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Note

Investigating the nature of branching in pectin by atomic force microscopy and carbohydrate analysis

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Abstract

Atomic force microscopy (AFM) has been used to investigate the nature of the long branches attached to pectin which were described in a previous report [Round, A. N.; MacDougall, A. J.; Ring, S. G.; Morris, V. J. *Carbohydr. Res.* **1997**, *303*, 251–253]. Analysis of the AFM images and comparison with neutral sugar and linkage analyses of the two pectin fractions suggest that the distribution and total amount of branches observed do not correspond with the pattern of neutral sugar distribution. It is thus postulated that the long chains consist of polygalacturonic acid, attached via an as yet undetermined linkage to the pectin backbone, with the neutral sugars present as short, undetected branches. This explanation would have important implications for the nature of 'in situ' pectin networks within plant cell walls and models of gelation in commercial extracted pectin, and the existence of significant branching will markedly influence the viscosity of extracted pectins.

Keywords: Branching patterns; Polygalacturonic acid; Plant cell walls, gelation; Viscosity

The pectic polysaccharides of the plant cell wall are amongst the most complex polymers known to exist,[1,2] and are implicated in a range of functions both vital to the plant and commercially important to the food and pharmaceutical industries.[2] The precise extent of this complexity and its role in the polymer's native environment, are still not fully understood. Techniques from many scientific disciplines have been used to study and characterise pectic polysaccharides for many years, and one of the conclusions of this extensive research is that the complexity that appears to hold the key to their role in the cell wall also makes pectins difficult to characterise analytically. Most currently used techniques rely on measuring and interpreting the colligative properties and characteristics of a population of polysaccharides. This approach must acknowledge the potential for underestimating the importance, both structurally and functionally, of the heterogeneous nature of a population of such complex molecules. The ability to examine individual polymers and analyse the degree of heterogeneity within a sample represents in itself an important contribution to the understanding of biological macromolecules. In addition, the study of individual polymers can be related to the models

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of average molecules constructed from the experimental data already extant, allowing the fine-tuning of these representations to more closely resemble reality. Recent preliminary work[3] has shown that AFM could reliably and reproducibly image pectic polysaccharides extracted from tomatoes. The images obtained in this preliminary study revealed a fraction of pectins containing long branches. This differs from the conventional picture of pectins as linear polymers containing 'hairy regions' composed of short neutral sugar side chains.[2]

Neutral sugar distribution varies between pectic fractions according to many factors, including plant species, type of tissue, degree of tissue maturation and method of extraction. By comparing AFM images of two pectic fractions extracted from unripe tomatoes using different methods, or known to have markedly different sugar compositions, it should be possible to draw some conclusions about the role of neutral sugars in determining the complete branching profile of the pectic fractions. The 1,2-cyclohexanediaminetetraacetic acid (CDTA) extraction releases pectin from the cell wall by complexing calcium. This frees pectin cross-linked by calcium and is considered to release pectin mainly from the middle lamella, and which is relatively rich in galacturonic acid. The subsequent extraction with Na_2CO_3 is then considered to free pectin by breaking ester linkages and to mainly release neutral sugar-rich pectins from the primary cell wall. Thus this work involves comparing two pectic fractions from the cell walls of tomatoes, each having a different distribution of sugar residues. In this way, any correlation between branch length distribution and neutral sugar content may be identified.

The results of sugar analysis of the sodium carbonate (Na_2CO_3)-extracted fraction can be compared (Table 1) to the results previously published for the CDTA-extracted fraction used in this study.[4,5] These results are typical of CDTA and Na_2CO_3 pectin extracts and are consistent with previous data reported[6] for unripe tomato pectin. The results of the sugar analysis show that the Na_2CO_3-extracted pectin contains nearly twice the amount of pectic neutral sugars as the CDTA extract, and agree with the conclusion that the Na_2CO_3-extracted fraction is richer in neutral sugars of the type expected to comprise the side chains of the rhamnogalacturonan regions, specifically arabinose and galactose.

