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Cell Wall Metabolism in Ripening Fruit

I. CELL WALL CHANGES IN RIPENING 'BARTLETT' PEARS[1]

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ABSTRACT

'Bartlett' pear (*Pyrus communis*) fruits were picked at the mature, green stage and ripened at 20 C. Fruits at different stages of ripeness (based on flesh firmness) were homogenized, and the sugar and uronic acid contents of cell wall and soluble polysaccharides were determined. Substantial amounts of galacturonic acid and arabinose were lost from the wall fraction as the fruit ripened. Most of this cell wall material was recovered, in an 80% (volume/volume) ethanol-insoluble form, from the soluble fraction of tissue homogenates. Structural analysis of ethanol-precipitable material indicates that it is an acidic (pectic) polymer-bearing side groups containing variously-linked arabinosyl residues.

Tissue softening generally accompanies fruit ripening. Softening is due, at least in part, to the dissolution of cell walls which results from ripening-associated changes in wall polysaccharides. A reduction in cell wall pectin content (usually measured as uronic acid) has been reported for virtually every ripening fruit (16, 17). This observation has led to a great many studies which have documented the involvement of pectolytic enzymes (chiefly PG[3]) in wall degradation (17). Decreases in cell wall neutral sugar content have also been described for ripening fruit (8, 11, 13, 21). These changes most often involve galactose and/or arabinose.

Losses of neutral sugars are generally observed by hydrolyzing cell walls prepared from fruit at different stages of ripeness and determining monosaccharide content (8, 13, 21). Many of the sugar residues in cell walls, including galactose and arabinose, are found in a variety of glycosidic linkages. For instance, galactosyl residues in polymers of the primary wall of sycamore can be linked through carbons three, four or six (10, 19). Thus, little information about the structure of wall polysaccharides involved in ripening-associated changes can be obtained from composition data alone. This, combined with the fact that few generalities about fruit cell wall structure can be made, makes reasoned speculation about the enzymes responsible for losses of neutral sugar from cell walls of ripening fruit difficult.

This study describes the loss of substantial amounts of uronic acid and arabinose from the walls of ripening 'Bartlett' pear fruits. In addition, structural analysis of pear fruit cell wall material which becomes water-soluble during ripening and of material solubilized by PG treatment of pear cell wall material provides information crucial to understanding the control of cell wall metabolism in ripening fruit.

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[3] Abbreviations: PG: polygalacturonase; TFA: trifluoroacetic acid.

MATERIALS AND METHODS

Source of Fruit. Mature green 'Bartlett' pears (*Pyrus communis*) were obtained from the orchard at Davis. Fruits were selected for uniformity of size and freedom from blemishes. Fruit were used immediately or stored (0 C) until needed. No fruits were stored for longer than 1 month.

Fruit Ripening. Lots of eight fruits of uniform size (total weight, 1.3–1.4 kg) were placed in 2-liter glass jars and held at 20 C. A stream of humidified air, maintained at a flow rate of 250 ml/min, was passed through the jars. Respiratory rate was determined according to the method of Claypool and Keefer (5) and ethylene was measured by gas chromatography (15). Changes in flesh firmness were determined using a UC fruit firmness tester (9) fitted with a 0.79-cm tip.

Preparation of Soluble and Cell Wall Fractions. Fruit at designated stages of ripeness (based on flesh firmness) were peeled and the cores were removed. The fruit flesh was diced and homogenized, in an equal volume of cold (4 C) distilled H_2O, for 1 min at full speed in a Virtis homogenizer. The brei was centrifuged at 10,000*g* for 10 min in a Sorvall model RC2-B refrigerated centrifuge equipped with an SS-34 rotor. The supernatant solutions and two distilled H_2O washes of the pellets were combined (soluble fraction).

The cell wall pellet was washed according to the technique of Talmadge *et al.* (19). The pelleted material was washed two times with 100 mM and with 500 mM K-phosphate (pH 7.0) and four times with distilled H_2O. All washings were carried out at 0–4 C. Centrifugation at 1,000*g* followed each washing. Supernatant solutions were discarded. The washed pellets were suspended in chloroform-methanol (1:1) and transferred to a coarse sintered glass funnel. The pellets were washed 3 times each with chloroform-methanol and acetone in order to remove residual lipid material. The pellet (crude cell wall) was air dried following the final acetone wash and stored over silica gel desiccant in a vacuum desiccator at 22 C.

