Native Structure and Degradation Pattern of Silk Sericin Studied by ^{13}C NMR Spectroscopy

Hidetoshi Teramoto,[†,‡] Aya Kakazu,[‡] and Tetsuo Asakura*[,‡]

New Silk Materials Laboratory, National Institute of Agrobiological Sciences, Okaya, Nagano 394-0021, Japan, and Department of Biotechnology, Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan

Received September 29, 2005
Revised Manuscript Received November 9, 2005

The cocoon filament of the *Bombyx mori* silkworm is composed of two silk proteins: fibroin and sericin. Sericin is a family of proteins synthesized exclusively in the middle silk gland (MSG)[1,2] and ranges in size from 65 to 400 kDa.[3,4] The sericin content is 20−30 wt % of the cocoon filament. Sericin envelops fibroin threads to glue them together and contains many hydrophilic amino acids including serine (ca. 35%),[5,6] which lends it high hydrophilicity[7] and sensitivity to chemical modifications.[8] The silk sericin plays important roles in the spinning process of the silkworm and the construction of a robust cocoon shell. Recently, several unique sericin properties, such as affinity to human skin and hair,[7] induction of heterogeneous nucleation of apatite,[9] and enhanced attachment of primary cultured human skin fibroblasts,[10] have been reported, which makes sericin an attractive bioresource to develop novel biomaterials.[11] As for biocompatibility, which is a critical issue for developing biomaterials, sericin has been previously implicated in the immune response mounted against silk sutures. However, a recent study has shown that soluble sericin as well as native silk fibers with sericin coating does not induce significant macrophage activation.[12]

Despite growing interest in sericin's function and applications, only limited knowledge about its characteristics has been obtained. It is well-known that sericin is easily degraded by heat or alkaline treatment during its separation from fibroin threads, making it difficult to study its native characteristics. Moreover, such degraded sericin exhibits poor mechanical properties, which hampers its application as a material.[13] Recently, a new strain of *Bombyx mori* silkworm named Sericin Hope was developed, which secretes sericin almost exclusively (98.5%).[14,15] The Sericin Hope silkworm enables us to obtain native sericin without degradation because the separation process from fibroin is not required.

In this communication, the Sericin Hope silkworm was used as a sericin source in order to investigate the structure of native sericin before spinning and after spinning using ^{13}C solution and ^{13}C solid-state NMR, respectively. Moreover, the structural changes of sericin in solution by heat treatment were monitored with solution NMR, which indicated that heat-sensitive sequences exist in sericin's primary structure.

Native Structure of Sericin before and after Spinning. Sericin solution stored in the MSG of the Sericin Hope silkworm was obtained, and the ^{13}C solution NMR was observed (Figure 1a). Although the aqueous solution was highly viscous, each carbon atom was observed as a sharp peak, indicating high segmental motion as reported for native liquid fibroin.[16] Most of the peaks except for those in the highly overlapped carbonyl region could be assigned on the basis of the previous assignment of fibroin[16,17] and small peptides.[18] The peak intensities assigned to the carbons of Glu, Phe, and Tyr residues were much stronger than those expected from the amino acid composition of sericin,[5,6] which indicates the presence of low molecular weight impurities. Actually, the peaks from the impurities were confirmed by observing ^{13}C solution NMR of the dialysate of native sericin solution (unpublished data). The Cα and Cβ chemical shifts of major amino acids in native sericin solution were in good agreement with those of pentapeptides (Gly-Gly-X-Gly-Gly) (Table 1), whose chemical shifts are representative of random coil structure,[18] demonstrating that native sericin before spinning takes essentially the random coil structure. However, when 8 M urea was added to the solution, only small lower field shifts of the Cβ peaks of Ser, Thr, and Asp residues were observed (Table 1), indicating the formation of intra- and/or intermolecular hydrogen bonding through the polar side groups of these residues in the native state without urea.

