Single-Molecule Force Spectroscopy on *Bombyx mori* Silk Fibroin by Atomic Force Microscopy

Wenke Zhang, Qiaobing Xu, Shan Zou, Hongbin Li, Weiqing Xu, and Xi Zhang*

Key Lab of Supramolecular Structure and Spectroscopy and Department of Chemistry, Jilin University, Changchun, 130023, P.R. China

Zhengzhong Shao

Department of Macromolecular Science and Laboratory of Molecular Engineering of Polymers, Fudan University, Shanghai, 200433, P.R. China

Michael Kudera and Hermann E. Gaub

Lehrstuhl für Angewandte Physik, Ludwig-Maximilians-Universität München, Amalienstr. 54, 80799, München, Germany

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A new atomic force microscopy (AFM)-based technique, single-molecule force spectroscopy (SMFS), was used to study the nanomechanics of *Bombyx mori* silk fibroin. Three types of force-extension curves were found in the system. A modified freely jointed chain (MFJC) model can fit two of the three types well, but the fit parameters are different. The third type of force curve, in which there exists a plateau, cannot be modeled by MFJC. These results may show that there are three kinds of conformations in the silk fibroin system and SMFS can "distinguish" different conformations of the polymer chains.

Introduction

Single-molecule force spectroscopy (SMFS) has allowed the study of nanomechanical properties of biomacromolecues[1−3] as well as synthetic polymers.[4−6] This technique, with piconewton sensitivity and subnanometer accuracy, is powerful not only in understanding the elastic properties of random coil polymers[4] but also in exploiting the conformational changes of polymers with suprastructures.[3,5−7] Some specific fingerprint information of nanomechanical properties of polymer chains has been obtained by using this method that cannot be achieved by conventional methods. For example, the SMFS spectra of 1,4-α-linked polysaccharides, such as amylose, heparin, and dextran, revealed a force-induced chair−twist boat conformational transition of individual glucopyranose rings that could be identified as a force plateau in force−extension curves.[8,9] Analysis of poly(vinyl alcohol) by SMFS revealed the force-induced conformational transition of a helical structure in the synthetic polymer system, which resulted in a kink at ∼200 pN in the force curves.[5] SMFS information can provide us with new insights into the mechanical properties of polymers that may help us to design and synthesize new high-performance materials. Moreover, according to the force fingerprint spectrum of amylose, Grandbois et al.[10] have been able to measure the rupture force of silicon−carbon and sulfur−gold bonds, which is not possible by conventional methods.

Silk fibroin is a widely used fibrous biopolymer produced by different silkworm species. In addition to its use as a starting material for textile applications, it has been investigated as a starting material for nontextile applications,[11] especially in the biomedical and biotechnological field. Many investigations on the conformation of silk in the natural fiber and in regenerated silk fibroin films have been made.[11−13] Silk fibroin consists of 18 kinds of amino acids, among which Gly, Ala, and Ser comprise ∼85%.[10] Three conformations have been found for silk fibroin; namely, random coil, α-helix, and *β*-sheet forms. Bhat et al.[12] and Tsukada[11] studied the conformation transition induced by solvents and other treatments by infrared (IR) spectroscopy and X-ray diffractometry. In addition, on the basis of theoretical conformation analysis of silk fibroin, model polypeptide with an Ala-Gly repeated sequence was given.[13] So far, no report exists on what the conformational transition caused by stretching a single fibroin chain looks like. Can we distinguish those different structures in the fibroin system by atomic force microscopy (AFM)?

* To whom correspondence should be addressed. E-mail: xi@mail.jlu.edu.cn.

