Artificial Spinning and Characterization of Silk Fiber from *Bombyx mori* Silk Fibroin in Hexafluoroacetone Hydrate

Juming Yao, Hiromi Masuda, Chenhua Zhao, and Tetsuo Asakura*

Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan

Received July 27, 2001

Introduction. Silk proteins are of practical interest because of their excellent intrinsic properties utilizable in biotechnological and biomedical fields as well as the importance of silkworm silks in the manufacture of high-quality textiles.[1−5] Moreover, moderate quantities of silks and silk-mimic biopolymers have been achieved due to the advances in molecular biotechnology and protein engineering.[6−8] However, production of useful materials, such as fibers, from these potential supplies of unprocessed biopolymers requires a detailed understanding of processing conditions necessary to induce the hierarchical structure responsible for the outstanding mechanical properties exhibited by native fibers. So the final hurdle on the way to the production of man-made silks now lies in the development of an appropriate spinning technology capable of converting these raw materials into high-performance fibers. It is advantageous to focus on the regeneration of natural silks because it can be used as a benchmark for evaluating the success of the spinning process. In addition, since silkworms produce filaments in only one size (ca. 1−2 deniers), twisted or braided yarns must be used when loads exceed a few grams. It would be desirable to produce silk fibers in deniers other than those found in nature that would be suitable for such applications as monofilament sutures. Unfortunately, the interstices of a multifilament yarn can be a route for infection. Thus, it is useful to propose a relevant artificial spinning process for the regenerated silk fibers.

Many reports have been published on the regeneration of silk fibers including spider silk.[9−16] Since the fiber properties depend on the conditions under which they have been obtained, the nature of the solvent and the postspinning treatments applied to the fibers may be the important factors, affecting the overall quality and properties of the fibers produced. Earlier, HFIP has been proposed as useful solvent for the regeneration of silk fibroin; i.e., a film with the silk I form prepared from the native silk fiber has been used for dissolving it for the preparation of the regenerated silk fiber.[9,12,14,16] However, *B. mori* and also a wild-type silkworm *S. c. ricini* produce silk fibers that are essentially insoluble in HFIP as is the fusion protein from these two silk fibroins obtained via genetically engineered syntheses.[17] Thus, new alternate solvents are required in order to produce the silk fibers effectively.

In this paper, we attempt to show that HFA-hydrate may be a solvent of choice for the preparation of regenerated silk fibroin fibers where HFIP exhibit limited applicability. A process for the artificial spinning of *B. mori* silk fibroin solution in HFA-hydrate was confirmed. Solid-state ^{13}C nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC), and wide-angle X-ray diffraction (WAXD) are used for the characterization of regenerated silk fibers obtained here.

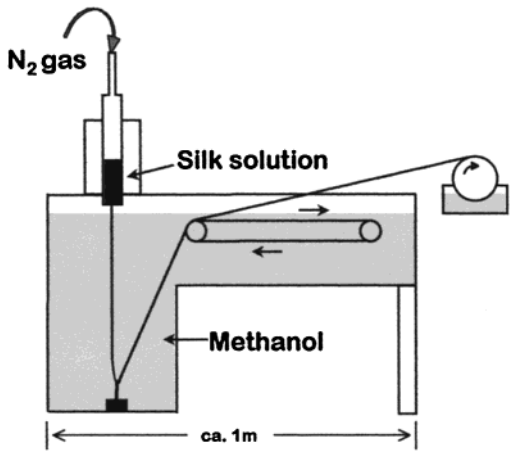


Figure 1. Brief view of the apparatus for the preparation of regenerated silk fibers from the solution of silk fibroin.

Experimental Section. a. Preparation of Regenerated Silk Fiber. Cocoons of *B. mori* were degummed three times with 0.5% (w/w) Marseilles soap solution at 100 °C for 30 min and washed with distilled water in order to remove another silk protein, sericin, from the surface of silk fibers. The silk fibroin fibers were then dissolved in 9 M LiBr at 40 °C. After dialysis against distilled water for 4 days, the solution was gently evaporated by forced airflow to a concentration of less than 2% (w/w). The aqueous solution of silk fibroin was cast onto plastic plates to prepare silk films with the silk I form.[18] Cocoons of *S. c. ricini* were degummed 5 times with 0.5% (w/w) sodium carbonate (Wako Pure Chemical Industries, Ltd.) to get silk fibroin fibers.[19] HFA-hydrate (HFA-trihydrate: $HFA{\cdot}3H_2O$) and HFIP were of analytical grade (Tokyo Kasei Kogyo Co., Ltd.) and used for the solvents of the silk films or silk fibers.

