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High-Resolution ^{13}C NMR Study of Silk Fibroin in the Solid State by the Cross-Polarization–Magic Angle Spinning Method. Conformational Characterization of Silk I and Silk II Type Forms of *Bombyx mori* Fibroin by the Conformation-Dependent ^{13}C Chemical Shifts

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ABSTRACT: High-resolution ^{13}C NMR (75.46 MHz) spectra of silk fibroins in the solid state were recorded by the cross-polarization–magic angle spinning method with emphasis on revealing conformational features of the dimorphic structures, silk I and II, of *Bombyx mori* fibroin prepared under different conditions. It was found that the ^{13}C chemical shifts of Gly, Ala, and Ser residues of silk II samples from *B. mori* fibroin by different preparations and of cocoon samples from several silkworms gave identical values with those of corresponding model polypeptides having the β-sheet conformation. Thus, identification of the silk II type form is easily performed by examining their ^{13}C chemical shift values. The ^{13}C chemical shifts of samples having the silk I form are significantly displaced from those of the silk II form, and can be used for diagnostic purposes. As expected, the ^{13}C chemical shifts of Ala and Gly residues of the silk I samples were identical with those of $(Ala\text{-}Gly)_n$II. However, we found that none of the ^{13}C chemical shifts predicted from the crankshaft model [Lotz, B.; Keith, H. D. *J. Mol. Biol.* **1971**, *61*, 201–215] in which Ala and Gly residues are close to the β-sheet and α-helix conformations, respectively, was in agreement with the ^{13}C chemical shifts of $(Ala\text{-}Gly)_n$II and silk I type form. Instead, we found that predicted ^{13}C chemical shifts from the loose helix proposed by Konishi and Kurokawa [Konishi, T.; Kurokawa, M. *Sen'i Gakkaishi* **1968**, *24*, 550–554] on the basis of the calculated ^{13}C contour map of chemical shifts for Ala residue are in good agreement with the displacement of the ^{13}C chemical shifts.

Introduction

In our previous papers, we have demonstrated that the ^{13}C chemical shifts of a number of polypeptides and proteins in the solid state as determined by the cross-polarization–magic angle spinning (CP–MAS) method are significantly displaced depending on their particular conformations such as the right-handed α-helix (α_R-helix), left-handed α-helix, (α_L-helix), β-sheet, ω-helix, 3_1-helix, and collagenlike triple helix.[1-6] Similar observation has been made independently by Kricheldorf and co-workers.[7-11] It is clear that the ^{13}C chemical shifts of amino acid residues are not mainly determined by the sequence but by the local conformation as defined by the torsion angles (ϕ and ψ) as well as the manner of hydrogen bonding.[1-6] This view was supported by our theoretical calculation of the contour map of ^{13}C chemical shifts utilizing FPT-INDO theory.[12] This finding permits one to use the conformation-dependent ^{13}C chemical shifts as an intrinsic probe to examine conformational behavior of polypeptides and proteins in the solid[1-6] and solution[13] states as viewed from the individual amino acid residues under consideration. In particular, we previously showed that the antiparallel β-sheet (silk II) of *Bombyx mori* fibroin and the α-helix form of *Philosamia cynthia ricini* fibroin in the solid state are well characterized by the conformation-dependent ^{13}C shifts of Gly, Ala, and Ser residues with reference to those of $(Gly)_n$I, $(Ala)_n$, and sequential model peptides.[3] In a similar manner, we demonstrated that the conformation of collagen fibrils can be analyzed by the conformation-dependent ^{13}C chemical shifts, referred to those of the appropriate model polypeptides, form II of $(Gly)_n$, $(Pro)_n$, and $(Ala\text{-}Gly\text{-}Gly)_n$ and polytripeptides of $(Pro\text{-}Ala\text{-}Gly)_n$ and $(Pro\text{-}Gly\text{-}Pro)_n$.[6]

Hence, it is now feasible to carry out conformational characterization of a number of other proteins by means of the CP–MAS NMR method, if the ^{13}C chemical shifts of suitable reference polypeptides are available. The major advantage in recording the ^{13}C NMR spectra in the solid state is that the polymorphic structure of polypeptides and proteins can be studied without any problems from the disruption of their conformations by dissolving in solvents. Furthermore, the ^{13}C chemical shifts in the solid state are in many instances free from conformational fluctuation as

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Table I
Characteristics of Fibroin Samples Prepared under Different Conditions

sample no.	starting material	treatment	conformation[a]
1	regenerated fibroin soln	precipitation by methanol	II
2	degummed silk	film from CF_3COOH soln	II
3	liquid silk	film immersed in methanol	II
4	C_p fraction[b]	dialysis of 9 M LiBr soln followed by lyophilization	II
5	A_p fraction[c]	same as above	II
6	regenerated fibroin soln	air-drying at 27 °C	I
7	liquid silk	standing in a refrigerator at 5 °C for 2 days	I
8	regenerated fibroin soln	air-drying at 50 °C	I
9	C_p fraction[b]	dialysis of 9 M LiBr soln for 4 h	I
10	A_p fraction[c]	same as above for 4 days	I
11	degummed [*Gly*-1-^{13}C]fibroin	same as above	I

[a] I stands for silk I type and II for silk II type. [b] Precipitate by digestion of fibroin with chymotrypsin. [c] Precipitate of fibroin with alkaline phosphatase.

encountered in the solution state and can be unambiguously related to the conformation as determined by the X-ray diffraction and other spectroscopic techniques.

