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PROPERTIES OF POLYGALACTURONATE AND CELL COHESION IN APPLE FRUIT CORTICAL TISSUE

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Abstract—Examination of the hydrodynamic properties of polygalacturonate fractions from unripe and ripe apple tissue suggested that the wall bound fraction was degraded during ripening but that the soluble fraction was not. Esterification of cell wall preparations with CH_2N_2 caused solubilisation of polygalacturonate. Acid MeOH caused more extensive solubilization, but this reagent hydrolysed arabinofuranosyl linkages. Both reagents reduced the cohesion of EtOH extracted apple tissue. This effect could also be achieved by treatment with sodium polyphosphate at pH 4 but not by EDTA or chaotropic agents. Free carboxyl groups on polygalacturonate probably maintain cell cohesion through co-operative binding of Ca^{2+} ions. The integrity of primary wall structure is thought to depend upon non-covalent bonding between cellulose, protein and polygalacturonate.

INTRODUCTION

Attempts to understand the molecular basis of softening of apple fruits during ripening have led to investigations of cell wall polymers [1–3], compositional changes during ripening [4, 5], and wall degrading enzymes [6, 7]. The results have been interpreted in the light of the developing understanding of plant primary cell wall structure [8–11].

The essential findings so far are (i) the major non-cellulosic polysaccharides of apple cell walls are a simple polymethylgalacturonate (PMG), probably localised in the middle lamella, and a branched PMG, with side chains of arabinose and galactose residues, probably concentrated in the primary wall [1, 2]; (ii) apple cell walls contain amounts of hydroxyproline rich protein which are small by comparison with other plant tissues, but this protein does not appear to be covalently linked to PMG [3]; (iii) during ripening a loss of galactose residues from the branched PMG [4] is probably catalysed by a β-galactosidase [6]; (iv) the increase of soluble unbranched PMG during ripening is associated with fruit softening [12] but it is not directly dependent upon the loss of galactose residues from the wall [6]; (v) apple cells do not appear to contain an endo-polygalacturonase but an exo-polygalacturonase is present [7]; (vi) the quantities of glucose-containing wall polymers do not change during ripening [5].

The present paper examines the possibility that cleavage of PMG chains is responsible for their solubilization and tissue softening and describes chemical modifications of cell walls which can simulate the same changes *in vitro*.

RESULTS AND DISCUSSION

Composition of cell walls and wall fractions

Cell wall material was prepared from unripe apples, picked prior to the commencement of the respiration climacteric and from similar fruit which had been allowed to ripen for a month off the tree during which time the respiration passed through the climacteric. PMG fractions were isolated in order to compare their physical properties before and after ripening. Soluble PMG was isolated from TCA extracts of fruit tissue to minimise contamination with protein and de-esterification by pectinesterase during extraction. Extraction of wall bound PMG is inevitably degradative and sodium polyphosphate (SPP) at pH 4 was used because it causes less damage to PMG at high temperatures than other extractants [1]. The PMG fractions were purified by ion-exchange chromatography on DEAE cellulose. Polysaccharide unretarded by DEAE cellulose was found in SPP extracts; this gave only arabinose on hydrolysis and was thought to have originated by cleavage of arabinofuranosyl linkages during extraction.

As expected [1] the soluble PMG from ripe apples had a low neutral sugar content whereas the wall bound fractions contained more arabinose, galactose etc. (Table 1). The soluble fraction from unripe fruit also had a

Table 1 Composition of cell walls and polymethylgalacturonate (PMG) fractions isolated from apple fruit cortex

	Molar ratio of component to galacturonic acid in Unripe apple Wall			Ripe apple Wall		
Component	Cell wall	bound PMG*	Soluble PMG*	Cell wall	bound PMG*	Soluble PMG*
Galacturonic acid	1	1	1	1	1	1
Rhamnose	0.126	0.009	0 135	0 075	0 039	0 031
Arabinose	0 777	0.293	0.332	0 950	1 100	0 153
Xylose	0.204	0.016	0 019	0 285	0.070	0 021
Mannose	0.081	0.003	0 004	0.088	0	0 016
Galactose	0 616	0.225	0 432	0 301	0 137	0.064
Glucose	1 70	0.124	0.188	1 60	0 248	0 017
Methoxyl	0.716	0.707	0 770	0.690	0 845	0 787
Galacturonic acid μmol/g fr. wt	22.9	12.9	1.8	16 4	8.37	6 0

* These figures are means for 2 or 3 portions of a single peak from a DEAE cellulose column

high neutral sugar content and could have been a precursor of the branched (primary-wall) PMG. The coefficient of variation of methanol estimations was about 5% and no significance is attached to variations in methoxyl contents. Estimation of free carboxyl groups by diazoethylation, followed by saponification and gas chromatographic detection of EtOH and MeOH was attempted. However diazo-ethane was considerably less reactive than CH_2N_2 and no more than 20% substitution of free carboxyl could be achieved in experiments with polygalacturonic acid and citrus pectin.

