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A ^{13}C CP/MAS NMR spectroscopy and AFM study of the structure of Glucagel™, a gelling β-glucan from barley☆

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Abstract

The structure of Glucagel™, a mixed-linked (1→3), (1→4)-β-D-glucan extracted from barley, was examined using ^{13}C CP/MAS NMR spectroscopy and atomic force microscopy (AFM). Results from ^{13}C CP/MAS NMR spectroscopy showed that Glucagel™ contained regions with two distinct conformations. In some of the regions the β-glucan chains associated to form a unique conformation, the A-conformation, while in the other regions the β-glucan chains were in an amorphous conformation. Dilute solutions of Glucagel™ were prepared for imaging by dissolving Glucagel™ in water at 90 °C. If the dilute solution was immediately deposited onto mica and the surface dried, then no fine detail was seen in the AFM image. However, when dilute solutions of Glucagel™ were left for several days before being deposited onto the mica surface, individual fibres could be clearly imaged. These results suggested that in gels formed from Glucagel™, junction zones occur because of the interaction of two β-glucan chains in the A-conformation.

Keywords: Glucagel™; β-Glucan; Barley; CP/MAS NMR; AFM

1. Introduction

Mixed-linked (1→3), (1→4)-β-D-glucans are mostly composed of cellotriosyl and cellotetrosyl residues separated by single (1→3)-β-linkages. They are cell-wall components in plants of the Poaceae family and are prevalent in the cell walls of certain cereal grains, particularly those of oat and barley. They accumulate in coleoptiles and leaves during cell expansion and decrease when growth has stopped [1–5]. Turnover of β-glucan during cell expansion is very rapid [2], which suggests that β-glucans are essential in cell-wall formation. There has, however, been little direct evidence of the role that β-glucans have in cell-wall expansion or for the molecular organisation of β-glucan in the cell walls. If the β-glucan is to function as a cell-wall component, then presumably there has to be some interaction between the β-glucan, either with itself or with other cell-wall components.

☆ Glucagel™ is a registered trademark of Industrial Research.

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Fincher and Stone [6] have suggested that there could be noncovalent interactions between cellulosic regions of the β-glucan to form junction zones as occurs with other cell-wall polysaccharides. There has been some evidence that junction zones do exist in β-glucan as concentrated solutions of β-glucan gel on centrifugation [7], and precipitates that are composed mostly of β-glucan are formed on freezing beer [8] or in beers that contain high levels of β-glucan [9]. It is also apparent from studies of solution properties that gel-like behaviour is a characteristic of β-glucans, especially β-glucans of low molecular weight [10,11].

We have recently reported [12] a two-step process, 'the Glucagel™ process' for isolating a β-glucan of high purity (~ 90%) from barley flour. The first step of the process is a warm-water extraction (below 55 °C) of the β-glucan from the flour. During this process, it appears that enzymes present in the flour cleave the β-glucan chains into lower molecular weight products. By altering the extraction time, it was found that different β-glucan products could be obtained with molecular weights varying from 14,000 to about 560,000 with respect to the pullulan standards. The second step in the process is the freezing and thawing of the extract, which forms a fibrous or gelatinous precipitate that can be separated and dried. Some 75% of the β-glucan in the flour is extracted during this procedure. The precipitate, Glucagel™, has functionality which has not been previously reported to occur in cereal β-glucans. It dissolves in water at temperatures above 60 °C to form transparent solutions which set to form soft translucent thermoreversible gels.

We have been interested in obtaining more information about the structure and properties of Glucagel™ using ^{13}C CP/MAS NMR spectroscopy and atomic force microscopy (AFM). NMR chemical shifts are very sensitive to the local environment about a nucleus. For solids, this means that different conformations of the same molecule can produce different chemical shifts. For instance, using ^{13}C CP/MAS NMR spectroscopy, it is possible to identify unambiguously in starch granules the A, B and V conformation of starch. AFM is a particularly useful technique for imaging individual molecules, including polysaccharides [13–17].

2. Results

NMR.—The solution NMR spectrum at 70 °C of Glucagel™ (Fig. 1(a)) was identical to that reported by Bock et al. [18] recorded at 57 °C. The resonance at 103.8 ppm has been assigned by Bock et al. to the C-1 carbon of a (1→3)-β-linkage, that at 103.6 ppm to the C-1 carbon of (1→4)-β-linkage, and that at 85.8 ppm to the C-3 carbon of a (1→3)-β-linkage.

The ^{13}C CP/MAS NMR spectrum of the dried Glucagel™ (Fig. 2(b)) consisted of a series of broad resonances. For the moistened Glucagel™ sample improved resolution was obtained, and if in addition, resolution enhancement was used in processing the FID, then a number of distinct resonances could be