Here we extend our approach to elucidate further conformational features of silk fibroins. It is well established that silk fibroins take several kinds of conformations depending on the species of silkworms and conditions of sample preparation.[14-17] In particular, it is known that fibroin from *B. mori* adopts dimorphs, silk II and silk I. It appears that the less stable silk I[18-23] is not well characterized as compared with silk II because of the experimental difficulty that an attempt to obtain more highly crystalline material easily leads to conversion to silk II. Lotz and Keith[24] proposed the crankshaft model in which Ala and Gly residues are close to the β-sheet and α-helix forms, respectively. On the other hand, Konishi and Kurokawa proposed the loose fourfold helical conformation whose residual translation is 2.27 Å.[22] As a novel tool for conformational characterization, it is emphasized that the ^{13}C NMR approach is a suitable means for studying samples with poor crystallinity for which the X-ray diffraction method is not practical.[25,26]

In this paper, we analyze the conformations of silk I and II samples of *B. mori* fibroin on the basis of the conformation-dependent ^{13}C chemical shifts, referred to those of $(Ala\text{-}Gly)_n$ and $(Ala\text{-}Ala\text{-}Gly)_n$. We find that the ^{13}C chemical shifts of Gly and Ala residues of silk I samples are identical with those of $(Ala\text{-}Gly)_n$II, in conformity with previous findings.[24] However, the crankshaft model of Lotz and Keith[24] is not acceptable as a conformational model of $(Ala\text{-}Gly)_n$II and silk I forms in light of the conformation-dependent ^{13}C chemical shifts of Ala and Gly residues so far accumulated. Instead, we found that the loose helix proposed by Konishi and Kurokawa[22] can account for displacements of the ^{13}C chemical shifts.

Experimental Section

Cocoons and Regenerated Silk. Cocoons of *B. mori* and *P. c. ricini* were obtained in our laboratory. *Antheraea yamamai* and *Antheraea mylitta* cocoons were the generous gift from Dr. M. Hagiwara of Tokyo University of Agriculture and Technology Koganei, Tokyo. [*Gly*-1-^{13}C]Fibroin cocoon (14% ^{13}C enrichment) from *B. mori* was prepared by feeding [1-^{13}C]Gly (95% enrichment, Shoko Tsusho Co., Tokyo) in addition to artificial diet to silkworm larvae of the fifth instar for an entire day. The cocoons were cut into small pieces for NMR measurements.

Raw silk or the cocoon of *B. mori* was degummed twice with 0.5% Marseilles soap (widely used for the degumming process in the silk industry) solution at 100 °C for 0.5 h, then washed with distilled water. The silk was dissolved in 9 M LiBr aqueous solution at 40 °C. The 6% regenerated fibroin solution was prepared by dialysis of the 9 M LiBr aqueous solution against distilled water, followed by centrifugation at 10000 rpm and concentrated gently with an electric fan. Fibroin samples 1–11 were obtained from degummed silk or liquid silk extracted from the silk gland of *B. mori* with the following treatments as summarized in Table I.

Silk II Type Samples. Sample 1 was precipitated by adding methanol to the 6% regenerated fibroin solution (1:1, v/v) and the precipitate was dried in vacuo at 27 °C. The degummed silk was dissolved in trifluoroacetic acid and dried with an electric fan (sample 2). Liquid silk from *B. mori* was immersed in methanol and dried with an electric fan (sample 3). Sample 4 was obtained from the precipitate when the crystalline fraction (C_p fraction) of *B. mori* fibroin obtained from chymotrypsin treatment was dissolved in 9 M LiBr solution and dialyzed against distilled water. Sample 5 was obtained from the precipitate when fibroin from *B. mori* was digested by alkaline phosphatase (A_p fraction). This sample was kindly provided by Dr. C. Hirao of Tokyo University of Agriculture and Technology. Samples 1–5 were considered as silk II type structure from the infrared spectra (amide I, 1630; amide II, 1530; amide V, 700 cm^{-1}).[23]

Silk I Type Samples. Sample 6 was prepared by air-drying of 6% regenerated fibroin solution cast over a film of poly(vinylidene chloride) at 27 °C. Sample 7 was prepared from liquid silk. After the middle silk gland from *B. mori* was allowed to stand in a refrigerator at 5 °C for 2 days, sericin was removed. The small pieces were used for the observation. Sample 8 was prepared by air-drying of 6% regenerated fibroin solution at 50 °C. Sample 9 was prepared by dialyzing the C_p fraction dissolved in 9 M LiBr solution against distilled water containing LiBr which was diluted progressively with water for 4 h. Prolonged dialysis caused the conversion to silk II form as viewed from the ^{13}C CP-MAS NMR spectra. Sample 10 was prepared with a similar manner to that of sample 9 but the A_p fraction was used together with extensive dialysis for 4 days. Sample 11 was prepared from regenerated [*Gly*-1-^{13}C]fibroin with a similar manner to that of preparation of sample 10.

Synthesis of Sequential Polypeptides. $(Ala\text{-}Gly)_n$ and $(Ala\text{-}Ala\text{-}Gly)_n$ were synthesized by the self-condensation reaction of di- and tripeptide *N*-hydroxysuccinimide (ONSu) esters, respectively, according to the procedure described in our previous paper.[6] The syntheses of the di- and tripeptide active esters were carried out by stepwise elongation using the dicyclohexylcarbodiimide (DCC)–HONSu method.[27-29] The synthetic route is shown in Scheme I.