(1) Rief, M.; Oesterhelt, F.; Heymann, B.; Gaub, H. E. *Science* (*Washington, D. C.*) **1997**, *275*, 1295.
(2) Rief, M.; Gautol, M.; Oesterhelt, F.; Fornandez, J.; Gaub, H. E. *Science* (*Washington, D. C.*) **1997**, *276*, 1109.
(3) Li, H. B.; Rief, M.; Oesterhelt, F.; Gaub, H. E. *Adv. Mater.* **1998**, *10*, 316.
(4) Li, H. B.; Liu, B. B.; Zhang, X.; Gao, C. X.; Shen, J. C. and Zou, G. T. *Langmuir* **1999**, *15*, 2120.
(5) (a) Li, H. B.; Zhang, W. K.; Zhang, X.; Shen, J. C.; Liu, B. B.; Gao, C. X.; Zou, G. T. *Macromol. Rapid Commun.* **1998**, *19*, 609. (b) Li, H. B.; Zhang, W. K.; Xu, W. Q.; Zhang, X.; Shen, J. C. *Macromolecules* **2000**, *33*, 465−469.
(6) Oesterhelt, F.; Rief, M.; Gaub, H. E. *New J. Phys.* **1999**, *1*, 1.
(7) Ikai, A.; Mitsui, K.; Furutani, Y.; Hara, M.; Mcmurty, J.; Wong, K. P. *Jpn. J. Appl. Phys.* **1997**, *36*, 3887.
(8) Li, H. B.; Rief; M.; Oesterhelt, F.; Heymann, B.; Gaub, H. E.; Zhang, X.; Shen, J. C. *Chem. Phys. Lett.* **1999**, *305*, 196.
(9) Marszalek, P. E.; Oberhauser, A. F.; Pang, Y. P.; Fernandez, J. M. *Nature* **1998**, *396*, 661.
(10) Grandbois, M.; Beyer, M.; Rief, M.; Clausen-Schaumann, H.; Gaub, H. E. *Science* (*Washington, D. C.*) **1999**, *283*, 1727.
(11) Tsukada, M.; Gotoh, Y.; Nagura, M.; Minoura, N.; Kasai, N.; Freddi, G. *J. Polym. Sci., Part B: Polym. Phys.* **1994**, *32*, 961.
(12) Bhat, N. V.; Ahirrao, S. M. *J. Polym. Sci., Polym. Chem. Ed.* **1983**, *21*, 1273.
(13) Oka, M.; Baba, Y.; Kagemoto, A.; Nakajima, A. *Polym. J.* **1990**, *22*, 416.

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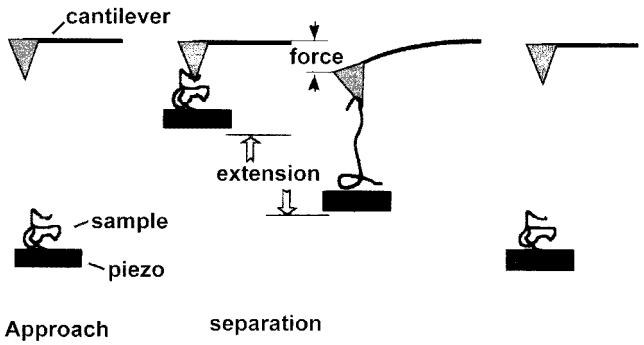
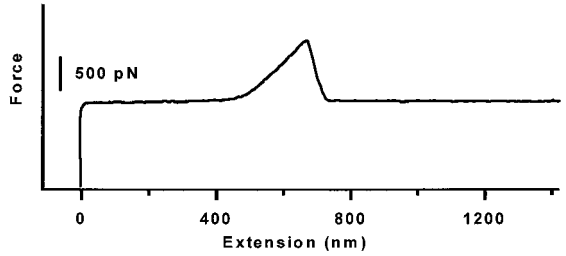
Scheme 1. A sketch of the SMFS experimental principle.





In this paper, we use SMFS to study the mechanical properties of a single silkworm fibroin. The SMFS spectra show a normal random coil formation, as well as unusual force curves whose shapes are similar to the force curves of polysaccharides.

