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Pectic Methyl and Nonmethyl Esters in Potato Cell Walls

IAIN M. MACKINNON,* W. GORDON JARDINE, NIAMH O'KENNEDY,†
CATHERINE M. G. C. RENARD,‡ AND MICHAEL C. JARVIS

Chemistry Department, Glasgow University, Glasgow G12 8QQ, Scotland, U.K.

Because pectins are released from potatoes and other plants under conditions that cleave ester linkages, it has been suggested that there are other galaturonoyl ester cross-links between pectin chains in addition to the known non-cross-linking methyl esters. A microscale titration method and a copper binding method were developed for the measurement of total polymer carboxyl (essentially pectic) ester content in potato cell walls. Relative to the uronic acid content of the cell walls, the degree of total esterification was 57−58%. Comparison with levels of methanol released on ester hydrolysis allowed nonmethyl uronoyl esters to be estimated to be 14−15% relative to total uronic acid. The possibility of nonmethyl-esterified linkages being formed in potato cell walls by a side-reaction catalyzed by pectin methyl esterase (PME) was investigated, but no increase in nonmethyl-esterified pectin was observed under conditions where pectin was being effectively de-esterified by endogenous PME activity.

KEYWORDS: ***Solanum tuberosum*; cell walls; pectin; methyl esters; nonmethyl esters; pectin methylesterase; texture**

INTRODUCTION

Pectic polysaccharides are characterized by a core chain in which galacturonic acid is the dominant monomer. A proportion of the galacturonosyl residues are esterified with methanol at the C-6 carboxylic acid group (*1*, *2*). Acetic (ethanoic) acid also forms esters with the O-2 and O-3 hydroxyl groups of pectic galacturonosyl residues (*2*), but these are ethanoyl, not galaturonoyl, esters. It has been suggested that other alcohols can form galacturonoyl esters in place of methanol (*3*). Kim and Carpita (*4*) introduced a gas chromatographic method for estimating the total ester content of pectin in cell walls, based on the reduction of esterified galacturonosyl residues to galactose (*5*). By determination of esterified methanol separately after saponification, the nonmethyl galacturonoyl esters can be estimated by difference. In this way, Kim and Carpita (*4*) in maize and McCann et al (*6*) in tobacco cell cultures have shown that the nonmethyl galacturonoyl esters in the pectic fraction are in significant amounts in both monocots and dicots.

Brown and Fry (*7*) isolated what appeared to be nonmethyl esters of galacturonic acid after enzymic hydrolysis of pectins but did not identify their alcohol components. The formation of alkali-labile amides between the ϵ-amino group of L-lysine and the carboxyl group of D-galacturonic acid was shown to be possible (*8*), so in principle amide as well as ester substituents might be present. Perrone et al. (*8*) presented evidence for the existence of such amide linkages between pectin and protein in dicot cell walls but at an abundance an order of magnitude lower than the nonmethyl esters quantified in maize by Kim and Carpita (*4*). It does not seem likely, therefore, that the majority of what have been described as nonmethyl esters are in fact lysine ϵ-N-amides. In this paper, we describe the carboxyl substituents as esters, consistent with their degree of alkali lability, but we do not exclude the possibility of amides.

In potatoes and other dicot species, alkaline hydrolysis sufficient to cleave methyl esters also solubilizes pectins (*9*−*15*). This would be consistent with the existence of intermolecular ester links, either between pectin and other polymers or between one pectic molecule and another (*3*). For example, the alcohol moiety might be O-2 or O-3 of a galacturonic acid residue in another pectin chain. Because no ester-cross-linked pair of galacturonic acid residues has yet been isolated from walls and characterized, the existence of such dimeric units remains conjectural. Hou and Chang (*16*) suggested that such intermolecular esters might be synthesized by a trans-esterification side-reaction of de-esterification catalyzed by certain isoforms of pectin methyl esterase (PME). However, the experimental support for this suggestion was based on a nonspecific method of total ester determination in which interference from acetyl esters is possible, and the enzyme preparations were not purified to homogeneity. We are not aware of other direct evidence of trans-esterification caused by PME activity. Recently, work by Chang and co-workers (*17*) has shown that the molecular size of commercial pectins increase in size after PME activity. However, circumstantial evidence against such a reaction occurring in potatoes is that no increase

* To whom correspondence should be addressed. Tel: 44 141 330 6571. Fax: 44 141 330 4888. E-mail: iainma@chem.gla.ac.uk.
† Present Address: Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, Scotland, U.K.
‡ Present Address: Station de Recherches Cidricoles−Biotransformation des Fruits et Légumes, INRA, B.P. 35327, 35653 Le Rheu Cédex, France.