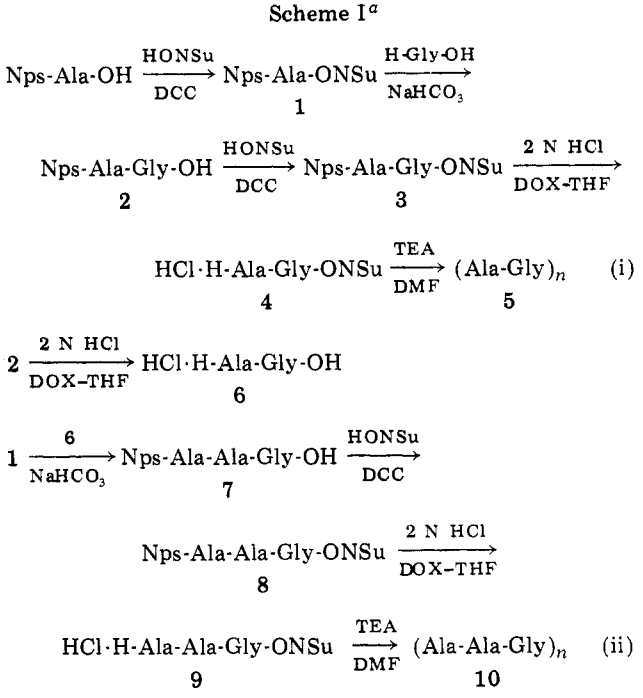
$(Ala\text{-}Gly)_n$. *o*-Nitrophenylsulfenyl-L-alanine *N*-hyroxysuccinimide ester (**1**) was prepared by a reaction of Nps-Ala-OH with HONSu in THF using DCC as condensing agent. The active ester was coupled with glycine in THF/aqueous $NaHCO_3$ system[6,30,31] to give Nps-Ala-Gly-OH (**2**). After being treated by the conventional procedure,[30] the solid product (**2**) was isolated and recrystallized and was converted to dipeptide–ONSu ester **3** in a similar manner to that described. The Nps-group of **3** was cleaved by treatment with 2 N HCl in DOX–THF (1:1) to give HCl·H-Ala-Gly-ONSu (**4**). The resulting dipeptide active ester **4** was purified as far as possible by recrystallization and polymerized in absolute DMF in the presence of TEA (1.2 equiv) to yield the sequential polypeptide $(Ala\text{-}Gly)_n$. The reaction mixture was poured into diethyl ether and the precipitate was collected by filtration, washed with hot methanol, and dried in vacuo. The conversion of $(Ala\text{-}Gly)_n$I thus obtained to form II was performed

Table II
^{13}C Chemical Shifts of Silk II Samples As Compared with Those of Model Polypeptides (±0.5 ppm, from Me_4Si)

	silk II					model polypeptides					
	sample 1	sample 2	sample 3	sample 4	sample 5	$(Ala)_n$[a]	$(Gly)_nI$[b]	$(Ser)_n$	(Ala-Gly)$_n$I	(Ala-Ala-Gly)$_n$	(Ala-Gly-Gly)$_n$I[c]
Ala											
C_α	48.3	48.9	49.1	48.7	49.0	48.2			48.5	49.1	48.7
C_β	19.0	20.0	18.6	19.2	19.2	19.9			20.0	20.4	21.6
C=O	171.7	172.5	171.9	170.7	170.7	171.8			171.9	171.1	171.2
Gly											
C_α	42.5	43.5	43.3	42.9	43.3		43.2		43.3	43.3	43.0
C=O	170.5	169.8	170.5	169.4	168.8		168.4		169.2	168.2	168.6
Ser											
C_α	54.0	53.1	54.0	54.8	54.0			54.4			
C_β	63.1	64.1	59.6	63.9	63.5			63.9			
C=O								171.2			

[a] Data taken from ref 2. [b] Data taken from ref 3. [c] Data taken from ref 6.

Scheme I[a]



[a] Nps, *o*-nitrophenylsulfenyl; HONSu, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; THF, tetrahydrofuran; DOX, 1,4-dioxane; DMF, *N*,*N*-dimethylformamide; TEA, triethylamine.

by dialyzing the polymer in 60% aqueous LiBr against similar solutions of LiBr which were diluted progressively with distilled water.[32] The dialyzed solution was then lyophilized after the small amounts of precipitates were removed. We found that $(Ala-Gly)_n$ from the precipitate gave similar ^{13}C chemical shifts with those of lyophilized powder from the solution.

(Ala-Ala-Gly)$_n$. Nps-Ala-Ala-Gly-OH (**7**) was prepared by reaction of Nps-Ala-ONSu (**1**) with **6** in THF/aqueous $NaHCO_3$ system,[30] then the Nps group of **7** was cleaved by treatment with 2 N HCl in DOX-THF (1:1) to give **9**. The tripeptide active ester **9** was purified by recrystallization from 2-propanol and diethyl ether and polymerized in DMF in the presence of TEA to yield $(Ala-Ala-Gly)_n$. The reaction mixture was treated with hot methanol and dried in vacuo.

$(Ser)_n$. Polyserine was purchased from Sigma Chemical Co. (degree of polymerization 5000, lot no. P5887) and used without purification.

^{13}C CP-MAS NMR Spectroscopy. ^{13}C CP-MAS NMR spectra[33,34] were recorded at 75.46 MHz with a Bruker CXP-300 spectrometer equipped with a CP-MAS accessory. Samples were placed in an Andrew-Beams type rotor machined from perdeuterated 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 buildup of signals from residual carbon signals from the rotor and probe assembly. In some instances, these undesirable signals were subtracted digitally from phase-corrected spectra with reference to those of the rotor without sample. Repetition time was 2 s, and spectral width and data points were 30 kHz and 4K points, respectively. Spectra were usually accumulated 2000–3000 times except for samples with ^{13}C enrichment to achieve reasonable signal to noise ratio. Chemical shifts were calibrated through external benzene and converted to the values from tetramethylsilane (Me_4Si).

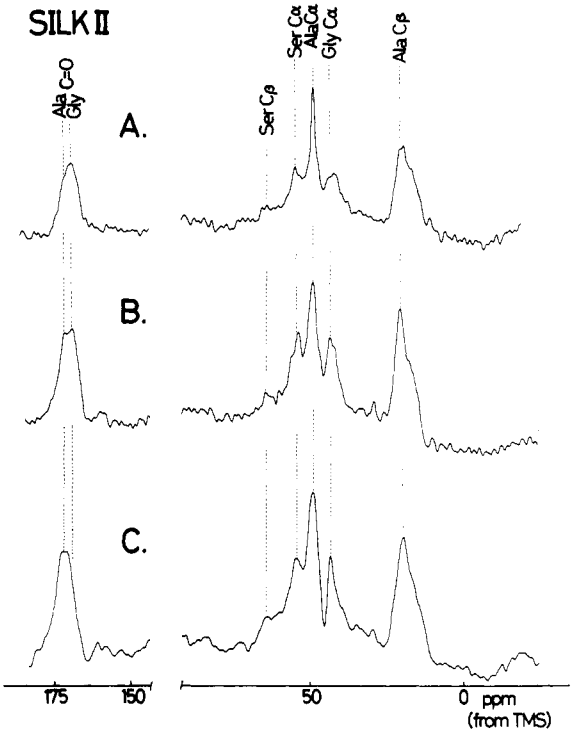


Figure 1. ^{13}C CP-MAS NMR (75.46 MHz) spectra of silk II type fibroins prepared under different conditions: (A) sample 1; (B) sample 2; (C) sample 3.

