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Characterisation of heterogeneous arabinoxylans by direct imaging of individual molecules by atomic force microscopy

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Abstract

Atomic force microscopy has been used to characterise populations of extracted water-soluble wheat endosperm arabinoxylans. The adsorbed molecules are extended structures with an estimated Kuhn statistical segment length of 128 nm, suggesting that they adopt an ordered helical structure. However, estimates of the molecular weight distribution, coupled with size exclusion data, suggest that, in solution, the polysaccharides behave as semi-flexible coils, with a Kuhn length of 16 nm. These data imply that adsorption of the arabinoxylan structures onto mica promotes formation of the helical structure. Adoption of this ordered structure is fortunate because it has permitted characterisation of branching observed in a small proportion ($\approx 15\%$) of the population of otherwise linear molecules. The degree of branching has been found to increase with the contour length of the molecules. Degradation of the polysaccharides with xylanase has been used to confirm that both the backbone and branches are based on β-(1→4) linked D-xylopyranosyl residues. © 2003 Elsevier Science Ltd. All rights reserved.

Keywords: Arabinoxylans; Pentosans; Atomic force microscopy; Kuhn length; Helices

1. Introduction

Arabinoxylans are important non-starch polysaccharides that can be isolated from cereal grains. The arabinoxylans are considered[1] to consist of a linear backbone of β-(1→4) linked D-xylopyranosyl residues containing individual α-L-arabinofuranosyl residues attached through O-2 and/or O-3 (Fig. 1). Other features present in extracted arabinoxylans[2] may be small amounts of uronic acids and the presence of ferulic acids, covalently linked through ester linkages to the arabinose, as shown in Fig. 1. It has been demonstrated that diferulic acid crosslinks are present in the arabinoxylan component of *Gramineous* plant cell walls, since the putative diferulate linkage has been isolated.[3] It has been suggested that these dimers may serve to cross-link arabinoxylans: A potential 5,5′ dimeric cross-link is shown in Fig. 1. Other types of diferulates have also been identified[4] and arabinoxylans are considered to contain 5,5′, 8-O-4′ and 8,5′ dimers. Arabinoxylan extracts from endosperm are partially water-soluble and result in viscous aqueous solutions. The origin of the viscous behaviour has been attributed to the formation of a rod-like structure in solution,[5] although recent detailed light scattering studies suggest that the polysaccharides can be described as semi-flexible coils.[6,7] Static and dynamic light scattering studies have reported[8] that the polysaccharides may not be simple linear structures, but may exhibit some degree of branching. The origin of this type of branched structure is obscure and, on the basis of the known chemical structure, should not arise from the arabinose component. However, branching could result from cross-linking of individual arabinoxylans through ferulic acid dimers. Therefore, one might expect the extracts to contain branched structures rather than purely linear polymers. The shape and size of individual molecules, and the extent of any potential cross-linking, would be important determinants of solution viscosity. The origin of the viscous nature of arabinoxylans is of considerable industrial importance. In bread making the viscous character is considered[9,10] to enhance the development of dough structures. However, cereal grains used in brewing, or for animal feed,

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are often treated enzymatically to breakdown the arabinoxylan structures that are considered to inhibit the industrial use of the material.[10]

Arabinoxylans are heterogenous in chemical structure and an analysis of their size and shape would benefit from the characterisation of individual molecules within a population. Atomic force microscopy (AFM) has been used successfully to characterise individual polysaccharides from land plants,[11–17] algal cell walls,[17–19] animal tissue[20,21] and bacterial sources.[22–26] Information has been gleaned on molecular branching,[12,27] molecular mass distributions,[17,28] and on various interactions involved in supra-molecular assembly,[25,29] cell wall structure[18,30] and polysaccharide gelation.[31,32] Preliminary studies have been reported[16] on the use of AFM to probe adsorption and desorption of arabinoxylans from mica surfaces. The present article describes the use of AFM to characterise the size and shape of individual arabinoxylan molecules, and to probe the possible existence of cross-linked or branched structures.

2. Results and discussion

Neutral sugar analysis of the polysaccharide sample confirmed the presence of the major sugars (98%) xylose and arabinose together with small amounts (2%) of the sugars glucose, galactose and mannose. Methylation analysis demonstrated the presence of *t*-xylose, (1→4)-linked xylose and *t*-arabinose residues linked at either the C-3 or C-4 positions on the xylose backbone. The level of backbone substitution with arabinose (0.55), the measured levels of ferulic acid (0.89 mg g^{-1}), di-ferulic acid (0.09 mg g^{-1}) and protein (1.8 mg g^{-1}) are consistent with values reported[33–35] elsewhere in the literature for water-soluble wheat endosperm arabinoxylans. The arabinoxylans were found to contain 5,5, 8-O-4′ and 8,5′ ferulic acid dimers.

The arabinoxylan samples could be fractionated by size exclusion chromatography (SEC). The protein content co-elutes with the carbohydrate suggesting that the protein is tightly bound, and possibly covalently attached to the polysaccharide. In order to study the polysaccharide alone the sample was protease treated to remove the protein. SEC fractionation of the protease treated sample confirmed the loss of protein and showed a net small decrease in molecular weight (Fig. 2). The protease treatment appears to reduce the presence of high molecular weight material (arrowed in Fig. 2) and increase the amount of low molecular weight material: possibly due to the breakup of aggregated structures releasing smaller sized polymer chains. For the treated material (Fig. 3) pullulan samples were used as reference materials. SEC Fractions 13–16 were collected and investigated by AFM.

