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Determination of the pattern of methyl esterification in pectin. Distribution of contiguous nonesterified residues *

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ABSTRACT

A method is described for determining the distribution of contiguous nonesterified galacturonic acid residues within pectins. First, the esterified galacturonic acids are converted to galactose by reduction with sodium borohydride, then the glycosidic linkages of the resulting galactose residues are cleaved selectively by liquid HF solvolysis. Separation and quantitation of the resulting galacturonic acid containing oligomers reveals the proportion of each stretch of contiguous nonesterified galacturonic acid residues in the original pectin. The distribution of nonesterified GalA in a pectin fraction obtained from cotton suspension culture cell walls with ~ 50% esterification appears to be far from random.

INTRODUCTION

Pectins, which have pronounced gel-forming properties, are extensively utilized in the food industry as thickening agents in food products. Prominent in pectins are homogalacturonan regions which have been implicated in gel formation. The galacturonic acid (GalA) residues in the homogalacturonan regions are often esterified with methyl groups. Most commercial pectins have been extracted from fruit pulp by hot acid extraction, causing them to lose their highly branched regions. This extraction procedure may also cause a decreased degree of methyl esterification (dm). Commercial pectins of dm above ~ 50% form gels under conditions of acidic pH and low water activity such as found in jams and jellies. Pectins of low dm (< 50%) form gels in the presence of Ca^{2+} ions. No detailed structural model of the junction zones between high-dm pectin chains has been reported, although involvement of both hydrophobic interactions and hydrogen bonds in the formation of junction zones has been shown by Oakenfull and Scott[2].

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A well accepted model for the gelling of low-dm pectins has been proposed involving cooperative Ca^{2+} crossbridges between adjacent nonesterified segments of two pectin chains[3]. For these junction zones to be stable, it was proposed that a minimum of seven adjacent Ca^{2+} crossbridges are needed, which would involve 14 contiguous nonesterified GalA residues in a 2_1 helin[3]. For high- or low-dm pectins, we can be sure that the distribution of methyl esterification will have a great influence upon the ability of pectin to form gels.

The biological functions of pectin in plants are still open to discussion, but pectins do make up a large proportion ($\sim 30\%$) of the polysaccharides in cell walls of most herbaceous plants (with the exception of grasses)[4]. Ca^{2+}-induced crosslinks may be involved in cell adhesion[5], cell wall extensibility[6], and overall tissue texture[5]. Despite the overall high dm of pectins in the growing parts of plants, there must be regions within the pectins capable of forming the Ca^{2+}-induced crosslinks since Varner and Taylor[7] found evidence for Ca^{2+}-binding pectins in crosssections from stems of *Bidens*, and Vreeland et al.[8] detected differential Ca^{2+}-dependent adsorption of fluorescent labeled oligomers of nonesterified GalA to walls of different cell types in leaves of *Dubautia* and soybean nodules.

Another case in which esterification can affect the behavior of pectins is that of enzymic degradation. Plants and plant pathogens produce enzymes to degrade pectins. These enzymes are prevented from acting by (or in the case of pectin lyase require) the presence of methyl esters. In ripening fruits such as tomatoes, the induction of methyl esterases precedes the induction of endopolygalacturonase[5].

Several methods have been used to investigate the pattern of esterification in pectin. Early methods have been reviewed by Taylor[9]. Subsequent methods involve the use of enzymes to produce characteristic oligomers as a result of the specificity of the enzymes[10,11] or involve ^1H or ^{13}C NMR spectroscopy[12,13], which allows nearest-neighbor frequencies to be determined. The results of these studies do not all agree with each other, except that acid or alkaline deesterification of pectins is random. Most of the studies, with the exception of those involving high-field ^{13}C NMR spectroscopy[13], indicate a nonuniform distribution of methyl esters in native high-methoxyl pectins.

We are proposing a purely chemical method for producing oligosaccharides containing the contiguous nonesterified GalA residues. These can be separated and quantitated to determine the frequency of each length of contiguous stretches of nonesterified residues. The method should give a more quantitative picture than the previous methods.

RESULTS AND DISCUSSION

From previously published work we knew (*1*) that it was possible quantitatively to reduce esterified galacturonic acid residues in pectins to galactose without significant demethylesterification or β-elimination[14], and (*2*) that it was likely the resulting galactosyl linkages would be very much more labile to liquid HF solvolysis

than those of galacturonosyl linkages[15]. Thus, HF solvolysis of samples reduced in this way, under ideal conditions, should lead to the production of oligomers of galacturonic acid with galactose at their reducing end ($GalA_n$-Gal oligomers), which represent the contiguous stretches of GalA residues between methyl-esterified residues (see Scheme 1). Separation and quantitation of these oligomers would reveal the distribution of nonesterified GalA residues within a particular pectin sample. To implement this procedure we needed to devise and prove methods for the following: (*1*) chromatographic separation of oligomers of $GalA_n$-Gal, (*2*) complete cleavage of all galactosyl linkages with no or insignificant cleavage of GalA linkages, and (*3*) quantitation of the various $GalA_n$-Gal oligomers.

