Preparation and Characterization of *Bombyx mori* Silk Fibroin and Wool Keratin

Yesim Iridag,[1] Murat Kazanci[2]

[1]*Department of Textile Engineering, Istanbul Technical University, Gumussuyu 34439, Istanbul, Turkey*
[2]*Max-Planck-Institute of Colloids and Interfaces, Department of Biomaterials, 14424 Potsdam, Germany*

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ABSTRACT: Silk and wool are well-known protein-based fibers. Their environmental stability, biocompatibility, and unique mechanical properties provide an important basis for using these natural proteins in biomedical applications. To use them as biomaterials in the form of fibers, films, or membranes, it is necessary to characterize these proteins in their solution and solid states because structural characteristics and morphological features have a great influence on the physical and mechanical properties of these new regenerated protein forms. Therefore, in the present study, silk fibroin and wool keratin were dissolved and their solution behaviors and secondary structures are analyzed and compared, using particle size distribution, molecular weight distribution (SDS-PAGE), Fourier transform infrared, and X-ray diffraction techniques. It was shown that keratin is more stable in solution and more amorphous in the solid state. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 100: 4260–4264, 2006

Key words: proteins; silk fibroin; wool keratin; FTIR, XRD, particle size distribution

INTRODUCTION

Naturally occurring fibrous proteins fall into one of two categories: highly extensible fibers such as the α-keratins (wools, hair, and other mammalian external appendages such as quills and horns); and fibroin fibers (various silks and spider webs). Among them, silk and wool, which are both high-quality natural protein fibers, have been widely used as high-quality textile materials. Silk has been used as a textile fiber for thousands of years because of its unique gloss, handle, and mechanical properties. Silk contains a fibrous protein termed fibroin that forms the tread corn and gluelike proteins termed sericin that surround the fibroin fibers to cement them together. Fibroin is a highly insoluble protein 90% of which are the amino acids glycine, alanine, and serine, leading to antiparallel β-pleated sheet formation in the fibers.[1] Once the silk fiber is solidified from the silkworm through a spinning process, it becomes a well-oriented and highly crystallized polymer. Silk fiber behaves like a thermoset polymer, although it is not all crosslinked. Therefore, concentrated chaotropic salts, which destabilize proteins in solution and increase their solubility, are required to dissolve *B. mori* silk fiber.[2] Keratins are fibrous proteins found in hair, wool, feathers, nail, horns, and other epithelial coverings. In wool, keratins occupy about 50 wt % of the cortical cells. At a molecular level, the most distinctive feature of keratins is the high concentration of half-cystine residues. Keratins may thus be regarded as three-dimensional polymers interlinked by S—S bonds between reduced keratin-monomeric units.[3]

The relative environmental stability of these families of natural proteins, in comparison to globular proteins, in combination with their biocompatibility, unique mechanical properties, and options for genetic control to tailor sequences, provides an important basis for exploiting them for biomedical applications.[4–6] To engineer these fibers for specific biomedical applications, they must be regenerated in such desirable forms as solutions, powders, films, gels, and filaments, depending on the preparation conditions and the field to which they will be applied.[7] To avoid problems with the conformational transitions of these protein fibers during solubilization and reprocessing from aqueous solution to generate new fibers and films, they should be characterized in their solution state Structural characteristics and morphological features, which greatly influence the physical and mechanical properties of these new regenerated protein forms, should be considered when these proteins are used as a biomaterial.

Most previous studies were of films cast from protein solutions so that the conformation of the protein molecules may have depended on the conditions of casting, for instance, casting temperature and concen-

Correspondence to: M. Kazanci (Murat.Kazanci@mpikg-golm.mpg.de).

tration.[8] However, few studies have investigated the behavior of these proteins in a solution environment[9] close to that of their native state. In a previous work, we studied the behavior of keratin proteins in different solutions.[10] The present study was focused on the structural aspects of silk fibroin and wool keratin proteins both in the solution state, using particle size distribution and molecular weight distribution (SDS-PAGE), and in the solid state, using Fourier transform infrared (FTIR) and X-ray diffraction (XRD) methods.

