Eur Food Res Technol (2006) 223: 78–82
DOI 10.1007/s00217-005-0138-3

ORIGINAL PAPER

Hongshun Yang · Hongjie An · Yunfei Li

Manipulate and stretch single pectin molecules with modified molecular combing and fluid fixation techniques

Received: 9 May 2005 / Revised: 2 August 2005 / Accepted: 14 August 2005 / Published online: 28 December 2005

Abstract Manipulating and stretching single pectin molecules offer convenience to determine the chain lengths and further illustrate the action between pectins and other macromolecules. Pectin molecules on mica surfaces, which were imaged in air with atomic force microscopy (AFM), can be aligned and stretched through applying modified molecular combing and fluid fixation techniques. Modified molecular combing showed more effective and powerful for manipulation than fluid fixation. Chelate-soluble pectin (CSP) can be straightened into line to one direction by molecular combing while sodium carbonate-soluble pectin (SSP) shows 'V' structures. The differences may be the result of the different parts of the pectins which touched the mica surface firstly during the dropping, and the lengths of the chains. CSP was relatively long and initially touched the mica with the end point while SSP was short and touched the mica firstly with its middle part.

Keywords Pectin · Atomic force microscopy (AFM) · Manipulate · Peach · Structure

Introduction

Plant pectin is a very important foodstuff and additive to food products. The quality attributes of fruits and vegetables are closely related to pectin structures and contents [1]. Traditional analytical methods like NMR [2], and biochemistry [3] have been used to illustrate the pectin structures of fruits and vegetables. However, due to a heterogeneous structure, pectin with varied structures or complex repeat units would tend to be averaged across the whole sample with most methods.

Atomic force microscopy (AFM) offers an opportunity to study individual molecules adsorbed onto a variety of surfaces without contrast agent or shadowing [4]. Visualization of the pectin structure has been reported [5–8], the pectin branching, molecular mass distribution, and the degradation mode of chain widths with time also have been illustrated. However, up to now one can only passively view the structural characteristics according to the states on the mica, pectin molecules often tangle with each other and it is not available to give exact statistical data on the chain lengths.

Manipulation of food macromolecules has a wide use in food science. For example, manipulation of pectin molecules would help to illustrate the effects of pectins on food quality and the concrete changes of pectins themselves during food process and storage. Extension of pectin molecules offers a chance to calculate the lengths of pectins. And further, aggregation of molecules or entanglement of a single linear molecule will result in potentially observable molecular features being obscured [9], manipulation makes the pectin strands not tangled and offers us to observe the reaction between pectins and other molecules directly.

Recently, with the rising interest in nanotechnology, manipulation of molecules in nanometer scale has become a hot scientific issue [10]. Reported manipulation on macromolecules (almost all are on DNA) can be divided into two groups. The former is about the sample preparation before imaging, mainly two methods can be used independently, one is 'molecular combing' [11, 12] and modified 'molecular combing' [13–16], the other is 'fluid fixation' [9] including gas flow [17]. The latter is used during the imaging, several research groups have attempted to isolate biological macromolecules with AFM tips. Precisely cutting, pushing, and folding of single DNA molecules by

The same contribution to the first author.

H. Yang · Y. Li (✉)
Department of Food Science and Technology, Shanghai Jiao Tong University,
2678 Qixin Road, Shanghai, 201101 P.R. China
e-mail: yfli@sjtu.edu.cn
Tel.: +086-21-6478-3085
Fax: +086-21-6478-3085

H. Yang
Institute of Refrigeration and Cryogenics Engineering,
Shanghai Jiao Tong University, Shanghai, 200030 P.R. China

H. An
Nanobiology Laboratory, Shanghai Jiao Tong University,
Shanghai, 200030 P.R. China

AFM have been illustrated, the operations including imaging, dissecting, and picking up were done with one AFM tip [16].

In this paper, sample preparation to manipulating the pectin molecules are introduced. Modified molecule combing and fluid fixation techniques were used to stretch chelate-soluble pectin (CSP) and sodium carbonate-soluble pectin (SSP). The principle of different AFM manipulation effects was illustrated.