The oligomers of $GalA_n$-Gal with $n = 1$ to at least 10 could be well resolved using the Dionex PA1 anion-exchange column eluted with a gradient of increasing sodium acetate concentration at pH 5.2. The oligomers could be detected by pulsed amperometry (in combination with postcolumn addition of sodium hydroxide; see Fig. 1B) or by UV absorbance (Fig. 1A) if they were derivatized with 2-aminopyridine (2-AP) prior to the chromatography. The 2-AP labeled oligomers eluted at slightly higher ionic strength than the nonlabeled oligomers. From previous work with homogalacturonan oligomers, we expected to see a regular pattern of peaks of decreasing spacing between oligomers as the oligomer length increased[16,17].

To assign the identities of the compounds that corresponded to the peaks in the chromatograms, an effluent splitter was installed between the column exit and the point of sodium hydroxide addition, and ~80% of the column effluent was directed to a fraction collector. The time difference between appearance of a signal at the pulsed amperometric detector and the arrival of a sugar at the fraction collector was determined using glucose by colorimetric assay[18] of the fractions in the fraction collector. The materials corresponding to peaks 1, 2, and 3 in Fig. 1 were collected and subjected to ^1H NMR and/or liquid secondary ion-mass spectrometry (LSIMS).

The material in peak 1 upon LSIMS showed ions at m/z 379.1 as would be expected for [GalA-Gal + Na]$^+$. That from peak 2 gave ions at m/z 555.2 and 577.2 corresponding to the masses of [$GalA_2$-Gal + Na]$^+$ and [$GalA_2$-Gal + 2Na

$$\text{-(GalAME)}_{n_a}\text{-(GalA)}_{n_b}\text{-(GalAME)}_{n_c}\text{-(GalA)}_{n_d}\text{-(GalAME)}_{n_e}\text{-(GalA)}_{n_f}\text{-(GalAME)}_{n_g}\text{-}$$

$$\downarrow \text{NaBH}_4,\ \text{M Imidazole pH 7}$$

$$\text{-(Gal)}_{n_a}\text{-(GalA)}_{n_b}\text{-(Gal)}_{n_c}\text{-(GalA)}_{n_d}\text{-(Gal)}_{n_e}\text{-(GalA)}_{n_f}\text{-(Gal)}_{n_g}\text{-}$$

$$\downarrow \text{HF (1\% } H_2O\text{), } -15^\circ C$$

$$(n_a - 1)\text{Gal} + \text{GalA}_{n_b}\text{-Gal} + (n_c - 1)\text{Gal} + \text{GalA}_{n_d}\text{-Gal} + (n_e - 1)\text{Gal} + \text{GalA}_{n_f}\text{-Gal} + (n_g - 1)\text{Gal}$$

Scheme 1.

