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# A HIGH RESOLUTION $^{13}C$ NMR STUDY OF SILK FIBROIN IN SOLID STATE BY THE CROSS POLARIZATION-MAGIC ANGLE SPINNING METHOD: CONFORMATIONAL CHARACTERIZATION UTILIZING CONFORMATION-DEPENDENT $^{13}C$ CHEMICAL SHIFTS

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Solid state high resolution $^{13}C$ NMR spectra of the silk fibroins from Bombyx mori (B. mori) and Philosamia cynthia ricini (P. c. ricini) together with those of appropriate model peptides were recorded by cross polarization-magic angle spinning method. We found by examining $^{13}C$ chemical shifts that anti-parallel β-sheet and α-helix forms were straightforwardly ascribed to the fibroins from B. mori and P. c. ricini, respectively.

It is well recognized that $^{13}C$ chemical shifts of an amino acid residue in peptides and proteins adopting unfolded conformation are effectively independent of all neighbors except proline residue.[1] Thus, any displacement of the $^{13}C$ chemical shifts, upon folding, from the values in unfolded conformation may be used as convenient probes of secondary folding, if existence of a sizable amount of the conformation-dependent $^{13}C$ shift is established. For this purpose, we showed that $^{13}C$ chemical shifts of solid homopolypeptides which exhibit conformation-dependent changes as large as 2-7 ppm between the α-helix and β-sheet forms may serve excellent reference data.[2,3] On the basis of such data, we previously revealed the folding behavior of calf thymus histone H1 in aqueous and 2-chloroethanol solution.[4] Thus, it is obvious that conformational characterization of peptides[5,6] and proteins is now feasible by means of this novel approach using the conformation-dependent $^{13}C$ chemical shifts in both solid and solution states.

Here we applied this approach to reveal the conformational feature of silk proteins.[7,8] Silk protein, mainly silk fibroin, is readily available in a pure form and the conformation in solid state has been extensively studied by X-ray diffraction[9] and infrared spectroscopy.[10] α-Helix, loose helix (silk I), anti-parallel β-sheet (silk II) and random coil conformations have been proposed depending on the species of silkworms and conditions of the sample preparation. In this communication, we demonstrate that the conformational features of fibroins are readily visualized by comparing $^{13}C$ chemical shifts with those of reference compounds.

B. mori and P. c. ricini were reared in our laboratory. The crystalline fraction of B. mori fibroin was precipitated from an aqueous solution treated with chymotrypsin. The silk fibroin film of P. c. ricini was obtained from the posterior divisions of the silk gland in the silkworm and dried at room temperature after the sericin was removed. The film and cocoon were cut into small pieces for NMR measurements. Polyglycine ($(Gly)_n$ I; β-sheet) was purchased from Sigma Chemical Company (Mw. 6000, lot 104c-0256) and checked by infrared spectroscopy.[11] $(Ala)_n$s, taking β-sheet

and α-helix forms, were described elsewhere.[3] BOC-(GSGAGA)-OH [BOC-(Gly-Ser-Gly-Ala-Gly-Ala)-OH] and BOC-$(GSGAGA)_2$-OBzl were synthesized by the stepwise elongation and fragment condensation methods. Single contact $^{13}C$ cross polarization-magic angle spinning (CP-MAS) NMR spectra[12-14] were recorded at 75.46 MHz by a Bruker CXP-300 spectrometer equipped with a CP-MAS accessory. Samples were placed in an Andrew-Beams type rotor machined from per-deuterated poly(methyl methacrylate) and spun as fast as 3-4 kHz. A contact time of 800 μs was chosen not as optimal but to avoid a build-up of signals from the rotor and probe assembly. Repetition time was 2 s. Spectral width and data points were 30 kHz and 4 K, respectively. Chemical shifts were calibrated indirectly through external benzene and expressed by ppm from tetramethylsilane. Spectra were usually accumulated 1000-2000 times.

Figure 1 shows the $^{13}C$ CP-MAS NMR spectra of cocoon from B. mori and of related polypeptides. These $^{13}C$ signals were assigned straightforwardly to the individual amino acid residues (Gly, Ala and Ser) indicated on the top trace, as referred to the peak-positions from the related peptides (Figs. 1C-F).[15] In contrast to previous $^{13}C$ CP-MAS NMR spectra of various proteins,[16-19] spectral resolution here seems to be greatly improved mainly because the spectra are recorded at the higher frequency 75.46 MHz. Under such conditions, line-broadening by $^{14}N$-$^{13}C$ dipolar coupling[20] is almost completely removed. Accordingly, $^{13}C$ peaks of carbonyl and $C_\alpha$ carbons are well resolved and can be used as probes of conformational characterization. Interestingly, peak-positions of Ala and Gly residues, including the carbonyl group, are in good agreement with those of $(Ala)_n$ and $(Gly)_n$, taking β-sheet[3,11] conformation within the experimental error (±1.4ppm)(Table 1). However, $^{13}C$ peaks of Ser residue from Ac-Ser-NHMe deviate slightly from those found in the cocoon. It appears that this compound is not sufficient as a reference of β-sheet form because of the end group at the C-terminal.