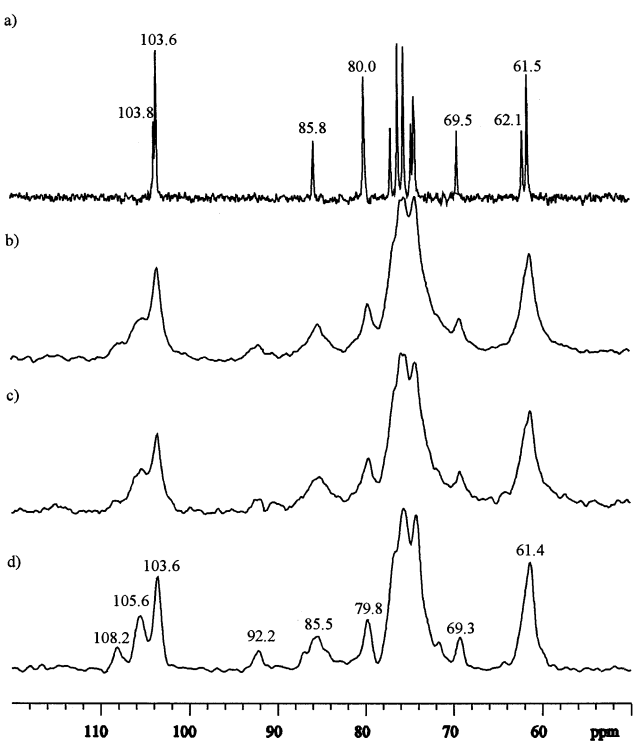

Fig. 1. (a) Solution spectrum of Glucagel™ recorded at 90 °C, (b) ^{13}C CP/MAS NMR spectrum of 5% w/w gel without resolution enhancement (number of transients is 20,000), (c) ^{13}C CP/MAS NMR spectrum of 23% w/w gel without resolution enhancement (number of transients is 3500), and (d) ^{13}C CP/MAS NMR resolution-enhanced spectrum of Glucagel™ with water added to give 66% w/w moisture content (number of transients is 1000).

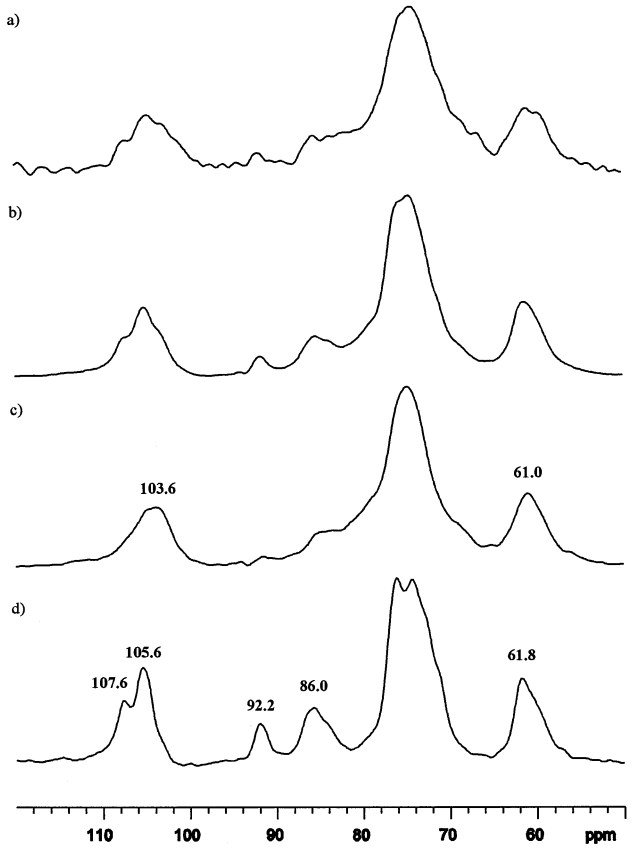

Fig. 2. (a) Spectrum of Glucagel™ with water added to give 66% w/w moisture content acquired with a single-pulse experiment, and (b) ^{13}C CP/MAS NMR spectrum of dry Glucagel™ and subspectra of the component of Glucagel™ that is (c) amorphous, and (d) in the A-conformation.

observed (Fig. 1(d)). Similar spectral features were observed in ^{13}C CP/MAS NMR spectra of the 5% w/w (Fig. 1(b)) and 23% w/w gels (Fig. 1(c)).

In the ^{13}C CP/MAS NMR spectrum of moistened Glucagel™ there are resonances that do not correspond to any resonance in the solution NMR spectrum. However, all of the resonances present in the solution NMR spectrum of Glucagel™ (Fig. 1(a)) were also present in the ^{13}C CP/MAS NMR spectrum, although not all of them were resolved in the ^{13}C CP/MAS NMR spectrum. In the C-1 region of the ^{13}C CP/MAS NMR spectrum, in addition to the peak at 103.6 ppm due to overlapping resonances of (1→3)-β-linked C-1 carbon and (1→4)-β-linked C-1 carbon, there were two resonances at 105.6 and 108.2 ppm, which were not present in the solution spectrum (Fig. 1(d)). In the solution spectrum, the C-3 resonance of the (1→3)-β-linkage occurs at 85.8 ppm. This peak was also present in the ^{13}C CP/MAS NMR spectrum as well as another peak at 92.2 ppm.

