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Textile-Fabric-Like Nanostructure Formation of Silk Protein by Self Assembling

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We have observed highly ordered nanoscale textile-fabric-like structure of natural fibrous protein, fibroin obtained from wild silkworm, *Samia cynthia ricini* (Eri silkworm) by Atomic Force Microscopy (AFM). It was also revealed that *S. c. ricini* fibroin molecules have rigid rodlike form. SDS-PAGE analysis showed that the *S. c. ricini* fibroin is a homodimer of 160kDa protein linked by a disulfide bond. The length and the diameter of observed rodlike molecules are in good agreement with the size estimated from the molecular weight. The AFM images also shows that molecules assemble in end-to-end fashion to form higher order structure. As there are many similarities between *Bombyx mori* silk fibroin and *S. c. ricini* silk fibroin, the structure formation mechanism is considered to be similar to that of *B. mori* silk fibroin, which assembles by electrostatic interaction of charged amino acid residues located on both ends of a molecule.
Key words: silk fibroin, protein, AFM, nanostructure, self-assemble

1. INTRODUCTION

Silk fibroin is natural fibrous protein and main component of silk fiber. Not only that silk itself is an important product, but also that fibroin is a good example of structure formation of biomaterials.

Fibroin is synthesized in the silk glands of a silkworm and stored in a weak gel state. When fibroin is spun into fiber, it transforms into lyotropic liquid crystal[1,2]. Once fibroin is spun into fiber, it becomes insoluble to water. Though such fiber formation mechanism is not fully revealed yet, it is anticipated that fibroin molecules self-assemble to form silk fiber.

We have tried to characterize the fibroin molecule by Atomic Force Microscopy and SDS-PAGE, and to observe the process of structure formation of fibroin molecules. This will provide us with important information not only on the silk fiber formation mechanism, but also on the structural information of biological systems, and will be beneficial to engineering applications concerned with nanoscale structures.

2. EXPERIMENT

For the AFM experiments, silk fibroin was obtained from the posterior silk glands (PSG) of 5th instar wild silkworms, *Samia cynthia ricini* (Eri silkworm). Fibroin gel was squeezed from the silk glands, thereafter the gel was immersed in distilled water, and the fibroin molecules gradually dissolved in distilled water at 5 C°. The obtained fibroin solution was then filtered by paper filter (mesh size 5 μm) 24 hours after removal from the silkworm. Thereafter, the polymer solution was diluted with distilled water to a concentration of 2×10^{-3} wt%, then the polymer solution was divided into two portions (A and B).

Portion A was filtered through a 0.45 μm filter one hour after the paper filtering, and further split into two samples. One portion of the polymer solution A. was cast over a newly cleaved mica surface immediately after filtering (sample No.1), and the other portion was cast 24 hours after second filtering (sample No. 2). Then both samples were allowed to dry in ambient air. Portion B was stored at 5 C° for 24 hours after paper filtering, and then also split into two samples. One portion was further filtered by a 0.45 μm filter and cast over mica surface (sample No. 3), and the other portion was cast over mica surface without further filtering (sample No. 4).

The Atomic Force Microscopy measurements were performed using SPI-3000S AFM system (Seiko Instruments Inc.) equipped with a SPA-300HV unit, and both contact mode and tapping mode measurement were performed. All scanned images were obtained using a 20 μm scanner. For contact mode images, an Olympus SN-AF01 cantilever and tip (Si_3N_4, a tip radius of 10 nm, triangular base, 100 μm long) with a force constant of 0.09 N/m was used. The scanning line frequencies ranged from 0.3 Hz for large image areas (10 μm square) to 2 Hz for smaller scale images. The loading force on the sample is smaller than 10^{-10}N which was determined from the force curve.

Tapping mode images utilized an Olympus SI-DF40 cantilever and tip (Si, a tip radius of 15 nm, rectangular base, 125 μm long) with a force constant of 40 N/m. The resonance frequency of the cantilever was 270 kHz, and the applied frequency was set on the lower side of the resonance frequency. All the measurements were performed in ambient air.

