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Measurement of Pectin Methylation in Plant Cell Walls[1]

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A procedure was developed to measure the degree of pectin methylation in small samples of isolated cell walls from nonlignified plant tissues or pectin solutions. Galacturonic acid was determined colorimetrically with the 3,5-dimethylphenol reagent. Methylation was measured by base hydrolysis of galacturonic acid methyl esters, followed by gas chromatographic determination of released methanol. Estimates of the precision of analysis of pectin and cell wall samples were made. The coefficient of variation for estimates of the pectin esterification in cell walls isolated from 10-g samples of cucumber tissue ranged from 7.7 to 13.2%.

KEY WORDS: pectin; cell wall; galacturonic acid; methanol; degree of methylation; gas-liquid chromatography.

Pectin in the cell walls of higher plants is esterified to a variable extent with methanol. Since plants also contain pectinesterase, changes in pectin methylation can occur in plants during development, in response to injury, or during processing and storage of fruits and vegetables. Methods available to measure pectin methylation include titration of the change in carboxyl groups after base hydrolysis of pectin (5), titration of pectin with polycations (1), and gas chromatographic analysis of methanol after hydrolysis of pectin (7). All of these methods require isolation of pectin from the plant cell wall before the extent of methylation can be measured. This requires multiple extractions of the plant tissue and results in incomplete removal of pectic substances from the cell wall (6). Knee (4) estimated the degree of pectin methylation of apples by measuring the total uronic acid in isolated cell walls colorimetrically, and the methanol content by base hydrolysis, followed by gas chromatography. This approach has the important advantage that the average methylation of the total cell wall galacturonic acid can be measured rather than limiting analysis to individual extracted fractions, which will not take into account nonextractable pectic polysaccharides. Another advantage is that cell wall from only a few grams of fresh tissue is sufficient for analysis. A small sample requirement also reduces the filtration time required in cell wall preparation so that analysis of larger numbers of samples becomes practical.

The approach that Knee (4) used has not been widely adopted, perhaps because only a limited description of the procedure was given and estimates of experimental errors were not provided. Also, Scott (2) developed a colorimetric determination of uronic acids with improved selectivity in samples with large amounts of neutral sugars, compared to the method of Blumenkrantz and Asboe-Hansen (3) which Knee (4) used.

The objective of this paper is to describe a detailed procedure to analyze the total uronic acid and methanol in isolated plant cell walls

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and in pectin solutions. The experimental errors of these measurements have been analyzed.

MATERIALS AND METHODS

Materials

Slicer-type cucumbers were purchased at a local market. Salt-stock cucumbers were obtained from cucumbers fermented in 6% NaCl with a controlled fermentation procedure (8). Sugar beets were provided by Dr. George Hogaboam, USDA-ARS, East Lansing, Michigan. Pectin samples were from Sunkist Growers Inc., Corona, California. Sodium polypectate was purchased from Raltech Scientific Services, Inc., Madison, Wisconsin. HPLC-grade methanol was obtained from Fisher Scientific Company (Raleigh, N. C.). Aldrich Chemical Company (Milwaukee, Wisc.) supplied the 3,5-dimethylphenol for uronic acid analysis. Isopropanol, acetone, 95% ethanol, and sulfuric acid were reagent grade.

Apparatus

Methanol release from cell walls was done in 3.0-ml Reacti-vials covered with a cap with a Tuf-Bond Teflon–silicone disc (Pierce Chemical Co., Rockford, Ill.). A cleaning sonicator was used to mix cell wall samples. Gas–liquid chromatography (GLC) of methanol was carried out with a Shimadzu Mini-2 gas chromatograph fitted with a flame ionization detector and a 5% Carbowax 20 M, 80/120 Carbopack B column (Supelco, Inc., Bellefonte, Pa.). A Hewlett–Packard HP-3390A integrator was used for analysis of the chromatograms. Colorimetric measurements were made with a Cary 219 spectrophotometer equipped with an automatic wavelength programmer.

Procedures

Cell walls for evaluation of analytical variables were prepared from cucumber mesocarp tissue and peeled sugar beets. The tissue was blended with 5 vol of 95% ethanol with a Tekmar blender. The blended tissue was filtered on a Büchner funnel with Whatman no. 1 filter paper. The insoluble cell wall fraction was resuspended in 70% ethanol (2.5-fold v/w, compared to the fresh tissue), blended, and filtered again. The cell walls were resuspended in 2.5-fold (v/w) acetone relative to the fresh tissue, blended again, and filtered as dry as possible on a Büchner funnel. The cell walls prepared in this way were dried in a vacuum oven at 25°C and stored in a desiccator until use.