Experimental Section

Solution-cast *Bombyx mori* silk fibroin films were prepared as follows. Degummed silk was dissolved in aqueous 9.3 M LiBr solution at 60−65 °C. The solution was put into the cellulose tube and was rinsed with running water for 20 h to remove most of the salt. Then the solution was dialyzed against deionized water at room temperature (15 °C) for ∼3 days (the water was changed every 2 h) and was filtered with Millipore filters (pore size 2 μm). The final concentration was about 0.1 mg/mL. Appropriate fibroin solution was deposited onto a $-NH_2$-group-modified quartz substrate and dried in air (∼20 h); then, the sample was rinsed with water and immediately used for experiments. We call this kind of sample natural fibroin (NF). The $-NH_2$-group-modified quartz slide was prepared as follows.[14] The quartz slide was put into a mixture of H_2O_2 and H_2SO_4 (30/70, v/v), and the solution was heated until there were no bubbles coming from the slide. After rinsing with deionized water and drying in an oven, the slide was put into a mixed solution of 3-aminopropyl dimethylethoxysilane and toluene (1/50, v/v) and kept in the solution for ∼8 h. After that, the slide was rinsed with toluene, chloroform, and then ethanol.

The force measurements were carried out in deionized water on a custom-built AFM setup. Silicon nitride cantilevers from Digital Instrument (DI, Santa Barbara, CA) were used. The spring constants of the cantilevers were calibrated by measuring their thermal excitation.[15,16] Measured values were ∼0.045−0.058 N/m. Scheme 1 shows a sketch of the SMFS experimental principle. The experimental details of SMFS by AFM have been described elsewhere.[1,3] In brief, silk fibroin was immobilized onto the glass substrate by physical adsorption. To do the force experiments, an AFM tip was brought into contact with the sample so that some molecules could adsorb onto the tip, due to nonspecific interaction between the polymer and the tip, and produce a connective bridge between the polymer and tip. During separation of the tip and the sample, the molecule is stretched. At this time, the deflection−extension curve was recorded and converted into a force−extension curve.

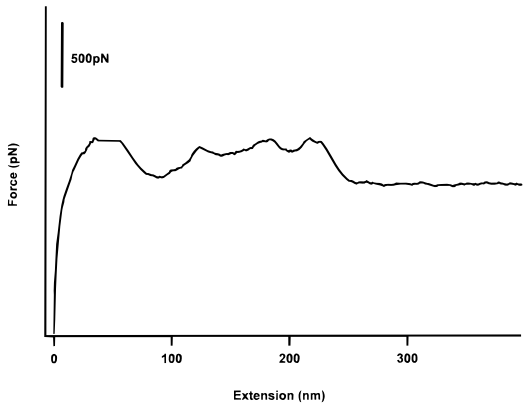


Figure 1. The force−extension curves of a bundle of chains of silk fibroin attached to a glass substrate.

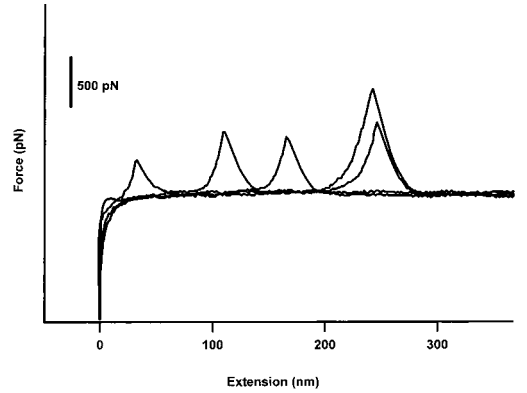


Figure 2. Typical force curves of type 1 on regenerated silk fibroin in water.

