	4·2

EtOAc–C_5H_5N–H_2O as developing solvent. Plates were sprayed with aniline phthalate [12] and ninhydrin reagents, and a spot which reacted with both was found with a mobility relative to glucosamine of 1·47.

Soluble glycoproteins

The material extracted in 5 mM phosphate from Me_2CO extracted apple tissue was subjected to ion exchange chromatography on DEAE cellulose with a phosphate concentration gradient [11]. Protein was eluted in three overlapping peaks, the first two of which were associated with carbohydrate peaks. Uronic acid was detected in the second protein–carbohydrate peak. However, the proportion of polyuronide eluting in this region was known to be affected by extraction conditions [11]. Ion exchange chromatography of extracts prepared in various ways (Table 3) showed that when added or endogenous pectinesterase was allowed to act during extraction polyuronide tended to be eluted at higher phosphate concentrations and to separate from the glycoprotein. Refluxing in acetone, before extraction, extraction in the presence of detergent, to which the esterase is very sensitive, or extraction in the absence of chloride ions, which activate the enzyme, all resulted in greater association between polyuronide and glycoprotein.

A 5 mM phosphate extract from 100 g unripe apple tissue was concentrated by ultrafiltration, allowed to de-esterify after addition of NaCl to 0·1 M, dialysed and applied to a DEAE cellulose

Table 2. Analysis of protein extracted from apple fruit tissue in various ways

Extraction sequence	Extractants in numerical sequence	Composition of material extracted (μg g^{-1} fr. wt) Hydroxy-proline	Amino acid
(a)	1 Acetone 2 5 mM Phosphate	3·46	120
	3 M KCl (pH 7·5)	2·42	213
(b)	1 0·2 M Tris phosphate 0·01 M DIECA (pH 8)	4·24	748
	2 M KCl (pH 7·5)	1·21	140
(c)	1 0·2 M Tris phosphate 0·01 M DIECA (pH 8)	3·89	658
	2 0·2 M Tris base (pH 10)	1·30	275
(d)	1 Water	2·68	352

Extractions were performed on preclimacteric Cox's Orange Pippin apples harvested 13/9/73. Extractives were dialysed and freeze-dried prior to hydrolysis. DIECA, $Et_2N{\cdot}CSSNa$.

Table 3. State of polyuronide extracted from acetone extracted apple tissue under various conditions

Acetone extraction	Aqueous extractant	Percentage of polyuronide eluting With glycoprotein	As free polyuronide
At 2°	0·1 M Tris HCl (pH 7·5)	6	94
	0·1 M Tris HCl 0·1% SDS	45	55
	5 mM Phosphate (pH 7·0)	47	53
Under reflux	0·1 M Tris HCl (pH 7·5)	31	69
	0·1 M Tris HCl 0·1% SDS	57	43
	0·1 M Tris HCl + PE	12	88

Preclimacteric Wagener apples were used in these experiments: acetone extracted tissue was incubated 3 hr at 25° with the aqueous extractants. PE indicates the addition of 2U citrus pectinesterase. SDS, sodium dodecyl sulphate.

Extractives were dialysed before application to DEAE cellulose columns and eluted with a phosphate gradient and with continuous automated analysis of total carbohydrate and uronic acid [11].

Table 4. Composition of soluble glycoproteins from apple fruit tissue separated on a column of Sephadex G-200

Component residue	Percentage composition of pooled fractions eluted between (ml/100 ml column volume)			
	40–56	56–69	81–94	102–107
Amino acids	15·8	11·8	85·0	87·3
Hydroxyproline	3·1	2·3	0·4	0
Hexosamine	0	0	0·4	0·4
Arabinose	17·0	21·8	2·2	0·4
Xylose	0	0	0·5	1·0
Rhamnose	9·4	8·1	1·0	0
Galactose	32·4	37·3	4·3	0·4
Glucose	5·1	4·6	2·0	6·5
Mannose	0	0	0	0·6
Uronic acid	17·2	14·1	4·2	2·8
(Relative amounts of fractions)	(10)	(10)	(6)	(5)

