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Supramolecular Structure and Conformation of a (1→3)(1→2)-β-D-Glucan from *Lactobacillus suebicus* CUPV221 as Observed by Tapping Mode Atomic Force Microscopy

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Tapping mode atomic force microscopy (TM-AFM) has been used to analyze the supramolecular structure and conformation of the (1→3)(1→2)-β-D-glucan produced by *Lactobacillus suebicus* CUPV221 isolated from cider. Solutions for TM-AFM observation were prepared by dispersing the solid glucan in distilled water and in alkaline aqueous solutions. It was found that from the distilled water at 10 mg/L or higher concentrations, the (1→3)(1→2)-β-D-glucan forms networks. The heat resistance of the networks depends on the concentration. From the alkaline aqueous solutions, different supramolecular structures were observed depending on the pH. From the weakest alkaline solution, a fairly rough morphology with a high density of spikelike growth features was revealed. As the ionic force of the medium increased, the sizes of the spikelike growth features diminished, and even many disaggregated fibers could be found. At 0.4 M NaOH (pH 13.16), the aggregates had disappeared almost totally. NaOH aqueous solutions (0.1 and 0.4 M) were used to carry out the study of conformation. At 0.1 M NaOH, the aggregates were partially detached, and many free microfibers were found to which a helical conformation could be assigned due to their stiffness and rodlike character. At 0.4 M NaOH, the beginning of the dissociation of the helical structures was seen.

KEYWORDS: (1→3)-β-D-glucan; lactic acid bacteria; atomic force microscope; supramolecular structure; conformation; thermal behavior

INTRODUCTION

Polysaccharides are relatively complex carbohydrates. They are biopolymers made up of many monosaccharides joined together by glycosidic bonds. They are important constituents of plants and microorganisms, where they perform a variety of functions (*1*). (1→3)-β-D-glucan polysaccharides are chains of D-glucose molecules, with the six-sided D-glucose rings connected at the 1 and 3 positions. They are found in the cell walls of yeast, fruiting bodies of fungi, and as exopolysaccharides (EPSs) in the culture medium of some bacteria. It has been shown that these (1→3)-β-D-glucans have broad biological activities such as antitumor, antibacterial, antiviral, and anticoagulatory effects (*2*). The biological activity of these biomacromolecules is strongly dependent upon either chemical or physical properties as well as conformation or structural features, which also depends on the environmental conditions (*3*). Besides, these (1→3)-β-D-glucans have a growing interest because of their applications in the dairy industry. They may act as both texturizers and stabilizers. A better understanding of the structure−function relationship of (1→3)-β-D-glucans remains a challenge to further improve applications of these biopolymers to better satisfy the consumer demand for appealing, tasty, and even healthier products (*4*).

It has been reported that some species of lactic bacteria are able to synthesize EPSs, which are secreted into the surrounding environment (*5*). Some strains of *Pediococcus parvulus* have been described as β-glucan-producing bacteria in cider (*6*) and wine (*7*). In our laboratory, an EPS-producing *Lactobacillus suebicus* CUPV221 strain has been isolated from a ropy cider of the Basque Country (Spain). On the basis of chemical and spectroscopy data, the polysaccharide secreted by this strain has the trisaccharide repeating unit presented in **Figure 1** (*8*).

The objective of our research is to know the supramolecular structure and conformation of the (1→3)(1→2)-β-D-glucan from *L. suebicus* CUPV221. Atomic force microscopy (AFM) has been applied successfully to visualize a range of polysaccharides including Curdlan (*9*), schizophyllan (*10*), scleroglucan (*11*), carragenans (*12*), oat β-glucan (*13*), etc. In these mentioned works, different AFM techniques and different methods of preparation of samples have been used. To develop the present work, tapping mode AFM (TM-AFM) has been used by the drop deposition method to prepare the samples.