Table 1. Cα and Cβ Chemical Shifts (ppm from External TMS) of Major Amino Acids in Native Sericin Solution, 8 M Urea-Denatured Sericin Solution, and Pentapeptides (Gly-Gly-X-Gly-Gly)[a]

	native sericin	8 M urea-denatured	GGXGG[b]
Ser Cα	55.69	55.68	55.9−55.8
Cβ	61.07	61.24	61.5−61.3
Gly Cα	42.62	42.53	42.8−42.4
Thr Cα	59.31	59.25	59.4−59.2
Cβ	66.93	67.11	67.5−67.1
Asn Cα	50.68	50.66	51.0−50.5
Cβ	36.15	36.22	37.0−36.4
Asp Cα	51.71	51.68	51.9−51.7
Cβ	38.44	38.67	39.0−38.7

[a] X = Ser, Gly, Thr, Asn, and Asp. [b] Chemical shift data were taken from ref 18.

Structural transition of sericin during spinning is of great interest because it is closely related to sericin's function in the spinning mechanism of *Bombyx mori* silk. The ^{13}C solid-state NMR spectrum of the Sericin Hope cocoon after spinning was observed (Figure 1c). For comparison, the spectrum of sericin film prepared from native sericin solution was also observed (Figure 1b). Although a detailed analysis of the CP/MAS spectra was difficult due to peak broadening and overlapping, Ser and Gly peaks could be obviously distinguished because of their abundance. Sericin film exhibited Ser Cα, Ser Cβ, and Gly Cα peaks at 55.3, 61.3, and 42.3 ppm (Figure 1b), which were respectively close to those of native sericin solution at 55.7, 61.1, and 42.6 ppm (Figure 1a). This observation indicated that sericin film is a largely random coil structure. Interestingly, the ^{13}C CP/MAS spectrum of the Sericin Hope cocoon was quite similar to that of sericin film (Figure 1c), indicating that sericin remains a largely random coil structure even after spinning and undergoes no remarkable structural transition during spinning. A similar conclusion has been previously obtained by FTIR analyses.[19] The observation that sericin exhibits no remarkable structural changes during spinning is quite in contrast to fibroin, which undergoes a drastic structural transition from silk I to silk II structure.[20]

Sericin has been considered to act as a kind of lubricant in the fiber formation process. Kataoka and Uematsu investigated

[†] National Institute of Agrobiological Sciences.
[‡] Tokyo University of Agriculture and Technology.
* To whom correspondence should be addressed. E-mail: asakura@cc.tuat.ac.jp.