CELL WALL ANALYSIS

Enzyme Treatments. Starch was removed from the cell wall preparation by treatment with α-amylase. Wall material was suspended in 20 mM K-phosphate (pH 7.0) containing 1 mg amylase (type III, Sigma) per ml. A drop of toluene was added to suppress microbial growth. Incubation lasted for 48 h at 37 C with continuous shaking. Following incubation cell wall material was sedimented by centrifugation at 1,000*g* and the supernatant solution was discarded. The pellet was washed two times in distilled H_2O and lyophilized.

Ten mg of α-amylase-treated cell walls were suspended in 4 ml 50 mM Na-acetate (pH 4.5) containing approximately 20 units of PG. The PG used in this work is an endoenzyme purified from a commercial pectinase preparation derived from *Aspergillus niger* (Pectinase, technical grade; ICN Nutritional Biochemicals) according to the procedure of Labavitch and Rae (14). A drop of

Table I. *Carbohydrate Composition of Cell Walls of Pear Fruit at Different Stages of Ripeness*
Data are expressed on a per pear basis so as to give a picture of the dynamics of change in individual fruits.

Pressure Test	Carbohydrate Composition								
	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Noncellulosic Glucose	Uronic Acid	Cellulose
kg	*mg/pear*								
8.2	50	35	980	590	21	300	50	1,040	1,020
4.5	33	30	780	490	28	215	35	1,000	1,010
2.3	30	30	400	565	25	150	30	680	1,020
1.1	28	31	450	500	30	150	31	302	1,000

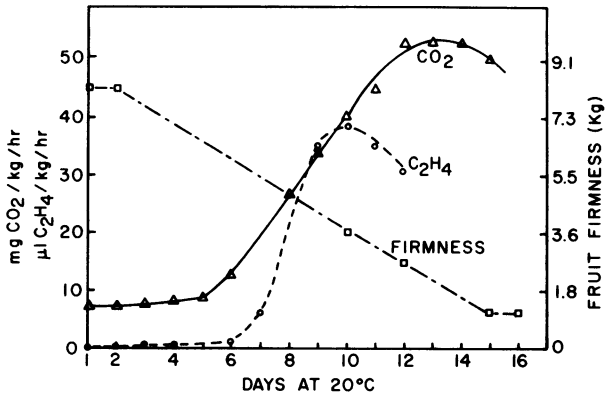

FIG. 1. Relationship between respiration rate, ethylene evolution, and fruit firmness in ripening 'Bartlett' pears.

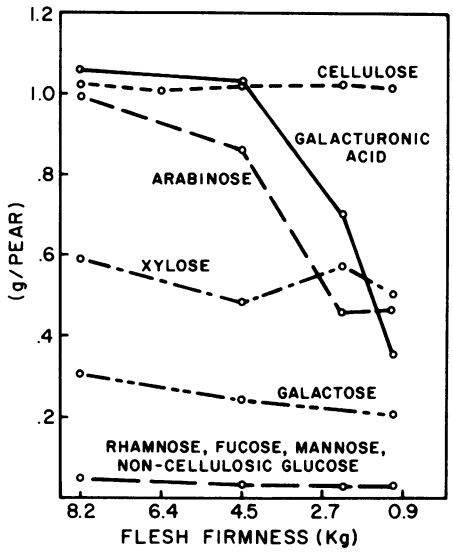

FIG. 2. Changes in cell wall composition during the ripening of Bartlett pears. The pattern of change and the amounts present are similar for rhamnose, fucose, mannose, and noncellulosic (2 N TFA-soluble) glucose (refer to Table I).

toluene was added and the reaction mixture was incubated at 37 C for 48 h with continuous shaking. Cell walls were pelleted and washed as for α-amylase. The supernatant solution and water washings were combined (PG-soluble fraction). Extracted cell walls were lyophilized.

Carbohydrate Analysis—Gas Chromatography. Neutral sugar compositions of samples of cell walls and solubilized material were determined according to the technique of Albersheim *et al.* (3). Glycosidic linkage compositions of extracted polysaccharides were determined by gas chromatographic analysis (19) of samples methylated according to the method of Hakomori (7) as modified by Sandford and Conrad (18).