Results

Silk II Type Samples. Figure 1 shows 75.46-MHz ^{13}C CP-MAS NMR spectra of silk II samples from *B. mori* fibroin prepared under different conditions. NMR signals arising from the major amino acid residues are well resolved and easily assigned to peaks of Gly, Ala, and Ser residues taking the antiparallel β-sheet conformation.[3,7] As an alternative reference of Ser residue taking the β-sheet conformation,[10] we also recorded the ^{13}C CP-MAS NMR spectrum of $(Ser)_n$ as shown in Figure 2. The ^{13}C chemical shifts of Gly, Ala, and Ser residues of silk II samples are summarized in Table II together with the values of $(Ser)_n$

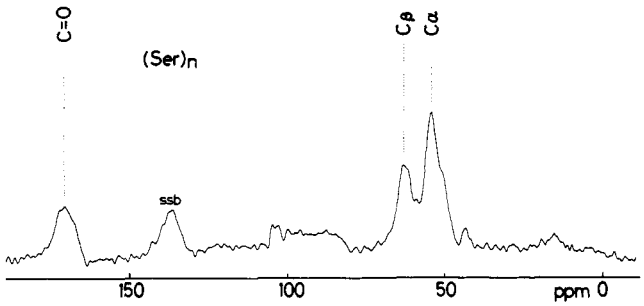


Figure 2. ^{13}C CP–MAS NMR (75.46 MHz) spectrum of $(Ser)_n$ in the solid state.

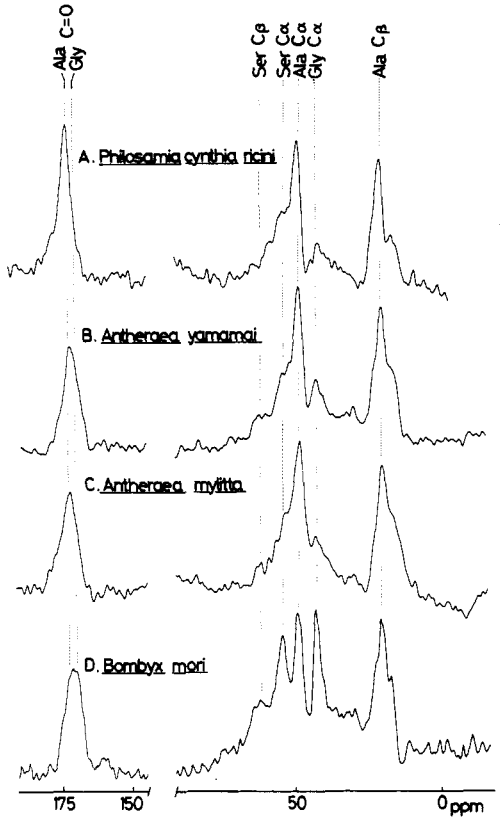


Figure 3. ^{13}C CP–MAS NMR (75.46 MHz) spectra of cocoons from various silkworms: (A) *P. c. ricini*; (B) *A. yamamai*; (C) *A. mylitta*; (D) *B. mori*.

and model polypeptides previously described.[2,3] Obviously, characteristic peaks of the β-sheet forms of the cocoon samples from various silkworms are well resolved and identical with those of Table II within the experimental error (Figure 3). A small peak is evident at the right-hand side of the Ala C_β signal, which was previously assigned to the α-helix form by Kricheldorf et al.[7] However, this peak is more likely to have originated from silk I type form, as will be discussed later. We also recorded the ^{13}C CP–MAS NMR spectrum of [*Gly*-1-^{13}C]fibroin cocoon from *B. mori* (Figure 4B). The ^{13}C chemical shift of the carbonyl carbon (14% enrichment) is exactly at the same position of the highermost peak of the carbonyl region (168.6 ppm) which was previously ascribed to Gly C=O signal.[2]

Silk I Type Samples. Figure 5E illustrates the ^{13}C CP–MAS NMR spectrum of silk I type samples from the C_p fraction (sample 9) prepared by careful dialysis of LiBr solution against a similar solution containing LiBr which was progressively diluted, followed by lyophilization of the solution after the precipitate was removed by centrifugation. It is noteworthy that the ^{13}C signals of Gly, Ala, and Ser residues are also well resolved. A part of the carbonyl signal is significantly displaced downfield as shown by peak a. In addition, the C_α signals of Ala and Ser residues of silk I (peaks b) are displaced downfield by about 2 ppm as compared with those of silk II (peaks a), whereas the

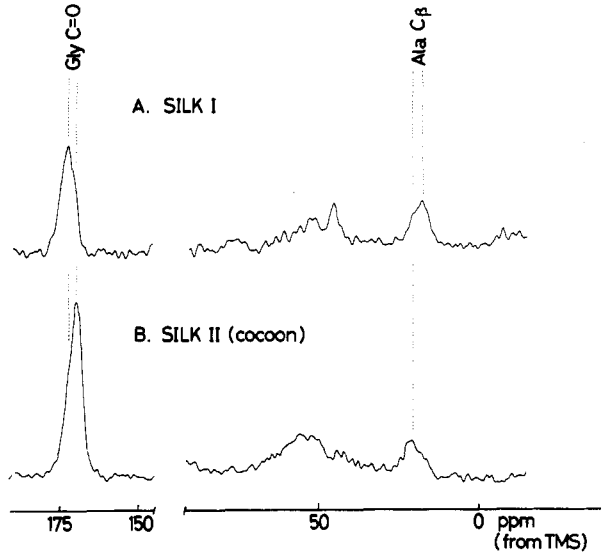


Figure 4. ^{13}C CP–MAS NMR (75.46 MHz) spectra of [*Gly*-1-^{13}C]fibroin from *B. mori*: (A) silk I type (sample 11); (B) silk II (cocoon). Number of transients = 500.