For SDS-polyacrylamide gel electrophoresis (SDS-PAGE) experiments, fibroin was obtained from 5th instar *S. c. ricini* silk gland, posterior silk gland (PSG)

and middle silk gland (MSG). Protein from PSG was squeezed from tissue, then washed twice with water. Thereafter the protein was dissolved in H_2O, 20mM Tris-HCl (pH 8.0) + 5M Urea, H_2O+30mM DTT, and 20mM Tris-HCl (pH 8.0) + 5M Urea + 30mM DTT at 4 C° for over night, respectively. Protein from MSG was obtained in a weak gel state, and was cut into small pieces and then immersed in each buffer in the same conditions as PSG samples.

Protein concentration of each sample was adjusted by BCA method to 2mg/ml. We added SDS-sample buffer (final concentration; 25mM Tris-HCl (pH 6.5), 5% Glycerol, 1% SDS, 0.05% BPB) to each sample without 2-Mercaptoethanol and the samples were loaded on the 10% SDS polyacrylamide gel (SDS-PAGE). After electrophoresis, gel was stained by 5% CBB and after silver stain plus (Bio-Rad).

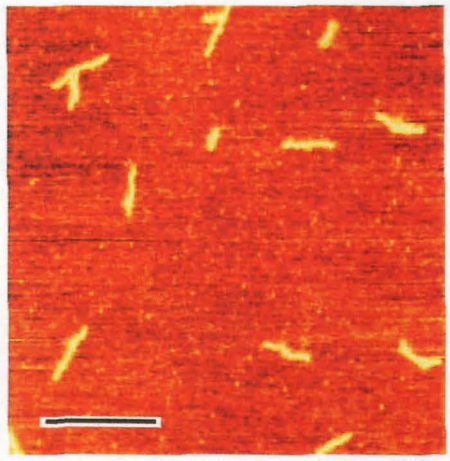

Figure 1. AFM image of *Samia cynthia ricini* fibroin sample, cast over mica immediately after filtering. Tapping mode, mica substrate, scale bar : 500nm.

3. RESULTS AND DISCUSSIONS

Fibroin solutions obtained from *S. c. ricini* were cast on mica surface under various conditions and observed by AFM. In the AFM images of sample No.1, many long, straight objects were observed (see Figure 1). The heights of these objects was nearly identical (0.4 nm), and the length was about 300 nm, and the width of the rods is 10 nm. Taking account the effect of tip radius, the observed objects have a rodlike shape, 0.4nm in diameter and about 200nm in length.

These rodlike objects are considered to be a single molecule of fibroin. To confirm this hypothesis, we have tried to analyze *S. c. ricini* fibroin by SDS-PAGE, and the result was shown in Figure 2. Only 2 bands are observed on PSG lanes (Figure 2. lanes 1,3), distinct one and very faint one. The distinct band can be observed on both PSG (lanes 1,3) and MSG lanes (lanes 2,4). It is obvious that fibroin is synthesized in the PSG and that the major contents of silk gland is fibroin. Therefore, the distinct band should be assigned to *S. c. ricini fibroin*. The fact that hardly any other bands were observed in PSG lanes shows the purity of this sample is very high. Therefore, the observed rods in Figure 1 can be assigned to *Samia cinthia ricini* fibroin.

Figure 3 shows the tapping mode image of sample No. 4, which was cast 24 hours after paper filtering, where Fig. 3(a) is a topographic image and Fig. 3(b) is its phase image. We can observe a highly ordered textile-fabric-like, or "nanofabric" structure, which seems to consist of warp and weft. Because this structure was observed in topographic image, phase image, and also in contact mode image (figures not shown), it is obvious that this structure was not artificially formed while observations. The size of this nanofabric is 2 μm in