GC was performed with a Sigma 3 instrument (Perkin Elmer) fitted with dual nickel columns (120 × 0.2 cm o.d.) containing 0.2% ethylene glycol succinate, 0.2% ethylene glycol adipate, and 0.4% silicone XF-1150 on Gas-Chrom P. Separations were achieved with a 25 ml/min carrier gas (N_2) flow and 1 C/min temperature programming; from 145 to 185 C for alditol acetates and from 115 to 185 C for permethylated alditol acetates. The integration of chromatographic peak areas and calculation of sugar concentrations was carried out with a Sigma 10 data system (Perkin-Elmer). The identity of chromatographic peaks was determined by co-chromatography against standards and combined GC-MS performed at the Facility for Advanced Instrumentation (University of California, Davis).

Carbohydrate Analysis—Colorimetric Assays. Uronic acid concentrations in soluble preparations were measured according to Blumenkrantz and Asboe-Hansen (4). Uronic acids in cell wall preparations were measured as above (4), as modified by Ahmed and Labavitch (2). Pentose in column fractions was determined using the orcinol reaction (6). Because orcinol also produces color following reaction with uronic acids, quantitative measurements of arabinose in pooled column fractions were made by GC.

The cellulose content of cell wall samples was determined using a modification of the technique of Updegraff (20). Cell wall samples were hydrolyzed two times with 2 N trifluoroacetic acid (1 h, 121 C). The insoluble residue was then dissolved in 67% (v/v) H_2SO_4 and assayed for hexose as described by Updegraff (20).

Soluble Fraction Analysis. Aliquots from the combined supernatant solution and cell wall pellet washings were made 80% (v/v) in ethanol and held overnight at 2 C. Precipitated material was collected by centrifugation (1,000g, 5 min). The precipitated material was dissolved in 50 mM Na-acetate (pH 5.0) and applied to a column of DEAE-Sephadex that had been equilibrated in the same buffer. The column was eluted with 100-ml portions of pH 5.0 acetate containing 0, 100 mM, and 500 mM NaCl. Column fractions were assayed colorimetrically for uronic acids (4) and pentose (6) and, ultimately, by GC (3). The fractions containing the most prominent peak were pooled, dialyzed against distilled H_2O, and lyophilized. The lyophilized preparation was dissolved in 10 mM Na-acetate (pH 4.0) and applied to a column of Bio-Gel A-1.5m. Column fractions were assayed as above and fractions containing the most prominent peak were pooled, dialyzed against distilled H_2O, and lyophilized.

RESULTS

Freshly harvested, mature pear fruits were held at 20 C, and the rates of CO_2 and ethylene production were monitored. A typical climacteric pattern was observed (Fig. 1). The firmness of the flesh declined steadily, beginning a few days after placement at 20 C.