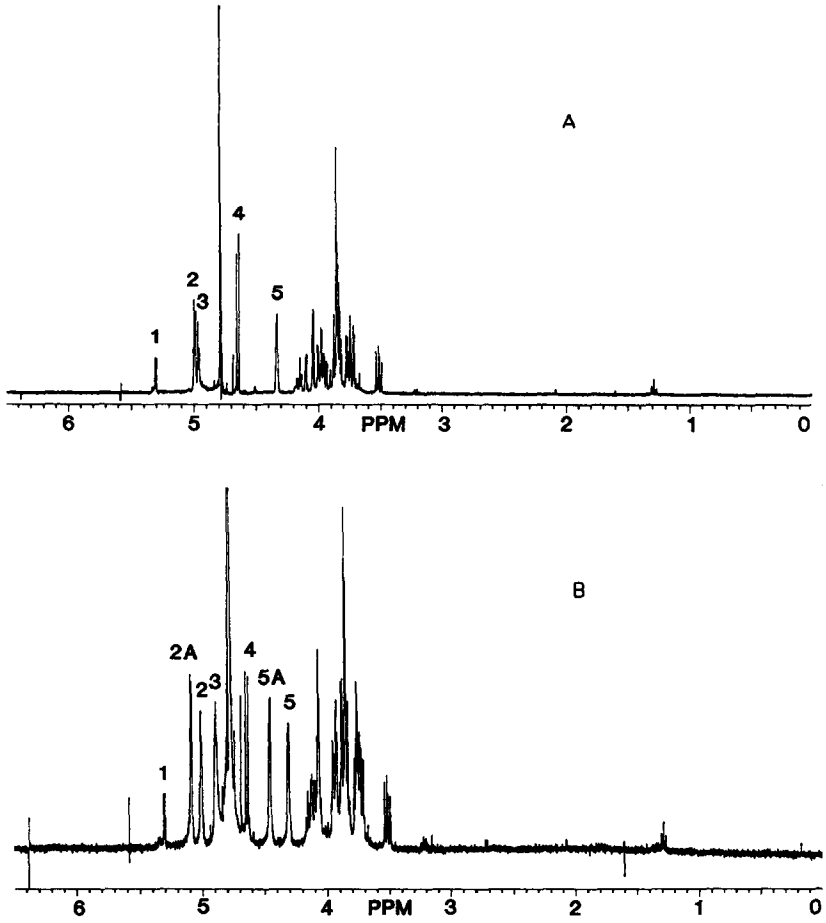
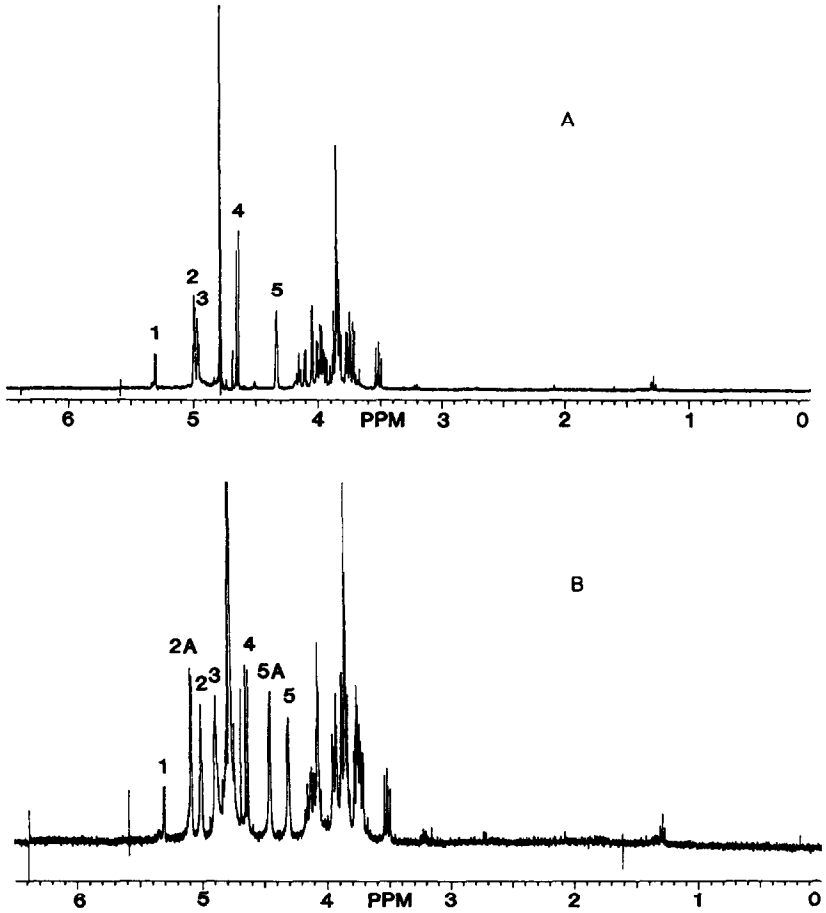


Fig. 2. A. ^{1}H NMR spectrum of the material in peak 1 of Fig. 1 identified as GalA-Gal. The numbered resonances are assigned as: 1, H-1 of the reducing α-Gal; 2, H-1 of the nonreducing α-GalA linked to either α- or β-Gal; 3, H-5 of the GalA; 4, H-1 of the β-Gal; and 5, H-4 of the GalA. B. The corresponding spectrum of the material in peak 2 identified as $GalA_2$-Gal; 1, H-1 of the reducing α-Gal; 2A, H-1 of the nonreducing terminal GalA; 2, H-1 of the internal GalA linked to either α- or β-Gal; 3, H-5 of GalA; 4, H-1 of the β-Gal; and 5A, H-4 of the internal GalA shifted downfield relative to resonance-5 because of its linkage at this position to the nonreducing end GalA; and 5, H-4 of the nonreducing end, GalA.

Oligomers of homogalacturonan can be generated from pectic acid by autoclave-induced autolysis or by partial endopolygalacturonase hydrolysis[20]. Standard di- and tri-GalA are commercially available from Sigma Chemical Co. Thus, it is easy to identify some of the homogalacturonan series rigorously and identify the higher oligomers from the regularity of the decrease in peak spacing as the oligomers get longer (Fig. 3, peaks 6, 10, 12, and 14). As one would expect for chromatography at pH 5.2 on an anion-exchange column, the $GalA_n$-Gal oligomers, having a lower charge-to-mass ratio, elute earlier than the $GalA_n$ oligomers.