Table 1
Sugar analysis [a]

	gal	ara	rha	glc	xyl	fuc	man	galA
CDTA	101	29	7	5	1	1	<1	710
Na_2CO_3	184	63	7	8	1	1	3	565

[a] All values are expressed as 'anhydro sugars', μg/mg dry weight; results for the CDTA fraction taken from Ref. 4 with permission.

For both the CDTA and Na_2CO_3 extracts the main backbone sugar was identified as (1→4)-linked galacturonic acid by methylation analysis. The presence of terminal galactose, terminal arabinose, (1→5)-linked arabinose, (1→4)-galactose and (1→2,4)-linked rhamnose confirmed the expected result that the majority of the neutral sugars were present as linear (1→5)-linked arabinan and (1→4)-linked galactan chains attached to the pectin backbone through the (1→2)-rhamnose residues. As discussed in detail elsewhere[4,6] the remaining small quantities of neutral sugars present as (1→4)- and (1→4,6)-glucose and terminal-xylose can be attributed to the presence of a small amount of a xyloglucan, those present as (1→2,4)- and (1→3,4)-galactose attributed to a Type 1 arabinogalactan, with the (1→3)-, (1→6)- and (1→3,6)-galactosyl residues attributed to a Type 2 arabinogalactan or, more likely, a contaminating arabinogalactan protein.

Typical AFM images of the sodium carbonate extract (Fig. 1) show a mixed population of single polymers and aggregates, distinguishable by height measurements. The aggregates are present even at low dilutions where few single polymers can be seen, suggesting that they are not simply superpositions or entanglements of polymers caused by the reduction of solvent volume during drying down on to the substrate, but are multi-polymer complexes held together by intermolecular interactions. The image is representative of the results obtained reproducibly from dozens of samples. The distribution of the single poly-

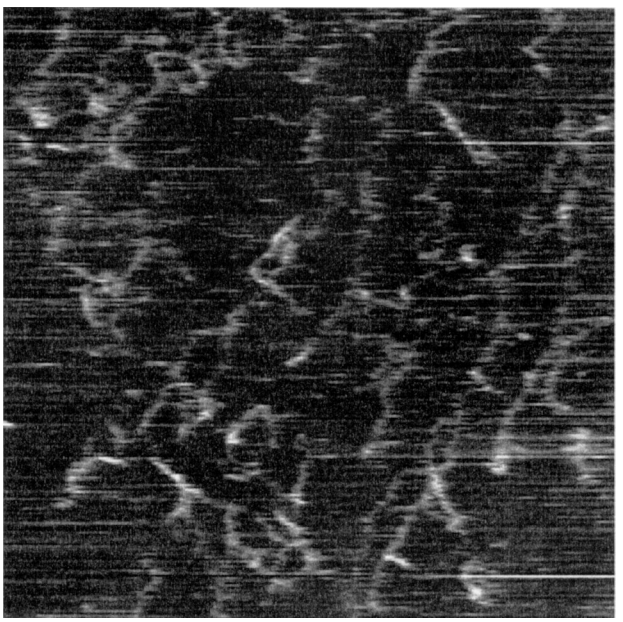

Fig. 1. A typical 1 μm^2 image of CDTA-extracted pectin on mica.

