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# A $^2H$ NMR Study of [*Ser*-3,3-$^2H_2$]- and [*Ala*-3,3,3-$^2H_3$]- Silk Fibroins in the Solid State. Role of Side-Chain Moiety in Stabilization of Secondary Structure

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An attempt was made to prepare [*Ser*-3,3-$^2H_2$]- and [*Ala*-3,3,3-$^2H_3$]-fibroins by feeding respective amino acids DL-[3,3-$^2H_2$]-serine (Ser) and [3,3,3-$^2H_3$]-alanine (Ala), respectively, to silkworms, *Bombyx mori* and *Philosamia cynthia ricini* together with daily artificial diet. We recorded $^2H$ powder-pattern NMR spectra of [*Ser*-3,3-$^2H_2$]- and [*Ala*-3,3,3-$^2H_3$]-cocoons and fibroins (10—20% deuteration) by means of the quadrupole echo method. It was found that separation of the $^2H$ quadrupole splittings is 125 and 39 kHz, respectively, for [*Ser*-3,3-$^2H_2$]- and [*Ala*-3,3,3-$^2H_3$]-cocoons and fibroins, respectively. Accordingly, it was concluded that side-cain reorientation of Ser residue is virtually frozen irrespective of differences in conformations of silk I and silk II, suggesting that the hydroxyl groups in these cases might be hydrogen-bonded to C=O or NH groups in intra or interchain. On the other hand, side-chain methyl group of Ala residue in [*Ala*-3,3,3-$^2H_3$]-fibroin turned out to undergo rapid three-fold rotation about the $C_\alpha$–$C_\beta$ axis, as viewed from data of the quadrupole splittings.

Silk fibroin from *Bombyx mori* is a fibrous protein whose major amino acid composition consists of Gly, Ala, and Ser residues (over 85%) and its ratio Gly:Ala:Ser is 3:2:1.[1-4] Lucas et al.[5,6] showed the presence of the following long hexapeptide sequence for the crystalline fraction ($C_p$ fraction) obtained as precipitate (60% of the original material) after digestion by chymotrypsin: Ser–Gly–Ala–Gly–Ala–Gly. Accordingly, X-ray powder diffraction patterns of this hexapeptide and its polymer were found to be similar to those obtained in powdered cocoon samples.[7,8] This model could be further simplified to repetition of Ala-Gly sequence, if the difference of side chains in Ala and Ser were ignored. To support this view, secondary structure of silk I and silk II from *B. mori* fibroin turned out to be very similar to that of forms II and I of poly(alanylglycine), respectively, on the basis of X-ray powder diffraction studies.[2,3,9] We further confirmed this conclusion on the basis of the similarity of $^{13}C$ chemical shifts of Gly and Ala residues between corresponding forms of silk and poly(alanylglycine),[10] because displacement of the $^{13}C$ chemical shifts due to a conformational change are found to be very useful as a novel means to examine conformational features of a variety of peptides, polypeptides, and proteins.[11-18]

Nevertheless, it seems important to clarify the role of side chains in Ser residues in stabilization of these secondary structures in the solid state, because half of Ala residues is replaced by Ser in *B. mori* fibroin. If the amount of Ser residues is smaller than this ratio, $\alpha$-helix form is stabilized instead of silk I form, as in *P. c. ricini* fibroin. To gain insight into the manner of side-chain orientation, it has been demonstrated that analysis of the quadrupole splittings in $^2H$ NMR spectra of $^2H$-labeled samples provides one unequivocal means to assess molecular reorientation or motions.[19-21]

In the present paper, we aimed to analyze the manner of side-chain reorientation in Ser residues of silk fibroins as compared to that of Ala residues, on the basis of $^2H$ NMR spectra of [$^2H$]-labeled fibroins from *B. mori* and *P. c. ricini* which were prepared by incorporation of deuterated amino acids, DL-[3,3-$^2H_2$]-serine (Ser) and L-[3,3,3-$^2H_3$]-alanine (Ala), to artificial diet. We found from analysis of $^2H$ NMR spectra that reorientation of side chains in Ser residues is virtually frozen, for any form of fibroin (silk I, silk II and $\alpha$-helix), as a result of plausible involvement of OH groups in hydrogen bonds which cause stabilization of these individual conformations. On the contrary, separation of the motionally averaged quadrupole-splittigs of Ala residue is 39 kHz due to the presence of the three-fold rotation of the methyl groups and is found to be identical among various types of backbone conformations such as silk I, silk II and $\alpha$-helix forms.