Analysis of small samples of tissue was done by adding 5 vol of 95% ethanol with a 10- or 20-g sample of blended tissue. The sample was blended for 1 min with a Tekmar homogenizer and filtered on a small Büchner funnel through Whatman no. 1 filter paper. To the Büchner funnel, 2.5 vol of 70% ethanol was added and removed by suction. The insoluble walls were finally washed with 2.5 vol of acetone and filtered as dry as possible. Cell walls were dried in a vacuum oven and desiccated until use.

Titrimetric analysis of methylation of pectin samples was done using the procedure of Schultz (5).

Pectin Methylation Analysis Procedure

a) Uronic acid analysis. Colorimetric determination of uronic acids was done with 3,5-dimethylphenol reagent (2). Dry cell walls (17.5 mg) were placed in 16 × 100-mm test tubes, wetted with 750 μl 95% ethanol, and sonicated for 3 min. Two equal aliquots (1.75 ml each) of chilled (0°C) 72% sulfuric acid were added to the cell walls. The tubes were mixed and chilled after addition of each aliquot. This procedure was required to prevent charring of the cell walls, which reduced the amount of uronic acid in the sample. The tubes were sonicated for 3 min, heated at 50°C for 10 min, and cooled in an ice bath. A 500-μl aliquot was diluted to 2.5 ml with cold water. A 1.0-ml sample of this diluted solution was mixed with 1.0 ml cold 6 N NaOH to make the solution alkaline for uronic acid de-

methylation. For the color reaction, 125 μl of the alkaline solution was mixed with 125 μl 2% NaCl, to minimize color development with neutral sugars (2). Two milliliters of cold, concentrated H_2SO_4 was added, mixed, and placed in ice water. Samples were then heated at 70°C for 10 min and cooled in tap water. A 100-μl aliquot of 3,5-dimethylphenol (0.1% in glacial acetic acid) was added and mixed for color development. After 15 min at room temperature, the absorbance was read at 450 and 400 nm. The absorbance difference between these wavelengths was used to calculate the galacturonic acid content. D-Galacturonic acid (0–125 nmol in the reaction mixture) was used for the standard curve. The galacturonic acid content calculated in this way was multiplied by the factor 1.12, determined by Scott (2) to take into account a lower color yield from galacturonic acid polymers compared to galacturonic acid.

Pectin samples were dissolved in water (3.6 mg/ml). Cold, concentrated H_2SO_4 (3.5 ml) was added in two equal aliquots to 1.0 ml of pectin solution. The samples were chilled in ice water after each H_2SO_4 addition. Tubes were heated to 50°C for 10 min and cooled. Subsequent steps were the same as described for cell wall samples.

b) Methanol analysis. Dried cell walls (17.5 mg) were weighed into a 3-ml Pierce Reactivial. The cell walls were wetted with 2.5 ml 5 mM citric acid, pH 5.0, 0.1 M NaCl buffer. The vial was closed and then 200 μl 1.0 N NaOH was injected into the vial with a Hamilton syringe. The sample was sonicated for 3 min and refrigerated overnight. On the next day, 300 μl of 82.5 mM citric acid solution was added to lower the pH to approximately 7 for GLC analysis. Finally, 330 μl of 25 mM isopropanol was injected into the vial as an internal standard for gas chromatography. The sample vial contained very densely suspended cell walls. However, a sample could easily be taken with a 10-μl syringe without plugging the syringe with cell walls, because the wall particles are larger than the i.d. of the syringe needle. Samples (2 μl) were injected into the chromatograph and run at a constant temperature of 80°C. Methanol concentrations were calculated relative to the isopropanol internal standard.

Pectin samples were dissolved in water to give a 5 mg/ml pectin solution, then 1.0 ml was transferred to a reaction vial. Citric acid, NaCl buffer (pH 5.0) was added to the vial such that the total volume was 2.5 ml, and the final concentration of citric acid was 5 mM and NaCl 0.1 M. The analysis then was done as described above.

Data Analysis

The degree of methylation is the ratio of the moles of methanol in a sample divided by the moles of galacturonic acid multiplied by 100 (5). Since both terms of this ratio have significant experimental variation, the coefficient of variation for the degree of methylation was calculated by the so-called δ method (9)

$$\text{Var}(dm) = \text{Var}(M)\cdot\left(\frac{1}{U}\right)^2 + \text{Var}(U) \times \left(\frac{M}{U^2}\right)^2 - 2\cdot\text{Cov}(M, U)\left(\frac{1}{U}\right)\left(\frac{M}{U^2}\right),$$

where Var (dm) = variance of the degree of methylation, M = moles of methanol, U = moles of uronic acid, Var (M) = variance of methanol analysis, Var (U) = variance of uronic acid analysis, and Cov (M, U) = covariance of methanol and uronic acid analyses. The covariance was assumed to be zero, so the third term was zero. The percentage coefficient of variation for the degree of methylation was then

$$CV_{(dm)} = \frac{\sqrt{\text{Var}(dm)}}{(dm)} \times 100$$

where dm = degree of methylation.

