Analysis of Structural Properties and Formation of Sericin Fiber by Infrared Spectroscopy

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Attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy was applied to sericin fiber spun by Sericin-hope silkworms, developed to produce silk protein sericin in large amounts, and to a native sericin solution before spinning. Polarized ATR-FTIR measurement showed little orientation of sericin molecules in sericin fiber. Secondary structures of sericin fiber and native sericin were analyzed by Fourier self-deconvolution and curve fitting. The drying process of the native sericin solution was also followed to determine the effect of dehydration on sericin conformation. These analyses showed that B-sheets in native sericin increased with drying and that the secondary structure of air-dried sericin was similar to that of sericin fiber. These observations suggest that drying is a significant factor in the structural transition of sericin during fiber formation and that sericin undergoes only modest structural changes by spinning compared with fibroin.

Key words: Sericin, fiber formation, ATR-FTIR, orientation, secondary structure

INTRODUCTION

The Bombyx mori cocoon consists of fibrous protein, fibroin, and a sticky protein, sericin, which envelops fibroin threads to glue them together (reviewed by Fedič et al., 2002). Sericin is produced exclusively in the middle silk glands and classified into at least six proteins of different lengths generated by alternatively splicing the primary transcripts of two sercin genes, Ser1 and Ser2 (Okamoto et al., 1982; Michaille et al., 1990; Garel et al., 1997), and ranges in size from 65 to 400 kDa (Sinozawa, 1979; Gamo, 1982). Such properties of sericin as high hydrophilicity and adhesion are due to its high serine content and large proportion of several polar amino acids (Komatsu, 1975; Gamo et al., 1977). Native sericin has not been extensively studied, however, probably because of its sampling difficulty. Its physicochemical properties must be determined to clarify functions of sericin and to obtain a detailed understanding of the silk fiber spinning mechanism.

We have used Sericin-hope, a silkworm race recently been improved by Yamamoto et al. (2001, 2002) from the mutant of Bombyx mori, Naked Pupa (Nd), as an experimental material for studying the characteristics of sericin in its native state. Since the posterior silk glands of Sericin-hope are degenerated, it secretes little fibroin. Despite this deficiency, Sericin-hope spins fiber (termed "sericin fiber" below) to make a thin cocoon. The spun fiber breaks very easily because it consists almost exclusively of sericin (sericin content is 98.5%, Yamamoto et al., 2002).

Using 13C solid-state NMR spectroscopy, Asakura et al. recently clarified the structure of Bombyx mori silk fibroin before spinning (silk I) and after spinning (silk II), and the mechanism of the structural transition during fiber formation (Asakura et al., 2002 and references therein). They suggested that the structural change of fibroin from silk I to silk II is explained by the shift of hydrogen bonds caused by stretching along the fiber axis in spinning. We expect sericin fiber to be formed similarly after undergoing various perturbations during fiber formation, and anticipated that significant information about sericin could be obtained by studying the structural changes sericin undergoes during spinning.

We analyzed sericin fiber spun by Sericin-hope and native sericin before spinning using attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy. Infrared spectroscopy is suitable for obtaining structural information on macromolecules and proteins because it easy to measure different forms such as fiber, film, and solution in different environments. Almost no work on the structural analysis of sericin using infrared spectroscopy has been reported, whereas some studies reported for fibroin (Suzuki, 1967; Sonoyama et al., 1997; Miyazawa et al., 2000; Tretinnikov and Tamada, 2001). The aim of this study is to clarify characteristics of sericin by investigating the structure and formation of sericin fiber using infrared spectroscopy. We first measured polarized ATR-FTIR to determine the molecular orientation of sericin fiber and did the same for fibroin fiber for comparison. Then, we analyzed the secondary structures of sericin fiber and after spinning, and followed the drying process of native sericin solution. We found that sericin fiber spun by Sericin-hope silkworms is almost randomly oriented in

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structure and is formed quite differently from fibroin.

**MATERIALS AND METHODS**

**Materials**

*Bombyx mori* larvae (Sericin-hope) were obtained from Sericultural Science Laboratory, National Institute of Agrobiological Sciences (NIAS). Larvae were reared on mulberry leaves at 24-27°C.

**ATR-FTIR**

ATR-FTIR spectra were recorded using a Jasco Herschel-350 Fourier transform infrared spectrophotometer with a liquid-nitrogen-cooled mercury-cadmium-telluride (MCT) detector and a SmIR Technologies single-reflection ATR attachment with a diamond prism. Spectra were recorded at a resolution of 4 cm\(^{-1}\) in 64 scans. Fourier self-deconvolution, second-derivative, and curve fitting procedures were conducted using a Jasco Spectra Manager.