In order to avoid aggregation effects, induced by freeze-drying, the fractions collected from the SEC column were concentrated by rotary evaporation to about 5 g mL^{-1} and 2 μL of this solution deposited

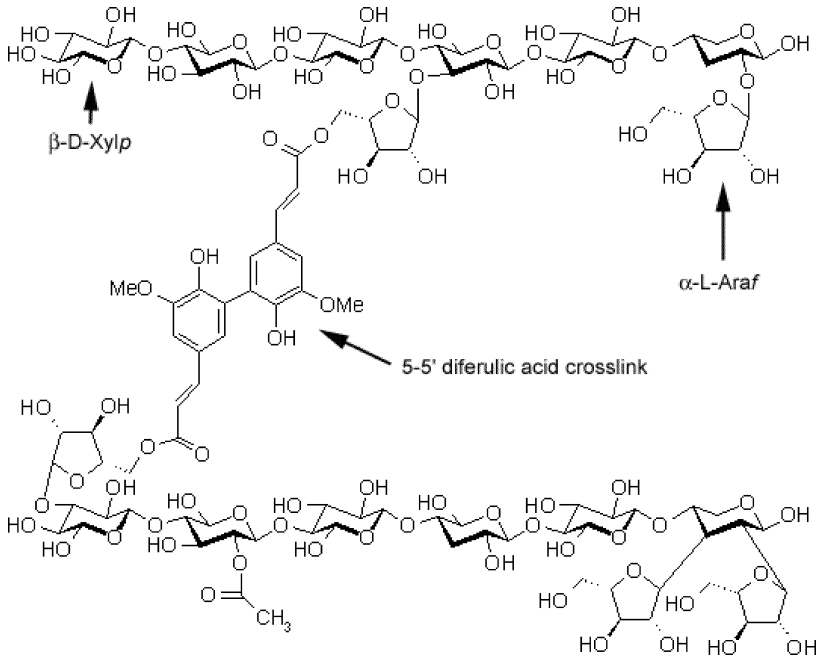

Fig. 1. Depiction of the main structural features predicted to be present within wheat arabinoxylans. The structure shown contains β-(1→4)-D-xylan backbones that are substituted with single and/or double α-L-arabinofuranoside moieties and, as an example, shows covalent cross-linking through a 5,5′ diferuloyl moiety. In reality, the arabinoxylans backbones would be longer than shown (i.e., the xylose–diferulic acid ratio would be greater). α-L-Ara*f* = α-L-arabinofuranose, β-D-Xyl*p* = β-D-xylopyranose.

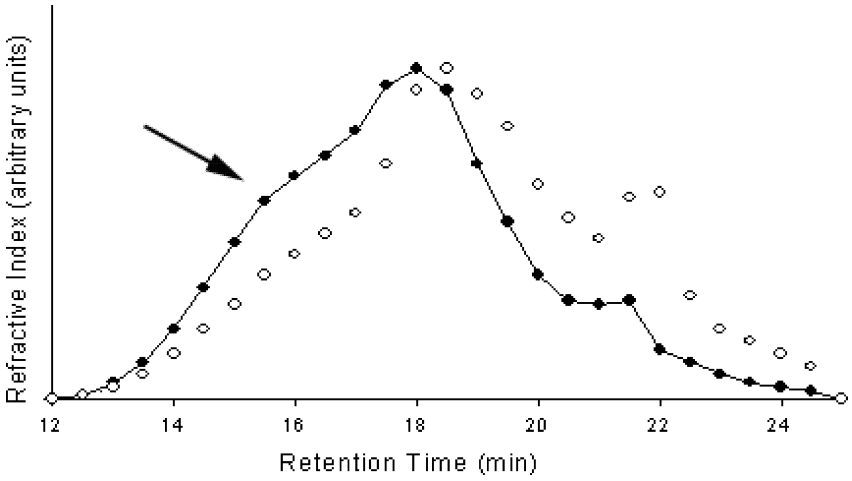

Fig. 2. SEC data illustrating the effects of protease treatment of the arabinoxylans. A sample of water soluble wheat flour arabinoxylan was treated with protease and the untreated (●) and treated (○) samples analysed using a Polysep G5000 column (2.5 × 85 cm, 2 mM ammonium bicarbonate buffer, flow rate 0.5 mL min^{-1}, 20 °C) with refractive index detection. The high molecular weight region (arrowed) is decreased by the action of the enzyme resulting in an increase of the amount of low molecular weight material.