10.1021/ma0521147 CCC: $33.50
Published on Web 11/25/2005

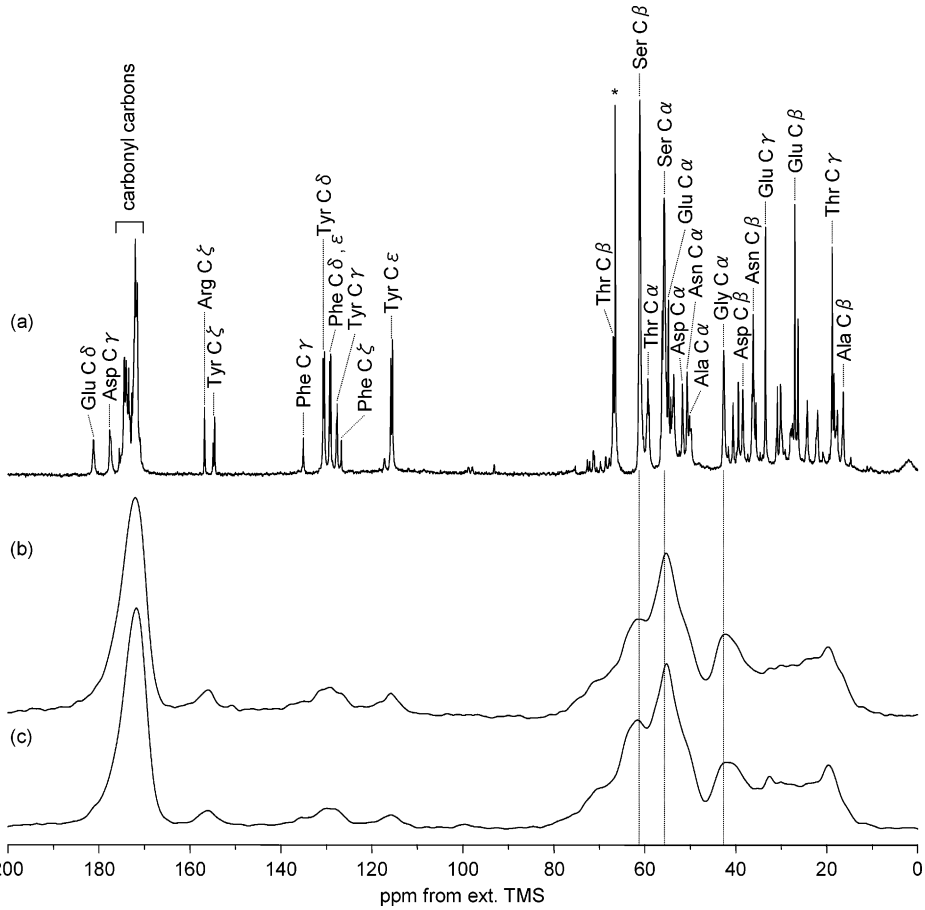

Figure 1. (a) ^{13}C solution NMR spectrum of native sericin solution derived from the MSG of the Sericin Hope silkworm (∗: 1,4-dioxane as an internal standard) and ^{13}C CP/MAS NMR spectra of (b) sericin film prepared by drying native sericin solution and (c) Sericin Hope cocoon.

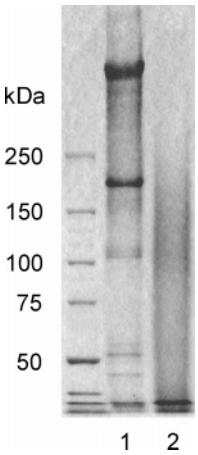

Figure 2. SDS-PAGE pattern of the regenerated sericin solution from the Sericin Hope cocoon before (lane 1) and after (lane 2) autoclaving at 120 °C for 20 min.

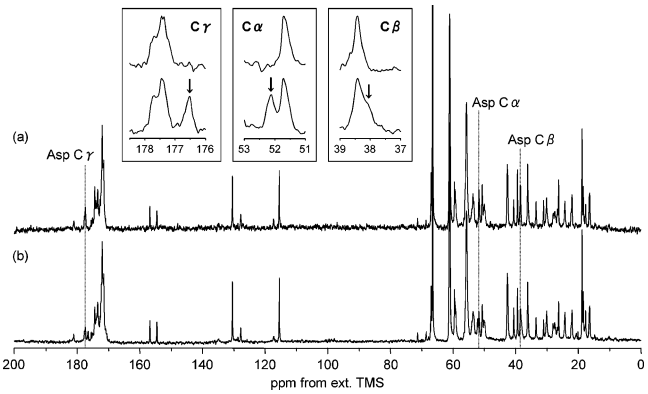

Figure 3. ^{13}C solution NMR spectra of the regenerated sericin solution prepared from the Sericin Hope cocoon (a) before and (b) after autoclaving at 120 °C for 20 min. Enlarged views around Asp Cγ, Cα, and Cβ peaks are represented in the insets.

the critical shear rates to transform sericin and fibroin into β structure and showed that sericin is difficult to undergo structural transition by shear stress compared to fibroin.[21] Although the present results obtained by using Sericin Hope silkworm cannot be directly correlated with general silk spinning process, the observed characteristic behavior of sericin must reflect its lubricating action.