Pears with a flesh firmness of 8.2, 4.5, 2.3, and 1.1 kg were

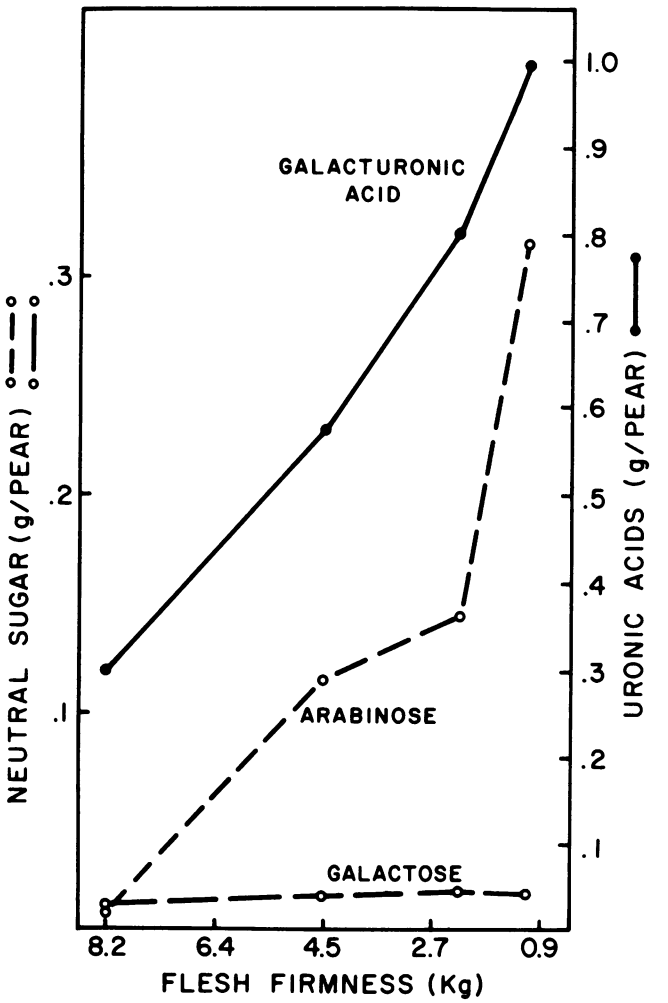

FIG. 3. Changes in carbohydrate components identified in the 80% (v/v) ethanol precipitable portion of the supernatant (soluble) fraction of pear fruit homogenates. Rhamnose, fucose, xylose, mannose, and glucose are also found in this fraction. They are present in lesser amounts than galactose and these levels do not change with tissue softening.

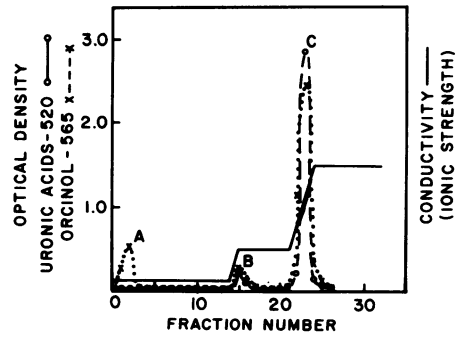

FIG. 4. DEAE-Sephadex chromatography of the 80% (v/v) ethanol precipitable fraction of the supernatant fraction from pear fruit homogenates. The sample, dissolved in 50 mM Na-acetate (pH 5.0) was applied to a column (1.5 × 10 cm gel bed) equilibrated in the same buffer. The column was eluted with 100-ml portions of 50 mM acetate containing 0, 100, and 500 mM NaCl. Changes in buffer ionic strength are indicated by the conductivity curve. Fractions of 10 ml were collected and were assayed for uronic acids (4) and pentose (6).

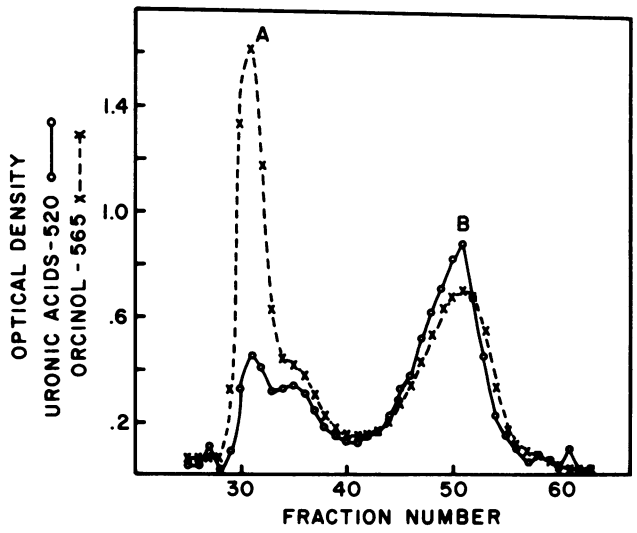

FIG. 5. Fraction C from the DEAE-Sephadex fractionation of the 80% (v/v) ethanol precipitable fraction from the supernatant of pear fruit homogenates (refer to Fig. 4) was dissolved in 10 mM Na-acetate (pH 4.0) and applied to a column of Bio-Gel A-1.5 m (2.3 × 90 cm gel bed) equilibrated in 10 mM acetate. Fractions of 5 ml were collected and assayed for uronic acids (4) and pentose (6).

chosen for analysis. Cell walls were prepared from these fruits and the neutral sugar, uronic acid, and cellulose contents of the wall preparations were determined (Table I). While the amount of each of these constituents changes somewhat during ripening a few features deserve mention (Fig. 2). Cellulose composition of pear fruits does not change as the ripening fruit softens. In contrast, the uronic acid content of the fruit declines sharply as the flesh softens below a pressure reading of 4.5 kg. Most of the noncellulosic neutral sugars decrease as the fruit softens. The clearest of these losses is seen in arabinose which accounts for close to 50% of the noncellulosic neutral sugars of the wall of the firm fruit.