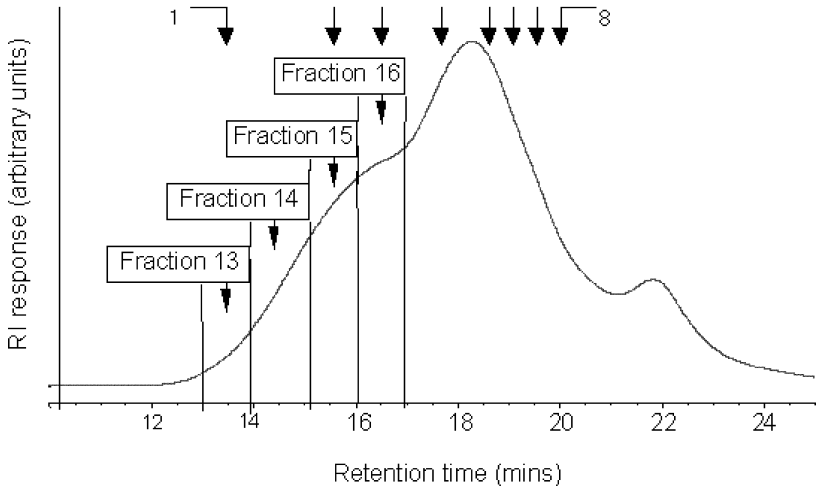

Fig. 3. Fractionation of wheat flour water-soluble arabinoxylans by SEC. The sample was fractionated on a Polysep G2000 column (2.5 × 85 cm, 2 mM ammonium bicarbonate buffer, flow rate 0.5 mL min, 20 °C) of the unfractionated arabinoxylan. Fractions were obtained by collection at time intervals from 13 to 16 min (Fractions 13–16), and the elution monitored using a refractive index detector. Arrows indicate elution retention times of the pullulan standards of molecular masses 1.66×10^6, 3.88×10^5, 1.86×10^5, 1.00×10^5, 4.8×10^5, 2.37×10^4, 1.2×10^4, 5.8×10^3 (1–8, respectively) are indicated.

onto freshly cleared mica substrates. The samples were placed in the liquid cell of the AFM and imaged in contact mode under butanol. The quality of the AFM images obtained was found to depend on the ionic strength of the buffer used in the SEC fractionation. At ionic strengths above 2 mM ammonium bicarbonate the AFM revealed what appeared to be aggregates of polymeric material clustered around the boundaries of dendritic shaped structures (Fig. 4). This effect disappeared if the salt content was lowered to 2 mM. It is believed that at high salt contents the ammonium bicarbonate crystallises on the mica substrate, concentrating the polymeric material and depositing it at the edges of the crystals. Subsequent sublimation of the salt results in the clustered polymeric material revealing the outline of the crystal ghosts. At 2 mM salt individual polymeric strands were observed (Fig. 5).

A portion of Fraction 13 was treated with xylanase in order to confirm that the polymeric material observed by AFM was purely arabinoxylan. The enzymatic digestion was monitored by SEC, measurement of reducing sugars, and by AFM. SEC fractionation showed the reduction in molecular weight by xylanase treatment (Fig. 6) and this was accompanied by an increase in the concentration of reducing sugars from 0.04 to 1.2 μmol of xylose. Xylanase treatment was

found to progressively reduce the size of the strands observed by AFM: after 5 min digestion the number average chain length had been reduced to 42% of the original chain length and, after 10 min incubation, no chains were visible on the substrate. These studies confirmed that the material under investigation was arabinoxylan.

Having established that the polymeric material was arabinoxylan, individual SEC Fractions (13–16) were then imaged and analysed. The contour lengths of the individual molecules were measured and are displayed in Fig. 7. In determining the molecular lengths individual molecules were defined as strands that were not entangled with, or overlapping other strands, and which lay entirely within the scanned area. A total of 1200 molecules were measured. The data is displayed in Fig. 7. Successive fractionation by SEC should lead to a decrease in molecular weight: As expected the peak length is observed to decrease from Fraction $13 \rightarrow 16$. However, each fraction showed a spectrum of chain lengths that could be well represented by a skew distribution.

The majority of arabinoxylan molecules were found to be linear chains, but a small fraction of the polymers were branched. Single linear chains (S) and branched chains (B) are illustrated in Fig. 5. Fig. 5(a) also shows an example of a more complicated 'H' shaped branch structure (H). Branched structures were distinguished from overlapping molecules by measuring the heights of the chains. In general the heights of the chains were about 0.4 nm rising to 0.8 nm when two chains crossed over one another. At genuine branch points the height remained unchanged. For all the samples examined the total fraction (relative number) of branched chains was about 15%, with about 1% of these branched chains containing more than one branch. Examples of branched structures are shown in Fig. 5. Branches appear to be randomly located on the backbones and the likelihood of a branch occurring on a particular chain was shown to increase with increasing total contour length of the molecule (Fig. 8). Individual arabinose sugars are too small to be observed attached to the backbone and, given that only terminal arabinose residues were observed by methylation analysis, the branches cannot be long arabinose chains. The fact that xylanase treatment of Fraction 13 completely degraded all the chains suggests that the branches are composed of $(1 \rightarrow 4)$-linked xylose residues.

The majority of the chains are linear, highly extended structures. This extension is unlikely to result from shearing due to deposition, or interaction with the mica, because similar extended structures were observed when arabinoxylan samples were imaged by tapping mode AFM under buffer.[16] Generally for random coil polymers the AFM images reveal time averaged spherical structures,[36] whereas helical polysaccharides are static extended structures. In some instances it has been possible to confirm the existence of the helix by direct visualisation[37] of the pitch of the helix. Studies[38–41] on stiff polymers such DNA and succinoglycan have shown that values of the persistence length can be determined from AFM images. Using the procedure described by Balnois and coworkers[41] the Kuhn statistical segment length for the adsorbed arabinoxylans was found to be 128 nm. The magnitude of this value suggests that the arabinoxylans are adsorbed onto the mica as helical structures.