Materials and methods

Materials and pectin extraction

'Jinxiu' yellow peaches (*prunus persica* L. Batsch.), a clingstone, nonmelting variety with average weight of 255 g (n=60), at a preclimacteric stage, were bought from the market within 5 h harvested in August from an orchard in Fengxian, Shanghai, China. The water-soluble pectin (WSP), CSP, and SSP were extracted according to the method of Zhou et al. [18]. Peaches were peeled and flesh at each time was used for extraction. The flesh was boiled in ethanol, then was decanted by filtration, and the solid residue was transferred to ultra purified water (Milli-Q Biocel Pure Water Equipment, Millipore Co. Ltd., France). After 2 h extraction, supernatant was collected (WSP) and the pellet was washed with acetone, then with 1 chloroform: 1 methanol (v/v). The pellet was resuspended in trans-1, 2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA), shaken and centrifuged, and the supernatant was collected (CSP), and then the pellet was extracted twice more with 10 mM CDTA. The pellet was resuspended in 20 ml, 50 mM Na_2CO_3 and 20 mM $NaBH_4$ at 4°C for 18 h, then 2 h at room temperature, was centrifuged, and was extracted twice more with the above Na_2CO_3. The supernatants were adjusted to pH 7.0 with acetic acid and SSP was obtained. Detailed procedures can be seen in our previous reports [7, 8]. The three kinds of pectin fractions were refrigerated and stored below −18°C.

Sample preparation for imaging

The pectin fractions were recovered to room temperature naturally, then strongly dispersed with Vortex, and a small volume (about 10 μl) of the pectin solutions was pipetted briefly (about 5 s) onto the freshly cleaved mica surface.

Three groups were divided according to the differences of manipulation exerted.

1. The solutions without manipulation;
2. The solutions manipulated with fluid fixation, that means, a continuous convective water flow exerted. The velocity of the convective flow within the droplet increases gradually, and by adjusting the droplet size, the stretching forces can be optimized [9];
3. Modified molecular combing was exerted, with a glass cover slip to comb the solutions and an extra pressure with a finger on the glass cover slip while combing. The manipulation should be accomplished in about 10 s, in case of the conglomeration of pectin molecules.

Then, the above solutions were air dried or dried in a dust-free enclosure. The concentrations of pectin solutions can be diluted to get images of individual polymers.

AFM manipulation

Nanoscope IIIa AFM system(Veeco/DI, Santa Barbara, CA) was manipulated in a glove box at 30–40% RH and 23–25°C. The relative humidity inside the glove box was controlled by silica gel and stabilized for at least 5 h prior to AFM observation. Tapping mode was carried out equipped with a Si_3N_4 cantilevered scanner with a 12×12 μm^2 scan size, a 4 μm vertical range, and a scan speed about 1–2 Hz. The integrity of the AFM tip was verified by imaging a reference standard with a known roughness of 5–7 nm [7, 8, 19].

AFM image analysis

The bright and dark areas in the image corresponded to peaks and troughs in the pectin chains. Different scales were used in the vertical and horizontal scale. AFM images are shown in the height mode without any image processing except flattening. Branched structures were distinguished from overlapping molecules by measuring the heights of the chains. In general, the heights of the chains were raised to two times when two chains crossed over one another. At genuine branch points the height remained unchanged [7, 8].

Results and discussion

Usually, biological samples must be strongly attached to an atomic flat matrix surface so that they are immobile in a buffer solution and not swept away before imaging with AFM. For pectin molecules, the surfaces should have the following characteristics: (i) they meet the requirement of being molecularly or atomically flat; (ii) they have a strong binding ability to pectin; (iii) their preparation is simple and convenient; (iv) they are stable in a buffer solution (better in aqueous) for a long time [13].

Pectin molecules were soft and easily tangled together in nature status after being deposited on the mica surface and air-dried. Most of the molecules were in conglomeraion (Figs. 1A and 2A), even the solution had been treated with long time dispersion.

Only few images about the separate pectins can be viewed (Figs. 1B and 2B), they were distributed randomly like soft threads. Most of them showed curved or coiled shapes, just like DNA molecules reported by Hu et al. [10].

With "fluid fixation" manipulation, molecular elongation and fixation were partially accomplished. The orientation