Results and Discussion

To make a single-molecule experiment successful, we tried to use a normal glass slide [poly(tetrafluoroethylene) and quartz substrate], but we could not obtain clean force−extension curves. Figure 1 shows an example of the force curves, indicating a bundle of stretched chains. Finally, we found that the $-NH_2$-group-modified quartz slide is suitable for this system. Furthermore, when the sample was dried, the probability that we can stretch a polymer chain became larger, possibly because of the strong binding of the polymer thin film to the substrate. To obtain single-chain stretching, we controlled the touch force between tip and sample during the approaching process. When the force was too large, multichain stretching was observed; when the force became small, it was difficult to pick up a polymer chain. In other words, we adopt the so-called "fly fishing-mode" method to effectively avoid multichain stretching. The facts that the force curves can be normalized and superimposed and the segment elasticity and Kuhn length of the polymer chains are the same can be regarded as the standard to confirm that a single chain was stretched.

Figure 2 shows some typical force curves (noted type 1) of NF measured on different samples using different cantilevers in deionized water. Because the silk fibroin was adsorbed on substrate by physical adsorption, the distribution of molecular chain length was disperse. Moreover, we were not able to control the point at which the polymer chain was picked up, so the contour length of the polymer chain between the cantilever tip and the

(14) Haller, I. *J. Am. Chem. Soc.* **1978**, *100*, 8050.
(15) Florin, E. L.; Rief, M.; Lehmann, H.; Ludwig, M.; Dornmair, C.; Moy, V. T.; Gaub, H. E. *Biosens. Bioelectron.* **1995**, *10*, 895.
(16) Butt, H. J.; Jaschle, M. *Nanotechnology* **1995**, *6*, 1.

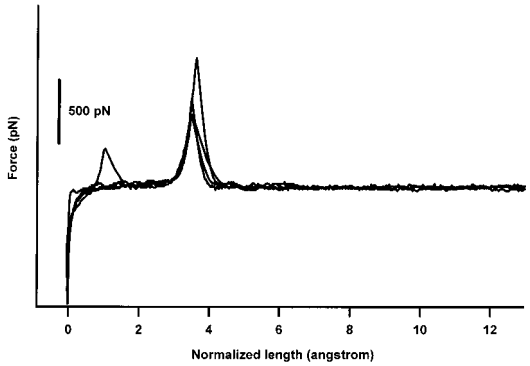


Figure 3. The force curves of silk fibroin shown in Figure 1 as normalized by their contour lengths.

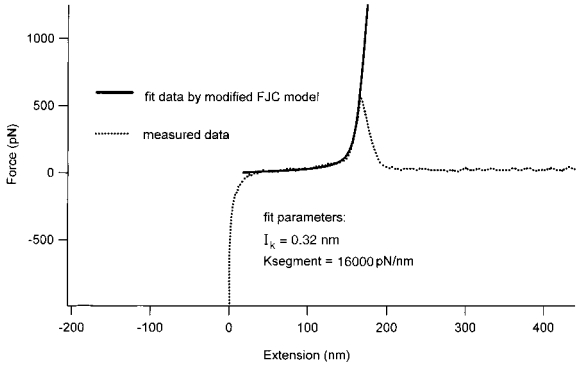


Figure 4. Comparison of experimental data of type 1 with the MFJC model.

substrate was disperse too. These kinds of force curves have similar properties; that is, despite the difference in contour lengths, they exhibit similar characteristics (e.g., the force rises monotonically with the extension until a rupture force is reached). To compare the force−extension relationship of polymers of different contour lengths, the force−extension traces were normalized by their lengths and were superimposed and plotted in Figure 3. The normalized result shows that the force curves can be normalized and superimposed very well and the elastic properties of silk fibroin chains scale linearly with the contour length. This finding corroborates that the elasticity of single fibroin molecule was measured and provides a rigid criterion for differentiating single- and multiple-molecular bridges. It has been shown that the elasticity of single polymer coil can be well described by an extended Langevin function shown as following, which is derived from a modified freely-jointed-chain model (MFJC model): $x(F) = [\coth(FI_k/k_BT) - k_BT/FI_k](L_{contour} + nF/K_{segment})$. Here x is the extension of the polymer chain (end-to-end distance), n is the number of Kuhn segments, equal to $L_{contour}/I_k$. The MFJC model treats the polymer as a chain of statistically independent segments of lengths I_k (Kuhn length), and the segment can be deformed under stress. The deformability of segments is characterized by a specific parameter, segment elasticity, $K_{segment}$. Such force−extension curves can be fitted well with a MFJC model (shown in Figure 4). The fit results show that the force−extension curves have the same segment elasticity (∼16 000 pN/nm) and Kuhn lengths (∼0.32 nm), although they have different contour lengths. In addition, there exists another type (noted type 2) of force curves that are similar to type 1 curves. Type 2 force curves can also be normalized and superimposed well, but the slope reflecting segment elasticity of the force curves in the high force regime are different, as shown in Figures 5 and 6. From