Concentrated de-esterified high MW material from a 5 mM phosphate extract of apple tissue was applied to a 10 × 1·5 cm column of DEAE cellulose and eluted with a phosphate gradient. Material absorbing at 280 nm, eluting in a peak at about 0·1 M phosphate, was concentrated and applied to a column (34 × 2·5 cm) of Sephadex G-200 equilibrated with 50 mM phosphate (pH 6·5) and eluted at 0·32 ml/min. Fractions (10 min) were collected and assayed for total carbohydrate [11] and A_{280}. From 40–69 ml/100 ml column volume a broad carbohydrate peak emerged which was divided at the maximum carbohydrate content to give two lots of fractions which were pooled (40–56, 56–69 ml/100 ml column volume). The carbohydrate rich peak was followed by two peaks with relatively high A_{280} from which fractions were pooled (81–94, 102–107 ml/100 ml column volume).

column. The glycoprotein fraction eluting at around 0·1 M phosphate was dialysed, freeze-dried and applied to a Sephadex G-200 column. The bulk of the carbohydrate eluted in a broad peak with a low *A* at 280 nm, after the void volume. This was followed by two peaks with high *A* at 280 nm and little carbohydrate. The small proportion of protein in the high MW peak was rich in hydroxyprolyl residues and the carbohydrate moiety contained galactose, arabinose, rhamnose and uronic acid residues (Table 4). The presence of the same sugar residues in the lower MW peaks was probably due to contamination by the higher MW components. A high content of α-amino acid residues with small amounts of xylose, glucose and hexosamine are thought to be characteristic of the lower MW components.

Alkaline degradation of wall-bound glycoprotein

After hydrolysis of apple fruit cell walls in 0·43 M $Ba(OH)_2$ [3], *ca* 70% of total hydroxyproline reacted as non-peptide bound. Reaction products were applied to a Dowex AGW 50X2 column and eluted with a gradient from 0 to 1 M HCl. Carbohydrate was detected in two peaks; the first at 0·15 M HCl was found to contain arabinose and hydroxyproline after hydrolysis, but without hydrolysis did not react colorimetrically as hydroxyproline; the second peak at 0·25 M HCl gave a reaction for hydroxyproline without hydrolysis, was found to contain arabinose after hydrolysis and GLC, and colorimetric estimation indicated an average of 4 arabinose residues per hydroxyproline residue. The first peak (A) which accounted for 18% of the hydroxyproline residues liberated by $Ba(OH)_2$ was probably glycosylated hydroxyproline which was peptide bound, while the second (B) was thought to be hydroxyproline tetra-arabinoside, and accounted for 74% of the hydroxyproline residues liberated. The remaining 8% of the hydroxyproline occurred in a peak eluted at 0·45 M HCl, associated with large amounts of ninhydrin-positive material, presumably free amino acids.

When 0·05 μmol samples of hydroxyproline arabinoside (B) were incubated with 2·4 U α-L-arabinofuranosidase (pI 6·5 enzyme from *Sclerotinia fructigena* [13]) at pH 4·8 for 24 hr at 20°, the reducing sugar released corresponded to 17% of the arabinose residues present. No reducing sugar was released in incubations of enzyme or substrate alone.

Products of hydrazinolysis [14] of apple fruit cell walls were applied to a Dowex AGW 50X2 column and eluted with a gradient from 0 to 0·25 M HCl. A broad peak of carbohydrate was eluted up to 0·1 M HCl followed by another peak at 0·2 M and although hydrazine interfered with estimation of hydroxyproline in hydrolysates, hydroxyproline could be detected in both peaks on TLC in two solvent systems. Material in the first peak eluted from the Dowex column contained galactose, arabinose, rhamnose and uronic acid in a molar ratio of 1:0·58:0·15:0·08. Gel filtration on Sephadex G-100 partly resolved two carbohydrate peaks, one at around 60/100 ml column volume without hydroxyproline and the other around the inclusion volume with hydroxyproline. Material in the second peak from the Dowex column contained galactose, arabinose, rhamnose and uronic acid in a molar ratio of

1:0·61:0·35:0·24. On gel filtration on Sephadex G-25 this material eluted as a broad peak of carbohydrate after the void volume. However, the maximum hydroxyproline content was found at about 60/100 ml column volume, similar to the elution volume of hydroxyproline tetra-arabinoside. The molar ratio of arabinose to galactose increased from 0·6 at the leading edge of the carbohydrate peak to 2·1 at its tail. Pooled hydroxyproline containing fractions eluted from a Sephadex G-25 column were incubated with 14U arabinosidase pI 6·5 for 20 hr at 20° and applied to the column again. After hydrolysis hydroxyproline was detected in a broad peak up to the inclusion volume of the column, suggesting that it was less extensively glycosylated than in the starting material. The bulk of the carbohydrate remained in the void peak.