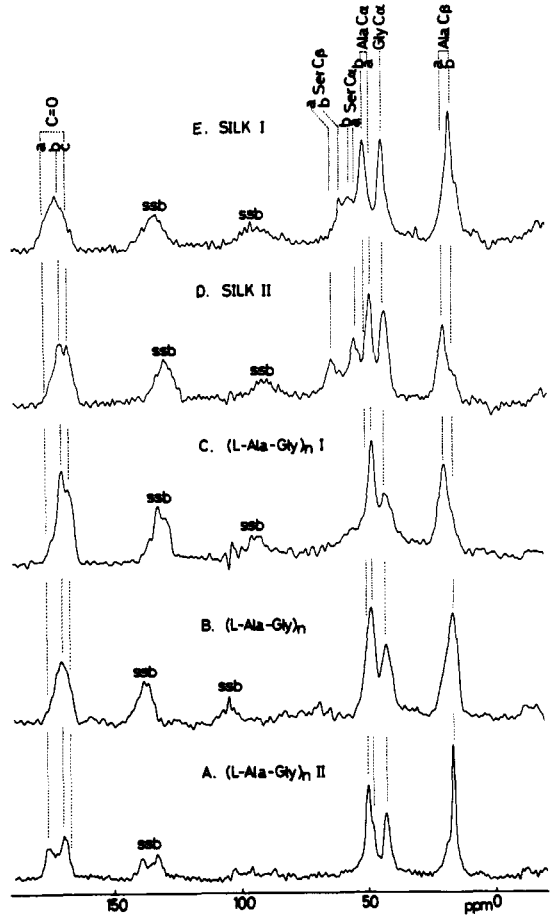


Figure 5. ^{13}C CP–MAS NMR (75.46 MHz) spectra of the crystalline (C_p) fraction from *B. mori* fibroin and $(Ala\text{-}Gly)_n$ in the solid state: (A) $(Ala\text{-}Gly)_n$II; (B) $(Ala\text{-}Gly)_n$ treated with dichloroacetic acid (DCA); (C) $(Ala\text{-}Gly)_n$I; (D) silk II from the C_p fraction (sample 4); (E) silk I from the C_p fraction (sample 9).

Table III
^{13}C Chemical Shifts of Silk I Samples Prepared under Different Conditions (±0.5 ppm, from Me_4Si)

	silk I					model polypeptides	
	sample 6	sample 7	sample 8	sample 9	sample 10	$(Ala\text{-}Gly)_n$II	$(Ala\text{-}Gly)_n$ treated with DCA
Ala							
C_α	49.7	50.1	49.1	50.5	50.3	50.5	49.3
C_β	15.8	15.7	15.8	16.4	16.6	16.6	16.6
C=O	172.5	171.5	171.9	176.3, 174.1[a]	177.5, 175.7[a]	177.1	173.7
Gly							
C_α	43.0	43.0	43.3	43.3	43.9	43.7	43.3
C=O				172.7	171.1	171.9	171.9
Ser							
C_α	56.0	54.4	54.0	56.8	55.8		
C_β	59.0	59.2	61.0	59.8	61.5		
C=O							

[a] A part of this peak (peak b of C=O group in Figure 5) could be ascribed to Ser C=O.

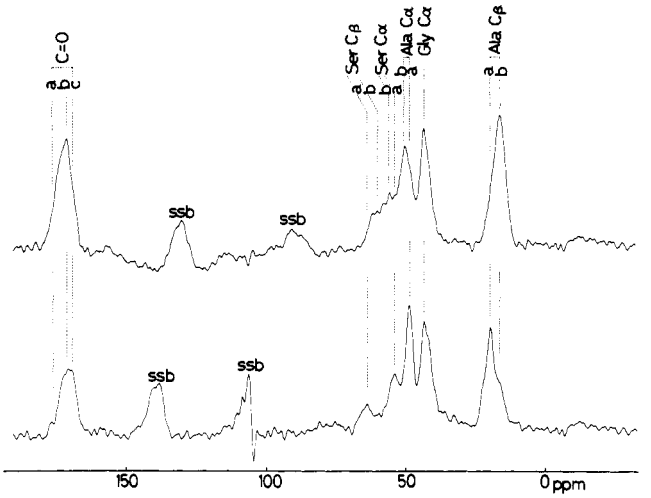


Figure 6. ^{13}C CP-MAS NMR (75.46 MHz) spectra of crystalline (A_p) fraction: (bottom) sample 5; (top) sample 10.

C_β signals are displaced upfield by 3.4–4 ppm (Table III). A similar result was obtained from the sample prepared from the A_p fraction (Figure 6). It is seen in the top trace of Figure 6 that a small amount of silk II form is present as manifested by peaks a of the Ser C_α and C_β carbons. Naturally, no such peaks are seen for silk I type form prepared from the C_p fraction (Figure 5E). The presence of the silk II type form in the former might be caused by the prolonged dialysis, because a long period of dialysis tends to convert the silk I sample to silk II form. It is clear from Figure 4A that the ^{13}C chemical shifts of the Gly carbonyl carbon of silk I type conformation (sample 11) resonate at 171.1 ppm, which is displaced downfield by 2.5 ppm as compared with that of silk II in the cocoon.