**Polarized ATR-FTIR of sericin and fibroin fiber**

Sericin fiber was obtained by pulling the fiber from mature Sericin-hope larvae during spinning and then wound on a CaF\(_2\) plate (1 × 2 cm) so that the fiber axis was aligned in one direction. Polarized ATR-FTIR were measured using a linear polarized infrared beam, recorded parallel and perpendicular spectra by placing samples on the ATR crystal so that the electric vector of incident radiation was parallel or perpendicular to the fiber axis. Although spinning rate may affect orientation of sericin fiber, it was quite difficult to take an accurate measurement of the rate because of the weakness of the fiber. We then measured polarized spectra of naturally spun sericin fiber and verified that they were almost identical with those of the manually drawn out fiber. Hence, we employed the manually drawn out fiber for analyses due to higher signal-to-noise ratio of the spectra, without considering the influence of the spinning rate on the orientation of sericin fiber in this experiment.

Fibroin fiber sample was prepared by degumming silk fiber drawn out from ordinary *Bombyx mori* larva in a 0.5 w/v% Na\(_2\)CO\(_3\) aqueous solution at 90°C for 20 min, and its polarized spectra were measured.

**ATR-FTIR of native sericin solution and its drying**

Middle silk glands were removed from mature Sericin-hope larvae and rinsed with milli-Q water. Native sericin solution from the silk gland was dropped onto the ATR crystal. Spectra were then recorded at five-min intervals for 60 min at room temperature until water evaporating from the solution reached equilibrium. We call the film-like sericin formed after drying "air-dried sericin".

**RESULTS AND DISCUSSION**

**ATR-FTIR spectrum of sericin fiber**

In representative ATR-FTIR spectrum of sericin fiber (Fig. 1), characteristic amide absorption bands of protein, amide I, II, and III, were clearly observed at 1637, 1510, and 1236 cm\(^{-1}\). Amide I absorption primarily represents the C=O stretching vibration of the amide group. Amide II absorption contains contributions from N-H bending and C-N stretching vibrations, and amide III mainly arises from C-N stretching and C=O bending vibrations. The band at 1398 cm\(^{-1}\) is attributable to C-H and O-H bending, and the band at 1053 cm\(^{-1}\) is assignable to C-OH stretching vibrations. These strong absorptions are due to side chains of serine residue making up about 30% of the constituent amino acids of sericin. The abundance of serine residues makes it difficult to obtain structural information from high wavenumber amide absorption at 3300 cm\(^{-1}\), which represents the N-H stretching vibration of the amide group, due to overlapping with the strong O-H stretching of serine hydroxyl groups at 3500-3200 cm\(^{-1}\).

**Polarized ATR-FTIR spectra of sericin and fibroin fiber**

From the polarized ATR-FTIR spectrum of sericin fiber (Fig. 2A), we obtained information on molecular orientation because the absorption intensity of oriented absorbing groups varies with the direction of the vibrational plane of the linear polarized beam. The absorption intensities of amide I, II, and III of sericin fiber are almost identical for parallel and perpendicular spectra (Fig. 2A). Spectral features of amide I and II absorptions were, however, slightly different from each other. The difference was probably due to the slight existence of oriented amide groups. We thus concluded that most sericin molecules

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**Fig. 1.** Representative ATR-FTIR spectrum of sericin fiber spun by Sericin-hope. Characteristic amide absorption bands of protein, amide I, II, and III, were clearly observed.
are not oriented in sericin fiber though only a small amount of oriented segments exists. In contrast, the absorption intensities of bands observed for fibroin fiber differ markedly for parallel and perpendicular spectra (Fig. 2B), indicating that fibroin fiber has a highly oriented structure. Molecular orientation and fiber formation of fibroin are caused by stretching in spinning as detailed by Asakura et al. (2002). Sericin fiber, however, showed almost no such orientation despite similar spinning. Such differences are probably due to the amino acid sequence of sericin, which differs markedly from that of fibroin (Ga-rel et al., 1997; Fedić et al., 2002).

**Secondary structure of sericin fiber**

Secondary structure analysis of protein using FTIR spectroscopy is an established technique (Miyazawa and Blout, 1961; Surewicz and Mantch, 1988; Surewicz et al., 1993; Jackson and Mantch, 1995). Amide groups in protein are involved in secondary structures such as β-sheets, random coils, α-helices, and turns. Since secondary structures are associated with a characteristic pattern of hydrogen bonding between amide C=O and N-H groups, it is expected that individual secondary structures will have typical amide absorptions. Amide I absorption, found in the 1700-1600 cm\(^{-1}\) region, is the most useful for determining protein secondary structures because it arises predominantly from C=O stretching vibration. Hence, we used the amide I region to analyze the sericin structure.

