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SHORT COMMUNICATION

The Influence of Ser and Tyr Residues on the Structure of *Bombyx Mori* Silk Fibroin Studied Using High-resolution Solid-state ^{13}C NMR Spectroscopy and ^{13}C Selectively Labeled Model Peptides

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Silk fibroin produced by *Bombyx mori* (*B. mori*) has good tensile properties for textiles. In the past decades *B. mori* silk fibroin has also come to the forefront as a biomaterials.[1] Two distinct structures in the solid state, silk I before spinning and silk II after spinning, have been proposed.[2] The conformation of silk I has been shown by us to have a repeated type II β-turn structure using solid state NMR approaches as well as X-ray diffraction analysis.[3] On the other hand, the conformation of the silk II has been proposed to be mainly an anti-parallel β-sheet. Recently,^{13}C solid-state NMR on selectively ^{13}C labeled 15 peptides labeled singly at different Ala methyl carbons was used to examine the silk II structure of $(AG)_{15}$ which was considered to be a model for the crystalline domain, with emphasis on a possible lamellar structure containing β-turns.[4,5] However, there are also Ser and Tyr residues and the relative content is reasonably high; 12.2% and 4.8%, respectively. They stand third and fourth behind the Gly and Ala residues.[2]

In this communication, in order to study the role played by Ser and Tyr residues in silk II structure of *B. mori* silk fibroin, we synthesized several ^{13}C selectively labeled model peptides containing Ser and Tyr residues in AG copolypeptide, mimicking the primary structure of *B. mori* silk fibroin. Especially, since the crystalline domain is $(AGSGAG)_n$ rather than $(AG)_n$, a more detailed structural analysis is possible when $(AGSGAG)_n$ is used. The ^{13}C CP/MAS NMR is used for the purpose.

EXPERIMENTAL

The model peptides were synthesized using solid-phase Fmoc chemistry on a fully automated Pioneer Peptide Synthesis System (Applied Biosystems Ltd.). All of the peptides were dissolved in 9M LiBr aqueous solution and then dialyzed against water (9M LiBr/dialysis treatment). The peptide $(AGSGAG)_5$ was also prepared by dissolving them in formic acid and then dried (Formic acid treatment).

The ^{13}C CP/MAS NMR measurements were conducted on a Chemagnetics CMX-400 spectrometer operating at 100 MHz for ^{13}C nucleus. A ^{1}H 90° pulse width of 5 µs duration was used with a 1 ms contact time and a 3 s repetition time. Approximately 15K FIDs were added to generate the spectra. The ^{13}C chemical shifts were calibrated indirectly through the methine peak of adamantane observed at 28.8 ppm relative to TMS at 0 ppm.[4,5] The peak fitting was performed as follows: After fixing the chemical shifts of three peaks of Ala Cβ carbon, the fraction and width of each peak was changed to reproduce the observed line shape by assuming Gaussian for each peak. The uncertainty of the percentages is about ±1%.

RESULTS AND DISCUSSION

Figure 1 shows Ala Cβ peaks in the ^{13}C CP/MAS NMR spectra of (a) **I**: $(AGSGAG)_5$, (b) **II**: AGSGAGAGSG[3-^{13}C]A^{11}GAGSGAGAGSG-AGAGSGAG and (c) **III**: AGSGAGAGSGAGAGSGAG[3-^{13}C]A^{19}GSG-AGAGSGAG after 9M LiBr/dialysis and formic acid treatments. The peak splitting of Ala Cβ carbon has been assigned previously in detail.[6] Namely, the highest-field broad peak at 16.7 ppm was assigned to a "distorted β-turn" and/or random coil where the torsion angles are distributed over a wide range of values. The other two peaks observed at 19.6 ppm and 22.2 ppm could be assigned to an anti-parallel β-sheet conformation. The change in the peak intensity of 16.7 ppm depending on the ^{13}C labeled position of $(AG)_{15}$ has been used for study of the lamellar structure.[4,5] In this study, two Ala positions in **I**, Ala^{11th} and Ala^{19th}, were selected for the ^{13}C labeling position because of relatively high distorted β-turn probabilities in the proposed lamellar structure of $(AG)_{15}$. The most remarkable difference between $(AG)_{15}$ and $(AGSGAG)_5$ after 9M LiBr/dialysis treatment was that $(AG)_{15}$ took silk I form,[4] but $(AGSGAG)_5$ took silk II form as shown in Figure 1 (left). This is clearly due to presence of Ser residue. The $(AG)_{15}$ with silk I form changed to silk II form after formic acid treatment[4] and therefore formic acid treatment was also applied to $(AGSGAG)_5$. The fraction of 16.7 ppm peak was 37% and 44% for [3-^{13}C]A^{11} and [3-^{13}C]A^{19} labeled positions, respectively, after 9MLiBr/dialysis treatment, and 31% and 36% for the same labeled positions after formic acid treatment. Thus, the formic acid treatment promotes increase in β-sheet structure of **I**. The latter values, 31% and 36%, are comparable with the corresponding values of $(AG)_{15}$ after formic acid treatment; 31% for [3-^{13}C]A^{11} and 34% for [3-^{13}C]A^{19}.[4]

Thus, $(AGSGAG)_5$ which is a better structural model for the crystalline domain than $(AG)_{15}$ can be speculated to form lamellar structure with silk II form. However, the fraction of 16.7 ppm peak is not 100%, means that turns also occur at other positions frequently in the lamellar structure as was discussed in $(AG)_{15}$.[5] The Ser Cβ peak which gives structural information on the Ser residue, suggests that the Ser residues in $(AGSGAG)_5$ took β-sheet judging from the chemical shift, 64 ppm, and the line shape.[7]

