Fingerprinting polysaccharides with single-molecule atomic force microscopy

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We report the use of an atomic force microscopy (AFM)-based force spectroscopy technique to identify, at the single-molecule level, the components of mixtures of polysaccharides. Previously, we showed that the elasticity of certain types of polysaccharides is governed by force-induced conformational transitions of the pyranose ring. These transitions produce atomic fingerprints in the force–extension spectrum that are characteristic of the ground-energy conformation of the pyranose ring and the type of glycosidic linkages. Using this approach we find that commercially available agarose and λ-carrageenan contain molecules that, when stretched in an atomic force microscope, produce a force spectrum characteristic of α–(1→4) D-glucans. We have identified these molecules as amylopectin or floridean starch, a storage polysaccharide in algae. Our methodology can identify individual polysaccharide molecules in solution, which is not possible by any other spectroscopic technique, and therefore is an important addition to the arsenal of analytical techniques used in carbohydrate research.

Most polysaccharides are obtained by simple extraction from the organism that produces them. Identification of the extracted polysaccharide and evaluation of its purity constitute a complex process that involves determining bulk sugar composition by chromatography and the type of glycosidic linkages by nuclear magnetic resonance (NMR) spectroscopy[1,2]. Here we propose a much simpler methodology for identifying the composition of polysaccharide samples by mechanically stretching their individual molecules in solution with an atomic force microscope, and recording their unique force–extension spectra.

The basis of single-molecule force spectra is the measurement of the elasticity of the molecule. The elasticity of polymers is entropic in origin and can be described by the freely jointed chain (FJC) or the wormlike chain (WLC) models[3–7]. Recent progress in techniques that allow mechanical manipulations of single polymer molecules[8–11] resulted in a number of spectacular measurements revealing that biopolymers (DNA, proteins, polysaccharides) rarely display purely entropic elasticity. Those studies showed that, at critical forces, many biopolymers undergo force-induced conformational transitions that greatly affect their elasticity[5,12–20].

By combining the AFM-based single-molecule force spectroscopy technique[8–11,13–20] with computational chemistry, we demonstrated recently that the elasticity of some polysaccharides (e.g., amylose, dextran, pullulan, and pectin) is governed by conformational transitions of the pyranose ring[18,19]. Force spectroscopy measurements have created a growing library of force spectrograms that can be used as molecular fingerprints to identify individual polysaccharides in complex mixtures. Here we demonstrate this approach to identify, at the single-molecule level, foreign molecules in samples of common commercial polysaccharides for which the composition is not easily determined[21-30].

Results and discussion

Overview of polysaccharide elasticity. Shown in Figure 1 are typical force–extension relationships (force spectra) of five different polysaccharides obtained by vertically stretching their single molecules in solution with an AFM instrument[13,14,17–20]. The force spectrum obtained on methylcellulose[21] displays the shape anticipated for a simple entropic polymer (FJC) (Fig. 1). However, all other molecules display pronounced deviations from the entropic elasticity visible as sudden changes in the curvature of their force–extension curves. Amylose and dextran[21] show a single transition that occurs at ~280 and ~850 pN, respectively, and that overstretches these molecules by ~17–19% of their final length[18]. Pullulan's spectrum is a linear combination of the spectra of amylose and dextran[18], in agreement with its structure being a combination of both. We demonstrated that the additional elongation of amylose, dextran, and pullulan involves force-induced flipping of the pyranose rings from their ground-energy chair ($^4C_1$) conformation to the boatlike conformation[18]. Similarly, we explained the two transitions in pectin at ~300 and 900 pN (Fig. 1) in terms of the force-induced two-step chair inversion transition in the α-D-galactopyranuronic acid ring, which increases the separation of the O_1O_4 oxygen atoms by a total of ~20% (ref. 19). Those results allowed us to develop a simple rule that relates the number of conformational transitions in force spectrograms of polysaccharides with the sum (per residue) of glycosidic and aglycon bonds in the axial (a) position: amylose (1a, 4e) has one transition; dextran (1a, 6x) has one transition; pectin (1a, 4a) has two transitions; and cellulose (1e, 4e) has none (e stands for an equatorial orientation, whereas x denotes a bond that is neither axial nor equatorial). Initial calculations of the pyranose ring structure placed under a stretching force readily predicted these transitions and their magnitude[18,19,31]. Furthermore, it was suggested that cellulose could undergo a forced compression from chair to boat that has not yet been observed[31].