EXPERIMENTAL

Preparation of samples

Washed, cleaned, and dried wool was obtained from Orma (Bursa, Turkey). Urea, 2-mercaptoethanol, sodium dodecyl sulfate (SDS), formic acid, and dimethylformamide (DMF) were used without further purification. The cleaned wool (2 g) was mixed with 7*M* urea (36 mL), SDS (1.2 g), and 2-mercaptoethanol (3 mL) and shaken at 50°C for 24 h at a natural pH. Water was distilled prior to use. Dialysis was performed using a regenerated cellulose membrane (Slide-A-dyzer, 3500 MWCO, Pierce), with water containing 0.08 wt % 2-mercaptoethanol changed regularly every 5 h, to obtain a colorless and clear solution of the reduced keratins.

Bombyx mori cocoons were obtained from Orma to produce regenerated silk fibroin solutions. The regeneration process utilized aqueous solutions of Na_2CO_3 (Sigma-Aldrich, Germany) in different concentrations. First, raw silk was boiled in an aqueous solution of Na_2CO_3 (1.1 g/L) with a ratio of 10% (w/v) raw silk at 95°C for 60 min. The silk was then transferred to another aqueous bath with 0.4 g/L of Na_2CO_3 and boiled for another 60 min. The fibers were washed with distilled water and dried at room temperature. Then 500 mg of air-dried, degummed silk fibers were dissolved in 20 mL of a $Ca(NO_3)_2 \cdot 4H_2O$—MeOH mixture at 80°C. The weight ratio of $Ca(NO_3)_2 \cdot 4H_2O$: MeOH was 75:25.[11] Impurities were removed by filtering the solution. The salt was completely removed by dialysis against distilled water for 3 days at room temperature through a regenerated cellulose membrane.

Then the protein solution (10 mL) was cast onto a glass plate and dried in a desiccator at 50°C for the preparation of regenerated film. The films were washed with distilled water to be peeled off easily from the plate for FTIR and XRD analysis.

Measurements

The aqueous solutions of the keratin and fibroin were subjected to one-dimensional slab SDS–polyacrylamide gel electrophoresis (PAGE) using a 10%–15% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) buffer, a kit of molecular weight markers (Sigma MW-SDS-70L), and tracking dye (Pyronin Y) at 250 V and 10 mA. The proteins were stained with Coomasic Brilliant Blue R-250 (Sigma) and analyzed by a densitometer.

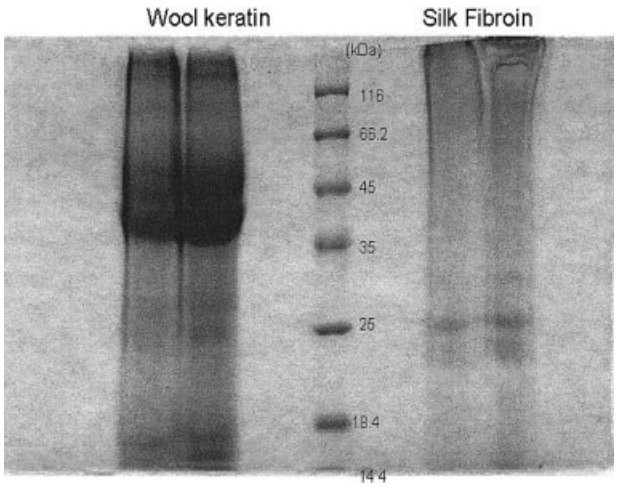

Figure 1 SDS-PAGE analysis of regenerated silk fibroin and wool keratin.

A Vertex 70 FTIR tester and a Hyperion 1000/2000 microscope were used to analyze the samples. The resolution of the infrared spectra was 2 cm^{-1}, and for each spectrum there were 32 scans, which were recorded from 800 to 3800 cm^{-1} in transmittance mode.

Particle size was measured by the laser beam scattering technique (Malvern Hydro 2000M/MU Particle size analyzer). The optic unit contained a 5 mW He–Ne (638 nm) laser.