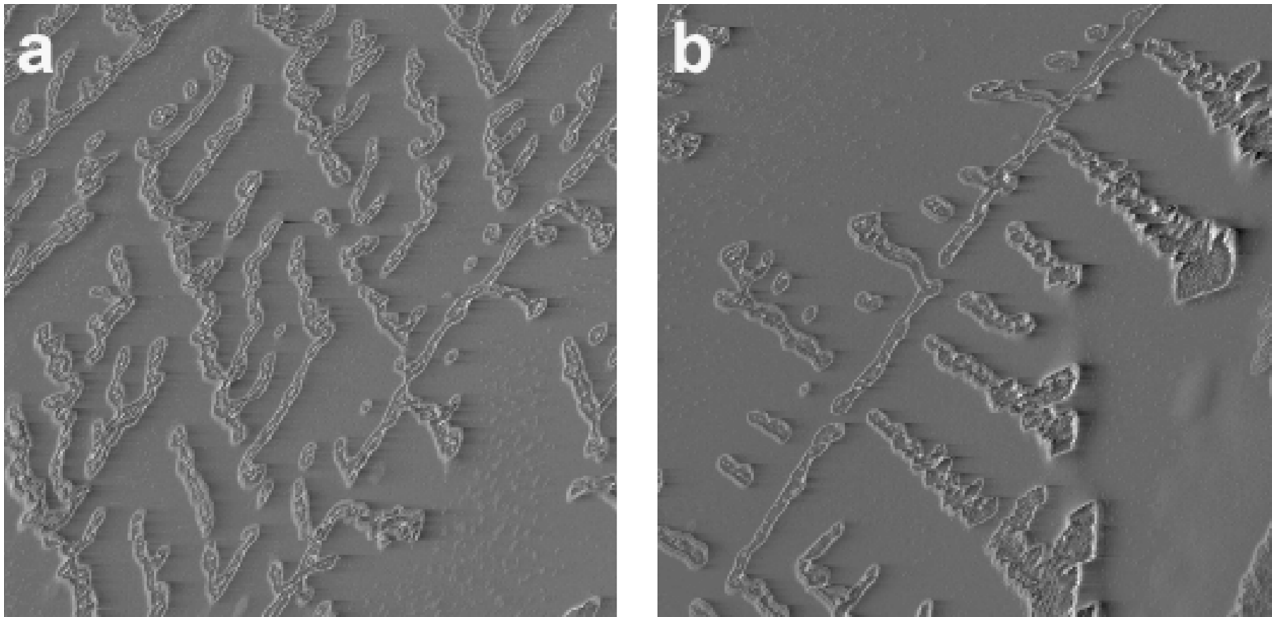

Fig. 4. AFM images (a and b) showing dendritic structures observed when the arabinoxylans were deposited onto mica from high salt (>2 mM) ammonium bicarbonate buffered solutions. Image sizes (a and b) 2×2 μm.