The losses of uronic acid and arabinose from the water-insoluble wall material total more than 1 g carbohydrate/fruit when mature green (8.2-kg pressure test) and overripe (1.1 kg) pears are compared. Because an awareness of the structure of the material that had been removed from the wall during ripening would provide information about the mechanism by which wall dissolution is controlled, an attempt was made to recover the lost cell wall material. Preliminary colorimetric and gas chromatographic analyses of the soluble fractions of pear fruit homogenates demonstrated that these fractions contained arabinose and uronic acid in amounts very similar to those amounts which had been lost from the wall (Fig. 3, compare with Fig. 2).

Soluble Fraction. A large amount of the uronic acids and virtually all of arabinose in soluble fractions could be precipitated by treatment with 80% (v/v) ethanol. The precipitated material was dissolved in buffer and subjected to ion exchange chromatography. Column fractions were assayed colorimetrically for uronic acids and pentose (Fig. 4). The most prominent peak, containing most of the uronic acids and arabinose, was eluted with the front of the 500 mM NaCl-containing buffer. The fractions containing this material (peak C, Fig. 4) were pooled, dialyzed, and lyophilized.

The lyophilized preparation was dissolved in buffer and subjected to gel filtration on Bio-Gel A-1.5m (Fig. 5). All of the arabinose in the preparation and a substantial amount of the uronic acids eluted with the column void volume (peak A), suggesting a mol wt of 1.5×10^6 daltons or greater. In addition, uronic acids (free of arabinose, according to gas chromatographic analysis) were eluted in fractions corresponding to a lower mol wt (peak B, Fig. 5).

Table II. *Carbohydrate Components of Mature Green Pear Cell Wall before and after Polygalacturonase Treatment*

The neutral sugar values were detemined by gas chromatography of the alditol acetate derivatives (3). The uronic acid values were determined by the method of Ahmed and Labavitch (1).

Sample	Carbohydrate Components							
	Rhamnose	Fucose	Arabinose	Xylose	Mannose	Galactose	Noncellulosic Glucose	Uronic Acids
	% of cell wall dry wt							
Mature green, no treatment	1.2	0.7	23.4	11.2	1.7	7.6	2.0	27
Mature green, PG-treated	1.3	0.5	13.8	18.0	1.8	4.2	1.8	4.5

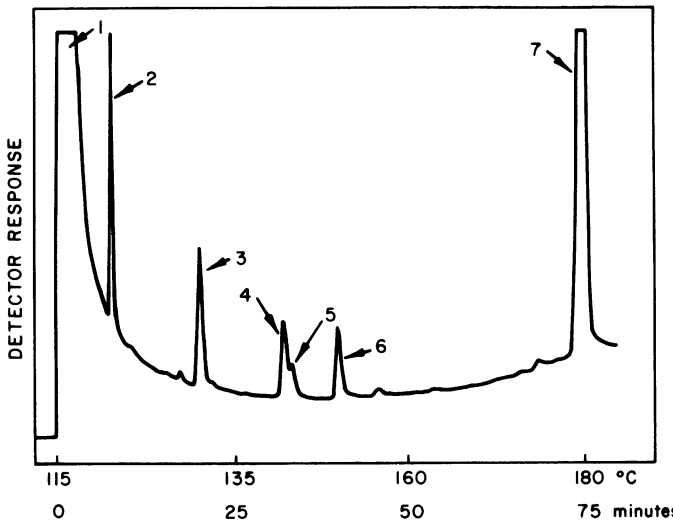

FIG. 6. Gas chromatogram of the permethylated alditol acetates produced upon derivatization (3, 8) of fraction A from the Bio-Gel A-1.5 m column (Fig. 5). The numbered peaks represent: 1, solvent; 2, terminal-arabinose (linked in polysaccharide only through carbon-1); 3, 1,5-arabinose (linked through carbons 1 and 5); 4, 1,3,5-arabinose; 5, 1,2,5-arabinose; 6, 1,2,3,5-arabinose; and 7, fully acetylated *myo*-inositol (internal standard).