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in alkali-extractable pectin occurs when potatoes are heated to activate endogenous PME (*13*).

The extent of esterification in potato tubers influences the texture of cooked potatoes (*14*, *15*, *18*). Methyl-esterified galacturonic acid residues do not contribute positively to the cohesion of the cell wall and middle lamella in the presence of divalent cations but can bring about a loss of cohesion because they are vulnerable to depolymerization by β-elimination (*18*). Blanching potatoes at temperatures between 50 and 70 °C, before cooking at 100 °C, results in the activation of PME and an alteration in the textural properties of the cooked potato (*19*). PME catalyzes the removal of methyl ester groups to expose C-6 carboxyls of pectic galacturonyl residues. In the conventional view, newly formed carboxylic acid groups can then potentially be cross-linked by divalent cations, causing an increase in the extent of cell adhesion on subsequent cooking (*14*, *19*, *20*). If PME also catalyzed a trans-esterification reaction (*15*) in potatoes, the creation of new covalent linkages within the cell wall could potentially make a large, and hitherto unrecognized, contribution to the increased cell adhesion observed in blanched and then cooked potatoes.

Here, we describe the measurement of total and hence nonmethyl esters by two methods that are simpler than that of Kim and Carpita (*4*), although less specific. Both exclude the possibility of acetyl interference. These procedures were applied to cell walls from potato tubers in which the solubilization of pectin under alkaline de-esterifying conditions is well-established (*10*, *12*). The potential for PME catalyzed trans-esterification reactions occurring was investigated by measuring any increase in nonmethyl ester linkages after blanching potatoes at two different temperatures within the PME activation range.

MATERIALS AND METHODS

Cell Wall Preparation. Potato cell walls were isolated by a procedure involving detergent extraction and buffered phenol inactivation of residual enzymes, cryo-milling, and enzymic removal of starch (*21*). Throughout the isolation, a mixed cation buffer (10 mM NaOAc, 3 mM KCl, 2 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 6.5) was used to mimic the ionic conditions found in the plant cell (*22*). Control of the cation environment of the cell walls throughout their isolation allowed losses of pectin from noncooked potatoes into solution to be kept below 0.5 mg of galacturonic acid per gram dried material (*21*). Residual starch content was quantified by enzymic analysis (*23*, *24*), allowing the residual cell wall weights to be corrected for starch and moisture so that results could be expressed on a net cell wall basis.

Commercial Pectin Samples. Rapid set citrus pectin was a gift from SKW (Beaupte, France); citrus pectin (P9561) with a degree of methylation of approximately 90% was obtained from Sigma (Poole, U.K.).

Activation of Endogenous PME (Blanching). Five potato tubers were hand-peeled and sliced to a uniform thickness of 5 mm ($\pm$0.5 mm). Slices weighing 100 g were heated for either 40 or 15 min in a beaker containing 300 mL of deionized water, which had been preheated to 55 °C (blanch 55 °C) or 70 °C (blanch 70 °C), respectively, in a water bath. After the potatoes were blanched, the heated potatoes were then cooled in 3 L of dH_2O for 20 min. Cell walls were then isolated, as described above.

Determination of Nonesterified Carboxyl Acid Content. *(a) Titration with Sodium Hydroxide.* The nonesterified carboxyl content was determined by equilibrating cell walls in acid so as to protonate all carboxylate sites, followed by a titration with 10 mM NaOH to pH 7.0. Cell walls were acidified with 10 mM HCl (in 70% ethanol at 4 °C overnight). The acid was removed by filtration, and the sample was washed with 70% ethanol. To avoid interference with the subsequent titration, residual HCl remaining in the washings was detected by precipitation of the Cl^- ions with aqueous $AgNO_3$. Washing was continued until no precipitate was observed. Following drying in acetone, 30 mg of cell walls were transferred together with 50 mL of degassed 0.1 M NaCl supporting salt into a round-bottom three-necked flask. The flask was fitted with a pH electrode, a septum through which alkali could be introduced, and a nitrogen gas inlet. The cell wall material was titrated, via a syringe, to pH 7.0 with freshly prepared 10 mM NaOH. All solutions used during the titration were degassed, and the titration was carried out under nitrogen to prevent interference from atmospheric CO_2.