RESULTS AND DISCUSSION

Determination of the degree of esterification requires the analysis of both the total galacturonic acid content and the esterified carboxyl

groups in a sample. A detailed procedure to do each of these analyses on insoluble cell wall samples or pectin solutions was described above.

Cell wall samples should be dried to have a low bulk density so the particles were accessible to the reagents in both analytical procedures. The procedures of Scott (2) were followed closely for uronic acid measurements. However, since he described certain analytical options, depending upon particular characteristics of the samples, the exact procedures required for nonlignified cell wall samples and pectin solutions were described. Care had to be taken in the addition of sulfuric acid to prevent charring of samples. Sonication of samples after sulfuric acid addition reduced the variability in the uronic acid analysis of duplicate samples, probably by allowing a more uniform exposure of the cell wall particles to the acid.

The approach to methylation analysis of pectin in cell walls was similar to that which has been used for pectin (5), in that the sample was treated with dilute base to hydrolyze the methyl esters. However, with a complex matrix like cell walls, analysis of methanol released rather than the increase in free carboxyl groups was used to measure methylation. A low concentration of citric acid (5 mM) was added to the solution used to suspend the cell wall samples because it acted as a buffer to keep the pH between 6 and 7 when the sample was acidified prior to gas chromatography. The NaOH concentration added was sufficient to raise the pH above 12 during hydrolysis of the ester groups.

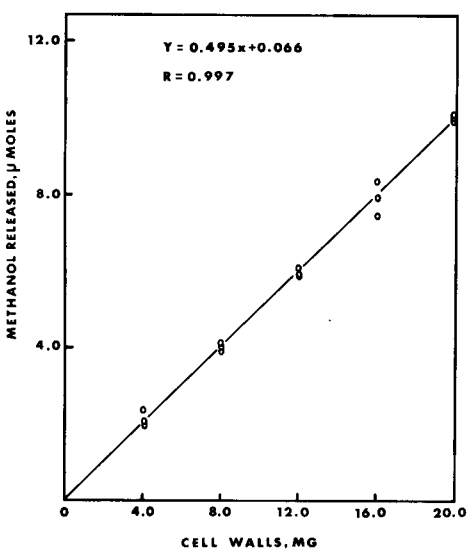

FIG. 1. Relationship between the amount of fresh cucumber cell walls subjected to base hydrolysis by the methanol analysis procedure and the methanol analyzed.

TABLE 1

COMPARISON OF ESTERIFIED CARBOXYL GROUPS IN COMMERCIAL PECTIN SAMPLES BY TITRATION OF CARBOXYL GROUPS OR GAS–LIQUID CHROMATOGRAPHY OF METHANOL AFTER BASE HYDROLYSIS

	Esterified carboxyl groups (mmol/g)	
Sample[a]	Titration[b]	GLC
Slow-set pectin	2.30 ± 0.04	2.16 ± 0.02
Rapid-set pectin	2.59 ± 0.01	2.36 ± 0.06
Polypectate	0.13 ± 0.02	0.22 ± 0.01

[a] Triplicate samples were analyzed.
[b] Schultz (5).

Precise analysis of methanol in cell wall samples required complete exposure of the cell wall particles to hydroxide ion. Several steps were taken to assure complete hydrolysis. First, the cell walls were washed with acetone before drying to get small particles which did not clump or case harden. Second, 0.1 M NaCl was added to the suspension solution. This was found to increase methanol release by 14%, compared to the use of water. Third, samples were sonicated to assure uniform initial wetting. Finally, the hydrolysis was done at 4°C for 24 h rather than 30°C for 30 min for pectin (5) to allow an extended period for wetting all particles in the suspension. Treatment of cell wall samples for 3 h with polygalacturonase (0.3 unit) or pectinesterase (1 unit), or a combination of both enzymes for 3 h, followed by NaOH hydrolysis, did not increase the release of methanol. This indi-

TABLE 2

ANALYSIS OF METHANOL, GALACTURONIC ACID, AND DEGREE OF PECTIN ESTERIFICATION FROM COMMERCIAL PECTIN SAMPLES AND CELL WALLS ISOLATED FROM FRESH CUCUMBERS, FERMENTED CUCUMBERS, AND SUGAR BEETS