Estimates for the intensity of each of the overlapping three C-1 peaks in Fig. 1(d) were obtained by fitting the resonances to Gaussian curves. Starting at the high-frequency end, the ratio of the intensity of the three peaks was estimated to be 1:2.1:2.4. The ratio of 1:2.1 for the two high-frequency peaks is similar to the ratio of (1→3)-β-linkages to that of (1→4)-β-linkages (1:2.3–2.7) found in barley β-glucans. Therefore, it appears likely that the peak at 108.2 ppm is a C-1 carbon of a (1→3)-β-linkage and that at 105.6 ppm is a C-1 carbon of the (1→4)-β-linkage. For the gels the peak at 108.2 ppm did not have an adequate S/N to allow an estimate of its intensity, but for the other two C-1 peaks the ratio was 1:1.0 for the 5% gel and 1:1.3 for the 25% gel.

Subspectra of regions within the dried Glucagel™ that have different proton relaxation times were generated from spectra acquired with and without a proton spin-lock [19,20]. The first subspectrum (Fig. 2(c)) contained a series of broad resonances, which have similar chemical shifts to the resonances found in the solution NMR spectrum of Glucagel™. In fact, the solution spectrum of Glucagel™ appeared similar to that in Fig. 2(c) if artificial line broadening of about 400 Hz was introduced.

The second subspectrum (Fig. 2(d)) contained those resonances at 107.6, 105.6 and 92.2 1 ppm that are absent from solution NMR spectra. As discussed above, the peak at 107.6 ppm is due to a C-1 carbon of a (1→3)-β-linkage and that at 105.6 ppm to a C-1 carbon of the (1→4)-β-linkage. The slight difference in peak maxima between the two spectra is due to differences in resolution. The peak at 92.2 ppm is most likely due to C-3 carbon of a (1→3)-β-linkage and that at 86.0 ppm due to C-4 carbon of (1→4)-β-linkage for a number of reasons. The peak at 86.0 ppm is in the region expected for a C-4 carbon of (1→4)-β-linkage, whereas the peak at 92.2 ppm is outside this region [21,22]. The ratio of the integrals for the peaks at 92.2 and 86.0 ppm was found to be 1:2.2, which is similar to the ratio of (1→3)-β-linkages to that of (1→

4)-β-linkage (1:2.3–2.7) occurring in barley β-glucans.

The peak at 92.2 ppm is the only peak in Fig. 2(d) that does not overlap significantly with any of the peaks in Fig. 2(c). The integral of this peak can therefore be used to obtain an approximate estimate for the amount of β-glucan associated with each region in the dry Glucagel™. The integral of this peak is about 3.6% of the total integral for the subspectrum of Fig. 2(d), but is only 1.6% of the total for the spectrum of the dried Glucagel™ (Fig. 2(b)). This suggests that about 40% of the total β-glucan observable by ^{13}C CP/MAS NMR comes from the region which contributes to peaks in the subspectrum of Fig. 2(d).

Determination of ^{13}C spin-lattice relaxation times (T_1) indicated that there were two different relaxation regimes present for the Glucagel™, one corresponding to each subspectrum. For peaks associated with the subspectrum in Fig. 2(b), $T_1(^{13}C)$ were < 0.5 s, but for the other peaks $T_1(^{13}C)$ were, where measured, of the order of 10 s (20 ± 2 s for the peak at 92.2 ppm and 12 ± 2 s for that at 105.6 ppm).

The spectrum of moistened Glucagel™ obtained with a single pulse sequence and MAS required delays of 60 s between pulses because of the long $T_1(^{13}C)$ of carbons in the A-conformation. The long delays meant that a relatively noisy spectrum was obtained even for accumulation times as long as 6 h. The ratio of the integral of the peak at 92.2 ppm to that of the total integral for spectrum of Fig. 2(a) was 1%. Thus, of the total β-glucan content of the Glucagel™ about 30% is in regions which contribute to the peaks present in the subspectrum of Fig. 2(d).

Atomic force microscopy.—For the first sample, deposited immediately onto the mica surface after the Glucagel™ solution was prepared, the image (Fig. 3(a)) consisted of a conglomerate of irregular structural features. When the sample was dried under vacuum, small spherical particles were observed (Fig. 3(b)) that ranged in size from about 20 to 50 nm in diameter. It was not possible to determine precisely the size of these particles from the particle diameter due to the inevitable broadening effects of the tip probe [23,24], which adds on a constant value of ~ 10 nm to the size of each particle. The height of the particles is unaffected by probe width and is, therefore, a more accurate reflection of the particle diameter [17]. Heights varied from about 3 to 30 nm. The average molecular weight for this sample, determined using gel-permeation chromatography, was about 142,000 with respect to the pullulan standards [12], although the molecular weight ranged from about 10,000 to 500,000. If it is assumed that the density of β-glucan is about 1.5 g mL^{-1} (similar to that of cellulose [25]), then the diameter of the particles can be calculated from the molecular volume (V):

$$V = \frac{M}{\rho N_A}$$

where M is the molecular weight, ρ is the density and N_A is Avogadro's number. Molecular weights of 10,000–500,000 correspond to molecular diameters of about 2.6–10 nm. Each particle therefore probably contains about 1–10 β-glucan molecules.

As the surface rehydrated, the round particles increased in volume and became more diffuse. After 2 days, it was apparent that a portion of the β-glucan had diffused across the surface of the mica (Fig. 3(c)).