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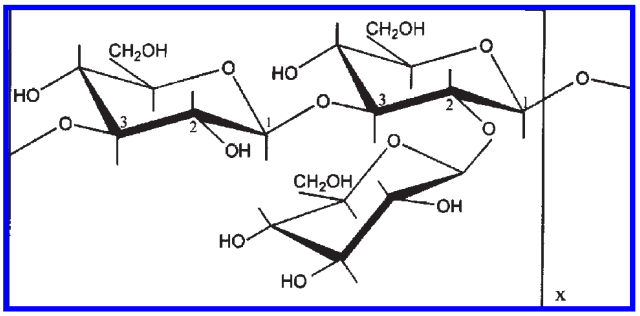

Figure 1. Repeat unit of the polysaccharide produced by *L. suebicus* CUPV221.

MATERIALS AND METHODS

Bacterial Strain and Media. *L. suebicus* CUPV221 was isolated from a ropy Basque cider. The strain was routinely cultured at 28 °C in MRS and stored at −80 °C with glycerol at 20% (v/v). For EPS production, a SMD broth was used without yeast extract, beef extract, or peptone, as these ingredients interfere with the β-glucan purification; it contained (in g/L of distilled water): glucose, 20; casamino acids (Difco), 10; sodium acetate, 5; Bacto yeast nitrogen base (BYNB) (Difco), 6.7; K_2HPO_4, 1; KH_2PO_4, 1; $Mg_2SO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.1; KCl, 0.45; diammonium citrate, 3.5; Tween 80, 1; adenine, uracil, thymine, and guanine, 0.005. Both sugar and BYNB were sterilized by filtering them through a 0.22 μm pore size Millex-GS filter unit (Millipore, Bedford, MA) and were added after autoclaving. The pH of the SMD medium was adjusted to 4.8 prior to sterilization.

Isolation and Purification of β-Glucan. The clear supernatant obtained by centrifugation for 30 min at 16000*g* was collected. Crude EPS was precipitated from the supernatant by the addition of 3 volumes of cold ethanol, followed by storage overnight at 4 °C. The polysaccharide was purified by precipitation with ethanol three times, and the final precipitate was resuspended in distilled water, dialyzed (M_w cutoff 12000−14000 Da) against distilled water for 48 h with water replacement twice a day, and finally lyophilized.

Samples Preparation for AFM Imaging. Several solutions of the (1→3)(1→2)-β-D-glucan were prepared in distilled water (100, 10, and 1 mg/L) or in alkaline aqueous solution (1 mg/L) at different pH values: 11.75 (5 mM NaOH), 12.86 (0.1 M NaOH), and 13.16 (0.4 M NaOH). The 100 mg/L water solutions were obtained by two different ways: with stirring or without stirring, both at room temperature during 24 h. Without stirring, the biopolymer was swollen and finally dispersed in the water. The alkaline aqueous solutions of different pH values were prepared by dissolving the β-glucan with slight stirring during at least 24 h. The AFM samples were made by the drop deposition method; 0.5 μL solutions were pipetted onto cleaved sheets of mica, and then, the solvent was naturally evaporated in a dust-free enclosure. During drying of the samples from the alkaline aqueous solutions, a slight flux of N_2 was applied to avoid sodium carbonate production by the reaction between sodium hydroxide and carbon dioxide from air.

AFM Imaging Procedure. The AFM scanning was performed at room temperature with a scanning probe microscope (SPM) (Nanoscope IVa, Multimode from Digital Instruments) operating in tapping mode. An extension of the tapping mode is phase imaging. The phase imaging measures the phase lag of the cantilever oscillations relative to the signal sent to the cantilever driver. The phase lag is very sensitive to variations in material properties, such as adhesion, viscoelasticity, and stiffness. In this way, the tapping mode extension has been used as a contrast enhancement technique. Samples were placed on top of a "J" piezoelectric scanner, the maximum *xy* imaging range of which is ~100 μm, and were scanned at a scanning frequency of 0.2−1 Hz using the MPP-12100 silicon probes of Veeco. Several specimens were scanned in different regions, and similar images were obtained, thus demonstrating the reproducibility of the results. All images are shown without any image processing except in some cases where horizontal leveling and contrast enhancement were used. The diameters of helical units and strands of the polysaccharide were measured with Digital Instruments Nanoscope IV Software version 5.12r5. The section command was used. Prior to making measurements, tilt and noise were corrected. The section command produced a profile of the surface. On the AFM image, a line was drawn, and a cross-sectional profile was displayed in the screen. With the cursors, the height of the microfibrils on the cross-sectional profile was selected, and reference markers were fixed on the image. The AFM software provided the information of the cross-sectional profile: vertical distance, horizontal distance, etc. At least 50 measurements were done. This method was accepted for the measurement of thicknesses and heights but not for the measurement of widths due to tip-broadening effects (*14*).