(b) Copper Binding. Nonesterified pectic carboxyl groups were also determined by a modification of the pectin determination method of Keijbets and Pilnik (*25*). Copper(II) ions bind to nonesterified galacturonic acid within the cell walls and are then removed by the addition of excess acid. The copper released by the acid gives a value for the nonesterified carboxyl content. Cell walls (75 mg) were equilibrated with Cu^{2+} ions by stirring twice in 30 mL of aqueous 2% copper sulfate for 30 min. Unbound copper was washed out with deionized water. Complete removal of copper was ensured by measuring the conductivity of the filtrate (when the conductivity was stabilized at <10 μS, the washing was considered complete). Ionically bound copper was subsequently removed at room temperature with 3×10 mL of 1 M nitric acid in 60% ethanol, and each 10 mL aliquot was in contact with the cell walls for approximately 5 min. The filtrate was made to 50 mL and analyzed for copper using flame atomic absorption spectroscopy (AAS) (Perkin and Elmer, 1100B) after a 1 in 10 dilution. Standards were made up in 4% ethanol so that the sample and standard matrixes were the same. Using AAS instead of the colorimetric method of Keijbets and Pilnik (*21*) allowed a significant improvement in sensitivity and precision.

Determination of Total Uronic Acid Content *(a) 3-Phenylphenol Assay*. The total uronic acid content of cell walls was determined by a modification of the 3-phenylphenol method of Filisetti-Cozzi and Carpita (*21*, *24*), using the heat of dilution of the sulfuric acid to drive the reaction (*27*). This method responds to both glucuronic and galacturonic acid. However, glucuronic acid exists only in trace amounts in potato cell walls (*10*, *12*), so therefore the glucuronic acid contribution to the measured total was neglected.

(b) Titration. Total pectic galacturonic acid was measured by first saponifying the cell walls in 0.1 M NaOH/70% ethanol at 4 °C overnight. The saponified cell walls were then acidified, washed, and titrated to neutrality in the same way as for determining nonesterified galacturonic acid. As well as retaining pectins in an insoluble form, washing with 70% ethanol prior to titration also removed interfering acetate ions cleaved by saponification; therefore, only polymer carboxylate groups contributed to changes in pH during the titration.

Determination of Methyl-Esterified Galacturonic Acid. Methanol released from cell walls or commercial pectins on saponification was determined by the Klavons and Bennett method (*28*).

Titration of Commercial Pectins. Approximately 2 g of pectin was acidified by leaving it overnight at 4 °C in 50 mL of 1% HCl in 70% ethanol. The material was filtered and then washed with 70% ethanol until no further AgCl precipitation was observed when $AgNO_3$ was added to the filtrate. The pectins were then dried by solvent exchange, three times with 96% ethanol, and followed by a further three exchanges with acetone. A total of 0.6 g of the acidified pectin was dissolved in 100 mL of degassed dH_2O and sealed in an Erlenmeyer flask with three drops of phenolphthalein solution. To prevent interference from atmospheric CO_2 prior to the subsequent titration, the flask was flushed for 2 h with N_2 gas under constant stirring. The acidified pectin was then titrated with 10 mM NaOH, under N_2 gas and constant stirring, to a pink end point. The pectins were then saponified with 20 mL of 0.4 M NaOH at room temperature for 1 h and then neutralized with 20 mL of 0.4 M HCl. The newly generated acidic functions were then titrated with 1 mM NaOH as before.

RESULTS

The titration method measures the total content of anionic sites (cation exchange capacity) in the cell wall, not merely the galacturonoyl free carboxyl content. This is also true of the copper binding method. Copper(II) ions bind specifically to pectic galacturonans, but when these are saturated, any other

Table 1. Determination of Total and Esterified Galacturonate in Commercial Citrus Pectins by Titrimetric and Colorimetric Methods[a]

	galacturonate content (mmol/g)					percent esterification	
	total galacturonate		nonesterified	total ester	methyl ester	total esters	methyl
	titrimetric A	colorimetric B	titrimetric C	titrimetric A–C	colorimetric D	100(A–C)/A	esters 100D/A
citrus pectin (SKW)	4.42 (0.01)	4.27 (0.05)	1.26 (0.01)	3.16 (0.02)	3.22 (0.06)	71.5 (0.1)	72.9 (1.3)
high Me pectin (Sigma)	4.18 (0.02)	4.13 (0.09)	0.41 (0.00)	3.77 (0.02)	3.75 (0.06)	90.2 (0.0)	89.7 (1.0)

[a] Total galacturonate was determined by titration and independently by the 3-pp colorimetric method (see Materials and Methods). Nonesterified galacturonate was determined titrimetrically giving the total ester content by difference from the titrimetric total galacturonate content. Methyl esters were determined by colorimetric assay of methanol after saponification. All analyses were in duplicate, and the range is shown in parentheses.