## Experimental

Silkworms of *Bombyx mori*, a hybrid between strains Nichi 140 and Shi 140, were reared with an artificial diet. During the period of 5 d before maturation, 2.5% DL-[3,3,-$^2H_2$]Ser (98 atom%, Sigma Chemical Company, USA; Lot No. 985-H) or L-[3,3,3-$^2H_3$]Ala (99.4 atom%, Sigma Chemical Company, USA; Lot No. 2010-I) were fed with an artificial diet (Silkmate 2M, Nippon Nosan Kogyo, Tokyo). Selectively deuterated cocoons thus obtained were degummed twice, to remove sericin, with 0.5% Marseilles soap solution at 100 °C, for 30 min. and washed by distilled water. Silk II sample thus obtained was dissolved in 9M LiBr aqueous solution (40 °C) and dialyzed against distilled water for 4 h and lyophilized (Silk I sample). Prior to measurements of $^2H$ NMR spectra of the individual forms,

these structural modifications were monitored by examination of infrared or high-resolution $^{13}C$ solid-state NMR spectra.

[*Ala*-3,3,3-$^2H_3$]-cocoon and fibroin from *P. c. ricini* were prepared by feeding 2.5% (w/w) L-[3,3,3-$^2H_3$]Ala to the silkworms together with an artificial diet during 5 d before maturation. At the same time, silkworms fed with [3,3,3-$^2H_3$]-Ala were excised and liquid silk was directly removed from the middle silk gland portion of *P. c. ricini*. The liquid silk thus obtained was dried gently over film of poly(vinyldene chloride).

Infrared spectra were obtained with a Shimadzu IR-435 spectrometer. 75.46 MHz high-resolution solid-state $^{13}C$ NMR spectra were recorded on a Bruker CXP-300 spectrometer by cross polarization-magic angle spinning (CP-MAS) method. Samples were contained in an Andrew-Beams type rotor machined from perdeuterated poly(methyl methacrylate) and spun as fast as 3—4 kHz by compressed air. In some instances, spinning side bands from quaternary carbons were suppresed by means of pulse sequence of the total side-band suppression (TOSS) by Dixon.[22] Chemical shifts were calibrated by the peak position of benzene at 128.5 ppm from TMS.

46.06 MHz high-power $^2H$ NMR spectra[19-21] were recorded on a Bruker CXP-300 spectrometer utilizing the quadrupole-echo pulse-sequence, $90^\circ_x$-$t$-$90^\circ_y$-$t$-echo.[23] The experiments were done on resonance so that negative frequencies of the symmetry spectrum are folded over on top of the positive frequencies on transformation. Samples were contained in a 10 mm sample tube placed in a transverse solenoid coil. Duration of 90° pulse was usually 4 μs. Spectral width, spacing of the two 90° pulses $t$, and data points were 250 kHz (125 kHz×2), 40—60 μs, and 2 K, respectively. Repetition time was 0.5 s. Spectra were usually accumulated more than 120000 times.