Fingerprints of elasticity of amylose and cellulose. The contour length of polysaccharide segments stretched by the AFM varies over a wide range. This is because the AFM tip picks molecules randomly with respect to their ends (Fig. 2A, C). The end-to-end distance (extension) of a stretched FJC polymer determined at a given force is

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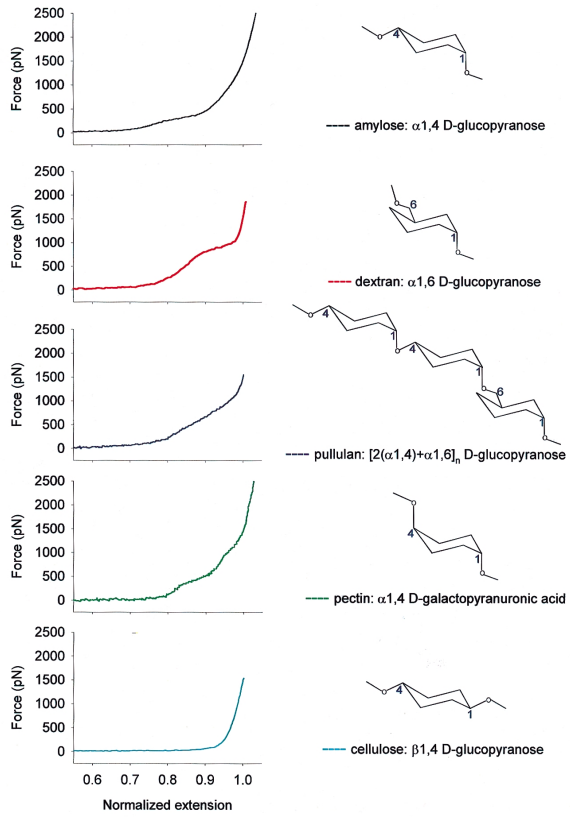

Figure 1. Fingerprints of elasticity of linear polysaccharides obtained by AFM. On the left, force–extension relationships of single polysaccharides in solution obtained by vertically stretching the molecules between a substrate and an AFM cantilever. On the right, the (simplified) monomer and the type of glycosidic linkages in the polysaccharide for which the force–extension curves are displayed on the left. All force–extension curves, except that of cellulose, display marked deviations from the purely entropic elasticity. These deviations are the "fingerprints" characteristic of monomers and linkages. All extensions were normalized by the molecule length determined at a force of 1,500 pN.

proportional to its contour length, L_c (refs 3–5, 13). Therefore, force spectrograms of FJC polymers that differ only by their contour length L_c superimpose when their extensions are normalized by the end-to-end length determined at a common force. We exploit this property of FJC polymers when we compare the recordings obtained from fragments of different lengths (various L_c). In Figure 2B and D we show two families of normalized single-molecule recordings obtained from amylose (A, B) and methylcellulose (C, D). We immediately note that all amylose recordings display the characteristic length transition (Fig. 2A), whereas none of the methylcellulose recordings display any significant deviation from entropic elasticity (Fig. 2C). We also observe that when we normalized the recordings from Figure A and C, the resulting traces superimposed reasonably well. We conclude that all the molecules in Figure 2A and B had a similar fraction of axial bonds, and that none of the molecules in Figure 2C and D had a detectable fraction of axial bonds. These observations are consistent with the known structures of amylose and cellulose. In addition, these results suggest that the samples we studied were pure, that is, did not contain foreign polysaccharides with different glycosidic linkages.