When water (10 μL) was dropped onto the mica surface of the vacuum-dried sample, and the sample was allowed to air dry, fibres appeared amongst a conglomerate of undifferentiated structure. One of these fibres was found to contain a loop, together with branching at the end of the fibre (Fig. 3(d)). Where the loop and branching occurred, the apparent thickness of the fibre decreased from about 13 to 11 nm. Although it was not possible to obtain the exact thickness of the fibre due to a combination of probe thickness and surface hydration effects [17,23,24], the presence of the loop suggested that the fibre was composed of two intertwined β-glucan chains.

For the second sample, where the Glucagel™ solution was left for 3 days at 4 °C before being drop cast, the AFM images were markedly different. Towards the centre of where the drop had been cast, the Glucagel™ had the appearance of a tangled network (Fig. 4(a)), while near the edge the Glucagel™ formed distinct particles (Fig. 4(b)). In amongst, and sometimes joined to, the parti-

cles were a number of fibres (Fig. 4(c)). Small area scans of these fibres (Fig. 4(d)) showed that the structure of the fibres was irregular. They contain small deviations away from the axis of the fibre. The height of these fibres was about 2 nm.

3. Discussion

^{13}C CP/MAS NMR spectroscopy can provide useful information about the molecular conformations of polysaccharides in the solid state. In some cases it is possible to directly confirm the presence of these conformation by AFM imaging of the polysaccharide.

Extra peaks apparent in the ^{13}C CP/MAS NMR resolution-enhanced spectra (Fig. 1(a)) of the moistened Glucagel™ suggested that some of the β-glucan chains were in a novel conformation in Glucagel™. The fact that subspectra can be generated (Fig. 2(c,d)) supported this conclusion, since it implies that

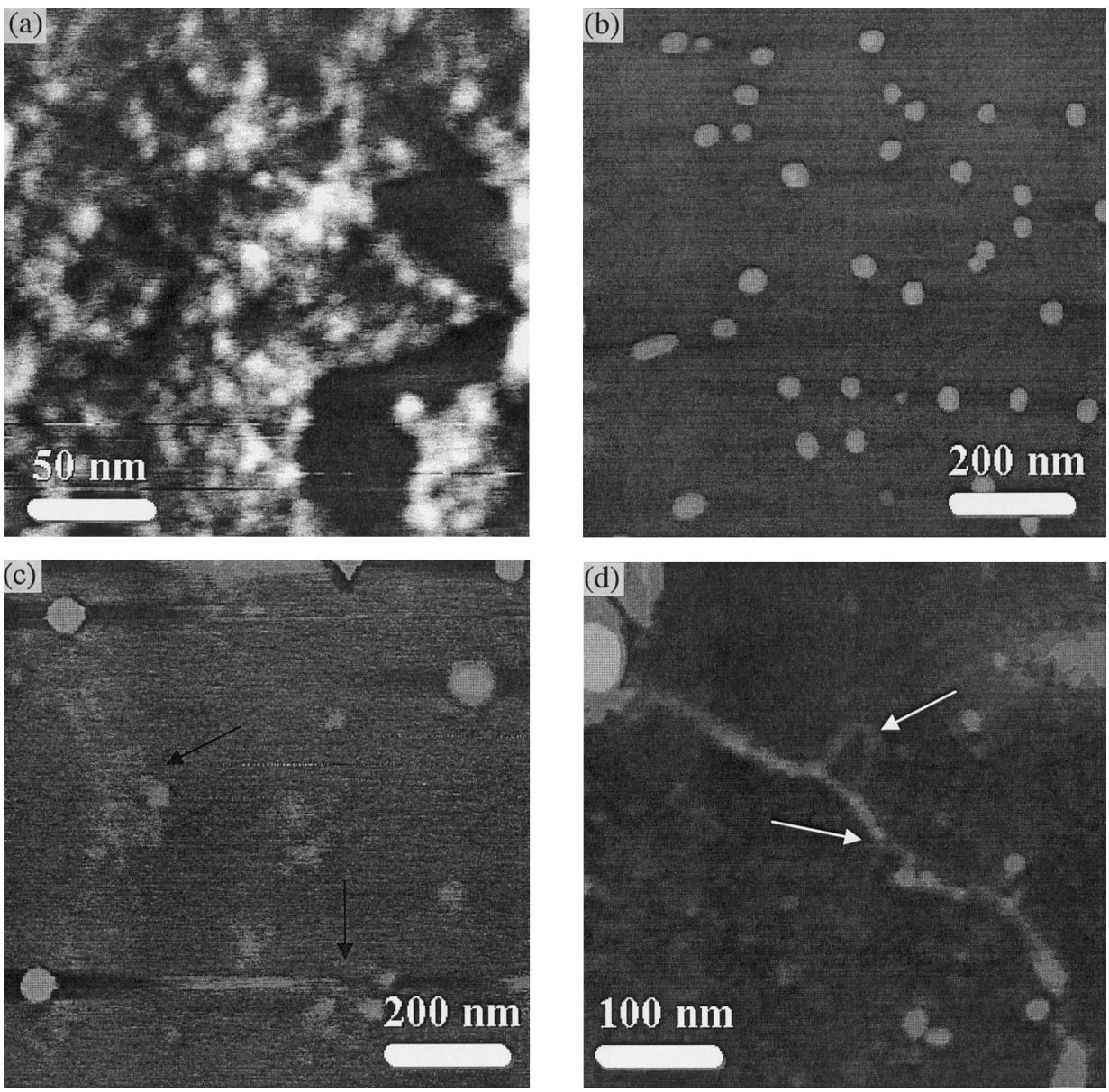

Fig. 3. AFM images in air of Glucagel™ (10 μg/mL) drop-cast onto mica. (a) Freshly prepared sample, (b) after vacuum drying, (c) after rehydrating in air for 3 days (arrows show some of the regions where the β-glucan has migrated across the surface), and (d) after rehydrating with a water droplet followed by drying (arrows show the loop within the fibre and splitting occurring at the end of the fibre).