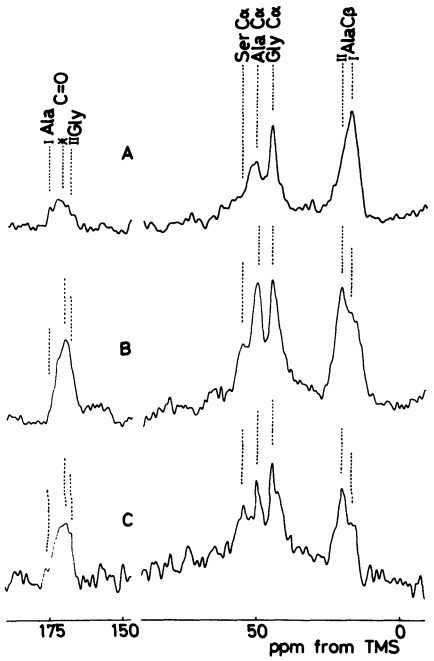


Fig. 1. 75.46 MHz $^{13}C$ CP-MAS NMR spectra of [*Ser*-3,3-$^2H_2$]cocoon and fibroin taking a variety of crystalline forms. The asterisked peak is ascribed to the carbonyl carbons other than silk I peak of Ala residue and silk II of Gly residue. A. silk I, B. silk II and C. cocoon.

## Results

We obtained [*Ser*-3,3-$^2H_2$]- and [*Ala*-3,3,3-$^2H_3$]-cocoons and fibroin samples from biosynthesis by feeding respective deuterated amino acids to silkworms and resultant degumming procedure, as described in the previous section.[24] Before recording individual $^2H$ NMR spectra of each samples, we first examined change of spectral features due to structural modifications by the $^{13}C$ CP-MAS NMR spectra, as demonstrated in Fig. 1. The $^{13}C$ CP-MAS NMR spectra of [*Ser*-3,3-$^2H_2$]cocoon or fibroin are not strongly different from those of undeuterated samples previously recorded,[10,14] because degree of deuteration at Ser $C_\beta$ carbon is at most 10—20%, as estimated by examination of high-resolution $^{13}C$ NMR spectra in aqueous solution. Apparently, expected conformational modification of the present samples was effectively achieved, because each silk I and silk II samples contain the respective forms as major components (≈80%), as manifested from the $^{13}C$ NMR peak positions of Ala $C_\beta$ or C=O peaks.

Figure 2 illustrates $^2H$ powder-pattern NMR spectra of corresponding [*Ser*-3,3-$^2H_2$]-cocoon and fibroin

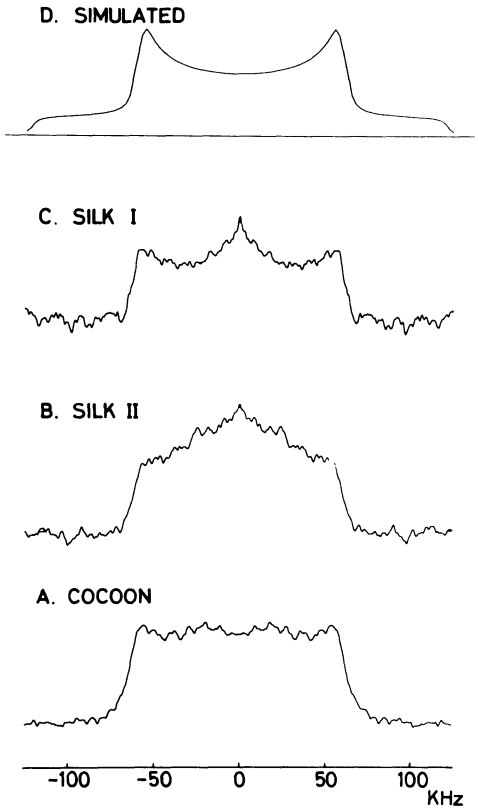


Fig. 2. 46.06 MHz $^2H$ NMR spectra of [*Ser*-3,3-$^2H_2$]-cocoon and fibrion. A. cocoon, B. silk II, C. silk I, and D. calculated spectrum with $e^2qQ/h=180$ kHz.