Polysaccharide mixtures. To test whether the AFM force spectroscopy can be used to identify the composition of mixtures of polysaccharides, we prepared a solution containing an approximately equal number of molecules of carboxymethylcellulose (CMC), carboxymethylamylose (CMA), and dextran. Table 1 summarizes the results of our AFM measurements performed on this mixture. On the average, 1 out of every 10 trial contacts between the AFM tip and the substrate results in a force spectrogram that is unambiguously interpretable. In the other 9 trials we either picked up no molecules at all (the most common case) or we picked up too many molecules of similar length that produced a complicated, uninterpretable spectrogram. Therefore, the efficiency of the method is ~10%. We could readily identify all three components of the polysaccharide mixture (Table 1). However, the frequency with which we observed these polysaccharides did not match their relative concentrations in solution, and depended on the substrate used to adsorb the molecules (Table 1, compare glass to gold). Hence, our method cannot yet provide reliable quantitative information regarding the composition of polysaccharide samples. Nevertheless, this method should be of immediate practical utility, providing qualitative information that is difficult to obtain by other methods.

Single-molecule force spectroscopy on agarose. Agarose is a linear galactan with mixed glycosidic linkages, namely α-(1→3) and β-(1→4) (ref. 21). Its idealized, canonical structure is shown in the inset in Figure 3A. Both the O-3 substituted monomer and the O-4 substituted 3,6 anhydro- α-L-galactopyranosyl unit are in the regular $^4C_1$ chair conformation[1,21]. In this conformation, all glycosidic and aglycon bonds are equatorial and are not expected to undergo a conformational transition under force. Hence, we anticipated that the force–extension relationship of agarose would follow simple entropic elasticity (FJC). Figure 3A shows normalized force–extension curves obtained on agarose molecules. In contrast to amylose or cellulose, these recordings display a variety of shapes and they generally do not overlap. However, there is a significant fraction of the recordings (marked in gray, 55% of the total number of recordings) that overlap well and are representative of simple entropic elasticity. We conclude that these recordings represent molecules with a structure that is consistent with the canonical structure of agarose (inset). Because most of these recordings were obtained on samples with a concentration ~100 times lower than the critical gelation concentration of agarose (~0.1% wt/vol; ref. 22), we argue that they reflect the elasticity of single agarose chains.

The rest of the recordings show significant deviations from the entropic elasticity, and they overlap less regularly. We conclude that these molecules must have a very significant number of axial bonds. It is known that in native agarose not all the ether rings on the 3,6-anhydro-α-L-galactopyranose residues are intact. These noncanonical residues relax to the chair conformation that has axial bonds at both C1 and C4. Such bonds can produce deflections in the force spectrogram[19], with the width of the deflection proportional to the number of residues with the axial bonds. However, Jol *et al.*[2], using ^{13}C NMR technique, determined that agar from *Gelidium* (our agar comes from *Gelidium*) contains no more than 3 mol% of these noncanonical residues. These residues, when stretched, could increase the contour length of agarose only by ~0.6%. However, the width of the deflection in the recordings of Figure 3A exceeds 15% of the contour length; therefore, deviations in agarose structure cannot explain our results.

Agarose molecules are involved in homophilic interactions leading to gel formation[21]. It is possible that occasionally we stretch bun-

Table 1. Frequency of AFM recordings in the 1:1:1 mixture of CMA, dextran, and CMC[a]

Substrate	CMA	Dextran	CMC	No. of trials
Glass	53 (62.4%)	4 (4.7%)	28 (32.9%)	790
Gold	5 (3.9%)	121 (94.5%)	2 (1.6%)	1,400

[a]First number is number of recordings; percentage in parentheses is percentage of successful events.