Hydrodynamic properties

Plots of viscosity number against concentration (Fig. 1) showed the same form as observed for polygalacturonic acid [13] although the values were generally higher. The viscometric properties were similar for each of the 2 or 3 portions eluted from a single extract applied to the DEAE cellulose column. Axial ratios [14] were calculated from limiting viscosity numbers, assuming that the molecules were prolate ellipsoids, a value of partial specific volume similar to that measured for citrus pectin (0.652 ml/g) and a level of hydration of 30%, similar to that of other polysaccharides [13]. The values ranged from 50:1 for the wall bound fraction from ripe apples to 100:1 for the soluble fraction from ripe apples. Three of the preparations showed little increase of viscosity number with concentration (Fig. 1); presumably the presence of neutral side chains prevented chain interactions at high concentration. By contrast the soluble PMG from ripe apples showed evidence of strong interactions and this was attributed to lower frequency of neutral residues in this polymer. That this polymer is freely extractable from fruit tissue and its production is associated with increasing ease of cell separation are at variance with its greater tendency to association. Presumably the wall bound polymers are involved in more stable interactions which are disrupted during extraction and cannot be reformed *in vitro*.

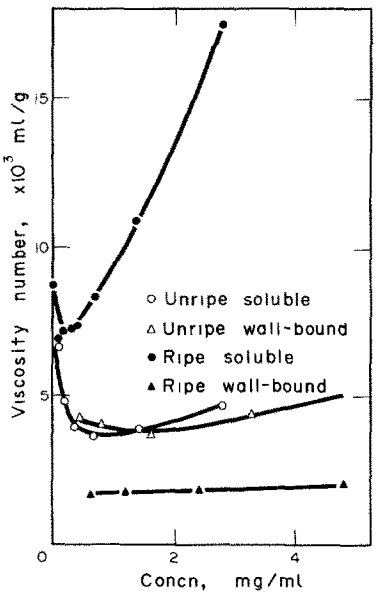

Fig. 1. Relationship of viscosity and concentration for polymethylgalacturonate (PMG) fractions from apple cortex.

Sedimentation analysis was carried out at a single low concentration as the material available was not sufficient for runs at higher concentration. Because the concentration was low the sedimentation coefficients obtained should be close to S_0. Three of the preparations gave slowly sedimenting peaks whose breadth suggested heterogeneity. The soluble preparation from ripe apples gave a sharp peak which scarcely broadened with time. This probably reflects a dependence of S upon concentration rather than greater homogeneity.

In calculating MWs from sedimentation and viscosity data [15] assumptions are made about molecular shape. The PMG molecules could exist as extended chains, probably with a helical conformation [16], or as loosely folded chains with a globular structure because of kinking introduced by rhamnopyranosyl residues interspersed in a polygalacturonosyl sequence [16]. The value of the constant used in calculation was a compromise between that for a prolate ellipsoid (β) [15] and a hydrated random coil ($\phi^{\frac{1}{3}p-1}$) [17]. This could mean that the MWs are in error by up to 10%.

When the MWs and corresponding DPs calculated from sedimentation and viscosity data were compared (Table 2), the wall bound fraction from ripe apples appeared to be degraded but the soluble fraction from the same fruit did not. This unexpected result could mean that the soluble material was released intact from the wall as a result of the degradation of another fraction or that while existing PMG was being degraded the soluble PMG was synthesized *de novo*.

Esterification treatments of cell walls

Diazomethylation of cell walls increased the degree of esterification of the PMG to *ca* 90%. This mild treatment caused an increased proportion of the PMG to become soluble on prolonged incubation in water (Table 3), suggesting that the presence of free carboxyl groups was necessary to the binding of this fraction in the wall. Walls treated with propylene oxide were found to contain alkali labile 2-hydroxypropyl groups in tenfold excess over the free carboxyl groups initially available, indicating that side reactions had taken place; the amount of PMG solublized (*ca* 30% total) was less than that in diazomethylated walls.

Treatment of cell walls with 0.5 M HCl in dry MeOH at

Table 2. Hydrodynamic properties and calculated features of polymethylgalacturonate (PMG) fractions from apple fruit cortex

Preparation	Limiting viscosity number (ml/g)	Sedimentation coefficient	MW	DP of main chain
Unripe apple				
soluble PMG	337	1.61	68000	210
wall-bound PMG	319	1.36	51000	202
Ripe apple				
soluble PMG	585	1.24	60000	280
wall-bound PMG	166	1.34	36000	100

Limiting viscosity numbers are means for 2 or 3 portions of a single peak eluted from a DEAE cellulose column. The first eluting portion was analysed in the ultracentrifuge.