samples from *Bombyx mori* taking silk I and silk II forms. Obviously, these $^2$H NMR spectra arose from superposition of at least two kinds of powder patterns, the wider and narrow components. It appears that no appreciable change in the spectral patterns of the wider components was noted among cocoon, silk I and silk II samples. In principle, any difference in spectral feature between the cocoon and silk II samples could be ascribed to the presence or absence of sericin which is a protein consisting of a large proportion of Ser residue. Therefore, it is now clear that there exists no appreciable change in the $^2$H NMR spectra of side-chain orientation as seen from the wider component in the deuterated fibroin and sericin. The spectral feature and the separation of peaks ($D_q$) due to the quadrupole splittings (125 kHz) were well reproduced by spectral simulation[33,34] assuming the quadrupole coupling constant $e^2qQ/h$ as 180 kHz, as shown in the top trace. In particular, the separation of peaks in the doublet signals is[19]

$$D_q = 3/4\,(e^2qQ/h) \qquad (1)$$

It is difficult, however, to record correctly the outer edges portion with separation of $2D_q$ (250 kHz) because of experimental limitation (spectral width 250 kHz).

On the contrary, we observed $^2$H powder-pattern NMR spectra with reduced spectral separation as large as 39 kHz for [3,3,3-$^2$H$_3$]Ala(A), silk II(cocoon; B) and silk I (C) of *Bombyx mori*, $\alpha$-helical sample obtained as dried liquid silk removed from the middle silk gland (D) and cocoon (E) from *P. c. ricini* (Fig. 3). Conformational feature of dried liquid silk sample from *P. c. ricini* is rather heterogeneous because of the presence of both the $\alpha$-helix (about 30%[35]) and random coil form (about 70%) (Fig. 4). Nevertheless, it is obvious that Ala residues are mainly involved in the $\alpha$-helix form, as far as the peak positions of the $^{13}$C CP-MAS NMR spectra are concerned (Fig. 5).[36] Consistent with our previous finding,[17] the peak positions of the $^{13}$C NMR peaks in Ala $C_\alpha$, $C_\beta$ and C=O carbons of [*Ala*-3,3,3-$^2$H$_3$]-fibroin in the solid state are in good agreement with those obtained from $(Ala)_n$ taking $\alpha$-helix form.

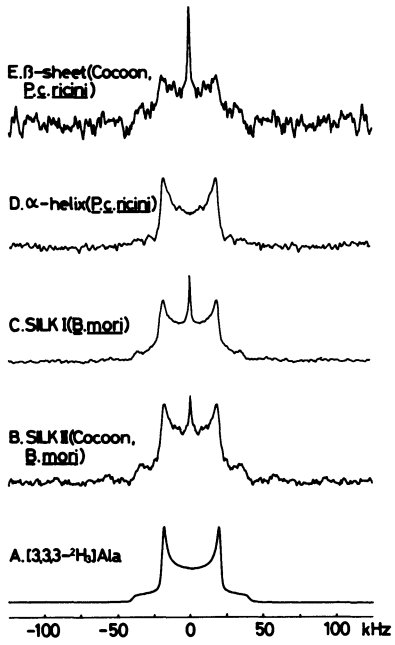


Fig. 3. 46.06 MHz $^2$H NMR spectra of [*Ala*-3,3,3-$^2$H$_3$]-cocoons and fibroins from *Bombyx mori* and *P. c. ricini*. A. [3,3,3-$^2$H$_3$]alanine, B. cocoon from *Bombyx mori*, C. silk I from *B. mori*, D. $\alpha$-helical portin of dried liquid silk from *P. c. ricini*, and E. cocoon of *P. c. ricini*.

## Discussion

It is conceivable that side-chain moiety of Ser in silk fibroins, $CD_2OH$ group, might participate in stabilization of secondary conformation in the solid state, because OH group has ability to form hydrogen bonding with C=O group of the same molecular

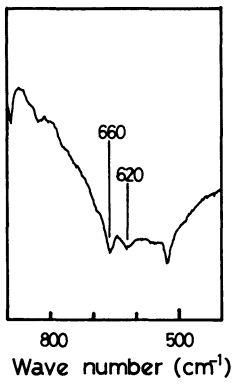


Fig. 4. Infrared spectrum of dried liquid silk from *P. c. ricini*.