X-ray diffraction (XRD) was done in microdiffraction mode with CuK_α radiation (λ = 1.5418 Å) on a DISCOVER D8 diffractometer (Bruker, Germany) with θ/θ geometry, a 2-mm-sized beam, and a two-dimensional detector (Bruker) with 1024 × 1024 pixels. Frames of data were collected for 1 min each, integrated, and merged to produce a diffraction pattern from 20° to 70° 2θ. The sample-to-detector distance was 1.3 m. The 2-D diffraction patterns were transformed into a 1-D radial average of the scattering intensity. DIFFRACplus EVA 7.0 software was employed in data analysis.

The FTIR and XRD results were analyzed using GRAMS32 software (Galactic Industries Corporation). The spectra were corrected only for outliers and were baselined using a 2-point fit.

RESULTS AND DISCUSSION

SDS-PAGE was used to determine the molecular weight of the proteins. Figure 1 shows the results of SDS gel electrophoresis of regenerated silk fibroin and

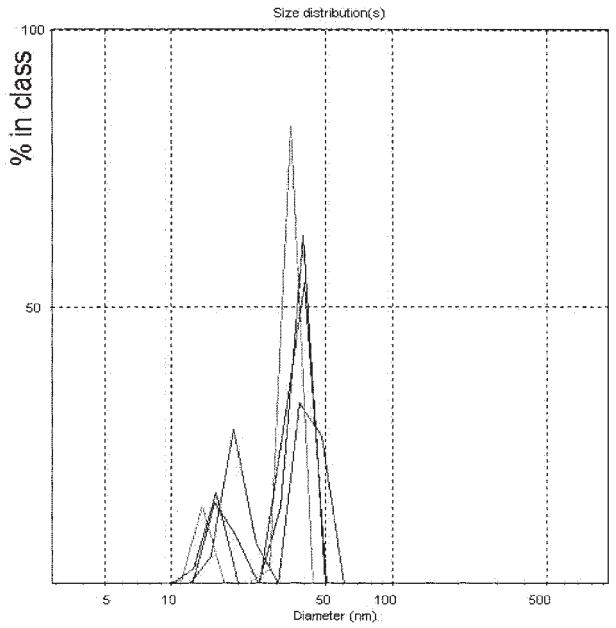

Figure 2 Particle size distribution of wool keratin.

wool keratin aqueous solutions. The results suggest that the regenerated protein solutions were composed of a mixture of polypeptides of several molecular weights. SDS-PAGE of the solution showed one major band (between 60 and 35 kDa) and one minor band (between 18 and 14 kDa) for the keratin solution and one minor band (between 25 and 20 kDa) for the fibroin solution. It is known that regenerated aqueous SF solution shows a broad dull band from 200 to 30 kDa and a clear sharp band at 25 kDa.[12] The former band might result from the degradation products of the heavy chains (350 kDa) of raw silk protein formed by degumming and dissolution in a solvent system, and the latter band at 25 kDa corresponds to the light chain of raw silk protein.[13] It was assumed that the high-molecular-weight fraction was derived from the microfibrils and the latter from the interfilament matrix in the keratin solution.[3] It was shown that the solutions were extensively depolymerized by scission of the main chains, resulting in a mixture composed of substances of heterogeneous molecular weight.[2,7]

The particle size distributions of the protein solutions were obtained using the laser-beam-scattering technique. Four samples from the keratin solution and two samples from the fibroin solution were taken and measured. The results are shown in Figures 2 and 3, respectively. The size distribution results were in good agreement with the results obtained from the molecular weight distributions. There were two particle size peaks in the range of 10–50 nm in diameter of keratin protein, as there was in its molecular weight distribution. There was a sharp major peak in particle size distribution of fibroin solution that was around 300 nm and a small peak that was more than 1000 nm in diameter. This means the molecular chains of SF in solution were more extended than the keratin chains. Therefore, SF molecules had a greater opportunity for intermolecular association, which was related to molecular entanglement, than did the keratin solution. This also suggested that silk fibroin molecules were mainly present in the form of a large protein complex even at concentrations as low as 0.1 wt % and that these elementary units form aggregated primarily by head-to-tail-type connections.[14] It was speculated that in the keratin solution the micelles were separated from each other by electrostatic repulsion, thereby retarding the aggregation of the protein molecules and oxidation of the nearby SH groups (to form S—S bonds).[3]