Degradation Pattern of Sericin. Sericin solution regenerated from the Sericin Hope cocoon under mild conditions exhibited distinct protein bands by electrophoresis (Figure 2, lane 1), demonstrating that no thermal degradation occurred during preparation. Heat treatment of the regenerated sericin solution in an autoclave at 120 °C for 20 min turned its electrophoresis pattern into a smear pattern (Figure 2, lane 2), which demonstrated that sericin molecules were degraded. The pH of the solution before and after autoclaving was 7.9 and 7.6, showing that the degradation occurred at around neutral pH. A similar smear electrophoresis pattern is observed for sericin separated from fibroin threads with water at high temperature and pressure.[22]

The ^{13}C solution NMR spectra of the regenerated sericin solution before and after heat treatment are shown in parts a and b of Figure 3, respectively.[23] Although the chemical shifts were almost unchanged by heat treatment, splitting of Asp peaks was clearly observed (Figure 3; expanded spectra in the insets).

Asp Cγ, Cα, and Cβ peaks were detected at 177.4, 51.7, and 38.4 ppm in both spectra (Figure 3a,b), whereas the spectrum after heat treatment exhibited new peaks marked by arrows at 176.5, 52.1, and 38.1 ppm near the original Asp peaks, respectively. The observed splitting of Asp peaks is due to hydrolysis of peptide bonds at N and/or C termini of Asp residues. It has been reported that Asp residue is prone to hydrolysis compared with others.[24−26] Since sericin contains many Asp residues (∼6%),[5,27] they might be hydrolyzed preferentially by heat treatment, which causes a remarkable degradation of sericin molecules.

The 38-amino acid repetitive motifs in sericin contribute largely to the mechanical strength of sericin materials because it is known that the repetitive motifs exhibit strong interchain interaction.[5,28] Asp residues are periodically contained in the repetitive region[5,27] and are weak points against heat treatment. We infer that the collapse of the repetitive region at Asp residues by heat treatment is the main cause for poor mechanical properties of hitherto known sericin materials.

In conclusion, we could successfully utilize the Sericin Hope silkworm to investigate sericin's characteristics in the native state using ^{13}C NMR spectroscopy. As a consequence, two major conclusions could be drawn: (1) Sericin forms a largely random coil structure in the native state in MSG and undergoes no remarkable structural changes during the spinning of silkworm. (2) Asp residues periodically contained in the repeated region of sericin might be a weak point toward hydrolysis by heat treatment. The sericin materials prepared from the Sericin Hope cocoon, such as film or gel, do not show thermal degradation and have excellent mechanical properties.[13,29]

Acknowledgment. We thank Dr. K. Mase, Sericultural Science Laboratory, National Institute of Agrobiological Sciences, for providing the Sericin Hope silkworms and cocoons. This work was partially supported by the Insect Technology Project from the Ministry of Agriculture, Forestry, and Fisheries of Japan.