dles of two or more interacting agarose chains, and breaking these complexes could possibly produce some irregular force–extension curves[17]. We note, however, that eight of these recordings (26% of all recordings, marked in blue in Fig. 3A) display a complex shape distinguishing them from the rest, and they have a very pronounced transition that is strikingly similar to the transition in amylose (Fig. 2A, B). Moreover, in this group, all the recordings overlap perfectly in every detail, even though they were obtained from molecules with contour lengths varying between 100 and 500 nm. The fact that these recordings scale so well, and display shapes very different from the characteristic discontinuous transition generated by breaking intermolecular complexes (e.g., xanthan[17]) contradicts the above-stated conjecture that they could be obtained on agarose complexes. Instead, we hypothesize that these recordings represent foreign molecules (a contaminant) structurally related to amylose. If these molecules are indeed α-(1→4)-D-glucan contaminants, they should be readily oxidized by periodate, and the horizontal deflection in the force spectrogram from these molecules should disappear as the glucopyranose rings are cleaved[18,19]. Periodate oxidation does not affect agarose because agarose does not have vicinal diols. Figure 3C shows the force spectrograms obtained on periodate-oxidized samples of agarose. It is striking that after oxidation the amylose-like fraction was completely eliminated from agarose (n = 108 recordings). This result supports further the assumption that the recordings of the amylose-like fraction cannot come from agarose complexes, because such complexes would not be affected by periodate.

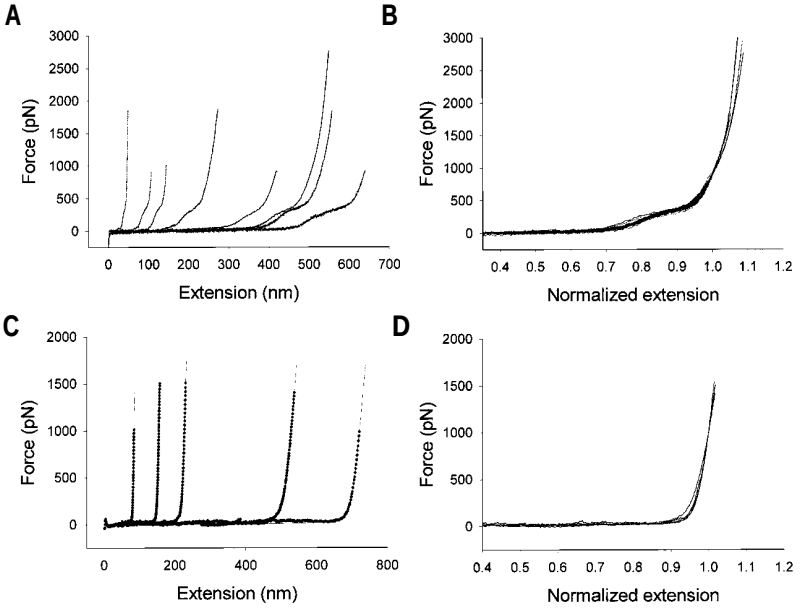

Figure 2. AFM force spectra obtained on amylose (A, B) and methylcellulose (C, D). In (A) and (C) we show force–extension relationships obtained by stretching fragments of different lengths. In (B) and (D) these relationships were normalized by the molecule's length determined at 900 pN (B, the number of recordings, n = 13) and 1,000 pN (D, n = 5). The thin solid lines in (C) are the fits of the FJC model to the data. The recordings were obtained from different samples, using different cantilevers, and after normalization they are found to superimpose, indicating (1) good reproducibility of our measurements, (2) stretching of single molecules, and (3) no foreign polysaccharides in these samples.