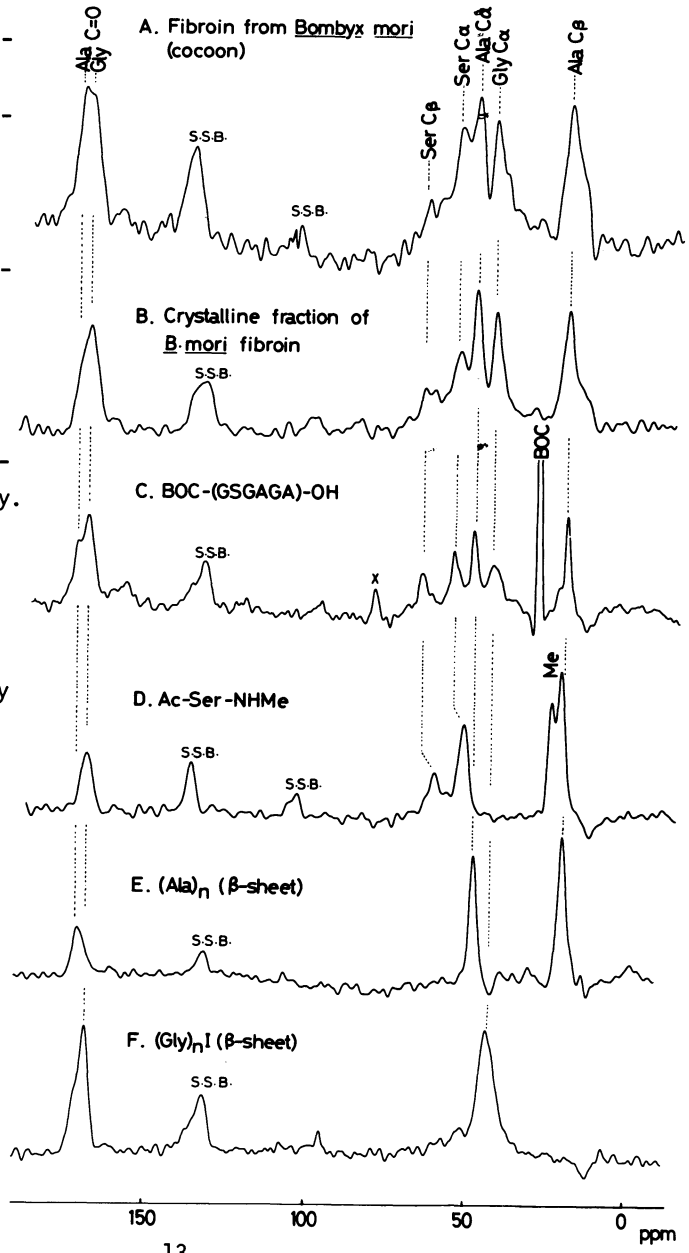


Figure 1. $^{13}C$ CP-MAS NMR spectra of cocoon and crystalline fraction of silk fibroin from B. mori and related model peptides. S.S.B. stands for the spinning side-band.

As an alternative reference, we examined $^{13}C$ CP-MAS NMR spectra of sequential peptides BOC-(GSGAGA)-OH (Fig. 1C) and BOC-$(GSGAGA)_2$-OBzl (spectrum not shown, but very similar to Fig. 1C) whose composition and sequence are similar to those of the crystalline fraction (Cp) of B. mori fibroin: Gly-Ala-Gly-Ala-Gly-Ser-Gly-Ala-Ala-Gly-[Ser-Gly-$(Ala-Gly)_n$]-Tyr, where n is usually 2.[7,8,21] As

Table 1. $^{13}C$ chemical shifts of silk fibroins as compared with those of model peptides (± 0.7 ppm, ppm from TMS)

| | Fibroins | | | | Model peptides | | | |
|---|---|---|---|---|---|---|---|---|
| | B. mori | | P. c. ricini | | β-sheet | | | α-helix |
| | cocoon | crystalline fraction(Cp) | cocoon | film | $(Ala)_n$[a] | $(Gly)_n$ | BOC-(GSGAGA)-OH | $(Ala)_n$[a] |
| Ala $C_\alpha$ | 49.7 | 48.7 | 48.7 | 52.4 | 48.2 | | 48.7 (48.9)[b] | 52.4 |
| $C_\beta$ | 20.2 | 18.9 | 19.8 | 15.4 | 19.9 | | 19.0 (19.6)[b] | 14.9 |
| C=O | 171.7 | 170.7 | 171.9 | 176.3 | 171.8 | | 171.2(172.1)[b] | 176.4 |
| Gly $C_\alpha$ | 43.9 | 43.0 | 43.9 | 43.9 | | 43.2 | 43.4 (43.5)[b] | |
| C=O | 169.4 | 169.4 | | | | 168.4 | 169.4(170.3)[b] | |
| Ser $C_\alpha$ | 55.0 | 53.9 | 54.8 | | | | 55.0 (54.9)[b] | (52.2)[c] |
| $C_\beta$ | 61.7 ~ 64.3 | 63.2 | | | | | 64.9 (64.5)[b] | (61.5)[c] |
| C=O | | | | | | | | (171.0)[c] |