The apparatus used for artificial spinning is shown in Figure 1. The *B. mori* silk fibroin HFA solution at 10% (w/w) was transferred to a pump fitted with a stainless steel 80 mesh screens. The viscosity of this solution at 50% radian was 18.3 P as determined with a mechanical spectrometer (RMS-800, Rheometric Far East Ltd.). The HFA solution was extruded through a stainless steel spinneret with 0.2 mm diameter and 1.2 mm length orifice using high-pressure N_2. Methanol was used as the coagulant since, the fiber prepared with this system is transparent. The as-spun filament passed through a 2 cm air gap before flowing into the methanol coagulation bath at room temperature. The extruded filament was soaked in the methanol bath overnight to allow the HFA to diffuse from the fiber before drawing. To improve the mechanical properties of the fibers obtained, the filament was then drawn to 3 times its original length in distilled water and steam-annealed at 125 °C for 30 min. The fibers after the postspinning treatments were immobilized on the bobbins to prevent recoil and dried overnight at room temperature.

* To whom correspondence should be addressed.

10.1021/ma011335j CCC: $22.00
Published on Web 12/04/2001

Table 1. Dissolving Ability of Silk Fibroins in HFA-Hydrate Compared with HFIP[a]

sample	HFA-hydrate	HFIP
B. mori silk fibroin film	2 h	2 days
B. mori silk fibroin fiber	2 months	no
S. c. ricini silk fibroin fiber	5 days	no

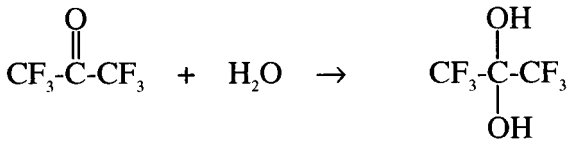
[a] The concentration was 10% (w/w) at 25 °C.

b. Fiber Characterization. The ^{13}C solid-state CP/MAS NMR spectra of the regenerated fibers were obtained on a Chemagnetics CMX 400 MHz spectrometer using a cross-polarization pulse sequence and with magic-angle spinning at 5 or 10 kHz. The experimental conditions were as follows: ^{1}H 90° pulse of 5.00−3.00 μs, contact time of 1 ms, pulse delay of 3 s. ^{13}C chemical shifts are recorded in ppm relative to TMS as the reference. DSC measurements were performed on a Thermoflex DSC8230D (Rigaku Denki Co., Ltd.) at a heating rate of 10 °C/min. The DSC range and sample weight were 2 mcal/s and 2.7 mg, respectively. The open aluminum pan was swept with N_2 during the course of the heating process. Wide-angle X-ray diffraction experiments were performed on a Rigaku fine-focus fixed tube generator with Ni-filtered Cu Kα radiation and a flat film camera with pinhole collimation. Well-aligned fiber bundles of *B. mori* fibers and regenerated fibers were mounted vertically at the exit of the collimator. Si and Ni standards were used for calibration. Typical camera lengths were 1.8 or 3 cm. Mechanical properties of the native and regenerated silk fibers were measured using a Tensilon tensile testing machine (RTM-100) at a temperature of 20 °C and a relative humidity of 65%. All samples were equilibrated in the controlled environment for at least 24 h before testing. The rate of crosshead was 50 mm/min on samples of 90 mm length with chart of 500 mm/min. Each value was the average of 15 measurements.

Results and Discussion. a. Solubility of Silk Fibroin in HFA. The solubility of silk fibroins was examined in HFA-hydrate at 25 °C and compared with that in HFIP (Table 1). It takes only 2 h to dissolve *B. mori* silk fibroin film with silk I form in HFA-hydrate, but it took 2 days in HFIP under similar dissolving conditions. Moreover, both *B. mori* and *S. c. ricini* silk fibers are soluble in HFA-hydrate but essentially insoluble in HFIP. Thus, the solubility of silk fibroins is dramatically improved in the case of HFA, which clearly indicates the advantage of the solvent system. There are no peaks due to degradation components in the ^{13}C solution NMR spectrum of the silk fibroin in HFA solution (data not shown). The *B. mori* silk films with the silk I form was soluble up to 20% (w/w) in HFA-hydrate at 25 °C, but solutions with a concentration of more than 10% (w/w) are very viscous and not suitable for artificial spinning. Thus, 10% (w/w) silk fibroin in HFA was used for artificial spinning.