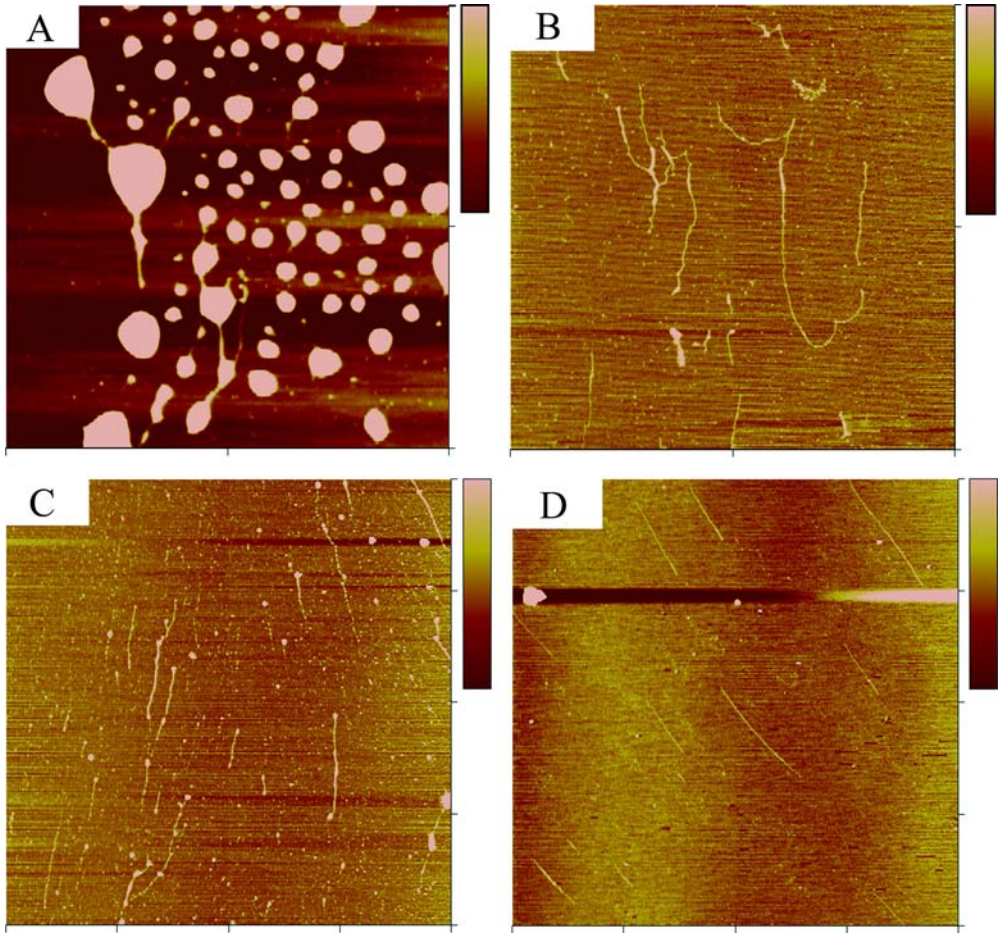

Fig. 1 AFM images of effects of manipulation on the chelate-soluble pectin from peaches. Height *bar*: 2 nm. **A** typical image without manipulation, image size: 5.0×5.0 μm. **B** untypical image without manipulation, image size: 5.0×5.0 μm. **C** image after fluid fixation manipulation, image size: 10.0×10.0 μm. **D** image after modified molecular combing manipulation, image size: 10.0×10.0 μm

of the aligned pectin molecules depends on the direction of the fluid flow during the process [15]. From the results, pectin molecules show a strong binding ability to the mica surfaces and could not be washed away by water fluid at a certain speed, indicating strong adsorption of mica to the pectin molecules, however, elongation of pectin molecules created by tilting the substrate was not available, which had successfully been applied on DNA molecules [9]. The actions between the pectin and mica might be electronic attraction like other macromolecules [13]. Generally, pectin molecules can be stretched to some degree (Figs. 1C and 2C), however, it is hard to get wholly-stretched molecules.

For molecular combing, extra pressure with a finger on the glass cover slip should be used while combing for getting stretched pectin molecules. The CSP molecules can be stretched thoroughly (Fig. 1D) and the molecule lengths could be plotted by the AFM software exactly (data not shown), the extra force might enhance the spreading speed of the pectin solution and thus the straight pectin patterns can be obtained [13]. However, for SSP molecules, it was almost impossible to get straight chains (Fig. 2D), indicating the different action mode between the two kinds of pectin and mica [10].

After being treated by the modified molecular combing method, the molecules changed their shapes and became straight and were aligned in the same direction (Fig. 1D), indicating that stretching force exerted during combing was efficient. The extension of the molecule is mainly due to the tension of the receding meniscus which is strong enough to lengthen the pectin [11]. Therefore, the principle of CSP stretching can be interpreted. (Fig. 3A).