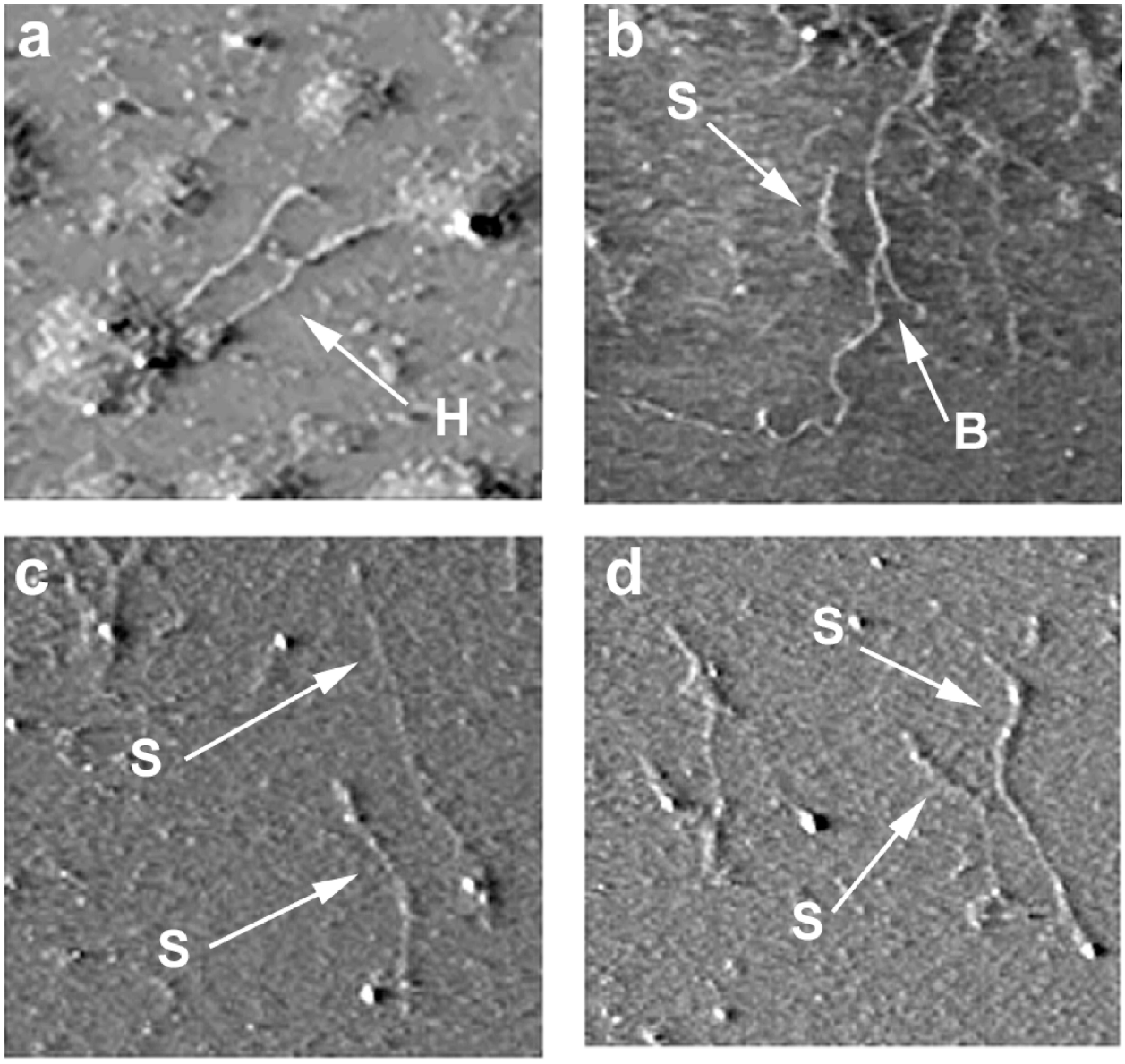

Fig. 5. AFM images showing the structural diversity of arabinoxylan molecules. Samples were deposited from 5 μg mL^{-1} solutions in 2 mM ammonium bicarbonate buffer. The images are error signal mode images and the images sizes are 1×1 μm. (a) shows an unusual 'H' shaped branch structure; (b) shows a linear (S) and a branched (B) molecule (arrowed) lying close to an aggregate; and (c and d) shows linear (S) molecules (arrowed).

The helical structures of arabinoxylans have been discussed by Atkins.[42] Thus the measured contour lengths can be converted into molecular weights by using the axial rise per sugar repeat unit of 0.5 nm, and an averaged mass per unit length based on the chemical composition of the chain (arabinose–xylose: 0.55:1). This analysis revealed a range of molecular weights for the fractions ranging from 6.8×10^4 to 9.4×10^5, with an overall number average molecular weight for the total sample of 3.6×10^5, with a standard deviation of $\pm 1.5 \times 10^5$. The weight average molecular weight was found to be 4.6×10^5. Given that the present fractionation procedures eliminates lower molecular weight material then this molecular weight value is consistent with the reported light scattering data[6–8] on arabinoxylans.

If the AFM images are considered to represent the conformation in solution, then the current data suggests that the arabinoxylans should be considered as high molecular weight worm-like chains. Although there have been suggestions[5] that arabinoxylans are rod-like in solution, the consensus opinion from light scattering studies[7,8] suggests that they are actually semi-flexible coils. Instead of estimating the stiffness of the polymers directly from the AFM images, it is possible to use the contour length determined from the AFM images to estimate the stiffness in solution. This estimate of stiffness can be obtained by comparing infor-

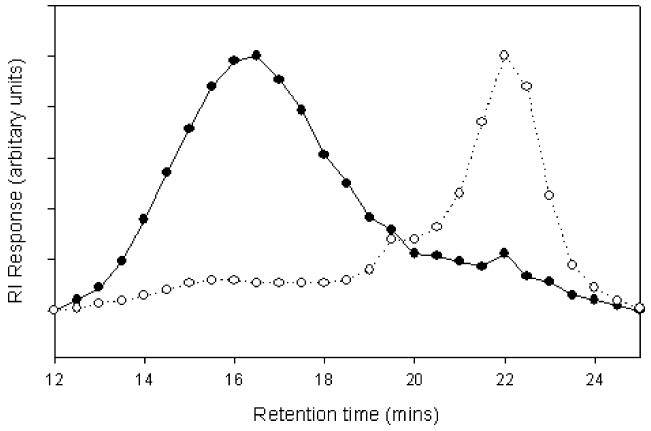

Fig. 6. Data illustrating the effect of xylanase treatment on the arabinoxylan. SEC curves obtained before (●) and after (○) xylanase treatment. Experimental conditions are the same as for the data shown in Figs. 2 and 3.

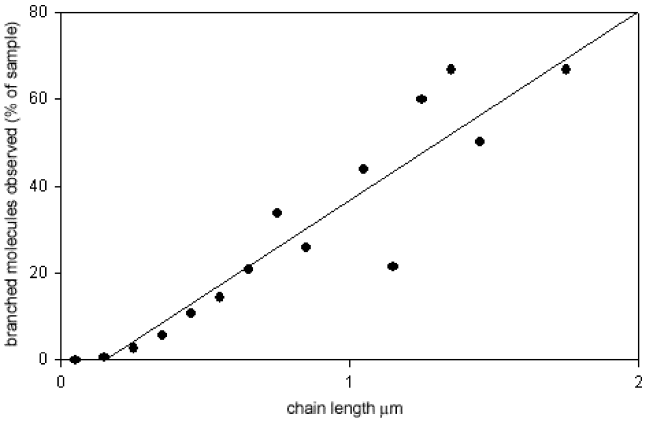

Fig. 8. Dependence of the percentage of branched molecules on the total contour length of the molecules. Data collected for 1200 molecules shows a strong correlation ($r^2 = 0.87$) between contour length and the probability that a polymer is branched.