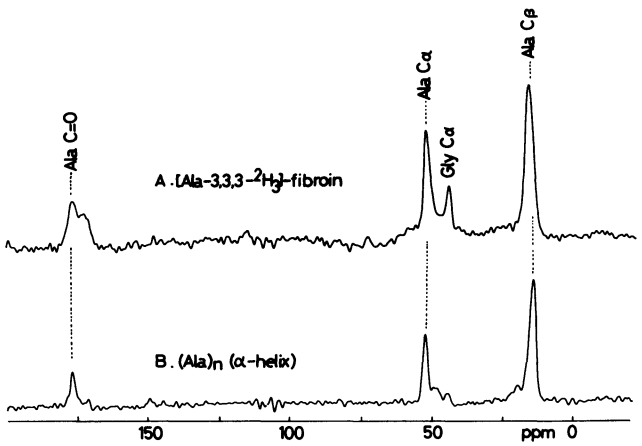


Fig. 5. $^{13}$C CP-MAS NMR spectra of dried liquid silk [*Ala*-3,3,3-$^2$H$_3$]-fibroin from *P. c. ricini* and $\alpha$-helical $(Ala)_n$. Spinning side bands were suppressed by the pulse sequence of TOSS.

chain. On the basis of survey of X-ray diffraction data on 19 or 43 globular proteins, Janin et al.[37] or Gray and Matthews[38] respectively, showed that approximately 70% of serine residue have their $O_\gamma$ atom within hydrogen-bonding distance (3.5 Å) of at least one carbonyl group, with an acceptable angular geometry for OH··O bond. They showed that the $\chi_1$ angle distribution of Ser side chain is almost equal among $g^-$ (38%), $t$ (28%), and $g^+$ (34%), suggesting the lesser steric hindrance created by the hydroxyl group, which in turn results in a smaller barriers for the rotation about the $C_\alpha$–$C_\beta$ bond.[37,38] Nevertheless, most of these observed hydrogen bonds involve a main chain carbonyl group as acceptor (83%), though side chain to side chain bonds may coexist with side chain to main chain bonds. It is further obvious that these hydrogen bonds are determined by the conformation of the main chain which fixes the position and orientation of the acceptor oxygens. In the case of the $\beta$-sheet form, intramolecular hydrogen bond can occur either between OH and C=O of the preceding ($i$-1) residue or of the same residue ($i$).[37] In the case of the $\alpha$-helix, similar hydrogen bond can be formed between the OH of $i$ th residue and C=O of $i$-4 residue.

It is now obvious that $^2$H NMR spectra of side-chain methylene ($CD_2$) group of Ser residues can be analyzed as static and the separation of the quadrupole splittings was also unchanged among the cocoon, silk II and silk I samples. This finding implies that side chain reorientation of Ser residue is virtually frozen irrespective of differences in backbone conformations (silk II ($\beta$-sheet) or silk I). Undoubtedly, the $\beta$-sheet or silk II form could be further stabilized by taking the above-mentioned intrachain hydrogen bonds. As to a silk I model of *B. mori* silk fibroin in the solid state, crankshaft-type model has been proposed by Lotz et al.[3,9] and later, three kinds of revised loose helix models have been reported.[39] Therefore, we examined any possibility of forming intramolecular hydrogen bonds between the OH group of given Ser residue and backbone carbonyl groups in the chain of partial sequence Ala–Gly–Ser–Gly–Ala–Gly–Ala taking several conformations for models of silk I form. Basically, it was found by model-building that most of these models are feasible to form intramolecular hydrogen bonds (<3.5 Å) between the OH hydrogen and the C=O oxygen of the Gly residue in the preceding ($i$-1) residue in a similar manner to the case of the $\beta$-sheet form.

Therefore, it is natural to consider that almost all of side-chain hydroxyl group of Ser residue in fibroin could participate in the formation of intra- or interchain hydrogen bonds. In addition, it appears that in cocoons side chain hydroxyl groups of Ser in sericin are mostly hydrogen-bonded to proton acceptors of fibroin in cocoon, as inferred from the data of Fig. 2. This view might be important in connection with understanding of a manner of sericin-fibroin interaction in the cocoons. Further, it is worthwhile to point out that there appears no difference in the dynamic aspect of these main chains, because particular motions, if any, could significantly influence the $^2$H NMR spectral features.