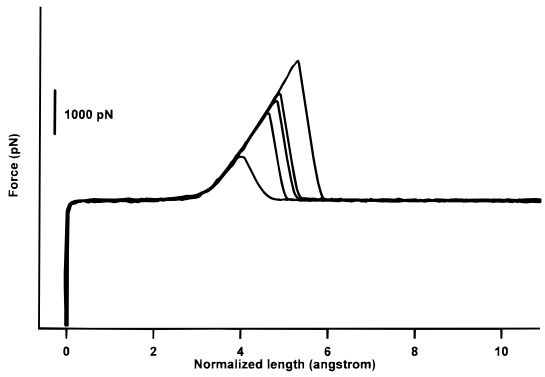


Figure 5. The normalized force curves of type 2.

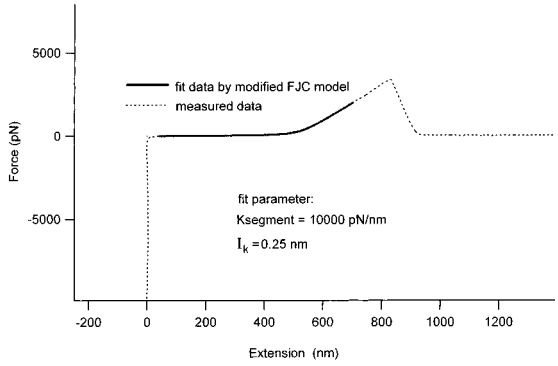


Figure 6. Force curves of type 2, superimposed with a MFJC fit curve; fit parameters are shown in the inset.

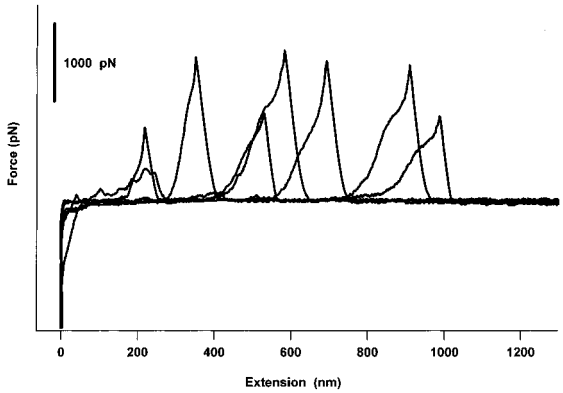


Figure 7. Force curves of type 3, with a plateau on regenerated silk fibroin.

these results we can deduce that the segment elasticity of type 2 is ∼10 000 pN/nm lower than that of type 1. This difference may indicate that there exist two kinds of random coil conformations for silk fibroin in water and the segment elasticities are different. This result is in agreement with the complexity of the composition of the silk fibroin.[10−13]

For the NF sample, there exist other kinds of force−extension curves exhibiting similar characteristics. First, the force rises monotonically with the extension, and then a plateau appears. The heights of the plateaus are different, ranging from 200 to 1500 pN. Next, the force rises sharply after the plateau and then drops to zero rapidly (see Figure 7). Although the height and the width of the plateau are different, the normalized force curves can be superimposed well in the high-force force regime above the plateau (see Figure 8). This result means that the overstretched states of the molecule are the same and the bridge between tip and substrate is of the same spring