mation obtained by the AFM analysis and SEC fractionation. The pullulan standards are highly flexible and will overestimate the molecular weight of the arabinoxylan. Extended arabinoxylan chains will occupy a larger volume than the random coil pullulans of 'equivalent mass'. To estimate the stiffness of the chains it is necessary to assume that, in the first instance, the chains are linear. The small fraction of branched chains will lead to a small underestimate of chain stiffness. SEC separates by volume. If the pullulan is pictured as a coil composed of n_0 segments of length l_0, where the contour length of the chain is $L = n_0 l_0$, then the radius of gyration (R_G) of the chain will be given by $[R_G = (n_0 \alpha^2 l_0^2)/6]^{1/2}$, with α dependent on segment–segment and segment–solvent interactions that determine the swelling of the coil.[43] Using the model adopted to analyse the light scattering data[7,8] then the arabinoxylan can be approximated by a stiff coil with n_K Kuhn statistical segments of length l_K. The contour length of the chain $L_K = n_K l_K$ and the radius of gyration will be $[(n_K \alpha^2 l_K^2)/6]^{1/2}$. Here it has been assumed that for both polysaccharides in water α will be the same. If the polymers separate at equivalent volumes then the radii of gyration should be equal, and hence $n_0 l_K^2 = n_K l_K^2$. The number average chain length of 570 nm for the arabinoxylans elutes at a pullulan molecular weight of $M = 2 \times 10^6$. If the mass per unit length (m_0) for pullulan is 324 nm^{-1}, then $m_0 L = M$ and $l_K = l_0 M/(m_0 L_K) = 10.8 l_0$. Assuming free rotation about the

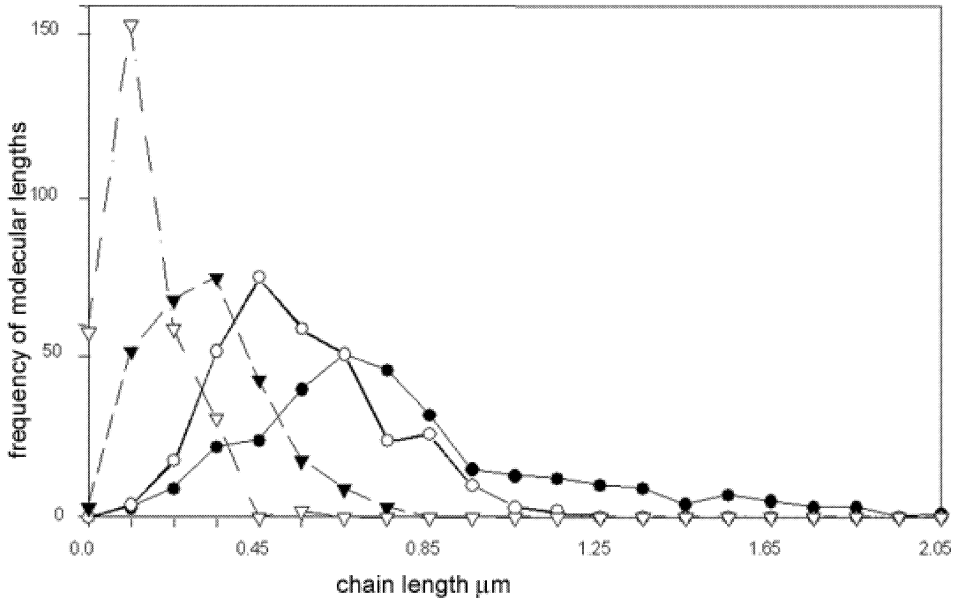

Fig. 7. Distributions of polymer contour lengths obtained for the SEC subfractions of the arabinoxylans Approximately 300 molecules were measured for each subfraction. (●) Fraction 13; (○) Fraction 14, (▼) Fraction 15 and (▽) Fraction 16.

(1→6)-linkage in the pullulan backbone, and taking the length of the maltotriose repeat unit as $l_0 = 1.5$ nm, suggests an estimate for $l_K = 16$ nm. This value is consistent with the values reported for arabinoxylans in solution determined from light scattering studies,[6–8] and is consistent with a locally stiff, semi-flexible coil structure in solution.

The discrepancy in the l_K values estimated for the arabinoxylans in solution, with that calculated for the deposited molecules, suggests that the act of depositing the polysaccharides onto mica promotes formation of the helical structure. This conclusion is important because it demonstrates that it is not possible to draw conclusions about the conformation or stiffness of polysaccharides directly from AFM images, unless it can be demonstrated that the conformation seen in the AFM is the same as that in solution. This would be true for AFM or electron microscopy unless the sample preparation had been designed to trap the solution conformation, or unless there was independent physical chemical evidence for the existence of the ordered conformation in solution. However, conversely, it is due to the formation of this ordered structure that it is possible to calculate the size, and observe the branching of the arabinoxylan molecules by AFM. The stiffness of the ordered structure inhibits motion of the polymer on the substrate allowing the details of the structure to be imaged. This is probably also true of the AFM images reported[12,27] for pectin molecules that revealed branching of the molecules.