In contrast, we found that the ^{13}C NMR spectra of samples 6–8 gave much broadened ^{13}C signals as compared with those of samples 9 and 10 (Figure 7). Nevertheless, the characteristic displacements of the ^{13}C peaks of Ala and Ser residues for silk I type form, as mentioned above, are clearly seen, although displacements of the carbonyl signals are less pronounced (Table III). It appears that such line broadening arises partly from the superposition of peaks from silk II on those of silk I type form, as encountered for sample 10 (A_p fraction). However, the major conformation present in samples 6–8 is silk I type in view of the present ^{13}C NMR data.

$(Ala\text{-}Gly)_n$. Parts C and A of Figure 5 illustrate the ^{13}C CP-MAS NMR spectra of forms I and II of $(Ala\text{-}Gly)_n$[24] as a model of the silk II and I, respectively. We also included the ^{13}C CP-MAS NMR spectrum of $(Ala\text{-}Gly)_n$ treated with dichloroacetic acid.[35] This form is not an

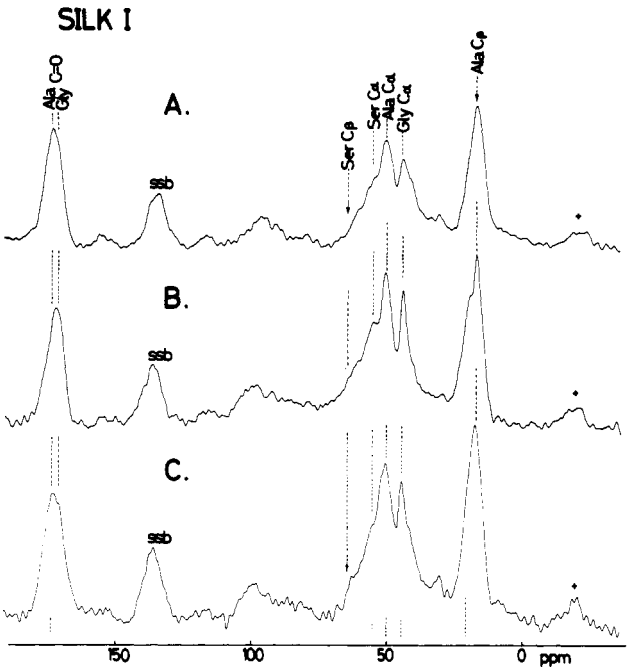


Figure 7. ^{13}C CP-MAS NMR (75.46 MHz) spectra of silk I samples from *B. mori* fibroin. (A) sample 6; (B) sample 7; (C) sample 8.

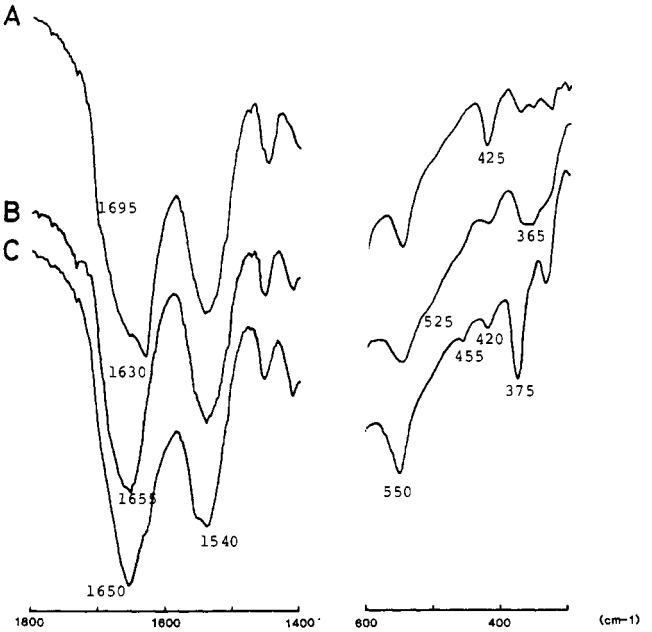


Figure 8. Infrared spectra of $(Ala\text{-}Gly)_n$: (A) form I; (B) $(Ala\text{-}Gly)_n$ treated with DCA; (C) form II.

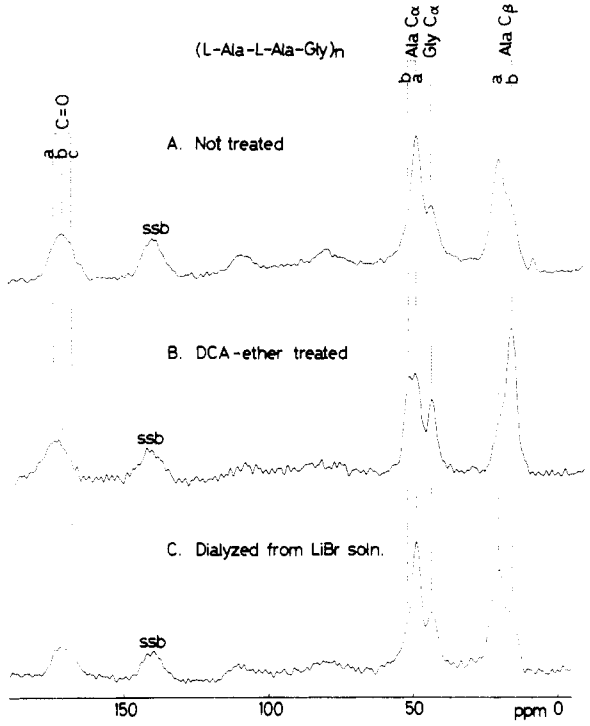


Figure 9. ^{13}C CP–MAS NMR (75.46 MHz) spectra of (Ala-Ala-Gly)$_n$ in the solid state: (A) not treated; (B) treated with dichloroacetic acid (DCA); (C) dialyzed from 9 M LiBr solution.