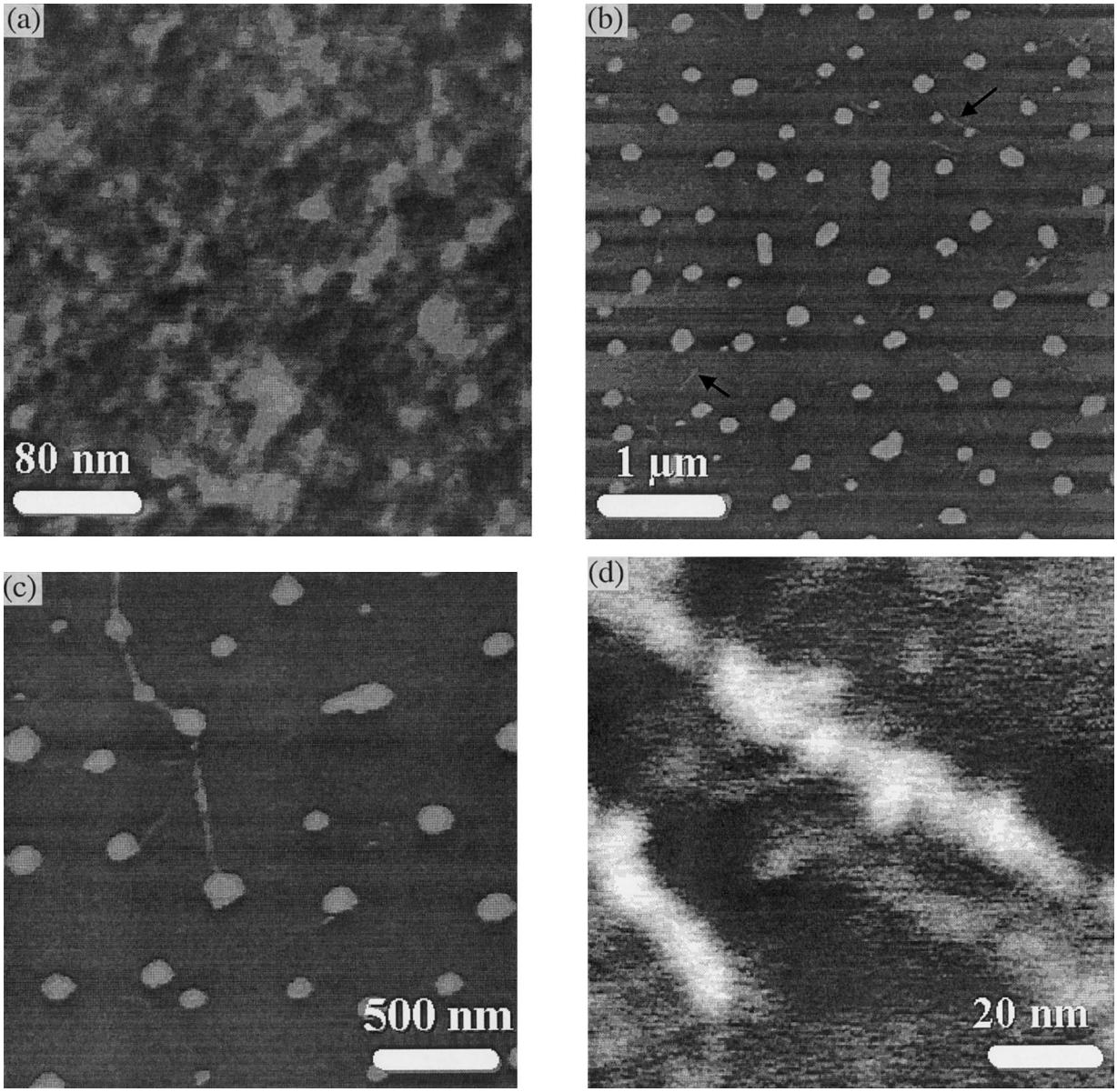

Fig. 4. AFM images in air of Glucagel™ (10 μg/mL) left at 4 °C for 3 days and then dropped cast onto mica. (a) Image from the centre region of the dried drop, (b) image of the outer region of the dried drop (arrows point to a few of the many fibres present). (c) and (d) show detailed images of various fibres.

there are regions within the dry Glucagel™ with different proton relaxation times, and, therefore, regions of the Glucagel™ that have β-glucan in different conformations. It was apparent that in one of the regions the conformation of the β-glucan was essentially amorphous. Resonances in the subspectra of this region (Fig. 2(a)) were very broad. They had chemical shifts similar to those of β-glucan in solution. However, the β-glucan chains in solution are in random conformations that rapidly change on the NMR time scale and hence narrow lines are obtained. In solid Glucagel™ these conformations are 'frozen out', resulting in broad resonances.

