Stability of Molecular Order in Silkworm Silk

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Silk spun by insects and spiders is providing materials scientists with useful lessons for the synthesis and assembly of high-performance fibers.[1,2] At the same time, biologists are comparing the evolutionary routes that have led to the diverse uses of silk proteins in nature.[3] In all these cases, the material of interest has been optimized for well-defined load-bearing applications. Optimization of material properties requires controlled spatial and orientational arrangement of the molecules over a range of length scales, in addition to a judicious choice of composition. It is therefore important that attempts to relate molecular order to material properties should be based on the *native* structure and properties of the material. In other words, the processes involved in specimen preparation should not significantly alter the microstructure before it, and the dependent properties, can be characterized. Here we report on the stability of molecular order in silkworm (*Bombyx mori*) silk under the conditions that are conventionally used to recover fiber from the cocoons.

Studying silkworm cocoon silk presents a particular challenge. The fibroin core (i.e., the source of the strength and stiffness of this material) is spun with an amorphous coating of sericin, which links individual fibers (brins) into twin-core bave and binds bave into the cocoon. To recover bave for studies of tensile properties—and for textile uses—the cocoons have to be degummed. The cocoons are boiled in water, or in dilute alkaline or soap solution, for periods of an hour or more.[4] The sericin is degraded and removed, at least to the point where it is possible to unravel bave from the cocoon, and it should be a concern that the fibroin may have also been altered to a point where its characteristics do not reflect those of the as-spun material. While insect cocoon silks are usually spun with a sericin coating, spider dragline silks are not. A comparison of their structure and properties is thus only meaningful if degumming does not significantly affect the molecular order in the insect fibroin. A recent study[5] reports qualitatively that degumming leaves the basic features of wide-angle X-ray patterns unchanged, but contradicts the results obtained by other researchers using the same characterization technique.[6]

The need arises for a characterization technique that can straightforwardly provide structural information about cocoon silk before and after degumming. The constraints on the technique are that it should (a) not require isolation or manipulation of single fibers, so that handling-induced microstructural change is avoided, (b) be unaffected by the presence of amorphous sericin, (c) provide an indication of molecular order that is not restricted to a single length scale, and (d) not depend on the structure remaining intact during the characterization process. On this basis, we chose differential scanning calorimetry (DSC) in preference to microscopy, diffraction, or spectroscopy. DSC can reveal whether boiling makes the silk microstructure more or less susceptible to breakup upon subsequent heating, and so can be used to detect molecular order changes induced by boiling. It does not matter that the specimen structure is destroyed during characterization by DSC—indeed, DSC provides information about the structure by quantifying the energy required to destroy it.

We performed DSC runs on as-spun *B. mori* cocoon silk, and on silk that had been boiled in distilled water (the least aggressive environment that can achieve degumming) for 2, 5, 10, 15, 30, or 45 min, or 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, or 7 h. All samples were blotted dry and stored overnight in a desiccator prior to loading into aluminum DSC pans. A fast heating rate is needed to maximize the temperature reached before significant degradation is able to occur, and enhances the ability of DSC to detect small endo- or exotherms. However, fast heating also limits resolution and can displace events to higher-than-equilibrium temperatures. A rate of 20 °C/min was used to achieve a balance between these competing factors. A typical result is shown in Figure 1. Two broad endotherms are recorded in all cases. The first is associated with the loss of residual adsorbed water.[7] The second corresponds to the loss of ordered regions in the microstructure.[8] A third, intense endotherm associated with sample degradation occurs outside the temperature range over which data were normally collected (Figure 2). A rescan of samples that have degraded does not show the endotherms exhibited during the initial scan. The breadth of the second endotherm partly reflects the fast heating rate, but will also reflect the statistical size distribution of the crystals, which have formed under nonequilibrium conditions. Similarly broad endotherms are exhibited by solidified fibers drawn from synthetic random copolyesters.[9] In the latter context, crystals also form as statistical "best matches";[10] their enthalpy of melting is independent of the rate of previous solidification, and we are therefore confident that our results from silk are not sensitive to the exact rate at which cocoons were spun.

For the loss of ordered regions, we find that previous boiling in water leads to no significant change in either the onset temperature (159 °C in scans performed at 20 °C/min) or the total enthalpy of transition (58 J/g), even if boiling is continued over a period of several hours. This result suggests that boiling leads neither to coarsening of the microstructure nor to a change in the volume fraction of ordered material. We recognize that these results may not be valid for degumming procedures performed in more aggressive (penetrating) media such as soap solution or dilute NaOH.