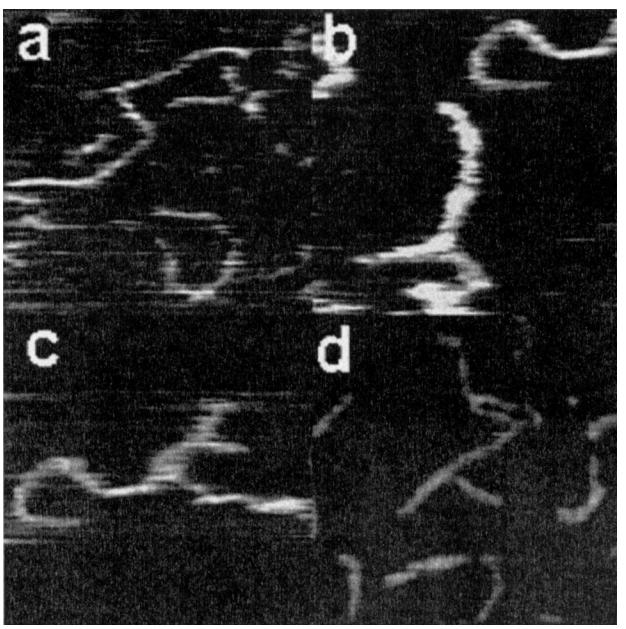

Fig. 2. Zoomed images of pectin molecules and features: (a) linear, unbranched polymer; (b) single branched polymer; (c) a multi-polymer complex resembling a singly branched, single polymer; and (d) a multiply branched single polymer. Image sizes for Fig. 2(a, b and d): 250nm^2; Fig. 2(c): 250 × 125nm.

mers reflect the heterogeneity of the pectin population. Linear polymers with contour lengths ranging in size from tens to hundreds of nanometres are present throughout the images collected, together with a proportion possessing branches. Linear (Fig. 2(a)), singly branched (Fig. 2(b)), multi-polymer complexes resembling branched polymers (Fig. 2(c)) and even multiply branched (Fig. 2(d)) single pectin molecules have been identified and can be seen in both the CDTA-extracted and Na_2CO_3-extracted fractions. Once a representative number of sample images have been collected and analysed, it is possible to produce histograms showing the contour length distributions. Fig. 3 shows the contour length distributions of the polymer branches and backbones for both the fractions studied in this work. Pectic polymers extracted with CDTA (mean backbone length approximately 350 nm) are about three times longer than those of pectin extracted by Na_2CO_3 (mean backbone length approximately 120 nm). This is qualitatively consistent with the different values of intrinsic viscosity observed[5] for the CDTA fraction ($[\eta] = 810$ g/cc) and the Na_2CO_3 fraction ($[\eta] = 309$ g/cc). The level of branching present in both extracts is similar: approximately 30% of the individual polymers identified in each fraction possess visible long branches. The pattern of relative branch lengths for the two fractions reflects that found for backbone lengths; branches in the CDTA fraction have mean lengths of approxi-

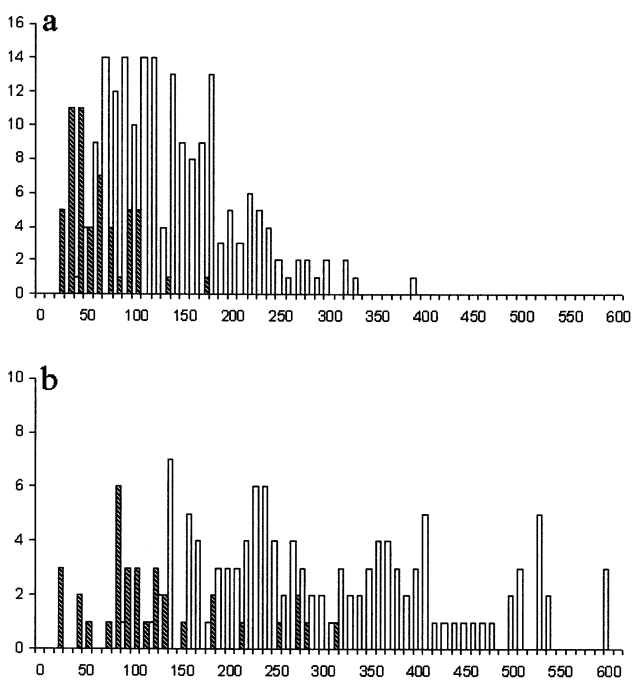

Fig. 3. Length distributions for (a) Na_2CO_3-extracted pectin and (b) CDTA-extracted pectin. Unfilled bars represent backbone contour measurements; filled bars are branch measurements. $n = 189$ and 117 polymers, respectively.

mately 150 nm whilst the branches in the Na_2CO_3 fraction measure on average 50 nm. It therefore follows that the ratio of backbone:branch length is also similar for both fractions, with the branches accounting for approximately 10% of total measured polymer length in both fractions. The distribution of polymer lengths reveals a number of broad peaks, but a well-defined periodicity does not emerge. The pattern of branch length and distribution relative to backbone length distribution (Fig. 3) applies to both fractions, despite the evident differences in their chemical composition.