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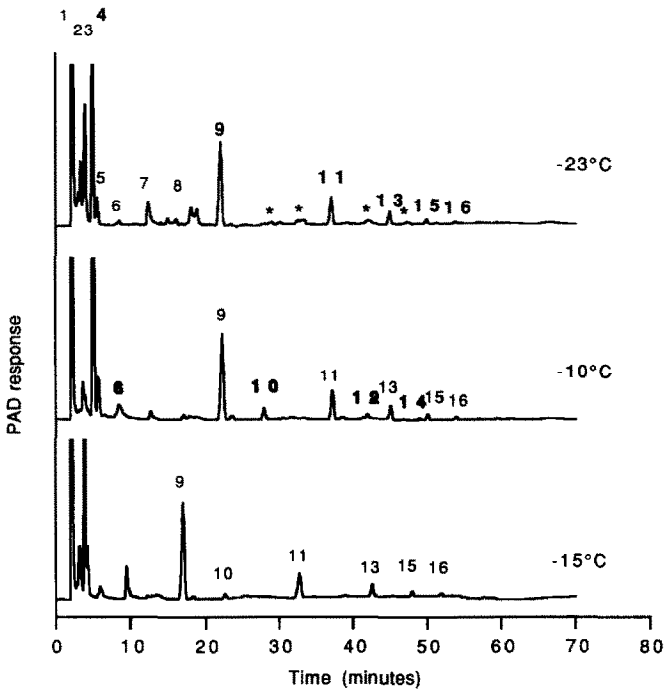

Fig. 3. Chromatogram of the oligomers derived from Sigma apple pectin using PAD showing the differences between runs conducted at −23, −10 and −15°C when using HF plus 1% H_2O to generate the oligomers. Peak 1, galactose plus other nonadsorbed material; 2, incomplete solvolysis products, probably GalA-Gal-GalA-Gal; 3, unidentified; 4, GalA-Gal; 5, GalA-Rha(1,2′:1′,2-dianhydride of β-rhamnose and α-galacturonic acid)[25]; 6, GalA; 7, unidentified; 8, a series of incomplete solvolysis products; 9, $GalA_2$-Gal; 10, $GalA_2$; 11, $GalA_3$-Gal; 12, $GalA_3$; 13, $GalA_4$-Gal; 14, $GalA_4$; 15, $GalA_5$-Gal; 16, $GalA_6$-Gal; and * additional unidentified solvolysis products. The chromatogram of the pectin treated at −15°C was run at a different time than those at the other temperatures and illustrates the sensitivity of retention times to the age of the column.

Being able to separate $GalA_n$-Gal oligomers from $GalA_n$ oligomers allowed us to optimize conditions in liquid HF to minimize the cleavage of GalA linkages. Fig. 3 shows chromatograms of reduced apple pectin from Sigma treated with liquid HF (1% water) at −23, −10, and −15°C. Although we predicted that all α-galactosyl linkages would be cleaved in liquid HF at −23°C (ref 15), we found that the reaction was not quantitative, as indicated by the appearance of several peaks in the chromatograms which corresponded to neither $GalA_n$-Gal nor $GalA_n$ oligomers. Peak 2 and the cluster designated 8 represent oligomers such as GalA-Gal-GalA-Gal. These are absent at the two warmer temperatures. At "excessively" warm temperatures, e.g., −10°C, that ensured complete Gal linkage cleavage, $GalA_n$ oligomers were produced, indicating some cleavage (albeit a small amount) of GalA linkages. An acceptably low level of GalA cleavage with essentially complete Gal cleavage was obtained using a reaction temperature of −15°C

for a duration of 30 min. Interestingly, treating cotton cell walls with anhydrous HF at 0°C gives rise to quite large (up to at least 30 residues in length) $GalA_n$ oligomers (Komalavilas and Mort, unpublished work).

For quantitative estimation of the proportion of blocks of various lengths of nonesterified GalA, we must be able to quantitate the $GalA_n$-Gal oligomers. The most convenient fractionation and quantitation would be chromatography on the Dionex PA1 column, followed by quantitation using pulsed amperometry. However, since it is impractical to generate pure standards for each oligomer expected, and there is evidence in the literature that the PAD does not give an equimolar response for oligomers of different length in the $GalA_n$ homologous series[16], we used end labeling with 2-AP for initial quantitation. We have previously shown for 2-AP labeled oligomers in the $GalA_n$ series that integrated areas of UV absorbance do give an equimolar response for oligomers in the range of from two to at least 20 residues in length[20]. We presumed that the oligomers ending in galactose would also give an equimolar response. To check that this presumption was correct, we compared the relative peak areas of the 2-AP labeled $GalA_n$-Gal oligomers produced from several pectins of known degree of esterification with those predicted to be obtained if the methyl esterification in the pectins were random. Several reports in the literature have suggested that fruit pectins are randomly esterified[12,13].