To analyze the structural changes of the (1→3)(1→2)-β-D-glucan with temperature, an external accessory of the Nanoscope IVa, which allowed heating of the sample up to 250 °C, was used. Height and phase images were captured simultaneously at several isothermal temperatures: 80, 125, 150, and 200 °C. The J-scanner was also used with the above-mentioned commercially available silicon probes. The scan rate was in the range of 1.0−1.5 Hz.

RESULTS AND DISCUSSION

Supramolecular Structure. Solutions with suitable solvents have to be prepared to carry out imaging of polysaccharides by AFM. (1→3)-β-D-glucans dissolve easily in alkaline aqueous solutions. Nevertheless, when alkaline aqueous solutions are used, different supramolecular structures can be observed depending on the pH, ranging from infinite microfibrils to spindle-shaped fibrils of various lengths (*15*). Pure water as a solvent of glucans is more debatable. There are some glucans widely accepted as insoluble in water, for example, Curdlan (*16*, *17*). For some others, such as water-soluble schizophyllan, the helical structure is such that the glucosyl side chains are on the outside of the helix. These side chains prevent the formation of large insoluble aggregates of triple helices through hydrogen bonding, and as a result, polysaccharides can be dissolved in water, in contrast to Curdlan (*18*). Our (1→3)(1→2)-β-D-glucan can be swelled by water until it molecularly disperses. Thus, we analyzed the supramolecular structure from aqueous solutions at different concentrations and from alkaline aqueous solutions at different pH values.

As suggested by other authors (*3*), it could be observed that the sample history decisively influenced the structural organization of the biopolymer. **Figure 2** shows the TM-AFM images of the samples obtained from the 100 mg/L water solutions with stirring (**A**) and without stirring (**B**). Two different ways of aggregation can be seen. On the one side, a matrix of fibers with different diameters, lengths, and orientations is observed for the samples from the solution with stirring (**Figure 2A**). The fibrous morphology is associated with a rigid rodlike behavior with a multistranded character (*11*). The fibers are formed by the aggregation of strands by means of stacking together side by side (*19*). On the other side, **Figure 2B** shows a three-dimensional network for the samples from the solution without stirring, where the (1→3)(1→2)-β-D-glucan swelled with water up to dispersion. This means that our (1→3)(1→2)-β-D-glucan at room temperature, at these particular conditions, forms a gel. Gelation involves aggregation of the rodlike helices through noncovalent associations (*17*). The constitution of gel networks in (1→3)-β-D-glucans is not surprising, considering the capacity of this polysaccharide chain to adopt an ordered hydrogen bond-dependent helical conformation. Gels based in such structures have been reported in others polysaccharides such as agarose, carrageenans, and gellan (*9*, *20*). These gels generated at low temperatures are traditionally referred to as low-set gels, unlike the gels formed at high temperatures, for example, Curdlan at 95 °C, which are termed high-set gels. The molecular mechanism of high-set gel formation is different from that of low-set gel formation (*21*). In high-set gels, there is cross-linking between the helix or

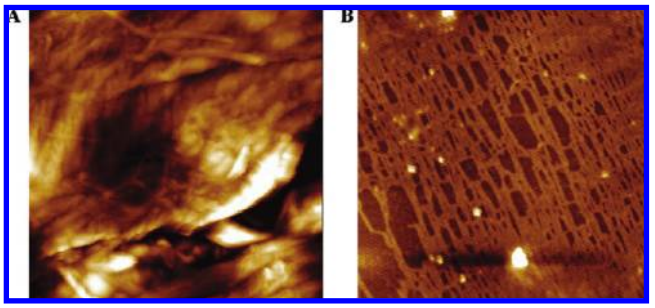

Figure 2. TM-AFM height images of (1→3)(1→2)-β-D-glucan. Images were obtained by depositing the glucan (**A**) from a stirred 100 mg/L aqueous solution, height image of 5 μm × 5 μm, and (**B**) from a 100 mg/L aqueous solution without stirring, height image of 2.5 μm × 2.5 μm, onto mica and scanning in air.