These results have shown that it is possible to characterise populations of pectin molecules, and individual molecules within a population, on the basis of their size and shape as derived from AFM images. Conventional sugar and methylation analysis provides compositional data for an 'average' member of this population, and is limited in its sensitivity to the effects of heterogeneity. These AFM studies have revealed unexpected heterogeneity of pectin molecules. For material extracted by selective and sequential methods from the cell walls of mature, unripe tomatoes, it has been found that both the CDTA and Na_2CO_3 fractions contain a mixture of aggregates and both linear and branched molecules. In both extracts, approximately 30% of the molecules are branched. The branching observed by AFM is unusual: Not all the pectin molecules are branched and those that are, have different numbers of branches. The presence of long branches means that it is not possible to derive simple relationships between, on the one hand, the measured average composition and molecular weight, and on the other hand, functional properties such as solution viscosity, without taking into account the contribution of the branched components.

It has been postulated that pectin possesses a backbone of polygalacturonic acid (smooth regions) containing rhamnogalacturonan regions with side chains of arabinose and galactose (hairy regions).[2] Given this, it might be expected that the relative proportions of backbone and branch length estimated by AFM should reflect the proportions of the backbone and branched sugars measured by chemical analysis. In the case of the CDTA extract, comparison with the total sugar and linkage composition suggests that the level of branching observed by AFM could be consistent with the composition. From the neutral sugar analysis, the CDTA extract contains about 15% arabinose and galactose, the principal sugars comprising the side chains and 84% galacturonic acid and rhamnose, the backbone sugars. This seems to compare well with the ratios by length of 10% branches and 90% backbone as determined by AFM. The discrepancy could be attributed to the presence of a number of short neutral sugar branches, which are difficult to detect by AFM. Thus, the suggestion based on previous methylation analysis,[4,5] that the pectic fraction extracted from unripe tomatoes by CDTA contains relatively few, relatively long side chains is consistent with the AFM images, with the proviso that the long chains are only present on a few pectin molecules.

There is no such relationship between arabinose and galactose content and branch length content in the case of the Na_2CO_3-extracted fraction. The proportion of polymer branch to backbone as measured by AFM is similar to that obtained for the CDTA extract, but the arabinose and galactose content is much greater. For the Na_2CO_3 fraction, the neutral sugar content (arabinose and galactose) is approximately 39% whereas the percentage total length present as branches, estimated from the AFM images, remains at approximately 10%. Thus comparison of the AFM images with the sugar analysis shows no strong correlation between branch distribution and sugar composition between the two fractions. The present chemical composition and linkage analysis is consistent with that reported elsewhere for ripe tomatoes,[4–6] which also suggested an increase in branching for the Na_2CO_3 fraction. Once again the discrepancy between the AFM data and the chemical composition could be explained by suggesting that the increase in branching is fully accounted for by an additional number of short chains undetected by the present AFM technique.

To account for the present data within a model that attributes branching in pectin en-

tirely to neutral sugar side chains, it thus becomes necessary to postulate two populations of branches: long neutral sugar branches (seen by AFM) and short neutral sugar branches (not observed by AFM). Given that the long branches have, therefore, to be considered as a different class of branches, it is possible to construct other models of the branched structure of pectins on the basis of the present results. The AFM data combined with the sugar analysis support the conclusion that all the neutral sugars are present as short branches. If these branches are all coiled or aligned along and around the rhamnogalacturonan region then they are likely to cause changes in height or width that are too small to confidently identify by AFM. In this case the AFM data suggests the existence of long branches of polygalacturonic acid, linked via an, as yet, undetermined branch point on the polymer backbone. The low level of 'long' branching present would make it difficult to determine chemically the nature of any direct linkage to the polygalacturonic acid backbone. One of the earliest analyses of the polysaccharides of the cell wall[7] did produce evidence of branched galacturonic acid residues, although the same authors dismissed such results as artefacts due to under-methylation. Further studies are needed, in which the AFM is used to characterise the products of chemical or enzymatic treatment of the pectins, designed to selectively cleave different sugar residues, and thus to identify the nature of the branches.