The potent solvent characteristic for the regeneration of silk fibers may lie in the following factors: (1) disrupting the strong hydrogen bonds in polymers within a short period of time, (2) no degradation of the polymer molecular chains, (3) solution stability over long periods of time with proper viscosity for fiber spinning, and (4) easy diffusion from the fibers. HFA-hydrate can be regarded as a fluoro alcohol, but it is actually a *gem*-diol.[20]

The use of hydrates of fluoroketones, as "special" solvents for synthetic polypeptides and some proteins,



has been reported in the literature.[21−26] HFA-hydrate has been proposed to be an ideal helix inducing and stabilizing solvent for peptides.[21−26] Actually, the aliphatic alcohols that contain a high percentage of fluorine are highly acidic[20,27] and thus would be expected to be stronger hydrogen-bonding acids. Because of the hydrophobicity of the trifluoromethyl groups, HFA molecules effectively seclude the peptide in a noninteracting environment in which intramolecular hydrogen bond formation is energetically favored.[26] As a result, HFA is expected to promote peptide helix formation in aqueous solutions. It is clear that large structural changes accompany the formation of regenerated silk fiber. The procedures of the silk fibroin solution extruded from spinneret and as-spun fiber coagulated in methanol may be attributed to this structural transition. The previous studies demonstrated that the shearing force from the spinneret can cause such structural transition,[27,28] and organic solvents like methanol may cause the silk fibroin to adopt a β-sheet.[3,4] Moreover, after extracting HFA from the as-spun fibers into methanol, the silk fibroin molecules to form a more stable β-sheet structure.

b. Characterization of Regenerated Silk Fiber. In ^{13}C CP/MAS NMR structural analyses of *B. mori* and *S. c. ricini* silk fibroins, the three crystalline forms of silk fibroins, silk I, silk II (β-sheet), and α-helix have been distinguished by the conformation-dependent ^{13}C chemical shifts of the respective amino acid residues.[1,18,19,29,30] Figure 2 shows ^{13}C CP/MAS NMR spectra of the regenerated silk fibers prepared from the HFA solution together with that of native *B. mori* silk fiber. The peak assignments have been reported.[18,29] The ^{13}C chemical shifts were 20.4 ppm for Ala Cβ, 49.4 ppm for Ala Cα, 172.3 ppm for Ala CO carbons, 63.7 ppm for Ser Cβ, and 54.8 ppm for Ser Cα carbons. These data clearly indicate that the Ala and Ser residues take β-sheet structure. The rate of the sample spinning was changed between the regenerated and native silk fibers as easily judged from the position of the spinning sidebands. The peaks from HFA marked by asterisk are still observed in the spectrum of the regenerated silk fiber (Figure 2a). So some HFA molecules are still bound with silk fibroin chain after immersing the fresh silk fibers in methanol overnight, indicating strong interaction between HFA and silk fibroin chains. However, the HFA molecules were eliminated after stretching process in water. The difference in the spectra between 100 and 125 °C steam treatment was observed in the spectral region between Gly Cα and Ala Cβ peaks. The Val Cβ peak at 30 ppm and Tyr Cβ peak at 37 ppm appeared in the former spectrum, but these peaks did not observe clearly in both the latter spectrum and the spectrum of native *B. mori* silk fibroin fiber. As mentioned below, crystal size will become larger after the steam-anealed treatment, and therefore the domain containing Tyr and Val residues seems to be incorporated into the growing crystal domain which consists of (Gly-Ala-Gly-Ser-Gly-Ala)$_n$.[1,18]

The difference in the structure between 100 and 125 °C steam treatment is observed more clearly in the DSC curves. When the steam temperature is 100 °C, the

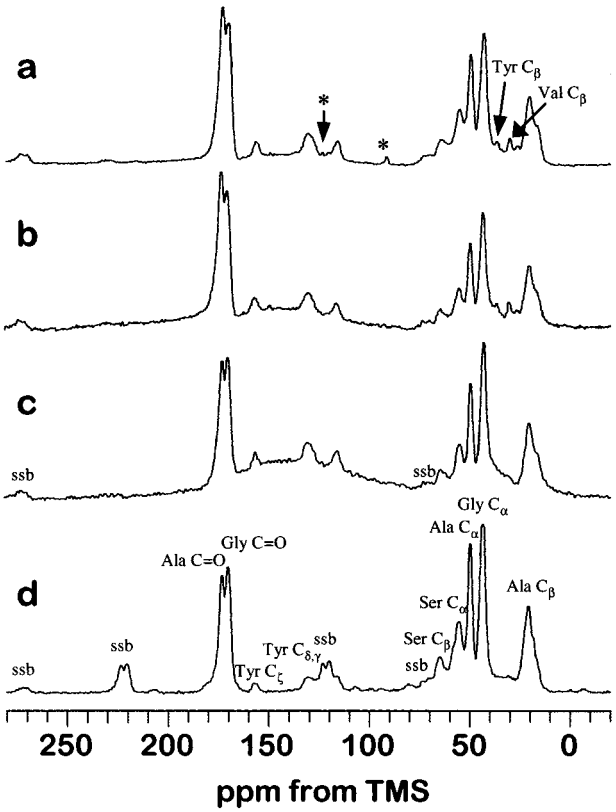


Figure 2. ^{13}C CP/MAS NMR spectra of (a) as-spun regenerated silk fiber, the regenerated silk fiber after postspinning drawing (×3, steam-annealed at 100 °C (b) and 125 °C (c) for 30 min), and (d) native *B. mori* silk fibroin fiber. The peaks attributed to HFA were marked by asterisks. The peak assignment was reported previously.[18,29] Asterisk indicates residual HFA peaks.