If the methyl esters within a pectin are completely randomly distributed, it is possible to predict how frequently each cluster size of contiguous nonesterified galacturonic acid residues would occur for any particular degree of esterification. The probability of a nonesterified residue occurring at any particular position is $[1 - (dm/100)]$ which we designate as p. The probability of two occurring adjacent to each other is p^2, for three together it is p^3, etc. The probability of an esterified residue at any particular position is $1 - p$. The products of the HF reaction after reduction of methyl-esterified GalA to Gal would begin at the nonreducing end with a GalA residue and end in a Gal residue (see Scheme 1). Obviously the sugar previous to the start of *each* of the oligosaccharides would have originally been galactose. The probability of obtaining, for instance, $GalA_2$-Gal would be $p^2(1-p)$. In the general case $GalA_n$-Gal would occur with a probability of $p^n(1-p)$. If we normalize to $GalA_1$-Gal we obtain the following expression:

$$\frac{\text{probability of } GalA_n\text{-Gal}}{\text{probability of } GalA_1\text{-Gal}} = \frac{p^n(1-p)}{p^1(1-p)} = p^{(n-1)}.$$

Since the probabilities will be reflected in the proportions of oligomers produced, one can predict the relative molar ratio of each oligomer to $GalA_1$-Gal or to any other oligomer in the series. In the case of the 2-AP labeled oligomers, normalization to $GalA_2$-Gal is necessary, because during the purification of the labeled oligomers there is not a quantitative recovery of the $GalA_1$-Gal oligomer. The loss of this oligomer is incurred because it does not carry a net negative charge after the 2-AP labelling and thus adsorbs to some extent to the extract clean column

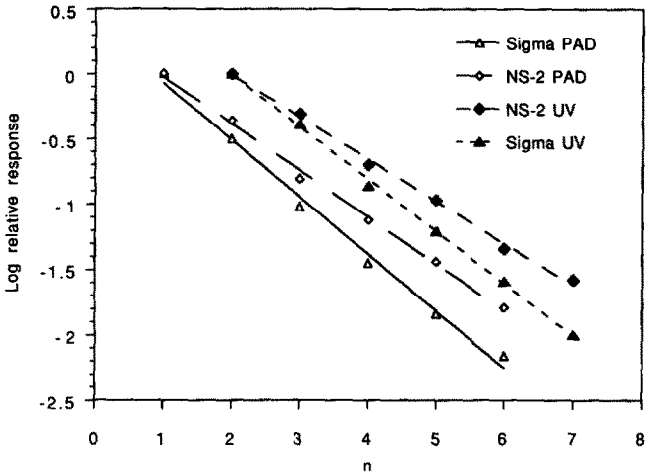

Fig. 4. Plots of the logarithm of the normalized integrated areas from oligomers produced from Sigma apple pectin (dm 61.54%) and NS-2 apple pectin (dm 50.87%) determined by either pulsed amperometry or UV absorption. Areas from the PAD were normalized to that of GalA-Gal. Those from the UV chromatogram were normalized to $GalA_2$-Gal. The lines drawn through the points are an equally weighted linear fit to the data.

that is used to remove the excess 2-AP before chromatography of the samples[21]. For 2-AP labeled oligomers, if the molar response of the UV detector is equal for all oligomers, a plot of $\log(\text{area}_n/\text{area}_2)$ vs. n should be linear with a slope of $\log p$ and a y intercept of $-2 \log p$. Fig. 4 shows that for apple pectin of two different degrees of esterification such plots are linear. The slopes of these plots are -0.40 for the Sigma pectin and -0.32 for the pectin designated NS-2. These slopes correspond to dm values of 60 and 52%, respectively. The dm values of these pectins as determined by the gas chromatographic method[14] were 61.5 and 50.9%, respectively. Plots for three other apple pectin samples also gave excellent linear fits, but with slopes different from what would be predicted from their dm values (see explanation below).

Fig. 4 also shows plots for the normalized PAD areas. These also are linear but with slightly steeper slopes. The increase in slopes is consistent with a uniform progressive decrease in sensitivity of the PAD with increasing oligomer length. We can modify the equation used to describe the plots obtained with the UV detector to take into account the decrease in response, assuming that the PAD response decreases by a factor of k for each additional residue in the oligomer. The response per mole for the PAD for the $GalA_n$-Gal oligomer can be predicted to be equal to the response per mole of $GalA_1$-Gal times $k^{(n-1)}$. Thus, if the normalized areas of the PAD are divided by the appropriate factor $k^{(n-1)}$, they should then be

proportional to the relative molar ratios of the oligomers. This leads to the general relation for the PAD that

$$\frac{\text{area GalA}_n\text{-Gal}}{\text{area GalA}_1\text{-Gal}} = (pk)^{(n-1)} \text{ and } \log\frac{\text{area GalA}_n\text{-Gal}}{\text{area GalA}_1\text{-Gal}} = (n-1)\log(pk).$$

This equation predicts that the slope of a plot of the log of the relative PAD responses will be steeper than that of the UV detector results by log k. For five different pectin samples of dm from 26 to 73% the mean k was 0.93 ± 0.03. Thus under our chromatographic conditions the response of the PAD falls by a factor of 0.93 for each additional residue in the oligomer. Unfortunately, k appears to change when different buffer systems (such as the sodium hydroxide–sodium acetate system often used with the PA1 column) are used for chromatography.