To confirm the molecular as well as microstructural stability of silk fibroin subjected to boiling, we used ninhydrin to test[11] for (the absence of) solubilized or degraded protein fragments in the boiling medium. Of course, the test will initially detect the sericin which is taken up by the water, but, assuming that sericin will eventually be removed and that any uptake of degraded fibroin by the (replenished) water will be continuous, a

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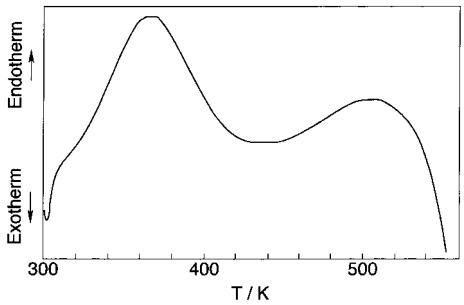

Figure 1. DSC trace of a 12.2 mg sample cut from an as-spun *B. mori* cocoon. The scan rate was 20 °C/min.

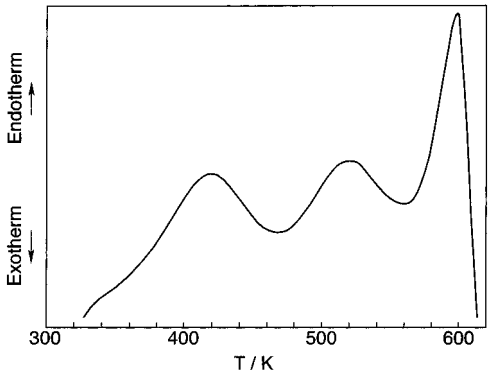

Figure 2. DSC trace of a 13.3 mg sample cut from an as-spun *B. mori* cocoon. The scan rate was 80 °C/min, allowing the full degradation endotherm to be displayed. The first and second endotherms are displaced to higher temperatures, compared to their positions in a 20 °C/min scan.

stage should be reached where any protein in the water originated in the fibroin core of the fiber. Silk samples (0.5 g) were boiled for 10 min in 50 mL of deionized water under reflux. The supernatant was recovered by filtration. The silk was washed three times with hot (∼70 °C) deionized water, which was combined with the filtrate and concentrated to a final volume of ∼10 mL on a Büchi rotary evaporator. The concentrate was spotted onto filter paper with a Pasteur pipet (typically eight spots per sample), and the paper was dried in an oven at 105 °C for 5 min. A 0.3 g sample of ninhydrin was dissolved into a solution of butanol containing 3 mL of glacial acetic acid; butanol was then added to obtain a final volume of 100 mL. A Pasteur pipet was used to spot approximately 0.1 mL of this solution onto the area of filter paper to be tested. Purple or pink spots developed on drying for the positive indication of protein. The silk was returned to boiling under reflux for a further 10 min, the entire procedure being repeated at 10 min intervals for a total of 130 min. The ninhydrin test showed qualitatively that the rate of loss of protein from the fiber decreases with cumulative boiling time, and failed to detect protein in the supernatant after a total boiling time of 110 min. Samples that were boiled for an additional 50 h (without interruption at 10 min intervals) also failed to detect protein in the supernatant. We interpret these observations as showing that the degradable fraction of sericin can be removed by boiling for nearly 2 h, while the fibroin core is stable over much longer periods of time.

The stability implied by the above results provides insight into the topology of the silk fibroin microstructure. The onset temperature of molecular order loss T_c is approximately 159 °C, so the temperature of boiling water corresponds to $0.86T_c$. In comparison, the stress relief temperature of nylon 6.6 injection moldings is $0.8T_c$;[12] i.e., the silk microstructure is significantly more stable despite both materials having similar crystallinity (approximately 60%[4,12]). We also note from Figures 1 and 2 that *B. mori* silk fiber does not exhibit the 175 °C glass transition which is undergone by fully amorphous silk.[7] Molecules in the amorphous fraction of silk fiber therefore appear to be constrained by the crystalline material. In nylons, and other conventional semicrystalline polymers, the crystalline regions are represented as individually surrounded by amorphous material. The stability of the silk microstructure suggests that the crystalline fraction is connected by extended chains throughout the microstructure. The significantly higher stiffness of *B. mori* silk in comparison to that of nylon[4] is consistent with this microstructural difference.

If the amorphous fraction is constrained as described, one would expect little or no change in optical birefringence in boiled versus native silk: there would be little scope for relaxing any molecular alignment retained in the amorphous phase after spinning, and our DSC experiments eliminate the likelihood of other types of microstructural change. (Note that differences in the degree of molecular alignment in amorphous material are one of the few microstructural characteristics that are difficult to resolve by comparing DSC traces. The loss of orientational order in amorphous material during a DSC run is not associated with an endotherm, and there is no discontinuous change in slope.) Birefringence measurements were performed by using the de Sénarmont method[13] to determine the optical retardation of fibers observed in a transmitted polarized light microscope. Two samples of native silk and two samples boiled in water for 50 h and then dried were characterized. For each sample this involved measuring retardation at three positions on each of 10 lengths of fiber; in other words, each value reported here represents an average of 30 readings. Allowance was made for the thickness of the nonbirefringent sericin layer in the native silk. The two native samples yielded birefringence values of 0.0239 and 0.0232, while the values after boiling for 50 h were 0.0226 and 0.0223. This decrease of less than 5% after boiling for over 2 days again demonstrates that the opportunity for microstructural change in *B. mori* silk at 100 °C is extremely limited.

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