α-helix as viewed from the conformation-dependent ^{13}C chemical shifts of Ala residue (C_α, 49.9; C_β, 16.9; C=O, 173.7 ppm) as compared with those of the α-helix form,[2] although the far-infrared spectrum as shown in Figure 8 is α-helixlike.[35] As expected from the previous finding by Lotz and Keith,[24] the ^{13}C chemical shifts of (Ala-Gly)$_n$II are in good agreement with those of silk I samples from the crystalline fraction (sample 9 and 10) as viewed from the similarity of ^{13}C chemical shifts in Ala and Gly residues (see Table III). The largest peak of the carbonyl group (peak b) of (Ala-Gly)$_n$II may clearly be ascribed to the Gly residue, because this peak position (171.9 ppm) is in good agreement with that of Gly C=O of silk I from [*Gly*-1-^{13}C]fibroin (171.1 ppm) (see Figure 4A). Naturally, the ^{13}C chemical shifts of Ala and Gly residues of (Ala-Gly)$_n$I are in good agreement with those of the silk II sample (see Figure 5C,D and Table II). Surprisingly, the C_α signal of Gly residue is not changed by going from the silk II to silk I and also from (Ala-Gly)$_n$I to (Ala-Gly)$_n$II. Such conformational insensitivity of the Gly C_α carbon was also noted in collagen and its model polypeptides.[6]

(Ala-Ala-Gly)$_n$. Figure 9 illustrates the ^{13}C CP–MAS NMR spectra of (Ala-Ala-Gly)$_n$[36,37] as a reference sample to examine whether or not any sequential effect could cause displacement of ^{13}C chemical shifts. Clearly, the sample without any treatment (Figure 9A) and also the sample dialyzed from LiBr solution (Figure 9C) resulted in the antiparallel β-sheet form (from infrared spectra, spectra not shown) whose chemical shifts are the same as those of (Ala)$_n$[2] and (Ala-Gly-Gly)$_n$I[6] (see Table II). Therefore, no sequence effects exist in a series of polymers, (Ala)$_n$ (β-sheet),[2] (Ala-gly)$_n$I, (Ala-Gly-Gly)$_n$I, and (Ala-Ala-Gly)$_n$, to the extent that the similar β-sheet conformation is retained. As judged from the far-infrared spectrum (375 cm^{-1}, the α-helix (major peak), and 440 cm^{-1}, the β-sheet (minor peak); spectra not shown), (Ala-Ala-Gly)$_n$ treated with dichloroacetic acid assumes an α-helical conformation, although a small amount of the β-sheet form is present. Therefore, the peak marked as a of Ala C_α should be ascribed to this β-sheet form.

Discussion

Silk II Type Conformation. The ^{13}C chemical shifts of silk II samples are in good agreement among five kinds of preparations, as summarized in Table II, although relative peak intensities as well as line widths are slightly different. The amino acid composition of *B. mori* fibroin is as follows: Gly, 42.9; Ala, 30.0; Ser, 12.2; Tyr, 4.8%.[17] However, it appears that the relative intensity of Gly C_α residue is smaller than that expected from the amino acid composition. Such intensity anomaly is seen in the relative peak intensity of Ala and Gly residues of (Ala-Gly)$_n$ in Figure 5 and is caused by the insufficient contact time of the cross polarization. Generally, correct relative peak intensities of the CP–MAS NMR spectra are obtained under the condition that proton spin–lattice relaxation time in the rotating frame ($T_{1\rho}$) < contact time < cross relaxation time (T_{CH}).[38] However, we did not attempt this optimization, in this paper, because we are mainly concerned with the displacements of chemical shifts. It is interesting to note that the ^{13}C signals of the C_α and C_β carbons of Ser residue in the crystalline fraction (Figure 5D and the bottom trace of Figure 6) give much higher peak intensities than those of the other preparations. In addition, the carbonyl ^{13}C signal of the C_p (crystalline) fraction is well resolved into two peaks. As a cause of such drastic change of the peak intensities of Ser residue, the following three points should be taken into account. First and most importantly, improved crystalline packing in the crystalline fraction as compared with that of whole fibroin prevents the presence of residues whose conformations are deviated from the β-sheet form. In other word, the presence of the unique conformation in the crystalline fraction results in the considerable narrowing of peaks, because the ^{13}C chemical shifts vary with their local conformations.[1-6] Second, amino acid composition of Ser residue in the C_p fraction is slightly increased as compared with the whole fibroin. Third, minor Tyr and Val residues which do not give rise to resolved signals but contribute as background in the 50–60 ppm region are substantially decreased in the crystalline fraction. Nevertheless, the contribution of the last two seems to be too small to account for such a change of the peak intensities of the Ser residue (23%).[15,17] Thus, the major conclusion from the present study is that the conformation of the Ser residue plays an important role in better crystalline packing.

The ^{13}C chemical shifts of Gly, Ala, and Ser residues of the silk II samples are in good agreement with those of the corresponding residues of homopolypeptides, sequential polypeptides, and other peptides taking the β-sheet conformation.[2,3] Therefore, the major portion of these samples assumes a β-sheet conformation, as described in the previous paper.[3] Naturally, the present ^{13}C NMR approach is not sensitive to the change of the intersheet distance as examined by the X-ray diffraction among polypeptides and fibroins from different silkworms.[15] However, this approach is very sensitive to the local conformations of individual amino acid residues, as manifested by the substantial change of the line widths of Ser residues among different preparations. This is also true for other residues such as Gly and Ala: the ^{13}C line widths of the Ala residues of fibroin treated with methanol (sample 1) suggest better crystalline packing. Further, crystalline packing is better for the sample treated with CF_3COOH (sample 2) as judged by the line widths of the Gly and Ala residues (see Figure 1).