In the other subspectrum, the resonances of the C-1 carbons for the (1→3)-β- and (1→4)-β-linkage, and the C-3 carbon of (1→3)-β-linkage (Fig. 2(b)) were absent in the solution NMR spectrum (Fig. 1(a)). This indicated that the β-glucan chains contributing to this subspectrum were associated in a new conformation. We have decided to call this new conformation the A-conformation, after similar nomenclature used for describing starch structures. The lines widths of resonances in

this region were also narrower than for the amorphous β-glucan, indicating that the A-conformation is more ordered. Gels formed from Glucagel™ have similar spectral characteristics to that of the moistened Glucagel™ itself (Fig. 1). Therefore, there are regions in gels where the β-glucan is in the A-conformation and regions where the β-glucan is in an amorphous conformation.

$T_1(^{13}C)$ relaxation data are consistent with Glucagel™ containing two regions. Carbons of the β-glucan in the amorphous conformation have relaxation times of about 0.5 s, whereas those in the A-conformation have relaxation times from 10 to 20 s. The relaxation time for the C-3 carbon of the (1→3)-β-linkage at 92.2 ppm was the longest at 20 s, perhaps indicating that this carbon is held rigidly in place.

In Glucagel™, there may be β-glucan components, particularly in the amorphous region, that are too mobile to cross-polarise and are therefore not 'visible' in the ^{13}C CP/MAS NMR spectrum. A single pulse, direct polarisation experiment can then be used to detect these components. The spectrum of moistened Glucagel™ (Fig. 2(a)) obtained with the single pulse sequence is similar to that of the dried Glucagel™ acquired using ^{13}C CP/MAS NMR spectroscopy (Fig. 2(b)). Shoulders at 103.6 and 61.8 ppm are relatively more intense in the spectrum acquired using the single pulse experiment suggesting that there are some components that are too mobile to cross-polarise even within the dried Glucagel™.

Of the 'visible' β-glucan in the ^{13}C CP/MAS NMR spectrum of the dried Glucagel™, about 40% is associated with the A-conformation. Of the total β-glucan in the Glucagel™, as observed in the single pulse experiment, about 30% is associated with the A-conformation. Thus even though the cellotriosyl and cellotetraosyl residues in β-glucan are arranged completely randomly, i.e., there is no regular repeating sequences [26], at least 30% of the β-glucan chains are in the A-conformation. It had been expected that the (1→3)-β-linkage in the molecule interrupts the extended cellulose-like regions, thus reducing its tendency to pack into stable aggregates [6]. However, it is clear from the generated subspectra (Fig. 2(b)) that the (1→3)-β-linkages must be directly involved in the A-conformation, since not only did the C-1 carbon and C-3 carbon of a (1→3)-β-linkage appear in the subspectra of the A-conformation, but their chemical shifts were different to those occurring in the subspectra of the amorphous component. Rather than the (1→3)-β-linkage interrupting interactions between chains, it appears that the (1→3)-β-linkage and associated carbons at C-1 and C-3 are in a fixed conformation that is an integral part of the A-conformation and that the conformation must extend across several of the cellotriosyl and cellotetraosyl residues.

The appearance of resonances in ^{13}C CP/MAS NMR spectra of polysaccharides that have no solution counterpart usually indicates that the polysaccharide is associated in a unique conformation. The appearance of resonances for β-glucan in the A-conformation indicates that there must be an association of the β-glucan chains, but the NMR results do not contain information about how the chains are associated. AFM studies of Glucagel™ could provide this information.

The structures of a wide variety of polysaccharides have been examined using AFM, particularly by the group at the Institute of Food Research, Norwich, UK [13–16]. Typically, samples are prepared by ensuring adequate dissolution of the polysaccharide in water. This may require filtering to remove any remaining gel-like portions of the dissolved polysaccharide. The polysaccharide is then deposited onto a freshly cleaved mica surface that is dried, and the surface is scanned under a precipitating alcohol such as *n*-butanol, which inhibits desorption or dissolution of the polysaccharide into the surface adsorbed water. More recently, Gunning et al. have reported imaging xanthan gum in air by using 'tapping' mode AFM [17]. They suggested that air drying is a relatively mild procedure that would be expected to preserve the native helical structure of xanthan gum.

To preserve the structure of Glucagel™ as much as possible, we likewise sought to image in air using tapping mode AFM. For samples that were cast onto the mica surface immediately after being dissolved, images were a series of undifferentiated blobs. If the sample was left to allow aggregation, then images of individual molecular strands were obtained.

Thus, for the samples that had been dissolved in water at 90 °C and then immediately deposited onto a mica surface and dried, no apparent structure was observed in the AFM image (Fig. 3(a)). This was perhaps not unexpected, as in solution the individual β-glucan molecules are highly mobile. Likewise, within the hydration layer on the mica surface, the β-glucan molecules are probably highly mobile. The surface image perhaps represents a time-average picture of these mobile chains.