As illustrated in Fig. 3, however, the quadrupole splittings of [3,3,3-$^2H_3$]Ala and [*Ala*-3,3,3-$^3H_3$]-cocoon and fibroins were equally reduced to 39 kHz, as a result of motionally averaged reduction in the quadrupole interaction of the methyl group. Apparently, these spectral patterns arose from the presence of axially symmetric field gradient. Therefore, the simplest such a motion is the three-fold rotation about the $C_\alpha$–$C_\beta$ axis. The separation of the doublet peaks due to the quadrupole interaction is then reduced to

$$D_q' = 3/8\,(e^2qQ/h)(3\cos^2\alpha - 1) \qquad (2)$$

where $\alpha$ is the angle between the C–D vector and $C_\alpha$–$C_\beta$ axis. The calculated quadrupole splitting $D_q'$ from this case is obtained as 42.6 kHz by using $\alpha$= 109° 44′ and this value is slightly larger than the observed value (39 kHz).[21] In other systems, the quadrupole splittings of deuterated methyl groups are in the range of 35 kHz for palmitic acid[40] and nonadecane.[41] Again, there appears no variation in the above-mentioned value among alanine monomer and fibroins of $\alpha$-helix, $\beta$-sheet, and silk I forms, reflecting the absence of major change in the dynamic state of main chains in these systems.

In conclusion, we successfully recorded powder-pattern $^2$H NMR spectra of [*Ser*-3,3-$^2H_2$]- and [*Ala*-3,3,3-$^2H_3$]-cocoons and fibroins taking various types of secondary structure. The quadrupole splitting for $CD_2OH$ side chain of Ser residue was found to be 125 kHz, indicating the presence of static side chain, whereas the splitting of the $CD_3$ side chain of Ala residue was reduced to 39 kHz, as a result of fast three-fold rotation. Accordingly, it is plausible that the hydroxyl groups of the side chains in Ser residue might participate in the formation of the intrachain hydrogen bonds which are effective in stabilization of particular forms irrespective of differences in backbone conformation in the cocoon, silk I, and silk II forms. Then, it is expected that the present approach might be useful as a new means to gain insight into molecular organization of fibroin samples as related to several kinds of physical properties of fibroins.

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24) It is already known that there is a possibility of conversion of fed deuterated serine or alanine to other amino acids such as glycine, alanine or serine within a body of silkworm (Ref. 25—29). It appears that the extent of amino-acid conversion is not larger than 10% for alanine and glycine, as inferred from our previous experience on biosynthetic experiment utilizing [1-$^{13}$C]-labeled amino acids (Ref. 30). However, it is well known that serine is the most important source of glycine biosynthesis in mammalian system (Ref. 31). In a similar manner, Muramatsu et al. showed that radioactivity of glycine rose to nearly 60% of that of [1-$^{14}$C]-serine by 20 min after injection to *B. mori* silkworms (Ref. 29). They showed that radioactivity from [1-$^{14}$C]-serine is retained only in the carboxyl carbons. This finding is consistent with that the conversion of serine to glycine in insect is catalyzed by serine hydroxymethylase in the presence of tetrahydrofolic acid (Ref. 32). In this connection, deuterium atoms from deuterated hydroxymethyl group in the side chain of serine are thus not any more retained in converted glycine by this reaction. By contrast, conversion of serine to alanine was very low (2%) and no other amino acids were synthesized from serine (Refs. 25 and 29). Accordingly, we neglected, in this paper, a plausible contribution from any converted amino acid residues. Further, it is emphasized that the present conclusion as to the orientation of side-chains in serine to be described later remains unchanged, even if deuterium atoms are incorporated into glycine to some extent by an unidentified mechanism from the deuterated serine (see text).

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