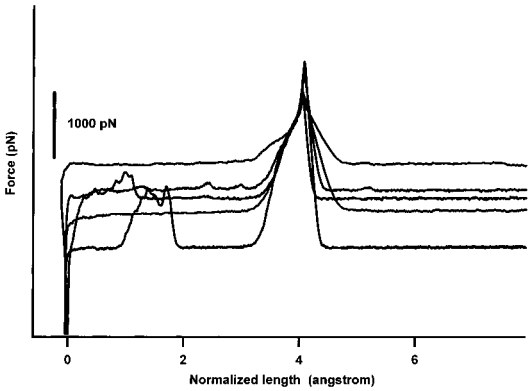


Figure 8. Superposition of the normalized force curves of type 3 in the high-force regime.

constants. This result may also support the fact that a single polymer chain was picked up. The molecules have the same state under high stress although the conformations at the relaxed state are different. The plateau may indicate that there exists strong intramolecular hydrogen bonding in the silk fibroin. This difference in height of the plateau may also indicate different proportion of intramolecular hydrogen bonding in the system. Yu et al. investigated the conformation change of single silk fiber in different parts of silkgland under stress and gave relevant model.[17] Their results show there are random coil and α-helix structures in the unstretched state. Due to the presence of strong intramolecular hydrogen bonding, the α-helix structure cannot be destroyed unless larger stress is exploited. Under stretching, the α-helix structure was destroyed gradually and turned into a more extended state. Under further stretching, the α-helix structure was completely destroyed and partially turned into a random coil conformation so that only β-sheet and random coil conformations existed in the fiber. These authors attributed the random coil conformation after stretching to the part that arised from the α-helix structure in the fibroin. In our results, it is possible that we sometimes stretch a single fibroin chain of the α-helix structure, and the plateau in the force curve could correspond to the unwinding of the α-helix structure.

(17) Yu, T. Y.; Li, G. X. *Acta Polym. Sinica* **1993**, *4*, 415.

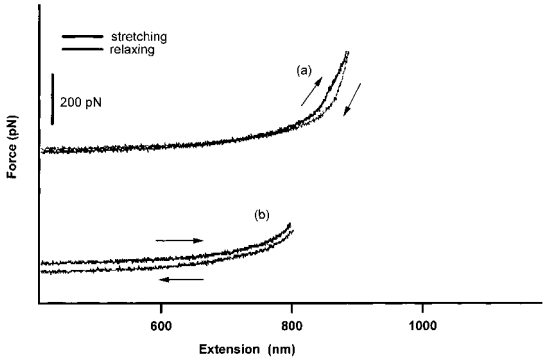


Figure 9. Stretching and relaxing traces of a single silk fibroin molecule (a) above the plateau and (b) below the plateau.

To see whether the conformational transition is reversible or not, we studied the cyclic process of stretching and relaxing. The force curves show that the process is irreversible at our experimental rate (0. 3−5.9 μm/s), as shown in Figure 9a. If the force is kept below the plateau in a subsequent deformation, the force curves show a reversible elasticity (seen in Figure 9b). This result proves that the conformational transition in the plateau regime is exhaustible.

In conclusion, three types of force curves were obtained on NF samples that may be attributed to conformational transition of different kinds of silk fibroin chains. For instance, force curves with a plateau correspond to the polymer chains in which strong intramolecular hydrogen bonding exists and force curves without plateau correspond to usual random coil parts. From the results, we can also see that SMFS can "distinguish" polymer chains of different conformations in the same system. However, the silk system is more complicated than expected. Moreover, the SMFS is in its developmental stage, and it takes time to accumulate data and correlate the "fingerprint" of force−extension curves with the micro- or nanostructures of polymers or biopolymers.

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