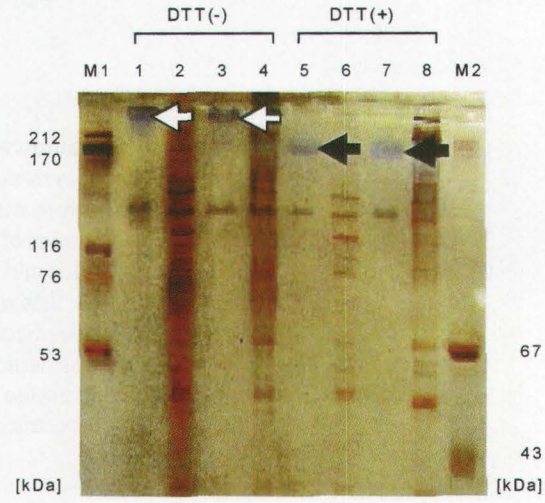

Figure 2. SDS-PAGE analysis of silk fibroin from posterior and middle silk gland of *Samia cynthia ricini*. Each contents were dissolved in water or Tris-Urea buffer, or each containing 30mM DTT, and samples were loaded on the 5% stacking gel and 10% separating gel without heat shock. Arrows indicate eri-silkworm fibroin. (M1,M2); molecular weight marker, (lane 1,5); PSG contents dissolved in water, (lane 2,6); MSG contents dissolved in water, (lane 3,7); PSG contents dissolved in Tris-Urea buffer, (lane 4,8); MSG contents dissolved in Tris-Urea buffer. lane 1 to 4; no containing DTT, lane 5 to 8; containing 30mM DTT in the solution.

width and some ten micrometers in length. The height of both the warp and weft is 0.4 nm, the same as the diameter of a fibroin molecule. This means that the molecules assemble in end-to-end fashion, otherwise the diameter of warp and weft would be greater than that of a single molecule. The distance between the neighboring warp and weft is 50 to 80 nm.

Similar structures are also observed in images of sample No. 2, which was stored 24 hours after 0.45 μm filtering (figures not shown). In contrast, we could not observe any rodlike objects nor larger structure on the sample No. 3. This means that the large structure was formed by self-assembling while the fibroin solution was stored, and that the "nanofabric" is not a part of tissue nor an *in vivo* structure. In the case of sample No.

3, molecules assembled together to form structures larger than mesh, and filtered off.

Here we tried to estimate the size of single fibroin molecule. Figure 2 shows that the protein main bands in both MSG and PSG samples were approximately 3.3 × 10^5 Da (Figure 2. DTT(-) lanes, white arrows). The DTT digested sample shows a single band in approximately 1.6 × 10^5 Da (Figure 2. DTT(+) lanes, black arrows). This shows that *S. c. ricini* fibroin molecule is composed of two equivalent part, that is, two 160kDa protein linked by a disulfide bond. This is a contrast to the similar protein, fibroin from *Bombyx mori* silkworm, which is a complex of heavy-chain (3.5 × 10^5 Da), light-chain (2.5 × 10^4Da) and P25 (2.7 × 10^4Da) [3].

Major components of *Samia cynthia ricini* fibroin are alanine (45%) and glycine (25%). Though the primary structure of *S. c. ricini* fibroin has not yet been completely sequenced, it is known to have polyalanine domains interleaved with glycine-rich domains[4]. The polyalanine domains of the fibroin have an α - helix conformation, which has been confirmed by NMR[5].

The molecular weight of *S. c. ricini* fibroin is 330kDa, which means fibroin consists of about 2.8 × 10^3 amino acid residues. α - helix form polyalanine have a diameter of 0.4nm, and the length of it is 0.15nm per unit amino acid residue[6]. Therefore the length of of *S. c. ricini* fibroin can be estimated as 300 to 400nm, which is in good agreement with the observed molecules as in Figure 1.

We have further observed a comb-like structure on the sample No. 4 (figures not shown) which was a long threadlike structure with many side chains. The distance between neighboring side chains is 50 to 80nm, and this is the same as that of "nanofabric" in Figure 3. We can also observe that such comb-like structure assembles side-by-side to form larger structure.

The nanoscale structure formation mechanism deeply depends on the molecular structure. The complete amino acid sequence of *S. c. ricini* fibroin is not yet fully revealed, therefore the detailed structure formation mechanism should be discussed after the *S. c. ricini* fibroin is fully sequenced. Because there are many similarities between *Bombyx mori* fibroin and *Samia cynthia ricini* fibroin, we can draw an analogy between *B. mori* fibroin and *S. c. ricini* fibroin.