original exothermic peak was observed at 123 °C, which is attributed to crystallization of amorphous silk fibroin accompanying the structural transition from random coil to β-sheet. Amorphous silk film has this exothermic peak at about 212 °C.[31] The endothermic peak was observed at 298 °C. On the other hand, after steam-annealing at 125 °C, the DSC curve of regenerated silk fiber showed a remarkable upward shift of the decomposition temperature by 5 °C with the absence of an exothermic peak.

The ^{13}C CP/MAS NMR spectra in Figure 2 are almost the same between the regenerated silk fiber after 125 °C steam treatment and native *B.mori* silk fiber, indicating their remarkably similar structure locally. The mechanical properties of fibers depend on the crystallinity and degree of molecular orientation which can also be explored by wide-angle X-ray diffraction (WAXD).[31] A similarity between the WAXD patterns of the two system reveals the structural similarity between the regenerated silk fiber and the native silk fiber (data not shown). Taken together, these results suggest that the regenerated silk fibers are well-crystallized and well-oriented to the fiber axis after artificial spinning and postspinning treatments, including drawing and steam-annealing, applied to the as-spun fibers in this work. Figure 3 shows a comparison between the stress−strain curves of the native *B. mori* silk fiber (a) and the regenerated silk fiber (b). Young's modulus is 61 cN/dtex for the native *B. mori* silk fiber and 54 cN/dtex for the regenerated silk fiber. Note that the size of the regenerated silk fiber is about 15−25 denier in this work, while that for native *B. mori* silk fiber is about

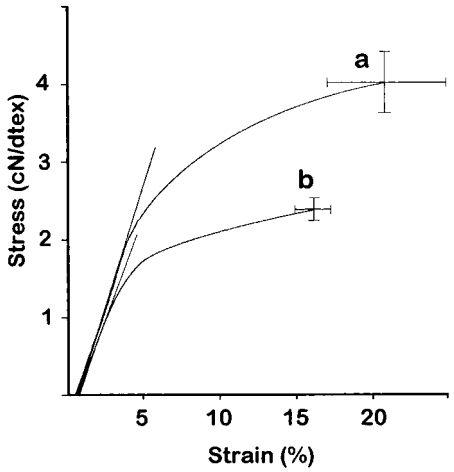


Figure 3. Stress−strain curves of (a) native *B. mori* silk fibroin fiber and (b) regenerated silk fiber (×3, steam-annealed at 125 °C for 30 min).

1−2 denier. Liivak et al.[14] found that there is a linear relationship between the logarithm of the maximum stress sustained by the fibers and the fiber size, and the regenerated fibers with the finest size approach the maximum stress measured for native *B. mori* fiber. In this work, although the maximum stress of the regenerated fiber is lower than that of the native fiber due to the larger fiber size, actually it is improved when compared with other regenerated silk fibers displayed in ref 14. This result suggests that the regenerated silk fiber produced with HFA solvent system is able of capturing the desirable key features of the native fibers.

Conclusions. The artificial spinning of *B. mori* silk fibroin was performed with HFA-hydrate as a spinning solvent. Although HFIP has been used as the spinning solvent previously, the HFA solvent system developed here establishes an advantage over the HFIP system. A 10% (w/w) HFA-hydrate solution of *B. mori* silk fibroin was extruded in methanol used as a coagulant solvent through spinneret with 0.2 mm diameter and 1.2 mm length orifice by high-pressure N_2 gas. After removal of HFA, the filament was stretched by about 3 times in distilled water and then steam-anealed at 125 °C for 30 min. The solid-state ^{13}C NMR spectra of the silk fibroin indicate that the regenerated *B. mori* silk fibroin fiber takes β-sheet structure after removal of HFA. The postspinning drawing is responsible for the preferential alignment of the polypeptide backbone as well as the β-sheet crystallites parallel to the fiber axis. At last, the steam-annealing must be responsible for the enlargement of crystal size. Thus, the regenerated silk fiber captures the main features of the native fibers by using HFA solvent system and relevant processing conditions.

Acknowledgment. The authors are grateful to Dr. S. Amiya and Mr. K. Enomoto at Kuraray Corp. for their helpful discussion in artificial spinning. T.A. acknowledges support from the Program for Promotion of Basic Research Activities for Innovative Biosciences, Japan.

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MA011335J