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Unexpected branching in pectin observed by atomic force microscopy

Andrew N. Round *, Alistair J. MacDougall, Stephen G. Ring, Victor J. Morris

Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

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Abstract

Pectic polysaccharides extracted from unripe tomato plant cell walls have been imaged with an atomic force microscope (AFM). The images obtained reveal for the first time a branched structure for tomato pectins that differs from that proposed for the neutral sugar side chains from enzymatic hydrolysis and sugar analysis. The branches are between 30 and 170 nm long and are relatively linear. This work demonstrates that the AFM is uniquely capable of unambiguously identifying, with minimal sample preparation, individual macromolecules within a heterogeneous population. © 1997 Elsevier Science Ltd.

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The functional properties of polysaccharides, both in their natural environment and in commercial applications, are closely related to their chemical and physical structures. However, complete structural analysis of complex and/or irregular polysaccharides is a difficult goal to achieve with present methods. The inherent irregularity of many biological polymers makes it difficult to obtain good crystals, and thus high-resolution crystallographic techniques cannot be used [1]. Currently, chemical analysis requires the degradation of the intact polysaccharide to a series of mono- or short oligo-saccharides [2], which immediately makes sequencing of the composite sugars problematic. This method is successful in determining the identity of the component sugars and the position of their glycosidic linkages, and, when coupled with selective enzymatic extraction, it can identify regions of distinct composition; however, it falls short of providing a complete monosaccharide sequence for any polymer that does not consist of simple, short repeat units. In addition, varying structures or complex repeat units do not lend themselves easily to determination in this way as the analysis is carried out over a large number of polymers and thus irregular sequences tend to be averaged across the whole sample. Physical characterisation methods such as light scattering and sedimentation analysis [3] also produce results that are the sum of the responses of a large number of polymers in a sample and are thus averaged over that sample; variation between individual polymers is difficult to quantify. The only current physical method that has the potential to allow investigation of the degree of variation present in a polysaccharide population is microscopy, and electron

* Corresponding author.

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microscopy has been used to study individual polymers [4,5]. However, this work has generally been carried out on replicas rather than directly on the samples themselves, and questions about whether or not the features observed are truly single polymers do not appear to have been adequately addressed. Because of these sorts of problems, very few biological polysaccharides have been fully characterised [1]. A case in point is that of the pectins [6], important constituents of the interfibrillar matrix in the cell walls of higher plants whose complicated and irregular structures have hampered a thorough investigation of the functional significance of their structural properties.

The atomic force microscope (AFM) affords an opportunity to directly image individual, intact pectin polymers and thus measure and appreciate the inherent variation within this class of polysaccharides. Its operation [7] and application to the study of polysaccharides [8] have been described elsewhere; briefly, a sharp tip on the end of a flexible cantilever is scanned over the sample surface, and the interactions between tip and sample are monitored to produce a three-dimensional image of the surface topography of the sample with subnanometer resolution with minimal sample preparation. We have obtained images from the AFM by depositing 1–3 μg/mL aqueous solutions of pectin (extracted from the cell walls of unripe tomatoes sequentially, using hot and cold cyclohexane-trans-1,2-diaminetetraacetate (CDTA) [9], followed by sodium carbonate at 20 °C) onto freshly cleaved sheets of mica and drying them in air before imaging in the DC (direct contact) mode under butanol.

A typical image of the sodium carbonate extract (Fig. 1) shows a mixed population of single polymers and aggregates that are distinguishable by height measurements. This image is representative of the results obtained reproducibly from dozens of samples. The distribution of lengths of the observable single polymers occupies a range between 30 and 390 nm and resembles a log-normal curve. Number-average and weight-average molecular weights can be calculated from the measured polymer contour lengths, and in this case $M_w = 50{,}000$. This figure is 2–3 times smaller than molecular weights estimated for similar samples using conventional techniques [10], and there are a number of possible contributory factors to this discrepancy, including the likelihood that previous estimates measured the aggregates seen in this image as well as the single polymers. Other reasons for underestimation of molecular weight include the following: (i) only confirmed single polymers have been taken into account — the aggregates may be single, large coiled polymers whose absence from the MW calculation would depress the value obtained; (ii) the AFM tip may have broken some of the fragile polymers, decreasing the average polymer length; and (iii) the lengths reported for the neutral sugar side chains found attached to the galacturonic acid backbone of pectins are too short to be seen in an AFM image — therefore, their contribution to the MW will not be included in a calculation based on visible contour lengths.