Fibroin protein in the regenerated silk and wool films was confirmed by FTIR spectroscopy. Figure 4 presents the FTIR spectra of protein materials, which showed characteristic vibrational bands at 1630–1650 cm^{-1} for amide I (C═O stretching), at 1540–1520 cm^{-1} for amide II (secondary NH bending), and at 1270–1230 cm^{-1} for amide III (C—N stretching). In addition, the positions of these bands indicate the conformations of the protein materials: 1650 cm^{-1} (random coil) and 1630 cm^{-1} (β-sheet) for amide I, 1540 cm^{-1} (random coil) and 1520 cm^{-1} (β-sheet) for amide II, and 1270 cm^{-1} (β-sheet) and 1230 cm^{-1} (random coil) for amide III.[15–17] These band shifts occurred because of the distinct hydrogen bonding states produced by the different conformations

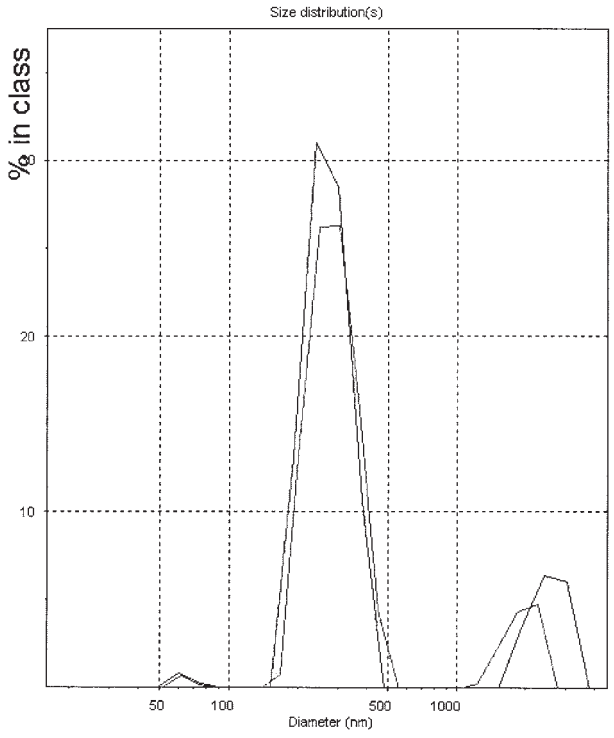

Figure 3 Particle size distribution of silk fibroin.

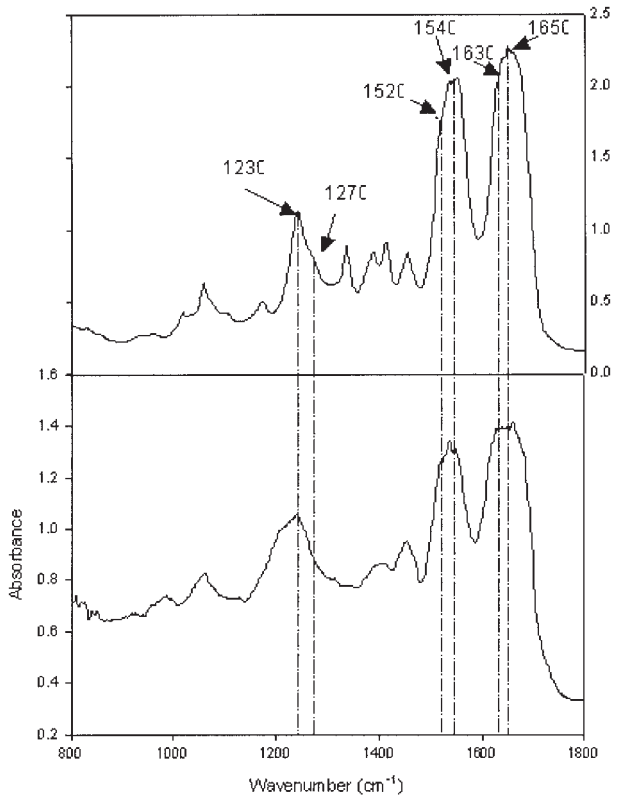

Figure 4 FTIR spectra of SF and Keratin films cast from their aqueous solutions.

adopted by the protein chains. Silk and wool can acquire mainly β-sheet and random coil confirmations.[4] The amide I band's intensity increased because of the increased presence of β-sheet confirmation in fibroin solution. The amide II band had an intensity close to that of the amide I peak. The amide III peaks showed that fibroin had crystalline and amorphous regions, unlike keratin, which had only an amorphous region. These results suggest that β-sheet crystallization was promoted in fibroin and that the formation of β structures in silk led to crystallinity.