Although the carbohydrate in the void peak gave a reaction in the carbazole–H_2SO_4 test [15] for uronic acids it was not retained by DEAE cellulose. Samples of citrus pectin were subjected to the hydrazinolysis procedure and applied to the Sephadex G-25 and Dowex columns. The colorimetric reaction of the polysaccharide was reduced by about 90% by hydrazinolysis; 30% of the remaining reactive material was unretarded by both columns and gave a magenta colour with sulphonated α-naphthol [16], indicating that it was rich in neutral sugar residues; the retarded material gave a brown colour, characteristic of uronic acids, though since it was retained by the Dowex column it must have been a basic derivative, possibly a hydrazide.

Enzymic degradation of wall-bound glycoprotein

No hydroxyproline residues were released from apple fruit cell walls on treatment with citrus pectinesterase. When de-esterified walls were treated with polygalacturonase pI 9·7 from *S. fructigena* [17] followed by endoglucanase from *Trichoderma viride* [18], 4–5% of total hydroxyproline was found in each filtrate. After these treatments walls were incubated in 5 mM phosphate (pH 7) at 20° for 20 hr and 13% of the hydroxyproline present in the original walls was found in solution. A further 36% was released on incubation with a protease preparation from *Streptomyces griseus*. This enzyme was also able to liberate hydroxyproline-containing material from untreated walls or from walls treated with polygalacturonase pI 4·6 [17] or polygalacturonase pI 9·7. If the walls were agitated vigorously (by magnetic stirrer) during polygalacturonase treatment they became more finely divided and more susceptible to protease attack than walls agitated more gently (by bottle roller). Under standard conditions the two polygalacturonase isoenzymes were equally effective as pretreatments to increase hydroxyprolyl residue release by the protease (Table 5).

Table 5. Release of hydroxyproline residues from de-esterified apple fruit cell walls by protease from *Streptomyces griseus*

Pre-treatment of wall	Percentage of total hydroxyproline released (a)	(b)	Percentage of (b) retained by DEAE cellulose
None	17	19	20
PG pI 4·6	28		
PG pI 9·7	27	52	15
PG pI 9·7 EG + buffer*		36	11

* 5 mM Phosphate (pH 7) released 13% of total hydroxyproline after PG (polygalacturonase) pI 9·7 and EG (endoglucanase).
(a) Agitated on bottle roller; (b) agitated by magnetic stirrer.

A small proportion of the hydroxyproline containing peptides released by protease were retained by DEAE cellulose (Table 5). This fraction from digests of de-esterified walls contained per unit weight of hydroxyproline, arabinose 5·0, xylose 0·5, galactose 4·7, glucose 3·4 and uronic acid 6·6. However, the uronic acid was probably a contaminant because a similar DEAE cellulose retarded glycopeptide fraction from polygalacturonase and endoglucanase treated cell walls contained no uronic acid residues. Other peptides which were unretarded by DEAE cellulose contained no uronic acid residues and were mostly excluded from Sephadex G-25. They were retained by Dowex AGW 50X2 and could be displaced in increasing amounts by successive elution with HCl, NaCl and NaOH (Table 6). These peptides contained arabinose, xylose and glucose residues.