Table 2. Total, Nonesterified, Total Esterified, and Methyl-Esterified Galacturonic Acid of Raw Potatoes, cv.s Marfona and Fianna[a]

	galacturonate content (μmol/g)							percent esterification		
	total galacturonate		nonesterified galacturonate			total ester	methyl ester	total esters	methyl esters	nonmethyl esters
	titrimetric A	colorimetric B	titrimetric C	copper D	mean (C + D)/2	mean E = A – (C + D)/2	colorimetric F	100E/A	100F/A	100(E – F)/A
cv Marfona	1062 (3)	1039 (44)	433 (20)	448 (22)	441	621	447 (10)	58.5	42.1 (0.9)	16
cv Fianna	1068 (13)	1045 (66)	451 (38)	457 (30)	454	614	456 (3)	57.5	42.7 (0.6)	15

[a] The total galacturonate content was measured by 3-phenylphenol assay and by titration with NaOH of saponified cell walls. Nonesterified galacturonate was measured on nonsaponified cell walls by titration and copper binding methods. The content of total esterified galacturonate was determined by subtracting the mean nonesterified galacturonic acid from the mean total galacturonate. Values in parentheses are the standard deviation of the mean. A standard deviation is not calculated for the mean nonesterified galacturonate determined by both titrimetric and copper methods, (C+D)/2, nor for calculated values dependent on this value, because it is not expected a priori to show a normal distribution around the mean.

anionic sites present will also bind copper. These include proteins and bound phenolic substances, but these are both at very low levels in potato cell walls (*12*). Binding of copper(II) ions to pectic galacturonans can exceed the stoichiometric ratio at neutral pH (*29*). Between pH 5.5 and pH 6.0, this problem is minimal, but pectic carboxlate groups are still essentially fully ionized.

Table 1 shows that for commercial pectins, where no evidence of nonmethyl esters has been noted (*2*), there was close agreement between values of total uronic acid and ester content determined by colorimetry and by titration. The slightly lower value determined colorimetrically could be due to either nonuronide anionic sites at trace levels or slight underestimation (*30*) of the polymeric galacturonide content by the colorimetric determination when calibrated with galacturonic acid, the standard method. For this reason, only the titrimetric value for total uronide was used in the calculation of the total ester content. There was close agreement between the total ester content determined titrimetrically and the methyl ester content determined colorimetrically.

Table 2 shows that the total uronic acid content of potato cell walls, measured colorimetrically, was likewise in agreement with their total anionic content measured by titration after saponification. This allowed us to regard the contribution of nonpectic anionic sites within the cell wall as negligible and to consider the titration values as a measure of nonesterified carboxyls and total pectic galacturonan. Close agreement was also found between the nonesterified galacturonic acid contents measured by titration and by copper binding (Table 2), provided that the pH was more tightly controlled during the Cu(II) binding and desorption steps than in the original Keijbets and Pilnik (*25*) procedure (data not shown). Interference from acetyl and conceivably other low molecular weight carboxylic substituents released on saponification from cell walls was deliberately excluded by the washing steps in 70% ethanol to leave only insoluble polymers. Such interferences were unlikely to occur in the commercial pectin samples because acetate esters are not detectable in commercial citrus pectins (*2*, *31*). All three approaches used here can therefore be considered as measuring pectic galacturonic acid content to an adequate approximation.

To test the existence of putative nonmethyl ester linkages within potato cell walls, the total acid, nonesterified, and methyl-esterified galacturonic acid contents were measured in two potato cultivars, cv's Marfona and Fianna (Table 2). Total esterification of the galacturonic acid was calculated by subtracting the nonesterified galacturonic acid content from the total galacturonic acid content (eq 1). The nonmethyl ester content can then be calculated (eq 2).

$$\text{total esterified Gal A} = \text{total Gal A} - \text{nonesterified Gal A} \quad (1)$$

$$\text{nonmethyl esterified Gal A} = \text{total esterified Gal A} - \text{methyl esterified Gal A} \quad (2)$$

The percentage total and methyl ester figures were expressed as a percentage of the total galacturonan value (Table 2). The mean total esterification for cv. Marfona and cv. Fianna was 58 and 57%, respectively, as compared to the methyl ester value of 43% for both varieties. Thus, there was 14−15% galacturonyl esterification that was unaccounted for by methyl esters in raw potato cell walls. The same methods, applied to extracted pectins (of citrus origin), showed that in this case all esters could be accounted for as methyl esters.

To establish if any new nonmethyl ester linkages were created by PME activation, cell walls were isolated from potato tissues when uncooked and after blanching at 55 or 70 °C. As in the previous experiment, the total, nonesterified, and methyl-esterified galacturonic acid contents were measured (Table 3). Because there was good agreement between the 3-phenylphenol and titration methods and between the titration and copper binding methods in the previous experiment, total and nonesterified galacturonic acid were measured by the 3-phenylphenol and copper binding methods only.