For cases in which the response of the PAD falls off in a geometric fashion with increasing length of oligomer in homologous series of other sugars, this relation may be useful to determine other PAD response correction factors. By comparing the relative areas of several of the 2-AP labeled members of the series by UV with their corresponding PAD areas, one should be able to determine the best k value. However, Ammeraal et al.[22] and van Riel and Olieman[23] have reported that for α-(1 → 4) linked glucan oligomers the molar PAD response increases in an irregular manner with increasing dp above 3. The factor k that we determine is strictly empirical since we do not even know what reaction, or reactions, are taking place at the surface of the electrode to give the PAD response.

We have isolated a homogalacturonan of relatively high dm (50%) from cotton suspension culture cell walls making use of its differential solubility in water vs. 80% ethanol after HF solvolysis of the walls at −23°C (ref 15). Subjecting this pectin preparation to the procedure described for the apple pectins, we obtained the results shown in Fig. 5. In this case it was essential to use the PAD for quantitation because, as noted above, the GalA-Gal oligomer labeled with 2-AP is not recovered quantitatively from the extract clean column used to remove the

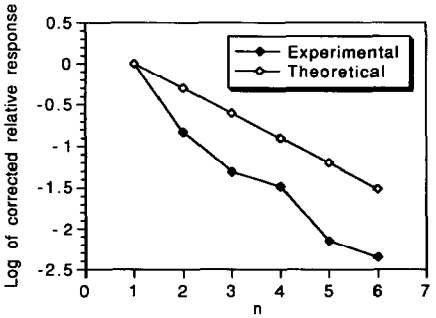

Fig. 5. A plot of the logarithm of the relative areas of the peaks obtained from cotton homogalacturonan by pulsed amperometry corrected by the relative response decrease factor and the plot predicted for a pectin of dm 50% with random distribution of nonmethyl esterified residues.

excess unlinked 2-AP reagent and this oligomer formed ~80% of the GalA-containing products. The pectin isolated from cell walls of cotton suspension cultures after treatment with HF at −23°C followed by water extraction did not show a random distribution of nonesterified GalA residues (Fig. 5). Comparison of the plot of the log of the relative PAD responses for the cotton pectin with the plot expected for a random distribution of esterification within a 50% esterified pectin immediately shows that the charged oligomers in this pectin are far from randomly distributed. The initial slope of the cotton plot would be consistent with 85% esterification of the sample if the pattern were random. Slightly more than 80% of the GalA-containing oligomers from this sample were GalA-Gal. If there were a strict repeating pattern of alternation between esterified and nonesterified residues, the degree of esterification would have to be 50%. Since the dm of the cotton pectin is 50%, this pattern of alternation should predominate, but there must also be small amounts of contiguous esterified residues to offset the decrease in overall dm contributed by the $GalA_2$-Gal and higher oligomers. If the pattern of esterification is close to being alternating and the homogalacturonan chain takes on an extended ribbon conformation, all of the negative charges would be on one side of the ribbon and the esters on the other. It is possible that the Ca^{2+} crosslinked "eggbox" structures proposed by Powell et al.[3] could form from one side of such a homogalacturonan ribbon but not the other.

It is likely that in some cases our methods described here may not show the entire distribution of lengths of contiguous nonesterified uronic acids. Since both methods of detection (UV for 2-AP labeled oligosaccharides and PAD for nonlabeled oligosaccharides) respond in a molar or submolar manner, as the oligomers get larger, for the same proportion of the mass of the initial pectin sample, the sensitivity for detection gets lower. Thus, long blocks of nonesterified GalA residues may go undetected. We are developing a postcolumn detection system (Thomas and Mort, unpublished work) which responds to sugar on a mass basis. The sodium acetate gradient described for separating the oligomers does not provide resolution above dp ~12. We are investigating the use of potassium oxalate gradients[16], in concert with the new mass-sensitive detection system, to solve this problem.