Binding of polyuronide to wall protein

Because of the lack of evidence for covalent attachment of polyuronide to wall protein the possibility of binding added polyuronide to wall

Table 6. Chromatography of DEAE cellulose unretarded glycopeptides released by protease from apple fruit cell walls, on Sephadex G-25 and Dowex 50

Sephadex G-25				
Elution vol. (ml/100 ml column vol.)	40–62	66–85	89–102	112–128
Percentage of hydroxyproline residues	61	16	4	8
Dowex AGW 50X2				
Eluant	H_2O	0·25 M HCl	M NaCl	0·1 M NaOH
Percentage of hydroxyproline residues	3	9	17	32

The material released by protease from PG pI 9·7 and endoglucanase pre-treated cell walls was applied to a DEAE cellulose column. Unretarded material was concentrated and applied to the Sephadex column, and the high MW peak from this was applied to the Dowex column. Percentages refer to the amount of hydroxyproline released by the protease represented in the eluted fractions.

protein was investigated. Cell walls were incubated with citrus pectin at pH 4 as previous work had shown that endogenous polyuronide was least soluble under slightly acidic conditions [11]. There was no evidence of binding of citrus pectin to untreated walls or walls pretreated with polygalacturonase isoenzymes pI 4·6 or 9·7.

Changes in wall protein during apple ripening

As shown in Table 1, there was little change in the distribution or total amount of hydroxyproline residues during fruit ripening. Since the extraction procedure resulted in contamination of walls with cytoplasmic protein, the increased solubility of protein in ripe fruit does not mean that wall protein is released during ripening. More work is necessary to determine whether the hexosamine residues are present in the wall before the significance of the decreases in their levels after ripening (Table 1) can be understood.

If the linkage or association of hydroxyproline-containing protein with other wall constituents changed during ripening it might be expected that the protein would become more susceptible to enzymic attack. However, the proportion of hydroxylprolyl residues released by protease did not change and arabinosidase caused similarly slight decreases in glycosylation of these residues in ripe and unripe fruit cell walls. Although lower glycosylation of hydroxyproline residues was observed in small scale $Ba(OH)_2$ hydrolysates of ripe fruit cell walls, this was not confirmed when gradient elution was used with a sample from ripe fruit.

Estimation of glycosylated serine residues was attempted by colorimetric estimation of serine in hydrolysates of alkali treated and untreated cell walls. Slight differences between apparent serine contents of treated and untreated walls were observed in unripe fruit and fruit ripened on the tree but not in fruit ripened off the tree (Table 7). However, the magnitude of the apparent serine content (up to 50% of amino acid residues) suggests that there was interference by other substances in these estimations.

DISCUSSION

Confirmation of the theory that cell wall structure in higher plants embodies "a protein–glycan network analogous with the peptidoglycan network of bacterial cell walls" [4], depends upon

Table 7. Properties of wall protein in ripe and unripe apple tissue

Wall prepared from	Pre-treatment	% HYP released by protease	$Ba(OH)_2$ % HYP released	Hydrolysis ratio Ara/HYP	Apparent serine content (μg per g fr. wt)
Unripe fruit	None	27·5	71·0	4·70	168
	AF pI 6·5		76·5	4·33	
	0·5 M NaOH				152
Fruit ripened off tree	None	28·2	90·5	3·88	195
	AF pI 6·5		100	3·65	
	0·5 M NaOH				195
Fruit ripened on tree	None	27·5	94·0	3·42	226
	AF pI 6·5		98·5	3·08	
	0·5 M NaOH				214

HYP, hydroxyproline; Ara, arabinose; AF, arabinofuranosidase.

the identification of covalent linkages between structural polysaccharides and proteins. These linkages might occur between soluble polyuronide and glycoprotein. The model derived from analysis of the extracellular polyuronide–arabinogalactan–glycoprotein complex from sycamore suspension cultures is consistent with the gross composition of many similar complexes. However, the insoluble extracellular complex from *Helianthus* cultures [10] differs in containing no galactose residues. Since the soluble polyuronide and glycoprotein in apple tissue can be separated after pectinesterase treatment of an extract they are not covalently linked. The aggregates of these components reported previously [11] contained much less hydroxyproline in relation to α-amino acids and, therefore, included a large proportion of protein with a little or no hydroxyproline. The same is probably true of the material which precipitated from M KCl extracts of apple tissue after dialysis. Presumably polyuronide can form ionic bonds with proteins and the aggregation could be stabilized in the presence of phenolics by the formation of "hydroxyquinoid chelate bridges" [4]. The aggregates [11] and precipitates prepared from apple tissue are clearly artifacts of preparation and the same may be true of soluble or insoluble aggregates from other plant tissues [8–10].