When CSP molecules in the chelator were dropped on the mica, the weight of the end part (M) would overweigh the long chain part (O, N), then the part of M would touch the mica surfaces first, it became fixed by adsorption force, the other parts of the strands (O and N) kept moving forward when molecular combing exerted, then the molecule was aligned in one direction.

However, compared to the CSP molecules, the relatively short chains of SSP molecules can be independently dropped on the mica surfaces without necessarily combining with the sodium carbonate [8]. In such a situation, the middle parts (O) of the chains would touch the mica surface first but rarely the end parts (M or N), 'V' shape strands would appear most (Fig. 2D). The reason was that after immediate adsorption of ON on the mica, the OM part would be varied according to the fluid orientation (for fluid fixation) or the direction of extra pressure (for molecular combing), then the result may be that OM and ON showed straight, however, not in the same direction (Fig. 3B). The schemes of practical alignments of the two kinds of pectin molecules are shown in Fig. 3C,D, which

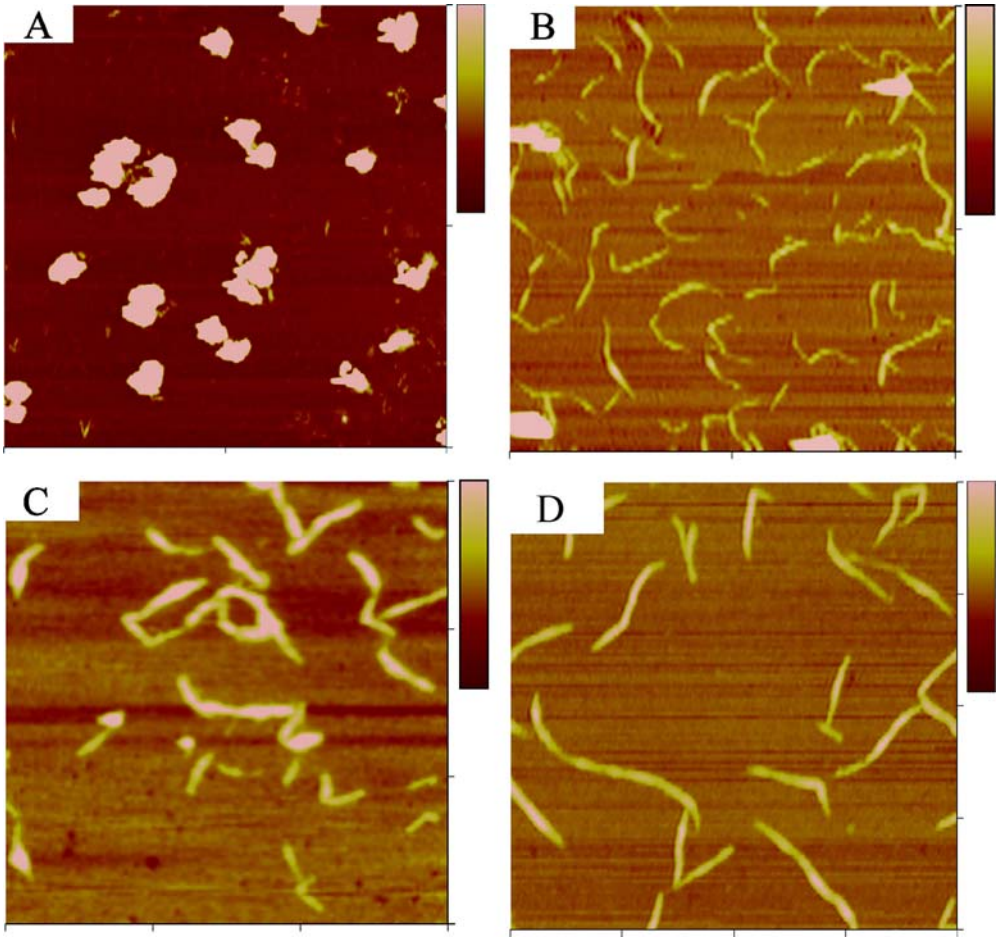

Fig. 2 AFM images of effects of manipulation on the sodium carbonate-soluble pectin from peaches. Height *bar*: 5 nm. **A** typical image without manipulation, image size: 5.0×5.0 μm. **B** untypical image without manipulation, image size: 2.0×2.0 μm. **C** image after fluid fixation manipulation, image size: 1.5×1.5 μm. **D** image after modified molecular combing manipulation, image size: 1.0×1.0 μm

were from Figs. 1D and 2D, respectively. Please note that the scales of the scan areas are different. The practical schemes of alignments were consistent well with the principle we proposed. When the extra pressure was little exerted, the results of manipulation may be similar to those of the fluid fixation actions (Figs. 1C and 2C). The WSP shows a similar result to CSP but is not discussed in detail.