In previous studies of arabinoxylans under buffer[16] the molecules appeared to move and change shape on the mica surface. These changes in shape, and the appearance of 'gaps' in the molecular structure, were attributed to desorption and readsorption of segments of the molecules during scanning. The present studies suggest that this de-sorption of segments of the molecules may involve partial denaturation of regions of the helix, allowing these portions of the chain to adopt their semi-flexible structure in solution.

The present studies have revealed branching of the arabinoxylan molecules. The fact that the molecules are completely degraded by xylanase suggests that the backbone and branches are both composed of β-(1→4)-linked D-xylose residues. If the branches are linked directly by glycosidic linkages then it will be very difficult to detect the nature of these linkages. Only a small fraction of the molecules are branched and the level of branching for these molecules is low. Hence the number of branch points will be too small to detect by conventional methylation analysis. Thus it is not possible to rule out the possibility of xylose–xylose or xylose–arabinose–xylose linkages as branch points.

One possibility is that the arabinoxylans may be cross-linked by di-ferulic acid residues. The arabinoxylans have been shown to contain di-ferulic acid and these linkages may be responsible for the branching of the polymers. Chemical analysis revealed the presence of 5,5′, 8-O-4′ and 8,5′ linkages. The content of dimers corresponds approximately to one linkage per 1400 backbone sugars, which is well in excess of the observed number of branched points. If ferulic acid dimers are responsible for branching then it is perhaps surprising that so few branched molecules have been observed. This also suggests that some ferulic acid dimers may play some previously undetected role, possibly in polymerising smaller arabinoxylan fragments into the linear chains visualised in the present study. Clearly there is a need for further studies to determine the distribution of ferulic dimers within and between molecules, and to test their role in cross-linking arabinoxylan structures. The location of specific structural features on individual chains requires imaging markers attached to those features along the chain. The original extracts of arabinoxylans from wheat flour contained protein. Fig. 9 shows AFM image of this protein (P) attached to arabinoxylan chains (AX). Clearly a protein specifically attached to a given structural feature could act as a probe for the desired structural element. If enzymes

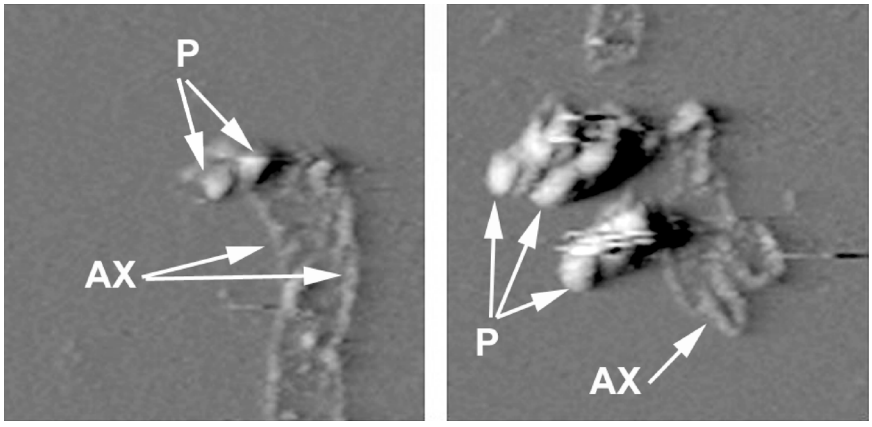

Fig. 9. AFM images showing protein (P) associated with arabinoxylan (AX) molecules. The pictures are error signal images with scan sizes of 500 × 500 nm. Note the protein may be aggregating the arabinoxylan chains.

could be modified to permit binding, but to inhibit hydrolysis, then such modified enzymes could be used as molecular probes to ascertain the location and function of dimeric ferulic acid residues in arabinoxylans. Similar such probes could be used to map other irregular structural features such as arabinose distribution, or the presence of block structures. It is intended to pursue this 'molecular mapping' line of research in order to further characterise the arabinoxylans and also other complex cell wall polysaccharides.

3. Experimental

3.1. Sample preparation

Water soluble arabinoxylans were isolated from wheat flour (variety *Soisson*; Unilever) as previously described[44] and stored as a freeze dried powder. For SEC and AFM the samples were dissolved in 2 mM ammonium bicarbonate buffer at a concentration of 2 mg mL^{-1}. Protein was removed by overnight incubation with protease (30 °C, immobilised protease K column: *Tritirachium album*; Sigma–Aldrich, Poole, Dorset, UK).

3.2. Chemical analysis

Neutral sugars were identified and estimated quantitatively by acid hydrolysis of the polysaccharide, followed by conversion of the products to alditol acetates, and their separation and identification by gas chromatography (GC).The procedures followed are those described by MacCormick and coworkers.[45] About 5 mg of vacuum dried material was weighed into a glass tube, 1 mL of 2 mol L^{-1} trifluoracetic acid was added, and the mixture heated in a sealed tube for 1 h at 120 °C. The acid was evaporated at reduced pressure (35 °C) and the residue treated with 50 μL of concentrated ammonia. 2-Deoxyglucose (200 mg) was added as an internal standard, followed by addition of 15 mg of $NaBH_4$ in 100 μL of 3 mol L^{-1} NH_3, and the mixture kept at 30 °C for about 1 h. The sample was then acidified with glacial acetic acid, cooled in ice and 1-methylimidazole (0.45 mL) and acetic anhydride (3 mL) added to the mixture. This sample was kept at 30 °C for 30 min, cooled in ice and then partitioned between water (5 mL) and CH_2Cl_2 (2×2 mL). The combined organic layer was washed with water (5×5 mL), separated and analysed by GC.