Bombyx mori fibroin is consists of mainly glycine (49%) and alanine (32%), and 77% of total amino acid residues are hydrophobic. In the case of *S. c. ricini* fibrion, 72% of total amino acid residues are hydrophobic, this ratio is close to that of *B. mori* fibroin (77%). The physical behavior also resembles each other, for example, Sol – Gel transition or liquid crystal formation. Furthermore, *B. mori* molecules have also rodlike shape, and form higher order structure by end-to-end interaction of molecules[7]. From the rheological experiments[8,9], the interaction between *B. mori* molecules are revealed to be electrostatic force.

On the analogy of *B. mori* fibroin, *S. c. ricini* wild silk fibroin is considered to form aggregates by an electrostatic interaction. The 10% of total amino acid residues of *S. c. ricini* are charged, and this ratio is higher than that of *B. mori*. Because such charged residues are located in non-helical domain, molecules form aggregate by electrostatic interaction of non-helical

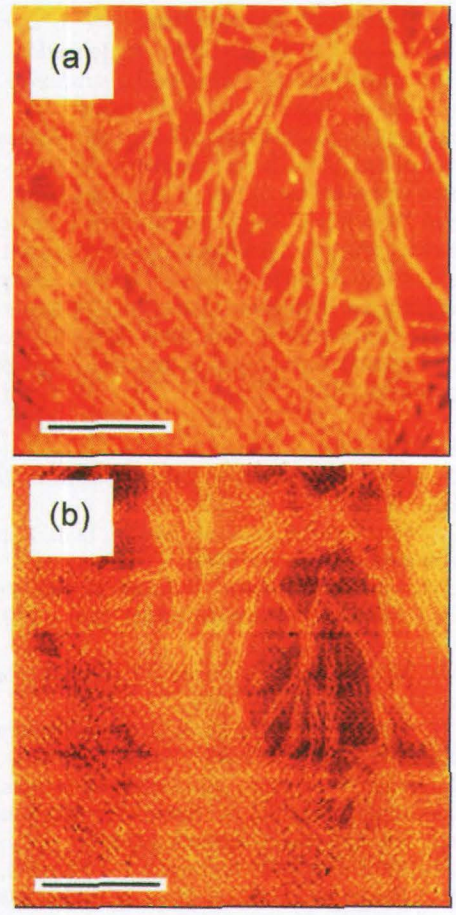

Figure 3. AFM image of *Samia cynthia ricini* fibroin sample, cast over mica 24 hours after filtering. (a): topographic image (b): phase image. tapping mode, mica substrate, scale bar : 500nm.

domain to construct highly ordered structure.

Because the structure formation mechanism that rodlike molecules assemble by end-to-end interaction is common to both kinds of fibroin, we can expect that *Bombyx mori* fibroin also form highly ordered structure. For the present, *B. mori* fibroin shows structures with relatively low order[7], but we can expect that *B. mori* fibroin also form highly ordered structure in optimum conditions.

The observed "nanofabric" are very similar to the higher order structure of nuclear lamina, from which the inner nuclear membrane is constructed[10,11]. Nuclear lamina consists of an intermediate-type filament protein, lamin. Lamin have both α – helical conformation domains and non - helical domains which are located on both ends of the helical domain. The lamin molecules form dimers by interaction between α – helical domains. Then these dimers assemble in a head – to – tail fashion to form long threadlike structures. This threadlike structures stick together side by side to form large fabric – like structure, though the structure formation mechanism of lamin is not clear [10-12].

4. CONCLUSION

To summarize this report, we have observed a highly

ordered "nanofabric" structure formed by *S. c. ricini* fibroin. Fibroin has an α - helix conformation, and assembles end-to-end to form rod-like structures. The highly ordered structure was constructed by self-assemble of fibroin molecules. Though the mechanism of structure formation is not completely revealed, it is supposed to be similar to that of *B. mori* fibroin. The observed "nanofabric" might be taken as a model of higher order structure formation of biomaterials, and this shows that self-assemble is one of the most important way of structure formation in biological systems. To reveal the mechanism of nanofabric formation would provide important information on structure formation of biological systems.

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