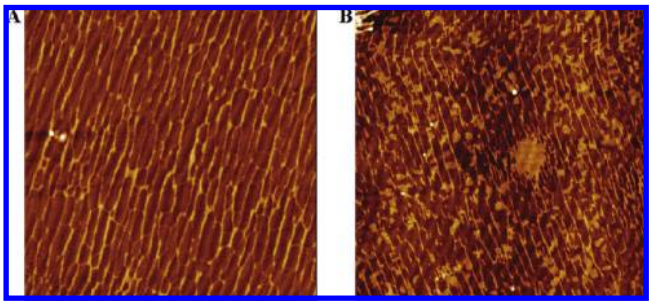

Figure 3. TM-AFM height images of (1→3)(1→2)-β-D-glucan. Images were obtained by depositing the glucan from a 10 mg/L aqueous solution without stirring onto mica and scanning in air. (**A**) Height image obtained at room temperature; height image of 2 μm × 2 μm. (**B**) Height image obtained at 80 °C; height image of 4 μm × 4 μm.

multistranded helices forming a three-dimensional network through hydrophobic interactions (*22*, *23*). In low-set gels, the strand associations are through hydrogen bonding. High-set gels have the properties of being much stronger and more resilient than low-set gels (*24*). Normally, a low-set gel melts on heating, whereas a high-set gel is stable against thermal treatments (*25*). However, when the analysis of the structural behavior with the temperature of our (1→3)(1→2)-β-D-glucan from the 100 mg/L water solutions was carried out, a heat-resistant gel was exhibited. At 150 °C, the network structure was maintained. This behavior is explained because, in spite of the network, it is supported by secondary weak interactions, and the stability is achieved by a large number of hydrogen bonds acting cooperatively (*26*). Nevertheless, when the study for samples from the 10 mg/L water solution was carried out, it could be seen that a gel was also formed (**Figure 3**), but it had worse heat resistance. Heating the sample at 80 °C reduced the cross-linking (**Figure 3B**), as this decrease of heat resistance was associated with the diminution of hydrogen bonding. Besides, many dotlike aggregates can also be found, which could be chains of polysaccharides in random coil configurations. As already has been observed in other (1→3)-β-D-glucans (*27*), the helical strands may unwind at high temperatures to give single chains (*17*). This means that for our (1→3)(1→2)-β-D-glucan, at these particular conditions, when exposed to high temperatures, the thermal energy of the strands leads to helix destabilization evolving to a partial structure in random coil.

It can be concluded that our (1→3)(1→2)-β-D-glucan from *L. suebicus* CUPV221 forms a three-dimensional gel network at room temperature in neutral medium. This conclusion is in accordance with a rheological analysis carried out in our laboratory for an identical polysaccharide, a branched 2-substituted (1→3)-β-D-glucan, produced by *P. parvulus* 2.6 (*28*). In this work, the existence of a physical network, a gel, in water solution at room temperature was also proved. Furthermore, two facts demonstrated that it was a low-set gel or weak gel. On the one hand, the viscosity decreased above a certain shear rate, and on the other hand, the viscous modulus (G'') was lower than the elastic modulus (G'), with a very slight dependence on frequency, a behavior associated with weak gels (*20*). This weak gel behavior, which has also been described in other β-glucans, such as the scleroglucan (*29*), favors the potential use of these polysaccharides as thickening agents from generally recognized as safe (GRAS) lactic acid bacteria (LAB).

To analyze the effect of the ionic strength of the medium on the supramolecular structure and packing arrangement of our (1→3)(1→2)-β-D-glucan, samples from alkaline aqueous solutions were scanned. Three different pH values were used, 11.75 (5 mM NaOH), 12.86 (0.1 M NaOH), and 13.16 (0.4 M NaOH), and **Figure 4** shows TM-AFM images of the different samples. The images of the sample from the weakest alkaline solution (**Figure 4A**) reveal a fairly rough morphology with a high density of spikelike growth features. Because of their dimensions, approximately 25 nm, these spikes are aggregates of fibers. As the ionic force of the medium increased, the size of the spikelike growth features diminished and even many disaggregated fibers could be found (**Figure 4B**). At pH 13.16, the aggregates had almost totally disappeared, and those that could be found did not have a definite form (**Figure 4**C). When the fibers were more deeply analyzed, it was observed that some fibers even had started unwinding, a process associated with the denaturation of the polysaccharide. This phenomenon will be approached in the next paragraph.