Hydroxyproline mono-, di-, tri-, and tetra-arabinosides have been identified in $Ba(OH)_2$ hydrolysates of cell walls from suspension cultures of many plant species [19]. Apple fruit is similar to other angiosperm tissues in that its cell walls yield predominantly the tetra-arabinoside on $Ba(OH)_2$ hydrolysis.

Although hydrazinolysis was thought not to cleave glycosidic bonds [14], this treatment does degrade polygalacturonide and galactosyl serine [5], both presumably by β-elimination [20]. Hydrazinolysis is therefore not suitable for deciding whether structural polysaccharides are linked to wall protein. Hydrazinolysis has the further disadvantage of generating positively charged fragments from wall polysaccharides, which can be confused with glycopeptides on ion exchange chromatography or electrophoresis. The heterogeneous basic material solubilized by hydrazinolysis of apple fruit cell walls could be partly separated into high MW galactose rich and lower MW hydroxyproline and arabinose rich fractions. The elution volume of the hydroxyproline rich material and the fact that it was susceptible to degradation by arabinosidase suggests that hydrazides of hydroxyproline arabinosides were present, which were not substituted with other sugar residues as those from sycamore suspension cultures appeared to be [14].

Although it had been claimed that a high proportion of serine residues in sycamore suspension culture cell walls were labile to mild alkali treatment [8], Lamport has convincingly shown that glycosylated serine residues in the same material are resistant to alkaline degradation [22]. This may explain the failure to detect alkali labile serine residues in apple cell walls.

Glucosamine has been found in a number of plant glycoproteins [23–25] but unidentified hexosamine in apple fruit cell walls was relatively easily released and could have been present in oligosaccharide chains, attached to glycoproteins like those found among soluble constituents. Glucose and xylose residues, which were also present in these low MW glycoproteins, were found in the glycopeptides released by protease from the cell wall. Whether these glycoproteins are wall constituents or cytoplasmic contaminants requires further investigation.

The release of hydroxyproline containing glycopeptides from plant tissue culture cell walls by *Streptomyces griseus* protease varies widely, depending upon the species and pretreatment of walls [21]. An unspecified proportion of the glycopeptides released by the protease from polygalacturonase and endoglucanase treated sycamore suspension culture cell walls was retained by DEAE Sephadex and contained uronic acid residues [8]. Similar material was not isolated from apple cell walls subjected to similar treatments.

The uronic acid found associated with hydroxyproline containing peptides after protease treatment of cell walls pretreated with pectinesterase alone could represent a covalent linkage of polyuronide to glycoprotein. However, if the proportions of this material in the extract reflect those in the whole wall this covalent binding would involve less than 0·5% of total polyuronide.

Greater release of hydroxyproline residues by protease after polygalacturonase treatment of cell walls suggests that polyuronide and wall protein

are physically associated in the wall so that removal of polyuronide aids access by the protease to its substrate. Polygalacturonase pI 4·6 from *S. fructigena* apparently selectively degrades an unbranched polyuronide component of apple fruit cell walls whereas polygalacturonase pI 9·7 degrades in addition polyuronide with neutral glycan branches [18]. Since these isoenzymes were equally effective as pretreatments to increase release of hydroxyproline containing peptides by the protease, it is probably the removal of the unbranched polyuronide which allows access to the protein. Although this polyuronide is solubilized during fruit ripening [18], wall protein does not become more susceptible to degradation by protease. Similarly arabinosyl residues on the glycoprotein did not seem to become more accessible to arabinosidase during fruit ripening.

EXPERIMENTAL

Preparations from Cox's Orange Pippin apples were the same as those used previously [18] unless otherwise stated.

Sources of enzymes. Citrus pectinesterase, arabinosidase pI 6·5, polygalacturonase pI 4·6 and polygalacturonase pI 9·7 from *Sclerotinia fructigena* and endoglucanase from *Trichoderma viride* were prepared and assayed as described previously [18]. Protease "Type VII" from *Streptomyces griseus* was purchased from Sigma Chemical Co. A unit of enzyme activity (U) released 1 μmol product per hr under the assay conditions [18]. None of the enzymes used contained hydroxyproline residues.