To decrease the surface mobility of the β-glucan chains, the sample was initially vacuum-dried. Surprisingly, small round particles were then observed (Fig. 3(b)). This suggested that in the absence of water, little adhesion exists between the β-glucan chains themselves, and between the β-glucan chains and the mica surface. Each of these particles appears to be formed from 1 to about 10 β-glucan molecules. The particles were not stable in air but rehydrated slowly, becoming significantly larger. After a few days a portion of the β-glucan was seen to re-form across the surface of the mica (Fig. 3(c)).

When the surface was briefly wetted and redried, fibres were observed. One of the fibres contained a loop, and splitting at the ends of the fibre was also observed (Fig. 3(d)). The loop probably exists because the sample was not in solution for a sufficient length of time for the loop to work its way out of the fibre. The existence of the loop suggests that β-glucan associates as two intertwined chains. The measured width of the fibre forming the loop (~ 10 nm) is about ten times the expected diameter of the β-glucan chain, due to tip effects [17].

It is interesting to note that once fibre formation had occurred, the β-glucan structure could then be imaged with considerably more detail. This is probably because the associated β-glucan chains are no longer mobile within the surface hydration layer. This is consistent with the results that Gunning et al. [17] obtained for xanthan gum. Xanthan assumes a very rigid single helical conformation that allows the fine molecular detail to be imaged with tapping mode AFM.

When the Glucagel™ solution was allowed to stand for a few days so that association of the β-glucan chains could occur, completely different types of structures were imaged. Towards the centre of where the drop was cast a fibrous network appeared (Fig. 4(a)), while towards the outer regions of the cast drop there appeared many particles interspersed with smaller fibres (Fig. 4(b)). The particles were about 231 nm across, which is four to five times larger than the particles obtained when a freshly prepared Glucagel™ solution was dropped cast onto mica then vacuum dried (see above). For a number of these particles, fibres appeared to originate from within the particle (Fig. 4(c)) suggesting that the particles could be formed from associated glucan chains that have perhaps curled up as the surface dried. The particles could also be microgels formed in solution from an aggregation of β-glucan chains that are associated in the A-conformation.

Although the fibres represent but a small weight fraction of the β-glucan on the mica surface, in quantity there are a large number of them, and they are probably representative of the type of interactions that can occur between the β-glucan chains. All the fibres (Fig. 4(b,c)) appeared to be well formed and reasonably straight. However, details of several of these fibres showed that the structure was not completely regular (Fig. 4(d)). Deviations away from the axis of the fibre can clearly been seen. The irregular nature of the fibre is probably a result of the random positioning of the cellotriosyl and cellotetraosyl units within the β-glucan chain. In certain regions of the fibre there is probably a good match between molecular units of the β-glucan chains. Where there is a mismatch say between a cellotriosyl and cellotetraosyl residue, a small deviation off the main axis of the fibre could form so that a match between residues was once again established. The β-glucan chains in the deviation would probably be in the amorphous conformation.

The height of the chains (~ 2 nm) is consistent with the fibres being formed from one or two polysaccharide chains [17]. That the width of the fibres is similar to that observed for the fibre containing the loop (Fig. 3(d)) indicates that it is probably formed from two chains.

Results of the AFM study appear to contradict the conventional wisdom that β-glucan has an extended conformation in solution. Undoubtedly this is true for high molecular weight β-glucans produced by traditional methods, since these form highly viscous solutions. But Glucagel™ is formed of a low molecular weight β-glucan that gels. For a gel structure to form, there must be some association and aggregation of β-glucan chains to form a more globular architecture. The globular structures observed by AFM are only observed after a solution of Glucagel™ has been left for several days, so that association and aggregation of β-glucan chains can occur. Where no aggregation has occurred, then no fine structure can be imaged, and the β-glucans appear as blobs on the mica surface. Under these conditions the β-glucan probably retains an extended conformation which cannot be imaged because of the its mobility within the hydration layer of the surface. Curiously, if the β-glucan is then dehydrated under vacuum, the preferred conformation is globular.

The fact that some of the β-glucan exists in the A-conformation in cereal grains possibly explains why a significant fraction (~ 20% for the Glucagel™ process) of the β-glucan remains unextracted during extraction into warm water, but is extracted at temperatures above 65 °C. Parts of the fraction that extracts at high temperature probably has regions where the β-glucan chain exists in the A conformation. This fraction would therefore not dissolve in water below temperatures of 60 °C, since this is the approximate melting temperature of gels formed from Glucagel™. The fraction that extracts at temperatures below 60 °C would contain β-glucan chains that were not self-associated to any extent.

Carpita et al. [5] have described β-glucan as 'possibly the principlal interlocking polymer' during cell-wall elongation, but there has been little evidence to show that β-glucan can 'interlock'. The results of this study show that β-glucan can self-associate or interlock and confirms the hypothesis of Fincher and Stone [6] that β-glucans form strong noncovalent interactions between themselves. In the cell walls of grasses, β-glucan chains probably form junction zones with other β-glucan chains. These junction zones may be formed from β-glucan chains that are associated in the A-conformation.