We suspect that the nature of hydrogen bonding, involving the O$^{\gamma}$H group of the Ser side-chain, might be critical to select silk I or silk II structures during the course of dialysis process against water. As reported previously,[8] the Ser side-chain residue is capable of forming intramolecular hydrogen bond involving the O$^{\gamma}$H group and the carbonyl group

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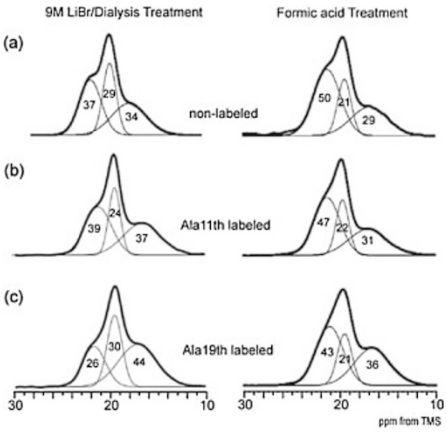

Figure 1. Ala Cβ peaks in ^{13}C CP/MAS NMR spectra of (a) **I**, (b) **II** and (c) **III** after 9M LiBr/dialysis (left) and formic acid (right) treatments.

Figure 2. The dominant structure of one Ser residue with inter-molecular hydrogen bonding formation (broken line) through the OH group in the side chain in copolypeptide.

of the Gly residue, presumably stabilizes the silk I form. However, multiple existence of AGSGAG sequence appears to modulate the pattern of the hydrogen bonding interactions involving O$^{\gamma}$H group of the Ser side-chains and the backbone C=O oxygen. Our ^{2}H NMR measurements of uniaxially aligned [3,3-$^{2}H_2$] Ser-labeled *B. mori* silk fiber with silk II form, indicated that the dominant conformer of the Ser side-chain is *gauche*$^{+}$ and this orientation could be a good candidate for facilitating intermolecular hydrogen bonds with the carbonyl groups on adjacent peptide chains, strongly favoring the β-sheet structure as shown in Figure 2.[9]

Figure 3 shows Ala Cβ regions in ^{13}C CP/MAS NMR spectra of (a) **IV**: (AGSGAG)$_4$AGYGAG, (b) **V**: AGSGAGAGSGAGAGSGAGAGSG-[1-^{13}C]A^{23}G[3-^{13}C]A^{25}GYGAG and (c) **VI**: AGSGAGAGSGAG[1-^{13}C]-A^{13}GYG[3-^{13}C]A^{17}GAGSGAGAGSGAG after 9M LiBr/dialysis treatment. When the 27th Ser residue was changed to Tyr residue in (AGSGAG)$_5$, the influence of the Tyr residue on the structure of the peptide was examined. Remarkable increase of random coil conformation was observed in the natural abundance spectrum of **IV** compared with the case of **I**. In order to examine the influence of Tyr residue on the local conformation of Ala residues near to the Tyr^{27th} residue, both the carbonyl carbon of Ala^{23th} and methyl carbon of Ala^{25th} were ^{13}C labeled. Here, the ^{13}C carbonyl carbon labeling was also performed to monitor change in the local conformation as much as possible (Data not shown). Because of broadening of two β-sheet peaks for Ala Cβ carbon, apparently broad single peak at 21 ppm was assigned to distorted β-sheet.[6] Then, Figure 3(b) can be reproduced by assuming two peaks and Ala^{25th} takes mainly random coil, but assumption of five peaks for Figure 3(a). Thus, one Tyr residue introduced in the (AGSGAG)$_n$ sequence in **IV** destroys largely the lamellar

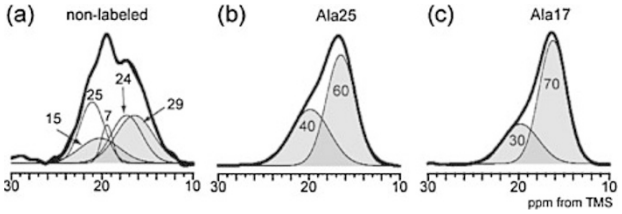

Figure 3. Ala Cβ peaks in ^{13}C CP/MAS NMR spectra of (a) **IV**, (b) **V** and (c) **VI** after LiBr/dialysis treatment.

structure. This indicates that the lamellar structure of (AGSGAG)$_n$ remains at the region apart from the Tyr residue. In order to examine change in the influence of Tyr residue on the structure of (AGSGAG)$_n$, the introduction of one Tyr residue was changed to the central part where both the carbonyl carbon of Ala^{13th} and methyl carbon of Ala^{17th} were ^{13}C labeled (Figure 3(c)). Although the local conformation of the ^{13}C labeling sites was also mainly random coil, it is noted that two Ala ^{13}C labeling sites in **VI** increase random coil fraction compared with those of two Ala ^{13}C labeling sites in **V**. The change in whole structure of both peptides, **V** and **VI**, can be monitored by the spectral change in non-labeled Ala Cα site: The non-labeled Ala Cα peak tends to shift from 48.7 ppm (β-sheet) to 50.0 ppm (random coil) in **VI** compared with the case of **V**. Thus, one Tyr residue introduced at the center of (AGSGAG)$_n$ sequence destroys the lamellar structure with β-sheet conformation effectively.

In conclusion, the presence of Ser residue promotes silk II structure, but Tyr residue destroys silk II structure largely depending on the position of the introduction in the chain.

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