We can envisage at least two mechanisms by which nonrandom distribution of esterification could lead to a plot predicted for a random distribution. The three apple pectin samples that provided linear plots of the log of the relative UV detector responses but with slopes not predicted by their dm values may be examples of these situations. (*1*) The pectin sample may contain two different domains, existing as intermixed polymers or within individual chains of the polymer. Pectins are known to consist of homogalacturonan regions (almost entirely GalA)[4] and "hairy" regions[24] (which probably correspond to what McNeil et al.[4] call rhamnogalacturonan I) in which rhamnose is interspersed frequently or at every second residue along the backbone with side chains often attached to them. In several different plant cell walls, the GalA-Rha repeated disaccharide in this

fraction appears not to be methyl esterified[25]. The sample of apple pectin designated Roth A gave a plot for the distribution of nonesterified GalA with a slope of −0.24, which corresponds to a dm of 42%, whereas by GLC analysis it was found to be only 25.6%. The rhamnose content for the Roth A pectin was found by GLC to be higher than the other pectin samples, and a large peak was observed in the HPLC chromatograms corresponding to the GalA-Rha dianhydride. The Roth A sample appears therefore to contain a significant content of rhamnogalacturonan I-like regions. It is likely that the GalA-Rha oligomers contribute to the total GalA content for this pectin without contributing to the methyl ester content. The dm estimated by the two different methods, one dependent on the ratio of total GalA to Gal and the other on the frequency of purified $GalA_n$-Gal oligomers, could be expected to differ in this case. (*2*) DeVries et al.[11] have proposed that apple fruit pectins immediately after their synthesis might have a pattern of methyl ester distribution of $[(GalAMe)_4\text{-}GalA]_m$, but that the pattern becomes less precise after deesterification. If the deesterification were random, it is likely that the distribution of nonesterified stretches of GalA would appear to become random sooner than the overall pattern of esterification. Thus, esterified clusters would persist. Since the esterified blocks in pectins are not accounted for in the present procedure, pectins of high degree of esterification may yield oligomers that give linear plots of the log of the relative detector responses, but with the slope expected for a lower degree of esterification. Plainly the distribution of lengths for contiguous methyl-esterified GalA residues should also be determined to obtain a complete picture of the distribution of esterification in the pectin. We are working out details of a procedure to do this in which, after the reduction of the esterified residues to galactose, residual GalA residues are destroyed using lithium metal in ethylenediamine, leaving homogalactan oligomers[26,27].

Given these two means by which a linear plot of the log of the relative detector response could be obtained for nonrandom distribution of esterification, we suggest that such plots should only be taken to indicate randomness if their slopes are what would be predicted by an independently measured dm.

The more general way to look at the distribution of nonmethyl esterified oligomers for a pectin is to compare the abundance of each oligomer produced using the methods described here directly with those predicted for a random distribution. In this way even small deviations (which would be minimized by conversion to logarithmic plots) can be discerned. Table I gives comparisons between the expected and experimentally derived proportions of $GalA_n$-Gal oligomers produced from three different pectins. For the two apple pectins, NS-2 and Sigma, whose plots gave slopes very close to those predicted for their dm values, the correspondence of oligomers is quite good and there are no obvious major deviations from the expected decrease in oligomer abundance. The decrease in oligomer abundance for Roth A apple pectin does not match the pattern expected from its dm (25%); instead, it matches more closely a pattern expected for a dm of 42%. This deviation from the expected decrease in oligomer abun-

TABLE I

Comparison of relative abundance of $GalA_n$-Gal oligomers obtained from three pectins to those predicted for randomly esterified pectins of the same dm

	NS-2		Sigma		Roth A		
	UV [a]	Predicted for dm 51% [b]	UV [a]	Predicted for dm 62% [b]	UV [a]	Predicted for dm 25% [b]	Predicted for dm 42% [c]
$GalA_2$-Gal	1	1	1	1	1	1	1
$GalA_3$-Gal	0.50	0.49	0.40	0.38	0.66	0.74	0.58
$GalA_4$-Gal	0.20	0.24	0.14	0.15	0.30	0.55	0.34
$GalA_5$-Gal	0.11	0.12	0.06	0.06	0.20	0.41	0.19
$GalA_6$-Gal	0.05	0.06	0.026	0.022	0.11	0.31	0.11
$GalA_7$-Gal	0.026	0.028	0.010	0.008	0.06	0.23	0.06

[a] Normalized oligomer abundance from integrated areas using UV detection. [b] Measured dm for this sample. [c] The dm values were deduced from the log–linear plot of this pectin.

dance for Roth A supports our hypothesis that two different domains, differing in distribution of methyl-esterified GalA, exist in this pectin.

EXPERIMENTAL

Materials.—Apple pectins were a gift from Dr. Benjamin Jones of Campbell Soup Company (Camden, NJ). Another apple pectin and imidazole were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium borohydride and 2-aminopyridine were purchased from Aldrich Chemical Co. (Milwaukee, WI). Cell walls were prepared from cotton (*Gossipium hirsutum* L. cv Acala 44) suspension culture cells as previously described[25].