[a] $^{13}C$ chemical shifts taken from ref. 3. [b] $^{13}C$ chemical shifts of BOC-(Gly-Ser-Gly-Ala-Gly-Ala)$_2$--OBzl. [c] $^{13}C$ chemical shifts of Ac-Ser-NHMe.

expected, peak-positions and intensities of these hexa- and dodeca-peptides are very close to those of the Cp fraction of B. mori fibroin (Figs. 1B and C; Table 1) and the fibroin in the cocoon (Fig. 1A). In addition, the $^{13}C$ shifts of the Ala and Gly residues of these hexa- and dodeca-peptides are in good agreement with those of $(Ala)_n$ and $(Gly)_n$, taking β-sheet form. However, there appears a small but definite difference in the linewidth of the Ser $C_\beta$ carbon. The linewidth of the Cp and cocoon is very broad compared with that of the reference peptide. This observation seems to be consistent with the previous X-ray diffraction data.[22,23] Namely, the a and b values of the Cp fraction and $(GSGAGA)_n$ are very similar but the c value of the former is slightly larger than that of the latter.[22] It appears that the larger value of the c-axis (inter-sheet spacing) in the Cp corresponds with the broadening observed for the Ser $C_\beta$ carbon of the Cp fraction. It is likely that such change in the linewidth is caused by superposition of signals arising from slightly different conformers as viewed from the Ser residue.

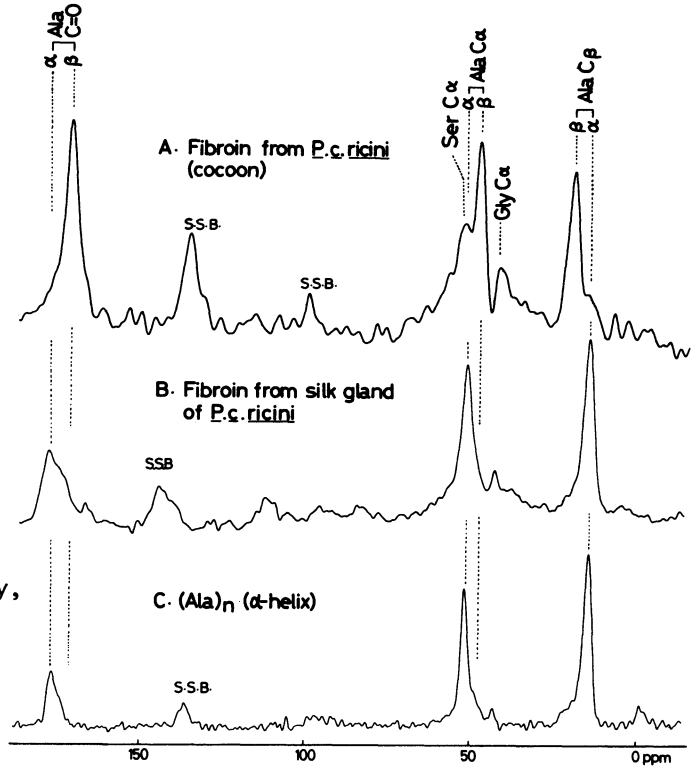


Figure 2 $^{13}C$ CP-MAS NMR spectra of cocoon (A), film (B) samples of silk fibroin from P. c. ricini and (C) $(Ala)_n$ taking α-helix form.

In a similar manner, we recorded $^{13}C$ CP-MAS NMR spectra of two kinds of preparations of silk fibroin from P. c. ricini (Fig. 2). Obviously, conformation of the cocoon sample is readily identified as the β-sheet form on the basis of the foregoing argument. On the contrary, note that three well resolved signals from the fibroin directly taken from the silk gland are ascribed to the $C_\alpha$, $C_\beta$ and carbonyl carbons of Ala residue (48.4% of the total amino acid residues) taking α-helix conformation (Fig. 2B), as compared with those of $(Ala)_n$ in the α-helix form (Fig. 2C and Table 1). Consistent with this finding, Asakura and Watanabe[24] found, on the basis of $^{13}C$ NMR spectra of a live mature larva of P. c. ricini, that the silk fibroin stored in the silk gland involves a significant amount of α-helix. The extent of Ala residue which takes part in the α-helix was determined as ca. 70%.[24] The presence of the α-helix conformation was previously confirmed by X-ray diffraction for the fibroin obtained directly from the silk gland of Antheraea pernyi[25] whose amino acid composition is very similar to that of P. c. ricini. However, the Gly, Ser and Tyr residues (33.2, 5.5 and 4.5%, respectively)[8] appear to give featureless broad signals underneath the strong peaks of Ala.

In conclusion, this new approach utilizing the conformation-dependent $^{13}C$ chemical shift is a potentially useful tool in analyzing the conformational features of silk fibroins as well as other proteins in solid state.

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