Force spectrum of l-carrageenan. λ-carrageenan, like agarose, is a linear galactan[21]. Its idealized simplified structure is shown in the inset of Figure 3B. In contrast to agarose, the O-4 substituted monomer has both glycosidic and aglycon bonds in the axial orientation. The O-3-substituted monomer has both glycosidic and aglycon bonds in the equatorial orientation. A chair inversion of the axial linkages of λ-carrageenan will extend the molecule under force[19]. Because only half of all monomers have axial bonds, we anticipated the force–extension relationship of λ-carrageenan to display a deflection, albeit this deflection should be about half (~10% of L_c) of the transition width in pectin (~20% of L_c, ref. 19), a homopolymer made entirely of $^{4a}C_{1a}$ monomers. In Figure 3B we show 24 normalized force–extension curves obtained on λ-carrageenan. As anticipated, all the recordings do display significant deviations from the entropic elasticity but they overlap poorly. We note that the recordings in Figure 3B group into two families. The recordings marked in black (n = 19, 80% of the recordings) all have a similar shape, are fully reversible, and superimpose well in the high-force region, but the length of the plateau varies. Like agarose, the real structure of λ-carrageenan deviates somewhat from its canonical form (Fig. 3B, inset) in that some of the O-4 substituted residues may be 3,6-anhydro-galactoses with the equatorial glycosidic and aglycon bonds, and the sulfation pattern may be less regular. Jol *et al*[2]. determined the content of 3,6-anhydro-galactose residues in λ-carrageenan to be <5 mol%. Based on this content we estimate that the width of the transition should be reduced (equatorial bonds) from ~10% of L_c (canonical structure) to ~9%. However, our measurements show that the transition width is actually >10% of L_c (as high as 25%), not shorter. Therefore, the structural variations cannot explain the range of the transition widths in this group of recordings.

In the second group, marked in red (n = 5; 20% of the recordings), all the recordings have identical shapes that are very different from the recordings marked in black: they superimpose in every detail in the whole force range; their transition is fully reversible; and they scale perfectly with the molecule length. In addition, we note that their shape is strikingly similar to that of amylose (Fig. 2A, B) and the amylose-like fraction in agarose (Fig. 3A). We hypothesize that similar to the amylose-like fraction in agarose, they represent foreign molecules (a contaminant) structurally related to amylose. In Figure 3D we show the force spectrograms obtained on samples of λ-carrageenan oxidized by periodate. Similar to the results on agarose, the amylose-like fraction was eliminated from λ-carrageenan (n = 70 recordings). As anticipated, the majority of molecules in the λ-carrageenan sample were not affected by periodate treatment because their force spectrograms (recordings marked in black, n = 50) still display the characteristic plateau the width of which is similar to that observed in the native carrageenan sample (inset). Interestingly, a new fraction of molecules whose force spectrograms are transition-less is now identified (Fig. 3D; marked in red, n = 20). These must be the glucan molecules whose pyranose rings were cleaved by periodate.

Floridean starch. To check whether the foreign molecules detected by our AFM measurements in agarose and λ-carrageenan are related to α-(1→4)-D-glucans we compared their force–extension curves to that of amylose and related glucans: CMA and amylopectin, a branched α-(1→4)-D-glucan (Fig. 4). It is remarkable that all the recordings in this figure superimpose very well in the whole force range up to the highest force reached of 2,500 pN. Thus we conclude that the foreign molecules of agarose and the foreign molecules of λ-carrageenan are very likely to have the backbone linkages identical to those of α-(1→4)-D-glucans.

Where do these glucans come from? It is well known that red algae, the source of carrageenans and agarose, also store in the form of intracellular-layered granules, a variety of starches (glycogen, floridean starch[2,23–28]). These starches are branched D-glucans with backbone linkages similar to those of amylose, namely α-(1→4). Therefore, it is likely that floridean starch gets co-extracted with