Conformational Study. The conformation is one of the most important factors of biomacromolecules and significantly affects and governs their functional activity and, therefore, the applications of the biopolymers (*30*). The established method to carry out conformational studies by AFM has been the preparation of the samples from alkaline aqueous solutions or organic solvent solutions at very low concentrations (*31*, *32*). In alkaline solutions due to the ionization of the hydroxile groups and the subsequent electrostatic repulsion between chains (*33*), a previous dissociation of the aggregates takes place, and then, as the alkalinity increases, the helix structure is believed to denature to yield individual disordered single chains (*34*, *35*). Thus, NaOH aqueous solutions at a concentration of 1 mg/L were used to develop the study. The minimal NaOH concentration required for the dissociation of the aggregates to occur was 0.1 M, and at 0.4 M, the beginning of the dissociation of the helical structures was observed.

Figure 5 shows a TM-AFM image and a section analysis of a sample from 0.1 M NaOH aqueous solution, pH 12.86. As can be seen in the image, **Figure 5A** at a pH of 12.86, the aggregates were partially detached, and many free microfibers could be found. Because of the stiffness and the rodlike character of these microfibers, a helical conformation can be assigned. In some of these microfibers, shown with black arrows in the image, the spiral conformation can even be *seen*. To obtain quantitative information from AFM images, trace analysis has been done for many types of polysaccharides (*14*), providing an estimate of the height of the macromolecule corresponding to its diameter (*31*). Nevertheless, some discrepancy between values by AFM and those determined by other techniques usually exists. McIntire et al. (*36*) found that for scleroglucan, the height measured by

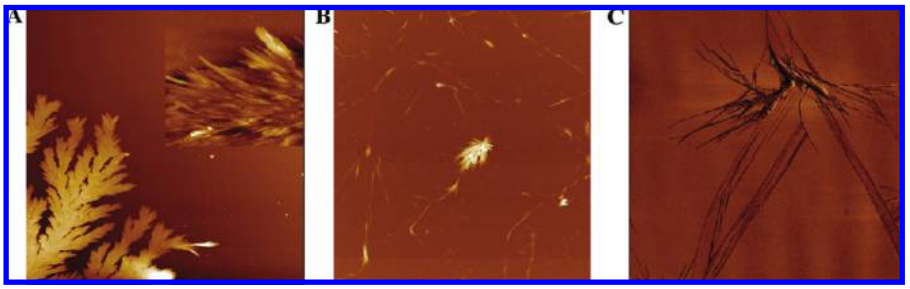

Figure 4. TM-AFM height images of (1→3)(1→2)-β-D-glucan. Images were obtained by depositing (1→3)-β-D-glucan onto mica and scanning in air from (**A**) 1 mg/L of 5 mM NaOH aqueous solution, pH 11.75, at height images of 40 μm × 40 μm and 5 μm × 5 μm; (**B**) 1 mg/L of 0.1 M NaOH aqueous solution, pH 12.86, at a height image of 50 μm × 50 μm; and (**C**) 1 mg/L of 0.4 M NaOH aqueous solution, pH 13.16, at a phase image of 10 μm × 10 μm. (The phase image is shown because of the high contrast in the height image due to the difference of heights between features.)