Liu et al. [14] reported high temperature may prohibit starch molecules conglomerating each other and spreading rapidly on the mica surfaces [14], however, considering the probable degradation during high-temperature processing, high temperature was not applied on pectins in our experiments.

Comparing the results of pectins before and after manipulation, it can be seen that the pectins were uniform and the structure was not damaged in the course of preparation and stretching, which indicates that pectin has a certain degree of stability.

Comparing with the DNA molecules, the peach pectins in our experiments showed a stronger binding ability to the mica surface, no need to graft other molecules or use other kinds of surfaces as DNA did [13]. The molecular combing for pectins should be quick with a vertical speed of at least 10 mm/s and the manipulation should be finished in about 5 s, while the speed was 0.3 mm/s and the manipulation needed was at least 5 min more for DNA molecules absorbed onto the APS (aminopropyl silatrane)-mica [10, 12]. No significant effects of the substrate surfaces on the spreading of the pectin solution or resulted tanglements were found, however, whether it is the same with the conformational changes that took place in liquid state imaging needs to be further investigated [20]. In our experiments, we focused on the pectins' genuine states in solutions, so the solutes of CDTA and sodium carbonate for CSP and SSP, respectively had not been dialyzed. Further work should be conducted for pectins without other solutes to investigate the effects of other solutes on the mode of adhesion to the mica surface.

In summary, we have developed a unique and novel method for positioning, stretching, and aligning of individual pectin molecules. The imaging was quite stable and reproducible. Future work will be focused on pectin length changes and interactions with other macromolecules during processing and storage. Specifically, pectins and macromolecules during different stages of processing and storage can be extracted, then manipulated by AFM, the lengths of manipulated pectins can be measured by AFM software easily, and the height information of AFM images of various macromolecules would illustrate their interactions. Given the convenient manipulation of the presented method, great applications will be expected in many fields of food science and technology.

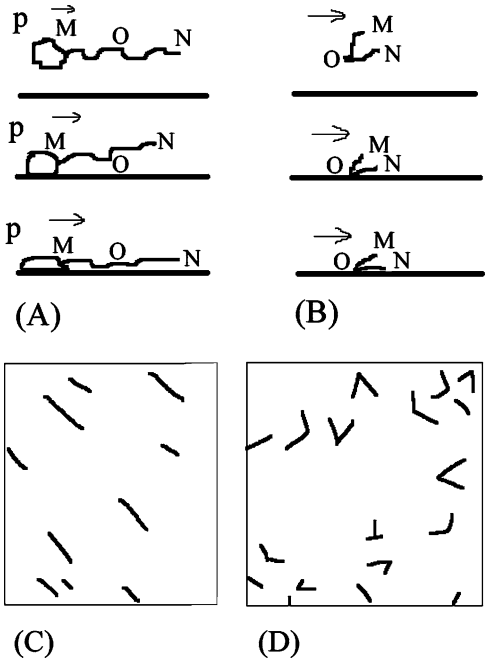

Fig. 3 Schematic images of theoretical and practical manipulation and aligning of the pectin molecules. **A** possible stretching pathway of chelate-soluble pectin molecules. **B** possible stretching pathway of sodium carbonate-soluble pectin molecules. **C** scheme of chelate-soluble pectin alignment after modified molecular combing manipulation. Image size: 10.0×10.0 μm. **D** scheme of sodium carbonate-soluble pectin alignment after modified molecular combing manipulation. Image size: 1.0×1.0 μm. Note: P, means chelator, CDTA; M, N, mean end points of pectin molecular chain; O, means the middle point of pectin chain

Conclusion

Pectin molecules on mica surfaces, which were imaged in air with AFM, can be manipulated through applying modified molecular combing and fluid fixation techniques. Modified molecular combing showed the effects of pectin molecules' manipulation more markedly than fluid fixation. CSP can be straightened into line to one direction by molecular combing while SSP shows 'V' structures. The end point of CSP and the middle part of SSP, which touched the mica surface first during dropping, and the different lengths of the two kinds of pectins may lead to the different effects after AFM manipulation.

Acknowledgements This research was supported by grants from the National Key Technologies R and D Programme (Project No. 2004BA527B) and the National Economy and Commerce Committee (Project No. 02CJ-12-07-03).

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