Sample[a]	Methanol/g (mmol)	CV[b] methanol/g (%)	Galacturonic acid/g (mmol)	CV[b] galacturonic acid/g (%)	Degree of esterification (%)	CV[b] degree of esterification (%)
Pectin A	2.40	4.87	4.63	7.23	51.9	8.73
Pectin B	2.26	3.94	4.65	7.28	48.5	8.29
Pectin C	2.43	0.41	5.55	3.98	43.7	4.00
Polypectate	0.248	11.7	4.57	5.17	5.42	12.7
Cucumber, fresh	0.608	2.62	1.126	6.14	54.0	6.69
Cucumber, fermented	0.234	6.07	1.435	1.66	16.3	6.26
Sugar beet	0.637	0.46	1.220	8.75	52.2	8.77

[a] Quadruplicate samples were analyzed.
[b] Coefficient of variation.

cated that the procedure adopted gave complete release of methanol.

After the hydrolysis period, samples were neutralized by the addition of citric acid so the sample injected into the GLC had a pH between 6 and 7. Isopropanol was added after neutralization as an internal standard for gas chromatography. Comparison of GLC analysis done the same day, as sample neutralization compared to the same samples rechromatographed 24 h later showed no difference in methanol content. Pectin, as well as cell

TABLE 3

EVALUATION OF ERRORS IN THE ISOLATION OF CELL WALLS, ANALYSIS OF METHANOL AND GALACTURONIC ACID CONTENT, AND CALCULATION OF THE DEGREE OF ESTERIFICATION FOR 10- AND 20-g SAMPLES OF BLENDED CUCUMBER TISSUE

Sample[a]	Cell wall dry weight (mg)	CV[b] cell wall dry weight (%)	Methanol/g cell wall (mmol)	CV[b] methanol content (%)	Galacturonic acid/g cell wall (mmol)	CV[b] galacturonic acid content (%)	Degree of esterification (%)	CV[b] degree of esterification (%)
Fresh cucumber								
10 g	180	7.5	0.452	5.4	0.748	10.3	60.5	11.6
20 g	349	3.8	0.429	4.4	0.772	6.1	55.6	7.5
Fresh mesocarp								
10 g	131	8.2	0.548	3.1	0.871	11.1	63.0	11.5
20 g	255	6.1	0.541	2.3	0.870	10.6	62.2	10.8
Salt-stock cucumber								
10 g	140	3.9	0.155	2.1	1.132	13.0	13.7	13.2
20 g	244	3.1	0.155	1.3	0.919	7.8	16.9	7.9
Salt-stock mesocarp								
10 g	98	7.1	0.155	3.5	0.991	6.9	15.6	7.7
20 g	204	7.2	0.147	3.0	0.986	6.5	14.9	7.2

[a] Quadruplicate samples of cell walls were prepared and analyzed.
[b] Coefficient of variation.

wall samples, could be used in the analysis. The only change required was that pectin samples were dissolved before addition to the reaction vials.

Figure 1 shows a linear relationship between methanol released and the amount of dried cell wall added to the reaction vial. Table 1 shows a comparison of the analysis of esterified carboxyl groups in a sodium polypectate and two pectin samples by titration (5) and by gas chromatography. Both techniques gave similar results with high precision. Though the results were statistically different, it was not known which was more accurate. The gas chromatographic method, however, had three advantages. It could be used for either soluble pectin samples or cell wall samples. It required only 5 mg of pectin per analysis, compared to 500 mg for the titration procedure. Finally, it gave a specific analysis for methanol, while titration does not distinguish between methyl esters of carboxyl groups and acetylation of galacturonic acid hydroxyl groups. In fact, the column used for methanol analysis could also be used to specifically detect acetyl groups released from pectin by modifying the conditions of chromatography.

Table 2 shows the results of methanol analysis, galacturonic acid analysis, and the calculated degree for esterification for quadruplicate samples of several pectin and cell wall preparations. Generally, the error in the galacturonic acid analysis was larger than for analysis of methanol. The error in estimation of the degree of methylation was slightly greater on a percentage basis than the largest error of the methanol or galacturonic acid analysis.

Since it was possible to do duplicate analyses of both galacturonic acid and methanol on a total of only 70 mg of cell walls, the errors involved in the preparation of cell walls from 10- or 20-g samples of cucumber tissue and subsequent estimation of pectin methylation were also analyzed. Results of the analysis of quadruplicate samples of cucumber tissue are given in Table 3. As would be expected, a slightly smaller error was observed with a 20-g, as compared to a 10-g sample. However, the error with either the 10- or 20-g sample was similar to that observed when analyses were made from a single large batch of isolated cell walls (Table 2). The errors were small enough to make this a useful procedure for the measurement of esterification changes in plant cell walls.

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