Table 3. Effects of esterification treatments on solubility of polysaccharides in cell walls from apple fruit cortex

Treatment	% Total soluble in H_2O				
	Gal UA	Ara	Xyl	Gal	Glc
None	26	14	0	15	1
CH_2N_2	55	2	4	2	1
MeOH–HCl	71	92*	7	91	18

* Present in MeOH–HCl supernatant.

room temperature [18], resulted in the solubilization of *ca* 70% of the galacturonic acid residues present and more neutral residues than the other treatments (Table 3). The presence of arabinose in the MeOH supernatant indicates that considerable hydrolysis of arabinosyl linkages occurred in this treatment.

The lower solubility of the branched PMG, observed in this and previous studies [1] suggest that it is bound by different linkages from those retaining the simpler, linear molecule. If it is a component of the matrix of the primary wall, it could be interacting with other wall components such as protein and cellulose. The solubilization of a portion of the branched PMG by 0.5 M HCl in MeOH could be due to cleavage of ester linkages attaching the carboxyl groups of galacturonate residues to hydroxyls on other polymers. Alternatively, the cleavage of arabinofuranosyl linkages by this reagent could implicate araban side chains in binding to other polymers.

Binding of polysaccharides to cellulose

Neither araban nor galactan bound to cellulose in a standard assay system [10]. The binding of crude apple pectin to an unsubstituted cellulose column was no more than 2% of the sample applied.

The linkage of PMG to hemicellulose in sycamore suspension culture cell walls is not acid labile [10]. However, branched PMG binds to DEAE cellulose more tightly than the unbranched molecule but binds more weakly after a mild acid hydrolysis [19]. This was thought to imply that araban side chains are involved in binding to DEAE cellulose. Binding in the cell wall would require an additional component to link cellulose and PMG. For example basic groups on the wall protein, which appears to be bound to cellulose fibrils [11], might form salt linkages with free carboxyls on PMG.

Chemical modification of cell cohesion

Unripe apple tissue extracted with EtOH and rehydrated was disrupted by a force of 6.5–7.5 N. Treatment with CH_2N_2 reduced the force required to 0.5 N and methanolic HCl reduced it to 0.4 N. Treatment with 50 mM EDTA in 0.1 M Na_2HPO_4 (pH 6.9), 8 M urea or 2 M KCl had no effect on ease of disruption. Incubation in 2% SPP (pH 4) at 20° for 16 hr reduced the force required to 0.4 N (0.1 M NaOAc buffer (pH 4) had no effect). Microscopic examination of esterified or SPP treated tissue revealed complete cell separation but little cell disruption, as judged by retention of starch grains within cells.

The effects of esterification treatments in reducing cell cohesion and increasing solubility of PMG show the importance of free carboxyl groups in middle lamella structure. Acid MeOH also reduces cell cohesion in strawberry fruit tissue, but the conclusion that methylation of PMG leads to softening [18] is not justified on this evidence. CH_2N_2 solubilises a PMG fraction with a low proportion of neutral residues, like that attacked by a polygalacturonase isoenzyme from *Monilinia fructigena* [2]. This adds weight to the suggestion that the unbranched PMG is important in middle lamella structure.

SPP probably acts as a chelating agent, removing Ca^{2+} ions from the middle lamella and cell wall. Although EDTA at pH 7 reduces cell cohesion of some plant tissues [18, 20–22] it is generally less effective than SPP [19]. Addition of excess Ca^{2+} reverses the macerating effect of SPP on apple and other tissue [19]. The Ca^{2+} ions could be located in 'egg box' junction zones [23] formed from parallel sequences of nonesterified galacturonosyl residues in chains which are predominantly esterified.

EXPERIMENTAL

Cell wall preparations. Two samples of Cox's Orange Pippin apples were harvested from 13-yr-old trees on M26 rootstocks at East Malling Research Station on 13 September 1974. Cortical tissue was separated from one sample and immediately frozen at −20° for subsequent analysis and from the other sample after 31 day's storage at 12°.

Soluble polysaccharides were extracted by disintegrating frozen tissue in 2% (w/v) TCA (2 ml/g tissue) and isolated after filtration (Whatman 541) by precipitation with 4 vol Me_2CO. The ppt was collected by centrifugation, washed by resuspension and centrifugation in Me_2CO–H_2O (4:1) ×4 and Me_2CO ×2 and dried.