GC analysis was carried out in triplicate on an HP 5800 series gas chromatograph, with a Thames Rtx-225 column (0.32 mm × 30 m). The carrier gas was helium at a flow rate of 3.0 mL mm^{-1} and the temperature programme was: 180 °C (1 min), +2 °C min^{-1} (12.5 min), 205 °C (30 min). Derivatives of external sugar standards were used to identify analytes and to calibrate response factors.

Linkages were determined by methylation analysis using a modification of the method of Ciucanu and Kerek.[46] Approximately 3–6 mg of vacuum dried material was suspended in Me_2SO (1 mL) by degassing and sonication under argon (50 °C, 3 h). Dry, powdered NaOH (0.25 g) was added to the sample, followed by further degassing and sonication. The sample was then cooled to 10 °C and 2 mL of MeI was added, the sample shaken and then sonicated (20 °C, 2 h). Excess CH_3I was evaporated and the mixture diluted with water (10 mL). The sample was eluted on a reverse-phase mini-column (Waters sep-pak C_{18} cartridge), washed with water, extracted with MeOH then $CHCl_3$–CH_3OH (1:1), and then evaporated to dryness. The product obtained was hydrolysed and converted to partially methylated alditol acetates (PMAAs) using the methodology described above. The mixture was separated using the same GC with the temperature programme: 55 °C (2 min), +45 °C min^{-1} (1.9 min), 140 °C (2 min), +2 °C min^{-1} (35 min), 210 °C (40 min). Analytes were identified by a comparison of their retention times with those of external and internal standards. The flame ionisation detector (FID) signal output was used to measure peak areas, and these were then converted to relative molar quantities using effective carbon response factors.[47] Identities of PMAAs were confirmed by GC–MS using an identical GC linked to a Fisons Analytical Trio 1S mass spectrometer (MS). Electron-ion (EI) mass spectra were obtained using a source temperature of 200 °C and an ionisation potential of 70 eV.

The ferulic acid and di-ferulic acid content of the arabinoxylans were estimated by HPLC[48] following saponification with NaOH (1 M, 16 h, room temperature and pressure).

Total protein content was determined using a Coomassie dye-binding assay[49] with bovine serum albumin (Sigma Aldrich) as a standard.

3.3. Size-exclusion chromatography (SEC)

Untreated and protease treated arabinoxylan samples were dissolved in 2 mM ammonium bicarbonate buffer at a concentration of 2 mg mL^{-1}. This buffer concentration was found to be optimum for subsequent AFM measurements. Samples (100 μL) were loaded onto a Polysep GFC P5000 column (Phenomenex, Macclesfield, Cheshire, UK), eluted at 0.5 mL min^{-1}, and the eluant monitored using a HP1047A refractive index detector (Hewlett Packard, Bristol, UK). A series of pullulan samples (Shodex P-82, New York, USA; molecular masses 166×10^4, 38×10^4, 18.6×10^4, 10×10^4, 4.8×10^4, 2.37×10^4, 1.22×10^4 and 0.58×10^4) were used as reference standards. Fractions were collected at 1 min intervals (Fig. 2).

3.4. Atomic force microscopy (AFM)

Fractions from the SEC column were concentrated by rotary evaporation to about 5 μg mL^{-1}. Samples (2 μL) were drop deposited onto freshly cleaved mica sheets. Typically samples were allowed to stand in air for about 10 min. The mica substrates were placed in the liquid cell of the AFM and imaged under redistilled butanol. Images were obtained using an East Coast Scientific AFM working in the dc contact mode. The apparatus was calibrated using etched silicon Ultrasharp calibration gratings from NTMDT, Zelenograd Research Instituite of Physical Problems, Moscow, Russia. The grating TGX01 was used for xy calibration and the gratings TGZ01-3 used for height calibration corresponding to step sizes of 25.5, 106 and 512 nm. Butanol was used to eliminate capillary condensation and to permit optimisation of the imaging force. SiN_3 cantilevers were used with a quoted force constant of 0.38 N m^{-1}. Both topographic and error signal mode images were recorded. Analysis of contour lengths was made using Image Tool (Uthscsa, San Antonia, TX, USA). To obtain a distribution of chain lengths approximately 1200 molecules were analysed. The analysis was performed on a series of scans and measurements were not made on aggregated molecules, molecules that overlapped one another and were confined to molecules contained completely within the scan area. Measurements of height were used to discriminate between branched and overlapping molecules. The persistence lengths of the adsorbed molecules were calculated using the methodology described by Balnois and coworkers.[41] The ECS software plane fits and re-normalises the AFM images. Image contrast was optimised using Adobe Photoshop software.

3.5. Enzymatic treatment

Xylanase activity was measured in the following manner. Arabinoxylan samples (2 mg mL^{-1}) were dissolved in 2 mM ammonium bicarbonate buffer and incubated with the xylanase (10 μg mL^{-1}, *Pseudomonas fluorescens* subspecies *cellulosa*) for 15 min at 30 °C. The reaction was stopped using dinitrosalicylic acid (DNS) reagent and the colour developed by boiling for 5 min. The absorbance was measured at 550 nm and the increase in reducing sugars was quantified using a xylose calibration curve (0–180 μg mL^{-1}).

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