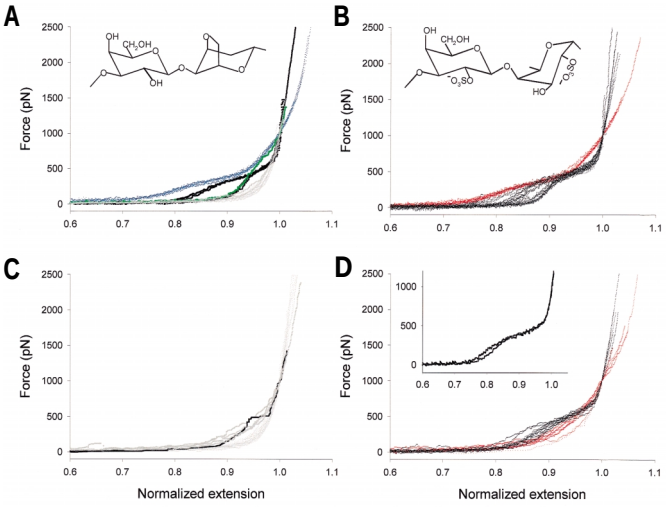

Figure 3. Force spectra obtained from native agarose and λ-carrageenan show multiple polysaccharide fingerprints. (A) Agarose recordings can be grouped into three major fractions marked in gray (number of observations, n = 17; 11 shown), black (n = 4), and blue (n = 8). The recording marked in green was a single measurement that did not belong to any of the main fractions. The inset shows the monomers and the glycosidic linkages in the canonical structure of agarose (α-(1→3), β-(1→4)). (B) The recordings from native λ-carrageenan show two distinct polysaccharide fingerprints. These recordings can be grouped into two fractions marked in black (n = 19) and red (n = 5). The inset shows the monomers and the glycosidic linkages in the canonical structure of λ-carrageenan (α-(1→3), β-(1→4)). Note the similar shape of the recordings marked here in red and marked in blue in Figure 3A. (C) Force spectrograms obtained on agarose sample subjected to oxidation by periodate (10 mM, 24 h). Only 1 out of 108 recordings (marked in black) displayed an atypical transition. All extensions were normalized by the length determined at 1,000 pN. (D) Force spectrograms obtained from λ-carrageenan sample subjected to periodate oxidation (5 mM, seven days). Note the absence of the recordings with the characteristic transition that were marked in red in (B) and appearance of a new fraction of transition-less recordings marked here in red. A total of 50 out of 70 recordings displayed a pronounced transition, whereas 20 did not display a discernible transition. The inset compares two recordings with the longest transition extracted from the fractions marked in black in (B) and (D). This comparison indicates that periodate does not affect significantly the width of the transition in λ-carrageenan. All extensions were normalized by the length determined at 1,000 pN.

agarose and carrageenan and is not fully eliminated during purification. This hypothesis is supported by a recent study of the composition of a variety of carrageenans and agars by Jol *et al*[2]. who used several analytical techniques, including high-performance liquid chromatography (HPLC) and ^{13}C NMR and found that agar derived from *Gelidium* contains up to 11 mol% of glucose (Table 4 in ref. 2). Jol *et al.* proposed that this glucose comes from "a starch component typically found in carrageenans and usually referred to as Floridean starch[2]". It is significant that agarose used in our study is also derived from *Gelidium*. Our methodology indicates that ~20–26% of molecules in the agarose and λ-carrageenan samples are glucans. It is twice as much as detected in agarose from *Gelidium*, by Jol *et al*[2]. (assuming that, on the average, galactans and glucans have similar molecular weights). This discrepancy can reflect the true difference in the starch content of Jol's samples and ours, or can be the result of overrepresentation of glucans in AFM measurements as discussed above (cf. Table 1). It is also likely that in addition to starch the studied samples were contaminated with other algal polysaccharide fractions, for example, kappa and iota carrageenans. However, the detection of these chemically similar molecules will possibly require combining the AFM methodology with chemical treatment that would convert these molecules into mechanically dissimilar polymers.

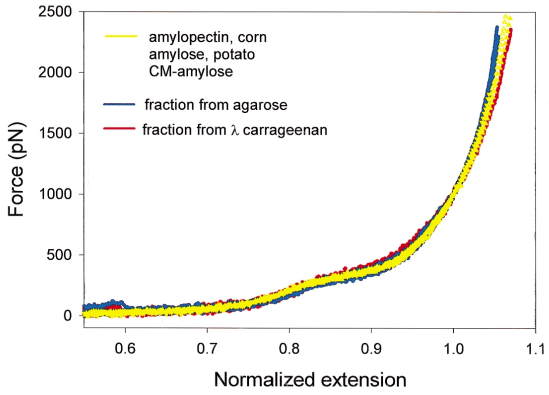

Figure 4. Superposition of force spectra from the amylose-like fraction of agarose (blue, n = 7), the amylose-like fraction of λ-carrageenan (red, n = 5), and from molecules with the amylose-like backbone linkages (amylose, CMA, amylopectin; yellow, n = 3). These data strongly suggest that the anomalous fractions observed in agarose and λ-carrageenan are amylose (amylopectin)-like contaminant molecules. All the recordings are normalized by the molecules' length at a force of 1,000 pN.