1. Experimental

Polysaccharide extraction.—Pectic polysaccharide from the pericarp tissue of mature green tomatoes (*Lycopersicon esculentum*, var. Rutgers) was extracted[4] using the chelating agent CDTA (50 mM, pH 6.8, 24 h), designed to remove polysaccharides held in the cell wall by Ca^{2+}-mediated crosslinks[8] followed by extraction using Na_2CO_3 at 1 and 20 °C, as described by Redgewell and Selvendran.[9] The fractions were stored at 4 °C as the potassium salt in aqueous solution, at a concentration of 1 mg/mL, until required. Residual cations were removed from the acidic fractions by stirring for 20 min over Dowex AG 50-X8 (H^+) and then the potassium salt was formed by adjusting the pH of a solution of the acidic polymer to pH 6.5 with 1 M KOH. $CHCl_3$ was added as a bactericide. The sample was not freeze-dried in order to minimise any aggregation of the polysaccharides.

Neutral sugar analysis.—Neutral sugars were released by Seaman hydrolysis[10] and reduced after neutralisation with ammonia.[11] The resulting alditols were acetylated and then analysed by gas chromatography.[12]

Linkage analysis of polysaccharide fractions.—The sugar composition and linkage analysis of the CDTA-extracted fraction has been reported by MacDougall et al.[4] The Na_2CO_3-extracted fraction was subjected to carbodiimide-activated reduction of its uronic acid residues as described by Rigby et al.,[13] prior to methylation following the method of Needs and Selvendran.[14] The carbodiimide reduction converts the galacturonic acid residues to deuterated galactose residues. The reduced pectic polysaccharide was then methylated, hydrolysed, reduced and acetylated to form alditol acetates, which were analysed by GC and mass spectrometry. The original proportion of galacturonic acid was then calculated from the ratio of the deuterated to non-deuterated galactose residues.

Atomic force microscopy.—The microscope used in these experiments was constructed by East Coast Scientific (Cambridge, UK). The operation of the microscope under the conditions described below has been discussed in detail previously.[3,15] For imaging, each pectin fraction was diluted to 1–3 μg/mL before the deposition of 2 μL onto freshly cleaved sheets of mica (ca 10 mm^2). The sample was then allowed to dry under ambient conditions before insertion into the liquid cell of the microscope. Triply distilled butanol (300 μL) was injected into the cell halfway through the sample approach sequence and images were obtained in direct contact mode. The use of a liquid as an imaging solvent reduces the influence of capillary forces arising between tip and surface; butanol was chosen in particular as alcohols are used to precipitate polysaccharides from solution, thus keeping the adsorbed

polysaccharide from desorbing during imaging. Topographic and error-signal mode images were collected simultaneously, and the imaging was repeated for a number of sample preparations. In excess of 100 images of areas 1–2 μm^2 were collected for each fraction.

Image analysis.—The heights of features in the images were measured using the AFM software supplied with the instrument (SPM 6.01, ECS, Cambridge, UK). For the length measurements, images were converted to TIFF files and analysed using NIH Image.

Detecting single polymers.—In order to support the use of a microscopy technique for investigating polymer heterogeneity at the macromolecular level, it is necessary to first demonstrate its ability to identify single polymers in a sample, and therefore to enable the rejection of aggregates from subsequent analyses. The way in which atomic force microscope images are constructed uniquely allows the direct measurement of parameters in three dimensions. This ability permits the rapid identification of features with dimensions incommensurate with those expected of single polymers, and also allows the user to distinguish branches on the polymer backbone from superpositions of two polymers upon each other. Measured heights of single strands vary between 5 and 7 Å between different sample preparations, due to differences in tip geometries and imaging forces, and by ± 1 Å within an image. At a true branch point, the height of the molecule will remain unchanged, whereas if two molecules cross over each other the measured height should double.[3] In practice, the experimentally observed height increase at an overlap is often less than double (but always greater than $1.5\times$) due to compression of the polymers by the AFM tip during scanning. Measuring the heights of apparent branching points ensures that superimposed polymers can be rejected from the analysis and only true branch points included. Height measurements also allow recognition of aggregated polysaccharides, which can then be omitted from any analysis.

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