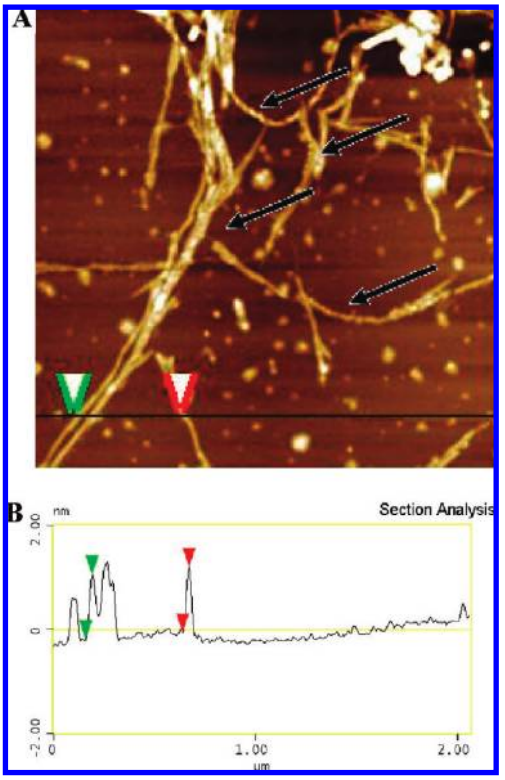

Figure 5. (**A**) TM-AFM height image of (1→3)(1→2)-β-D-glucan. The image was obtained by depositing the glucan onto mica and scanning in air from 1 mg/L of 0.1 M NaOH aqueous solution, pH 12.86, image of 2 μm × 2 μm. (**B**) Height profile of the cross-section highlighted in panel **A**. Markers on the image indicate the points where the measurements were taken.

AFM was approximately 67% of the thickness expected from X-ray fiber diffraction. They thought that the dissimilarity might result from the fact that the molecules were distorted by desiccation or by interaction with the mica substrate or that the molecules were partially embedded in a layer of water adhering to the mica surface. The diameter of our microfibers measured by the mentioned method (**Figure 5B**) was 1.8 nm. In literature, many values for the different polysaccharides have been reported (*14*). The most similar values to those obtained in our work have been for scleroglucan (*37*) and for xanthan (*38*), both polysaccharides that exist mainly as linear helical structures (*39*, *40*). Keeping in mind, besides, the results obtained in Congo Red analysis for the (1→3)-β-D-glucan, produced by *P. parvulus* 2.6 (*28*), where the results suggested that the glucan adopted an ordered hydrogen bond-dependent helical conformation in neutral and slightly alkaline aqueous solution, we considered that the observed microfibers correspond to units of helical strands.

Figure 6 shows height and phase TM-AFM images of a sample from the 0.4 M NaOH aqueous solution (pH 13.16). Phase imaging is a powerful extension of TM-AFM that can provide nanometer-scale information not revealed by height measurements. By mapping the phase of the cantilever oscillation during the tapping mode scan, phase imaging goes beyond simple topographical mapping to detect variations in composition, adhesion, friction, viscoelasticity, and perhaps other properties. In the present work, phase imaging has been used as a contrast enhancement technique to provide additional information and higher resolution. As can be seen in **Figure 6**, some of the microfibers were unwinding into smaller units. This behavior, again, was consistent with the rheological study of the (1→3)-β-D-glucan produced by *P. parvulus* 2.6 (*28*). In this work, a significant reduction of relative viscosity was observed in 0.4 M NaOH over time, showing also that, in this alkaline medium at room temperature, no hydrolysis of (1→3)-β-D-glucan took place. The viscosity decrease was associated with a denaturation process of the ordered conformation of the polysaccharide, suggesting that the hydrogen-bonding structure broke down gradually. The images of **Figure 6A** and **Figure 6B** are direct evidence of this suggestion as the helices consisting of helical strands are unwinding (**Figure 6A**) and keeping even in some strands the helical form (black arrows in the image of **Figure 6B**). The diameter of the strands was 0.8 nm (**Figure 6C**). In view of values by AFM of diameters of strands given by other authors for similar glucans with triple-helix conformations (*14*), we propose that our (1→3)-β-D-glucan could have a triple-helix conformation. Nevertheless, more precise quantification techniques, such as X-ray diffraction, must be used to confirm this statement.

It should be mentioned that the particles that can be seen in the images from the NaOH aqueous solutions (**Figures 5** and **6**) are due to sodium carbonate precipitation, in spite of the slight N_2 flux used during the drying of the samples.