Chromatography. DEAE cellulose and Sephadex G-200 chromatography were carried out as described previously [11]. Glycopeptides were separated by gel permeation chromatography on columns of Sephadex G-25 and G-100, equilibrated with 0·1 M HOAc, and by ion exchange chromatography on columns (7·5 × 1·0 cm) of Dowex AGW 50X2 (200–325 mesh, H^+ form), eluted first with H_2O and then with linear gradients formed from 50 ml H_2O and 50 ml 0·25 M or M HCl, as indicated.

Estimation of amino acids and hydroxyproline. Proteins and glycopeptides were hydrolysed in 6 N HCl at 105° for 16 hr, if necessary filtered on Whatman GF/A, to remove solids, evaporated *in vacuo* over P_2O_5 and NaOH and redissolved in H_2O. Amino acids were estimated with ninhydrin [26] and hydroxyproline as described by Leach [27]. For estimation of serine, hydrolysates were passed through columns (2 × 1 cm) of Dowex 50X8 (H^+ form), which were eluted with H_2O, followed by 2 N HCl to displace the serine. The eluate was evapd at 40°, redissolved in H_2O and serine was estimated as HCHO after periodate oxidation [28].

TLC of amino acids. Glycopeptides, hydrolysed in 6 N HCl as above, were applied to cellulose plates which were developed with MeOH–H_2O–C_5H_5N (8:20:5) or propan-2-ol–HCO_2H–H_2O (20:1:5) and sprayed with ninhydrin to visualize amino acids.

Estimation of carbohydrates. Carbohydrate in fractions from column chromatography was estimated with sulphonated 1-naphthol [16] and uronic acid with carbazole and H_2SO_4 [15]. Reducing sugar was estimated colorimetrically [29, 30]. For estimation of monosaccharide residues, samples were hydrolyzed in N HCl at 105° for 2 hr, if necessary filtered on Whatman GF/A, and evaporated over P_2O_5 and NaOH. Neutral sugars were separated and estimated by GLC of their TMSi ethers [11]. Hexosamine in similar hydrolysates was estimated, after purification on columns of Dowex 50, by the Elson–Morgan reaction [31].

$Ba(OH)_2$ hydrolysis. Samples of wall suspension were mixed with an equal vol. of H_2O and $Ba(OH)_2$ to 0·43 M, heated at 90° for 9 hr [3], and filtered through sintered glass (porosity 3). The filtrate was neutralized with 3 N H_2SO_4, centrifuged, and the supernatant was concentrated. Samples from 5 ml wall suspension (arabinosidase treated and untreated) were purified as for serine estimation above, before hydroxyproline [27] and carbohydrate [16] estimations. Hydroxyproline values were multiplied by 1·8 to correct for lower reaction by the tetra-arabinoside than free hydroxyproline; this factor was determined by analysis of the hydroxyproline tetra-arabinoside peak prepared as follows. Hydrolysates from 50 ml wall suspension were applied to a Dowex AGW 50X2 column and eluted with a linear gradient up to 0·5 M HCl. Carbohydrate [16], hydroxyproline [27] and amino acids [26] were estimated in the various fractions.

Hydrazinolysis. Wall suspensions were filtered, washed with Me_2CO and dried *in vacuo* over P_2O_5. Samples (100 mg) were heated in sealed tubes with 2 ml dry hydrazine and 20 mg hydrazine sulphate at 100° for 10 hr [32]. After removal of hydrazine in vacuo over H_2SO_4, residue was extracted with H_2O and the soln applied to a Dowex AGW 50X2 column, which was eluted with a gradient up to 0·25 M HCl. Fractions containing carbohydrate were neutralized with 10% dioctyl methylamine in $CHCl_3$, freeze dried and redissolved in a small vol. of H_2O before application to other columns.

Protease digestion. Mixtures containing, in proportion, 10 ml wall suspension, 10 ml 0·01 M phosphate (pH 7) and 5 mg protease were incubated under toluene at 30° for 24 hr. The suspension was filtered on sintered glass and centrifuged to remove fine debris.

NaOH treatment of cell walls. Wall suspensions were filtered, washed with Me_2CO and dried as above. Samples (50 mg) of wall were treated with 0·5 N NaOH for 0 and 24 hr at 20° before hydrolysis and estimation of serine as above.

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