Figure 5 shows the X-ray diffractograms (XRD) of the regenerated fibroin films. The X-ray results confirmed the FTIR data, showing the characteristic peak $2\theta \approx 20°$ (silk II), which indicated a β-sheet structure.[16] The XRD results for the regenerated keratin film showed a broader peak, shifted 2θ degrees with lower intensity scores, indicating that the keratin film was more amorphous than the fibroin film. There were also other high-intensity regions for both protein structures: $2\theta \approx 28°$ for fibroin and $2\theta \approx 32°$ and 34° for keratin. Most probably this pointed to the different types of crystalline structures present.

CONCLUSIONS

Compared with that of wool keratin, the protein structure of silk fibroin is the most studied by many researchers for different types of applications in industrial and biomedical fields. However, the brittleness and poor mechanical properties of regenerated fibroin materials have been two of the biggest obstacles to their utilization. Different researches have suggested different processing techniques[16] and coagulation with different polymers[18–20] to overcome these problems.

In the present study, we compared the data from wool keratin and silk fibroin protein structures. Since keratin protein and silk fibroin have similar structures, keratin protein might be a good alternative for the application that fibroin has certain shortcomings. To be able to compare these structures, we utilized XRD, FTIR, particle size distribution, and SDS-PAGE techniques.

The FTIR results revealed that the β-sheet crystallization of SF molecules were more pronounced than those of wool keratin molecules. This was confirmed by XRD, which showed that keratin had a more amorphous structure, a broader peak with less intensity. This might be evidence of less brittleness of keratin protein films. The particle size distribution results showed that the keratin solution was much more stable than SF solution, which had a tendency to form aggregates very easily. Molecular weight distribution results indicated two distinct peaks of keratin solutions. These results should be followed up and confirmed by mechanical tests. Nevertheless, this study has successfully shown the similarities and differences

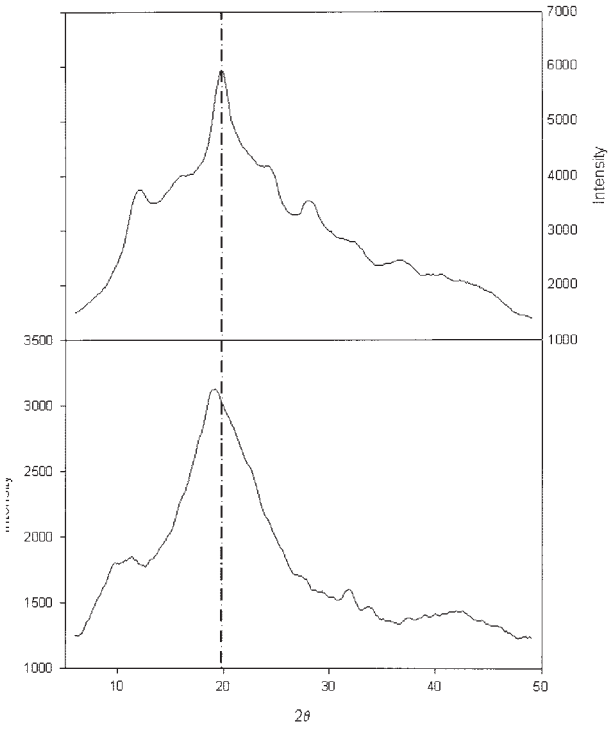

Figure 5 XRD patterns of SF and Keratin films.

in these two protein structures. Understanding the structures of these proteins will also help to understand other types of proteins that are becoming available in large quantities for use in different types of applications.

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