Cell walls were obtained by disintegrating tissue in 2 vol 0.2 M Tris-(hydroxymethylaminomethane)/Pi (pH 8) including 10 mM diethyldithiocarbamate at 2°. Insoluble material in this and subsequent stages was collected by a combination of centrifugation and filtration. It was washed with H_2O before resuspension in H_2O to which was added an equal vol of 0.2 M Tris HCl 2 M KCl 0.2% Triton X-100 (pH 7.5). After 3 washes with H_2O the residue was resuspended in $CHCl_3$–MeOH (1:1), filtered, washed with H_2O and resuspended in chloral hydrate–H_2O (3.5:1, w/w). After standing 48 hr at 20° to solubilize starch [4] the suspension was centrifuged; the pellet was washed by resuspension and centrifugation ×4 with H_2O and divided into a portion which was freeze dried and another which was stored at −20° as an aq. suspension.

To extract wall-bound PMG, aliquots of aq. suspension were extracted with Na polyphosphate (SPP, final concn 2% w/v, adjusted pH 4 with HCl) at 98° for 4 hr and filtered on sintered glass (porosity 3). The filtrates were dialysed against H_2O 24 hr at 2°.

DEAE cellulose chromatography. Soluble (TCA extract) and wall-bound (SPP extract) PMG were isolated by chromatography on 15 × 1.5 cm columns of DEAE cellulose in the phosphate form as previously described [1]. Each extract yielded a single broad carbohydrate peak eluting from 0.02 to 0.15 M Pi and pooled fractions were dialysed and concentrated by freeze drying.

Carbohydrate analysis. H_2SO_4 hydrolyses and preparation of alditol acetates were as described previously [2]. Alditol acetates were analysed by GLC on a 1.8 m × 4 mm column of 5% OV 275 on Chromosorb W at 225°. Galacturonic acid was estimated colorimetrically [24], directly in soluble fractions, and, after enzymic solubilization, in cell walls [25]. Me ester was estimated as MeOH after treatment of cell walls or polysaccharides with 0.5 M NaOH at 20° for 30 min, by GLC on 1.8 m × 4 mm columns of Porapak Q at 120°.

Physical measurements. Viscosity and sedimentation were measured on solns of polysaccharides in 0.1 M NaPi (pH 6.5) at 20°. A horizontal viscometer [26] with a capillary 200 × 0.4 mm was used with an applied pressure of 1960 Pa. Sedimentation

coefficients were estimated from photographs taken during a 3 hr run at 55000 rpm in a Beckman Model L centrifuge with a sample concn of 2 mg/ml. The partial specific vol of citrus pectin (Koch Light Ltd) was measured in soln in 0.1 M NaPi (pH 6.5) at 20° in a 10 ml pycnometer.

Esterification treatments. CH_2N_2 was generated from *N*-methyl *N*-nitroso *p*-toluenesulphonamide [27] and diazoethane similarly from the *N*-ethyl derivative [28]. Samples (10 mg) of cell wall were incubated at 2° in 5 ml of Et_2O–MeOH (9:1) soln of these reagents for 24 hr. The supernatant soln was removed and the residues allowed to dry.

Similar samples of cell wall were incubated at 20° for 6 days in 1 ml propyleneoxide–H_2O (9:1) after which they were freeze dried. Esterification was measured by saponification (0.5 M NaOH, 20°, 30 min) followed by spectrophotometric measurement of periodate consumption [29] by the supernatant against a standard of 1,2-propandiol. Samples of polygalacturonic acid were 80% substituted after a similar esterification treatment.

After esterification in 0.5 M HCl in MeOH for 24 hr at 20° the supernatant was removed for separate analysis and the residue washed with MeOH and dried at 20°.

To estimate solubilised polysaccharides 10 mg samples of treated cell wall were incubated in 5 ml H_2O under toluene for 16 hr at 20° and the supernatant was analysed after centrifugation, as described above.

Cellulose binding assays. Solns of araban (Koch Light) and galactan from potato tubers [30] were assayed for binding by incubating with a cellulose suspension followed by centrifugation [10]. Samples of H_2O soln of a crude apple pectin prepn [31] were applied to a 2.5 × 1.5 cm column of cellulose (Whatman CC 41) and eluted with H_2O under gravity. To recover bound material the column was eluted with 0.25 M NaPi 1 mM EDTA (pH 6.5). Carbohydrate in eluates was reacted with sulphonated α-naphthol reagent for colorimetric estimation [32].

Tissue cohesion tests. Discs of apple cortex tissue 15 × 2 mm were extracted with EtOH–H_2O (4:1) and washed with the appropriate solvent before transfer to CH_2N_2 in Et_2O [27], 0.5 M HCl in MeOH, or aq. reagents. After incubation 16 hr at 20° the discs were rehydrated if necessary and their resistance to disruption was measured with a 'penetrometer' with an 8 mm plunger applied to a stack of 5 discs [33].

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