The esterified galacturonic acid results were expressed as a percentage of the total galacturonic acid content (Table 3). After the potatoes were blanched at 55 °C, the levels of methyl-

Table 3. Methyl and Nonmethyl Esterification in the Pectic Fraction of Cell Walls from Blanched Potato Tissue, cv. Maris Piper[a]

	galacturonate, μmol/g of dry cell walls				percent esterification		
cooking treatment	total galacturonate colorimetric A	nonesterified copper B	total ester (A – B)	methyl ester colorimetric C	total esters 100(A – B)/A	methyl esters 100C/A	nonmethyl esters 100(A – B – C)/A
raw	1131 (58)	481 (13)	650 (59)	453 (7)	57 (6)	40 (2)	17 (6)
blanched 55 °C	1065 (44)	635 (18)	430 (48)	245 (9)	40 (5)	23 (1)	17 (5)
blanched 70 °C	868 (49)	341 (18)	350 (52)	229 (7)	40 (6)	26 (2)	14 (7)

[a] Total and nonesterified galacturonic acid were measured by the 3-phenylphenol and copper binding methods, respectively. Values in parentheses are the standard deviation of the mean.

esterified galacturonic acid in the cell walls fell due to thermal activation of PME. The degree of methyl-esterification also decreased after blanching at 70 °C, but to a lesser extent than after blanching at 55 °C. The extent of total esterification also fell, but the difference between total and methyl esters, i.e., the nonmethyl ester content, remained constant within the limits of error in this experiment. The precision was lower than that of the data reported in Tables 1 and 2 due to the use of the colorimetric, rather than the titration, method for the estimation of total uronic acid content.

DISCUSSION

The results presented here confirm by methods completely independent of that of Kim and Carpita (*4*) that nonmethyl esters do exist. While neither of the methods used here is highly specific, the relatively large quantity of nonmethyl esters detected makes it unlikely that artifacts such as other anionic components of the cell walls were contributing to the final value measured. Before our procedures are applied to cell walls from other plant tissues, however, the absence of such interferences should be verified. In particular, phenolic interference is possible in gramineaceous tissues such as maize (*4*).

Blanching to activate PME prior to cooking is a widely used method to improve the physical properties of cooked vegetables (*19*). Using the techniques developed, we have established that blanching potatoes and thus activating PME does not increase the proportion of C-6 galacturonyl nonmethyl esters caused by a trans-esterification reaction that occurs during PME activation in potatoes. This conclusion is in contrast to that of Hou and Chang (*16*) on bean sprouts, but—assuming that it was indeed galacturonoyl esters that they measured—they claimed that only some PME isoforms in bean had transesterase activity. Our data were obtained at two temperatures spanning the temperature range at which different PME isoforms were activated (*32*) and which is used in various industrial processes involving the blanching of potatoes (*33*). It seems likely, therefore, that the only effect of PME activation during the blanching of potatoes is the recognized process of removing pectic methyl ester groups producing, on further cooking, potatoes that exhibit greater cell adhesion than conventionally cooked potatoes.

Various ways can be envisioned in which alkali-labile covalent bonds could retain pectin in an insoluble form in the cell wall (*10*, *12*). There may be intermolecular ester linkages between a pectic molecule and an insoluble polymer, such as xyloglucan (*4*, *7*, *10*). Alternatively, two intermolecular cross-links per pectic molecule would be enough to form an infinite, insoluble network.

Caution is needed before the nonmethyl esters found here are equated with the intermolecular bonds responsible for the insolubility of pectins. Far more nonmethyl ester links were identified in these experiments than would be required to insolubilize the alkali-labile pectic fraction of the potato cell wall. Following a hot aqueous extraction procedure, which would depolymerize pectin by β-elimination, the presence of one such link per pectic fragment would be sufficient to retain these pectins in an insoluble form within the cell wall. It is possible that some of the nonmethyl esters measured are intramolecular rather than intermolecular. Intramolecular esterification across the monosaccharide ring to form a γ-lactone is not possible in pectins, but simple model-building experiments (data not shown) demonstrate that a lactone can be formed with the adjacent galacturonosyl residue in the direction of the nonreducing end of the chain.

These experiments show that methyl esters are not the only substituents on C-6 of potato pectins. A method for the identification of the alcohol(s) involved in nonmethyl esterification is urgently required.

Abbreviations Used. PME, pectin methylesterase; AAS, atomic absorption spectroscopy; cv, Cultivar; 3-pp, 3-phenolphenol.

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