4. Experimental

Glucagel™ preparation.—For AFM studies a highly purified sample of Glucagel™ was prepared by repeated freeze/thaws. A barley pollard flour (100 g, β-glucan content ~ 7.0%) obtained by Burley milling a breeder's selection, was mixed with water (500 mL) at 55 °C in a sealed plastic bottle and heated at 55 °C for 2 h. The bottle was shaken occasionally during this time. The mixture was centrifuged (5000*g*, 10 min), and the supernatant was decanted, frozen at −20 °C for 12 h, and then thawed. The Glucagel™ precipitate was recovered from the thawed solution by filtration through a sintered glass filter funnel (porosity 2) and washed with distilled water (2 × 100 mL). The Glucagel™ was redissolved in water (250 mL) at 80 °C and filtered while hot through glass-fibre filter paper to remove a small amount of a fine precipitate. The freeze/thaw step was repeated, and the Glucagel™ was again recovered from the thawed solution by filtration. The Glucagel™ was stirred with water (1 L) at room temperature for 1 h, and the solution was filtered to recover the Glucagel™. The Glucagel™ was purified once more by dissolving in water (250 mL) at 90 °C, filtering through glass-fibre paper, and then freeze/thawing the supernatant. The precipitate in the last freeze/thaw was recovered by filtration and dried at 80 °C in an oven. This yielded 1.46 g of Glucagel™, having an appearance similar to that of filter paper. The molecular weight was determined by gel-permeation chromatography as described elsewhere and is reported with respect to pullulan standards [12].

For ^{13}C CP/MAS NMR and solution NMR studies, a less pure Glucagel™ was prepared by a method similar to that detailed above, except that only two freeze/thaw steps were used.

Solution ^{13}C NMR spectroscopy.—Glucagel™ was dissolved in D_2O at 80 °C to form a 1.0% w/w solution. Solution ^{13}C NMR spectra were acquired at 125.7 MHz on a Varian Unity 500 spectrometer at 70 °C. The ^{13}C resonances are reported relative to an internal standard of Me_2SO ($\delta =$ 40.3 ppm).

^{13}C CP/MAS NMR spectroscopy.—Four samples were prepared for analysis by ^{13}C CP/MAS NMR spectroscopy: the dried Glucagel™ precipitate, Glucagel™ with water added to give 66% w/w moisture content (referred to as moistened Glucagel™), and 5% w/w and 23% w/w gels. The gels were formed by dissolving Glucagel™ in water at 80 °C, and cooling to room temperature. Samples were packed into 3.5-mm rotors fitted with tight end caps, smeared with silicone grease to prevent water evaporation, and spun at speeds up to 5 kHz in a MAS probe from Doty scientific. Spectra were acquired at 125.7 MHz on a Varian Unity 500 spectrometer. Resonances were recorded with respect to an external standard of Me_4Si. Spectra were resolution enhanced by applying a Lorentzian-to-Gaussian transformation to the FID.

Subspectra of regions within the dried Glucagel™ precipitate that have different proton rotating frame relaxation times were generated using the method of Newman and Hemmingson [19] as described by Morgan et al. [20] on a Varian Inova 200-MHz NMR spectrometer equipped with a 7-mm MAS probe from Doty Scientific. Two spectra were acquired: one was a normal ^{13}C CP/MAS NMR; the other was acquired in a similar manner but the cross-polarisation pulse was preceded by a 4000 μs proton spin-lock. Some peaks in the latter spectrum are relatively less intense compared to peaks in the spectrum acquired without the proton spin-lock. This is because these peaks are associated with carbons in regions where the proton relaxation times are faster. Therefore, subspectra can be generated from these two spectra by a weighted subtraction. The weighting was adjusted so that the more intense peaks were present in one subspectrum, and the less intense peaks were present in the other subspectrum.

To determine if there were components of the Glucagel™ that were too mobile to cross-polarise, spectra were acquired on the moistened Glucagel™ sample using a single-pulse, direct-polarisation sequence, together with MAS [27] on a Varian Inova 200 MHz NMR spectrometer. Delays between pulses were 60 s, which were at least $3 \times T_1$ of the slowest-relaxing ^{13}C. T_1 for ^{13}C were determined using the Torchia's pulse sequence [28] for the slower relaxing components and saturation–recovery for the faster relaxing components.

Atomic force microscopy.—Images were recorded in tapping mode on a Nanosope III atomic force microscope (Digital Instruments, Santa Barbara, CA). Tapping mode imaging in air was carried out at the cantilever's resonance frequency using a probe and cantilever unit composed of silicon (Nanoprobe, cantilever length 125 μm and resonance frequency 307–375 kHz). Scan rates on all images were 2 Hz. The drive vibration amplitude was 15–45 mV, generating a free cantilever vibration amplitude of 1 V.

Two samples were examined using AFM. The first sample was prepared by dissolving purified Glucagel™ in water (10 μg/mL) at 90 °C for 0.5 h. The solution was immediately deposited onto a freshly cleaved mica surface, air dried, and then imaged using AFM. Next, the sample was vacuum dried for 0.5 h at 10^{-1} mbar pressure and imaged again. The sample was then left for 2 days in a covered Petri dish, and the surface was imaged again. Finally, a drop of water was added to the mica surface, and the surface was allowed to air dry before being imaged.

For the second sample, Glucagel™ was dissolved in water (10 μg/mL) at 90 °C, and the solution was left at 4 °C for 3 days before being deposited onto a freshly cleaved mica surface. The surface was air dried and imaged.

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