Pectin reduction.—Commercial pectin preparations and cell-wall extracts were reduced (to convert methyl-esterified galacturonic acid to galactose) using $NaBH_4$ as described previously[14]. Reduced samples were desalted by EtOH precipitation and dried in vacuo. During borohydride reduction methyl-esterified galacturonic acid was quantitatively converted to galactose, and the dm was calculated as the ratio of newly formed galactose to the total galacturonic acid in the unreduced sample by GLC[14].

Hydrogen fluoride solvolysis.—Reduced samples (100 mg) were treated in Teflon vessels with liquid HF (10 mL), which was 1% (v/v) water using an apparatus described previously[15]. This apparatus keeps the highly corrosive HF completely contained until it has been rendered much less volatile and aggressive by complexation with Et_2O. Reactions were carried out at −15°C for 30 min. Reactions were initiated by addition of HF (chilled in a holding vessel to −20°C just prior to transfer) and terminated by submersion of the reaction vessel into liquid N_2. The temperature of the HF holding vessel and reaction vessel was monitored using a type T thermocouple thermometer, equipped with a flexible thermocouple probe encased in Teflon (Cole–Parmer Instrument Co., Chicago, IL). The HF was quenched by addition of dry ice cooled anhyd Et_2O (100 mL), allowing the frozen

HF to thaw and stir for 30 min. In preliminary experiments we found that addition of the HF at a temperature cooler than the targeted reaction temperature was necessary to avoid warming of the mixture above the targeted reaction temperature upon addition of HF. Termination of reactions by first submerging the reaction vessel into liquid N_2 to freeze the mixture and then adding dry-ice-cooled anhyd Et_2O, allowing the Et_2O–HF complex to form during thawing of the mixture, avoided warming of the mixtures above the targeted reaction temperatures. The Et_2O–HF was evaporated in vacuo, and the remaining material was dissolved into 100 mM AcOH, 1 mL per 10 mg, and incubated at 80°C overnight to hydrolyze the fluoro groups from the reducing ends of the oligosaccharides. Samples were then frozen, lyophilized, and utilized for high-performance anion-exchange separations.

High-performance anion-exchange chromatography.—Liquid chromatographic separations were carried out on a Dionex Bio-LC (Dionex Corp., Sunnyvale, CA). The system consisted of a gradient pump module, an eluant-degassing module, a pulsed amperometric detector (PAD), and a variable wavelength detector. Pulse potentials and durations used for the pulsed amperometric detector in this study were as follows: E_1, 0.05 V; t_1, 480 ms; E_2, 0.60 V; t_2, 120 ms; E_3 −0.60 V; t_3, 60 ms. The response time for the detector was set at 1 s. Separations were carried out using a PA1 pellicular anion-exchange column (4 × 250 mm) at 1 mL min^{-1}. Eluant *1* was water, eluant *2* was M NaOAc, pH 5.2. Samples (100 μg to 1 mg) were injected into a system equilibrated at 30 mM NaOAc (97% eluant *1*, 3% eluant *2*), and sample components were eluted after a 3 min lag period using a linear gradient of NaOAc from 30 to 200 mM over 27 min, then to 500 mM over 25 min, and after a 5 min hold, to 750 mM within 5 min, with a final 5 min hold. The system was allowed to equilibrate at initial conditions for at least 5 min prior to another injection. Chromatographic data were collected using custom built data loggers[28] and downloaded into a Macintosh IIx computer. Chromatographic data were viewed and quantitated using the program Analog Connection Chrom (Strawberry Tree Computer, Inc.). Peak identities were confirmed by ^1H NMR spectroscopy and by liquid secondary-ion mass spectroscopy of desalted fractions. Relative molar quantities of peaks were obtained by comparison of the pulsed amperometric response to the UV response for 2-aminopyridinylaminated samples, prepared as previously described[21].

Other procedures.—Gas chromatographic analysis of trimethylsilylated sugar derivatives[29] was conducted with a Varian 3300 gas chromatograph (Varian Associates, Palo Alto, CA) using a DB-1 fused silica capillary column (30 m × 0.25 mm, 0.25-μm film thickness: J&W Scientific Inc., Rancho Cardova, CA) and inositol as internal standard[14]. ^1H NMR spectra of selected chromatographic fractions were recorded at 400 MHz using a Varian XL-400 superconducting NMR spectrometer (Varian Associates, Palo Alto, CA) at 25°C with D_2O as solvent and TSP as internal standard (0.00 ppm) on 400 MHz operating frequency. Liquid secondary-ion mass spectrometry was conducted using a VG Instruments ZAB

2SE mass spectrometer in the positive-ion mode using thioglycerol as matrix, as described by Dell[30].

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