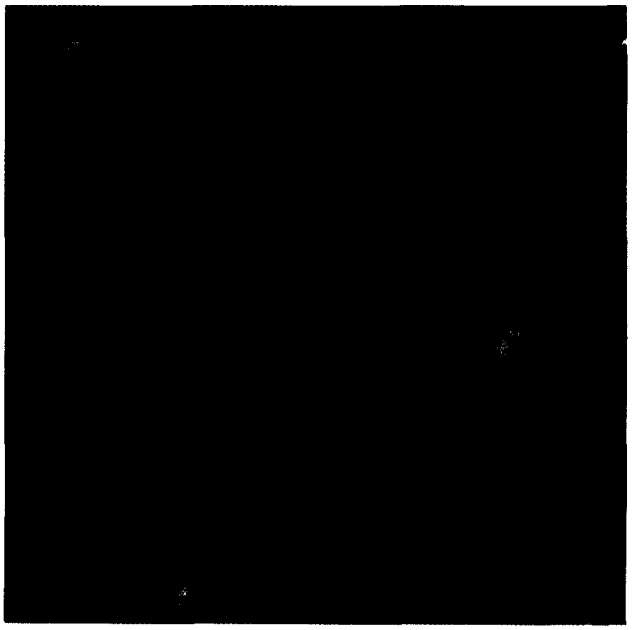

Fig. 1. A typical field of sodium carbonate-extracted pectin from unripe tomato (image size 1 μm × 1 μm). Note the coexistence of large aggregates (bright portions of image) with single polymers.

Unexpectedly, long branches are revealed to be attached to approximately 20% of the single polymers, with approximately 30% of these having more than one branch. Fig. 2 shows an enlarged example of one of these multibranched polymers with attendant height measurements to discount the possibility of overlapping between two polymers — single polymer strands have diameters of between 0.5 and 0.8 nm. The branches, which range in length from 30 to 170 nm, are much longer than the mean neutral sugar side-chain lengths inferred by comparing the amounts of neutral sugars with the amount of branched rhamnose in methylation analysis [11] of similar tomato pectins. There are two possible interpretations of the nature of these long branches: The first is that they represent the extreme end of a range of neutral sugar

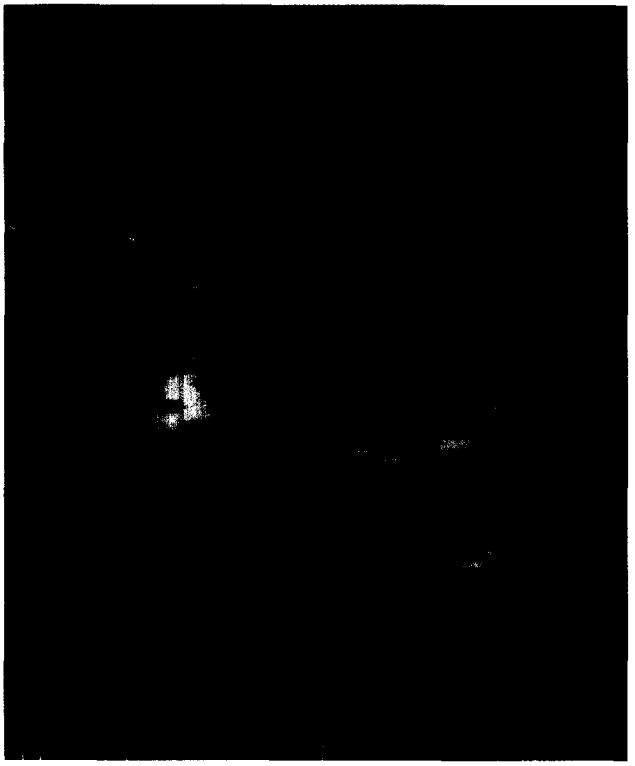

Fig. 2. An enlarged image of a single, branched polymer (image size 224 nm × 226 nm). The traces show the contours of the areas highlighted by the bars a (branch and backbone) and b (branching point); the height of the polymer chain is recorded in Angstroms. Note that branch, backbone and branching point of the polymer have similar heights (5.2, 5.2 and 5.9 Å, respectively).

side-chain lengths. Consideration of previous linkage analyses [11] suggests that if there is a non-mean distribution of neutral sugars attached to branched rhamnose, then there may be enough to account for the long chains revealed here and for a population of shorter chains that we would not expect to see using the AFM. However, there does not appear to be any evidence for substantially longer neutral sugar side chains from chemical analysis, when detection of such side chains would not appear to pose any problems. The identification of the observed branching as representing the neutral sugar side chains is therefore problematic. An alternative explanation is that the branches consist of galacturonic acid, attached to the backbone by either rhamnose or small amounts of a branched galacturonic acid. This latter interpretation is in agreement with the fact that the positions of linkages present in uronic acid residues are difficult to measure using standard analyses, and the number of branched galacturonic acid residues required to produce the number of branches seen here would be below present analytical limits of detection.

This preliminary account shows that the AFM is capable of revealing structural details that are not susceptible to any other method of investigation, and that irregularities in polymer structure that escape detection in whole-sample-based analyses can be identified when individual polymers can be examined. Tomato pectins are here revealed to possess long branches, the existence of which were previously unknown, and these new structural features may have an important bearing on consideration of structurally related pectin functionality. We are currently investigating the use of immunolabelling techniques coupled with AFM in order to identify these new branches as well as other specific regions of the pectin backbone, with a view towards refining our understanding of the complexity of these biopolymers.

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