Structural analysis of polysaccharides combines methodologies of carbohydrate chemistry, chromatographic and spectroscopic techniques[23], and x-ray diffraction[29]. These are laborious methods that measure properties of the bulk, and they average over all molecules. Here we demonstrate a methodology for studying polysaccharides[30] based on stretching single molecules in solution with an atomic force microscope. Our method registers the molecular fingerprint of force-induced conformational transitions within the pyranose ring[18,19,31] and offers the possibility of identifying individual molecules in solution. Our ability to detect starch impurities in agarose may have an immediate practical implication in that starch inclusions are known to interfere with the mechanical properties of agar gels[27].

Experimental protocol

Single-molecule force spectroscopy by AFM. We have described our AFM apparatus and the method for calibration of the AFM tip spring constant elsewhere[16].

Polysaccharides. Dextran (T500, T2000, Pharmacia, Uppsala, Sweden), amylose (A-0512, type III, from potato; CMA, C-4947; pullulan, P-4516 from *Aureobasidium pullulans*; methylcellulose, M-0387, degree of substitution (DS) = 1.5–1.9, Sigma, St. Louis, MO), and CMC (cat. no. 41,933.8, DS = 0.9, Aldrich, Milwaukee, WI) are linear or >95% linear (dextran) homopolymers of D-glucopyranose. Amylopectin (A-7780, from corn, Sigma) is a α-(1→4)-linked D-glucan with amylose-like branches every ~20 monomers that are attached at position 6 through a α-(1→6) linkage. Pectin (P-9135, from citrus fruits; Sigma) is a linear α-(1→4) D-galactouronan. Agarose (A-9539, from *Gelidium*; Sigma, supplied by BioWhittaker Molecular Applications, Rockland, ME) and λ-carrageenan (C-3889, Sigma) are linear galactans with α-(1→3) and β-(1→4) linkages. Dextran, pullulan, methylcellulose, pectin, and λ-carrageenan were dissolved in water at a concentration of 0.01–10% (wt/vol). Amylose was solubilized by wetting with ethanol (100 mg/ml), followed by treatment with sodium hydroxide (10%) and heating. Amylopectin was dissolved at 0.1% concentration by boiling in water. Agarose was dissolved in water by heating 1% solution in a microwave oven followed by extensive stirring at ~80°C, and subsequent dilution to the final concentration covering a wide range between 0.001 and 0.1%. A layer of polysaccharide molecules was created by drying a drop of these solutions onto glass coverslips followed by extensive rinsing. This procedure leaves a monolayer of polysaccharide molecules tightly adsorbed to the glass surface[17]. The measurements were carried out in water or in PBS buffer (pectin, agarose, and λ-carrageenan). In order to pickup polysaccharide molecules, an AFM tip was pressed down onto the sample for 1–3 s and at forces of 10–40 nN.

For measurements on a polysaccharide mixture we used carboxymethyl derivatives of cellulose and amylose because these molecules have similar solubility to dextran. We verified that these derivatives produce force spectrograms that are indistinguishable from their native counterparts (cf. Fig. 1). The polysaccharides had similar molecular weight distributions (500–700 kDa; according to manufacturer) and their segments picked up by the AFM had, on the average, similar length distributions. The polysaccharides were dissolved at 0.05% (wt/vol) before mixing the solutions at 1:1:1 ratio.

For ring cleavage of a putative starch contamination, samples of agarose and λ-carrageenan were treated with sodium meta-periodate (Sigma). After 24 h of oxidation of an agarose sample with 10 mM periodate, no starchlike molecules were recorded in the AFM experiments, whereas a seven-day treatment with 5 mM periodate was required to totally convert starchlike contaminants in the λ-carrageenan sample into acyclic products.

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