The amide I region of the parallel spectrum of sericin fiber (Fig. 3A) is broad. As mentioned, individual secondary structures of protein are expected to have typical amide I absorption. Because natural protein contains more than one secondary structure, absorptions overlap in the amide I region to yield a featureless band (Fig. 3A). Fourier self-deconvolution and second-derivative procedures have been frequently used to distinguish between individual overlapping absorptions (Byler and Susi, 1986; Surewicz and Mantch, 1988; Surewicz et al., 1993). The deconvolved and second-derivative spectra of the amide I region of sericin fiber are shown in Fig. 3B and 3C. The broad amide I region was resolved into nine distinct bands by these procedures and each band was observed at almost the same position in both spectra. To determine the proportion of individual resolved amide I components, we iteratively fitted Gaussian curves to deconvolved spectra, finding the deconvolved amide I band of sericin fiber to
be a superposition of the nine Gaussian curves (Fig. 4).

We assigned each separated amide I component to secondary structures based on previous studies (Miyazawa and Blout, 1961; Jackson and Mantsch, 1991, 1995) (Table 1). Bands at 1629, 1619, and 1610 cm\(^{-1}\) were assigned to β-sheets. The appearance of the low wavenumber absorption at 1619 and 1610 cm\(^{-1}\) indicates the formation of strong intermolecular hydrogen bonds between extended chains, often referred to as intermolecular β-sheets (Jackson and Mantsch, 1991, 1995). The band at 1697 cm\(^{-1}\), due to the higher wavenumber component of β-sheets, is typical of antiparallel β-sheets (Miyazawa and Blout, 1961; Jackson and Mantsch, 1991, 1995). The band at 1660 cm\(^{-1}\) is in the region typically seen for α-helices. Bands at 1650 and 1639 cm\(^{-1}\) were assigned to random coils, and the remaining bands at 1682 and 1670 cm\(^{-1}\) were assigned to turns (Jackson and Mantsch, 1991, 1995). The proportion of each secondary structure is shown in Fig. 5, showing the abundance of β-sheets and random coils in sericin fiber.

Secondary structure of native sericin and structural changes in drying

We conducted ATR-FTIR analysis of native sericin solution directly extracted from middle silk glands to determine the secondary structure of sericin before spinning. The protein spectrum was obtained by subtracting the large water absorption from the original ATR spectrum of native sericin solution at 3300 and 1640 cm\(^{-1}\), obtaining a straight base line from 2000 to 1750 cm\(^{-1}\) based on the method described by Dong et al. (1990). The amide I band was separated into individual components and the proportion of secondary structures was determined by Fourier self-deconvolution and curve fitting as described previously. We also followed structural changes in native sericin during drying by recording spectra until the native sericin solution dried to equilibrium (representative data at 0, 30, and 60 min drying, Fig. 6). The proportion of β-sheets apparently increased with drying, whereas that of α-helices and turns decreased, suggesting that dehydration induced intra- and intermolecular hydrogen bonds to form between extended chains, increasing the occurrence of β-sheets, which can form more favorably for sericin in dehydrated state than other structures.

Structural changes in sericin during fiber formation

Table 2 summarizes the proportion of secondary structures of native sericin, air-dried sericin, and sericin fiber. Compared to native sericin, β-sheets were more abundant in fiber, with a lower proportion of turns and α-helices, indicating that structural transitions occurred during spinning. Interestingly, the secondary structure of air-dried sericin, which did not undergo spinning, resembled that of sericin fiber. The similarity of secondary structure be-
clarify the correlation between the characteristics of sericin and its amino acid sequence in detail.

In this work, we conducted ATR-FTIR measurements of sericin fiber spun by Sericin-hope and native sericin from the middle silk gland. Spectroscopic analyses showed little orientation of sericin molecules in sericin fiber and that the air-drying of native sericin solution leads to a similar secondary structure to the spun fiber. These observations suggest that sericin undergoes only modest structural changes by spinning compared with fibroin. We assume that such differences between the two proteins are necessary for smooth silk fiber spinning: the difficulty sericin presents in undergoing structural changes is probably required for covering fibroin softly and for acting as a type of lubricant in spinning.

Sericin may be applicable to functional materials as a biological resource (reviewed by Zhang, 2002), making it important to clarify the physicochemical properties of sericin to better understand the spinning mechanism behind silk fiber and to clarify the possible application of sericin to biomaterials.

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