Supporting Information Available: Detailed experimental procedures including sample preparations and NMR measurements. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Fedič, R.; Žurovec, M.; Sehnal, F. *J. Insect Biotechnol. Sericol.* **2002**, *71*, 1−15.
(2) Craig, C. L.; Riekel, C. *Comp. Biochem. Physiol. B* **2002**, *133*, 493−507.
(3) Sinohara, H. *Comp. Biochem. Physiol.* **1979**, *63B*, 87−91.
(4) Gamo, T. *Biochem. Genet.* **1982**, *20*, 165−177.
(5) Garel, A.; Deleage, G.; Prudhomme, J. C. *Insect Biochem. Mol. Biol.* **1997**, *27*, 469−477.
(6) Takasu, Y.; Yamada, H.; Tsubouchi, K. *Biosci. Biotechnol. Biochem.* **2002**, *66*, 2715−2718.
(7) Voegeli, R.; Meier, J.; Blust, R. *Cosmet. Toiletries* **1993**, *108*, 101−108.
(8) Teramoto, H.; Nakajima, K.; Takabayashi, C. *Biomacromolecules* **2004**, *5*, 1392−1398.
(9) Takeuchi, A.; Ohtsuki, C.; Miyazaki, T.; Tanaka, H.; Yamazaki, M.; Tanihara, M. *J. Biomed. Mater. Res.* **2003**, *65A*, 283−289.
(10) Tsubouchi, K.; Igarashi, Y.; Takasu, Y.; Yamada, H. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 403−405.
(11) Zhang, Y. Q. *Biotechnol. Adv.* **2002**, *20*, 91−100.
(12) Panilaitis, B.; Altman, G. H.; Chen, J.; Jin, H.-J.; Karageorgiou, V.; Kaplan, D. L. *Biomaterials* **2003**, *24*, 3079−3085.
(13) Teramoto, H.; Nakajima, K.; Takabayashi, C. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 845−847.
(14) Yamamoto, T.; Mase, K.; Miyajima, T.; Hara, K. Japanese Patent No. 3374177.
(15) Yamamoto, T.; Miyajima, T.; Mase, K.; Iizuka, T. In *Annual Report 2002*; National Institute of Agrobiological Sciences: Tsukuba, Japan, 2002; pp 24−25.
(16) Asakura, T.; Watanabe, Y.; Uchida, A.; Minagawa, H. *Macromolecules* **1984**, *17*, 1075−1081.
(17) Asakura, T.; Watanabe, Y.; Itoh, T. *Macromolecules* **1984**, *17*, 2421−2426.
(18) Howarth, O. W.; Lilley, D. M. J. *Prog. Nucl. Magn. Reson. Spectrosc.* **1978**, *12*, 1−40.
(19) Teramoto, H.; Miyazawa, M. *J. Insect Biotechnol. Sericol.* **2003**, *72*, 157−162.
(20) Yamane, T.; Umemura, K.; Nakazawa, Y.; Asakura, T. *Macromolecules* **2003**, *36*, 6766−6772.
(21) Kataoka, K.; Uematsu, I. *Kobunshi Ronbunshu* **1977**, *34*, 37−41 (in Japanese).
(22) Zhang, Y. Q.; Tao, M. L.; Shen, W. D.; Zhou, Y. Z.; Ding, Y.; Ma, Y.; Zhou, W. L. *Biomaterials* **2004**, *25*, 3751−3759.
(23) The ^{13}C NMR spectrum of the regenerated sericin solution (Figure 3a) exhibited much smaller peaks assignable to Glu, Phe, and Tyr residues than native sericin solution (Figure 1a), showing that low molecular weight impurities can be removed during regeneration process. The Cα and Cβ chemical shifts of major amino acids in the regenerated sericin solution were in good agreement with those of native sericin solution, which demonstrated that sericin remained in a largely random coil structure after regeneration from the cocoon.
(24) Tomizawa, H.; Yamada, H.; Imoto, T. *Biochemistry* **1994**, *33*, 13032−13037.
(25) Frare, E.; Polverino de Laureto, P.; Zurdo, J.; Dobson, C. M.; Fontana, A. *J. Mol. Biol.* **2004**, *340*, 1153−1165.
(26) Hosia, W.; Griffiths, W. J.; Johansson, J. *J. Mass Spectrom.* **2005**, *40*, 142−145.
(27) Okamoto, H.; Ishikawa, E.; Suzuki, Y. *J. Biol. Chem.* **1982**, *257*, 15192−15199.
(28) Huang, J.; Valluzzi, R.; Bini, E.; Vernaglia, B.; Kaplan, D. L. *J. Biol. Chem.* **2003**, *278*, 46117−46123.
(29) Teramoto, H.; Miyazawa, M. *Biomacromolecules* **2005**, *6*, 2049−2057.

MA0521147