Colorimetric and gas chromatographic analyses of peak A revealed arabinose and uronic acids in the ratio of 8:5. Figure 6 shows the results of gas chromatography of this material after methylation, hydrolysis, reduction, and acetylation. Peaks corresponding to derivatives of nonreducing, terminal-arabinose and arabinosyl residues linked through carbons 1 and 5 and, in some cases, carbons 2 and/or 3 were identified. This indicates a backbone of 1,5-linked arabinosyl residues, some of which bear arabinosyl side groups at carbons 2 and/or 3. The methylation analysis provides no information about linkages involving uronosyl residues.

PG Treatment of Walls of Unripe Pears. A sample of cell walls from mature green (8.2-kg pressure test) pears was subjected to digestion by a purified preparation of endo-PG. The noncellulosic neutral sugar and uronic acid compositions of treated and untreated walls are compared in Table II. The PG treatment reduced cell wall uronic acid content substantially and cut wall arabinose content by approximately 50%.

The PG-soluble fraction was dialyzed against 50 mM Na-acetate (pH 5.0) and chromatographed on DEAE-Sephadex as was the 80% ethanol precipitatable material from the soluble fraction of tissue homogenates. As for the ethanol precipitate, the dominant peak was eluted at the front of the 500 mM NaCl-containing buffer. This peak material contained uronic acids and arabinose. Gas chromatographic analysis of the permethylated alditol acetates derived from this material revealed the same set of arabinosyl derivatives that is shown for the ethanol precipitate (Fig. 6).

DISCUSSION

The most obvious cell wall compositional changes that accompany 'Bartlett' pear fruit ripening are the substantial losses of uronic acids and arabinose (Fig. 2). These findings are in agreement with Jermyn and Isherwood (8) who studied the ripening of Conference pears.

The most rapid losses of uronic acid and arabinose from the insoluble wall do not commence until substantial tissue softening has occurred (Fig. 2). This could be an indication that the initial metabolism of cell wall pectic polysaccharides causes a decrease in tissue firmness without altering pectin solubility. Alternatively, other (undetected) changes in cell walls or alterations in cell membrane integrity may trigger tissue softening. The loss of cell wall uronic acids (pectin) during ripening is generally accompanied by an increase in water-soluble pectin (16). This is clearly the case in the 'Bartlett' pear (Fig. 3). The finding that a substantial amount of the arabinose that had been lost from the insoluble cell wall during ripening could be isolated as part of a large mol wt (Fig. 5), acidic (Fig. 4) polysaccharide is significant from a number of standpoints. The dicot cell wall model of Keegstra *et al.* (10) was developed from studies of polysaccharides extracted from cultured cells; all of its features have not been demonstrated in walls prepared from plant tissues. This is especially true for fruit tissues, although Knee (12) has suggested that the model holds for the apple. The column and gas chromatographic analyses reported here indicate the presence, in pear fruit, of a highly branched, pectic arabinan similar to that described for cultured sycamore cells (19). Because substantial amounts of arabinosyl residues are apparently linked to the uronosyl residue-containing polysaccharide there is no need to postulate the presence of a mechanism for wall arabinose removal that is distinct from the mechanism which effects solubilization of galacturonan.

PG treatment of walls from green fruit solubilizes a pectic arabinan very similar to that which becomes soluble during ripening. This adds support to the long held contention that PG plays a significant role in fruit softening (16, 17). The fact that the water-soluble arabinan is of large mol wt indicates that the pectin need not be extensively metabolized in order to be solubilized. Furthermore, because the ripening pear contains considerable PG (1) the structure of the pectic arabinan must provide protection for the backbone. This protection could be conferred by the presence of a high degree of esterification of the uronosyl residues in the galacturonan. Alternatively, the arabinosyl residue-containing component may consist of a series of short segments which are spaced at regular, close intervals along the backbone. In either

case, PG action would be restricted.

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