In conclusion, we have shown that the (1→3)(1→2)-β-D-glucan from *L. suebicus* CUPV221 forms gel network structures. From the alkaline aqueous solutions, different supramolecular structures were observed depending on the pH. From the weakest alkaline solution, a fairly rough morphology with a high density of spikelike growth features was revealed. As the ionic force of the medium increased, the size of the spikelike growth features diminished, and even many disaggregated fibers could be found. At pH 13.16, the aggregates had disappeared almost totally. Because of the stiffness and the rodlike character of fibers,

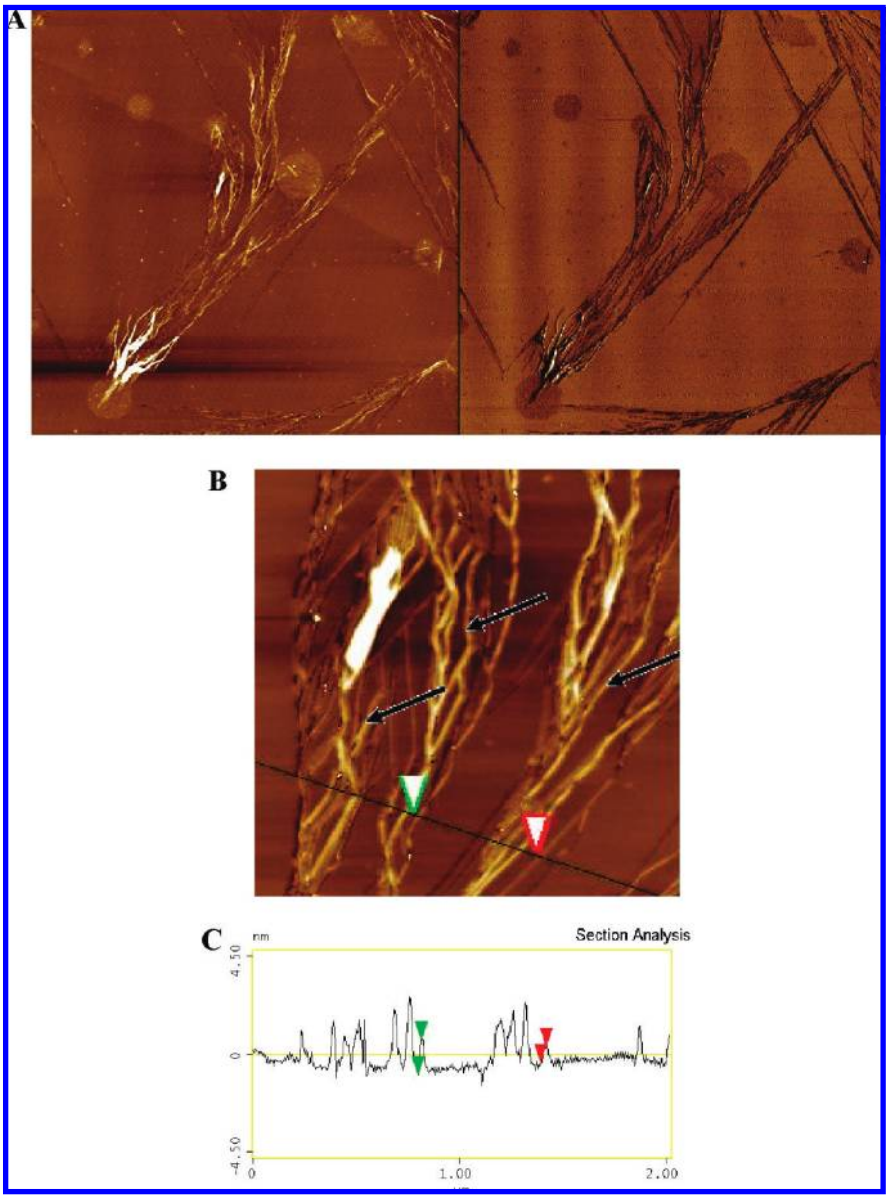

Figure 6. TM-AFM height (left) and phase (right) images of (1→3)(1→2)-β-D-glucan. Images were obtained by depositing the glucan onto mica and scanning in air from 0.4 M NaOH aqueous solution, pH 13.16. (**A**) Images of 12 μm × 12 μm. (**B**) Height image of 2 μm × 2 μm. (**C**) Height profile of the cross-section highlighted in panel **B**. Markers on the image indicate the points where the measurements were taken.

a helical conformation is assigned. At 0.4 M, pH 13.16, the dissociation of the helical structures takes place.

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