Silk I Type Conformation. Lotz and Keith[24] showed that the X-ray diffraction pattern of (Ala-Gly)$_n$II is iso-

Table IV
Conformation-Dependent ^{13}C Chemical Shifts of Ala Residue in Various Polypeptides (±0.5 ppm, from Me_4Si)

				collagenlike helix	
	α_R-helix[a]	α_L-helix[b]	β-sheet[a]	3_1-helix[c]	triple helix[d]
C_α	52.4	49.1	48.2	48.7	48.3
C_β	14.9	14.9	19.9	17.4	17.6
C=O	176.4	172.9	171.8	172.1	173.1

[a] $(Ala)_n$; ref 2. [b] Copolymers of D- and L-alanines; ref 2 and 12. [c] $(Ala\text{-}Gly\text{-}Gly)_n$II; ref 6. [d] $(Pro\text{-}Ala\text{-}Gly)_n$; ref 6.

Table V
Comparison of the ^{13}C Chemical Shifts of Ala C_β Signals of Various Polypeptides with the Calculated Shielding Constants for the Appropriate Torsion Angles[a]

		^{13}C shifts	
conformation	ϕ, ψ[b]	obsd	calcd[c]
α_R-helix	−48, −57	14.9	−63.0
α_L-helix	48, 57	14.9	−63.7
β-sheet	−142, 145	19.9	−65.7
3_1-helix	−80, 150	17.5	−64.7
silk I (loose helix)[d]	−25.5, 134.5	16.6	−64.0

[a] From the contour map of the ^{13}C chemical shifts of *N*-acetyl-*N*-methylalanine amide (ref 12). [b] Torsion angles in degrees. [c] Shielding constant in ppm (see ref 12 for details). [d] Model by Konishi and Kurokawa, ref 22.

morphous with silk I. They proposed a model of $(Ala\text{-}Gly)_n$II in which the conformation of the Ala and Gly residues are widely different and the torsion angles (ϕ and ψ) of the Ala and Gly residues correspond closely to those found in the β-sheet and α-helix forms, respectively (crankshaft model).[24] It is now possible to examine whether or not this model is acceptable in light of the conformation-dependent ^{13}C chemical shifts. As summarized in Table IV, it is recognized that the ^{13}C chemical shifts of Ala residues vary substantially among the polypeptides taking the α_R-helix, α_L-helix, β-sheet, and collagenlike 3_1-helix and triple helix.[2,6] As described already, such variation is not due to the specific sequence of amino acid residues but to the conformation. Further, these conformation-dependent ^{13}C chemical shifts of Ala residue are well reproduced by our previous calculation of the contour map of the ^{13}C chemical shifts of *N*-acetyl-*N'*-methylalanine amide as a model of $(Ala)_n$ by the FPT-I-NDO method as a function of the torsion angles.[12] In particular, the ^{13}C chemical shifts of Ala C_β of these polypeptides are in parallel with the shielding constants obtained from the contour map[12] (Table V). As to the C_α and C=O carbons, however, it is necessary to take account of the effect of hydrogen bonding, which makes the situation difficult for the conformers other than the α-helix and β-sheet.

If the crankshaft model is correct for $(Ala\text{-}Gly)_n$II, the expected ^{13}C chemical shifts of Ala residue taking the β-sheet conformation should appear in the following regions: C_α, 48.2; C_β, 19.9; C=O, 171.8 ppm.[2] In the case of Gly C=O, the expected peak position for the α-helix form is 172.5 ppm, from the value of $(Ala\text{-}Ala\text{-}Gly)_n$ treated with dichloroacetic acid. This value is very close to the value by Kricheldorf and co-workers,[7] obtained from the copolymer $H(Ala)_nGly^*(Ala)_mNHR$ ($n + m = 20$, * = 20% ^{13}C enrichment at the carbonyl group). None of the peaks predicted from the crankshaft model can explain the experimental ^{13}C chemical shifts of $(Ala\text{-}Gly)_n$II as summarized in Table III. The most serious discrepancy is that the Ala carbonyl signal at 177.1 ppm cannot be explained by this model. Therefore, it is obvious that the crankshaft model cannot be accepted for $(Ala\text{-}Gly)_n$II and silk I.

On the other hand, Konishi and Kurokawa[22] proposed a loose fourfold helical conformation whose residue translation is 2.27 Å, to account for the X-ray diffraction of silk I sample prepared from the C_p fraction. On the basis of the parameters (number of residues is 4 and unit height of helix is 2.27 Å), the torsion angles were calculated as $\phi = -25.5°$ and $\psi = 134.5°$ if the local conformations of all residues are assumed to be the same.[39,40] These torsion angles deviate slightly from the partially allowed region of the conformational map of Ramachandran and Sasisekharan.[39] Nevertheless, it is interesting that the Ala C_β ^{13}C shielding value expected for the torsion angles thus obtained corresponds to the observed ^{13}C chemical shift, as summarized in Table V. Therefore, the loose helix model seems to be preferable in accounting for the ^{13}C NMR spectra of $(Ala\text{-}Gly)_n$II and silk I type conformation, although further work is necessary to check the ^{13}C chemical shifts of Ala C_α and C=O carbons. Thus, more refined model building is also required to satisfy the X-ray diffraction pattern[22] and the present ^{13}C NMR data. As to the ^{13}C chemical shifts of Ala C=O at 177 ppm, hydrogen bonding perpendicular to the fiber axis[20] might account for such a downfield displacement.

Previously, Kricheldorf and co-workers[7] claimed that the presence of small amount of the α-helix conformation in crude silk from *B. mori* and tussah silk moth (*Antheraea mylitta*) is detectable by means of the ^{13}C chemical shifts of the Ala residue (15 and 177 ppm). As described above, such a peak is clearly seen in the cocoon samples (Figure 3). It is true that the α-helix conformation is dominant for Ala-rich fibroin such as *P. c. ricini* or *A. mylitta* in the liquid silk of the silk gland[41,42] and film prepared from the liquid silk.[3] Thus, it is natural to expect that the α-helical content of Ala-rich fibroin should be much higher than that of *B. mori* fibroin. On the other hand, the α-helical contents of *B. mori* and *A. mylitta* fibroins were 10 and 5%, respectively.[7]

It should be emphasized that the ^{13}C chemical shifts of Ala C_β and C=O carbons in the α-helix form are very close to those of the silk I type form (see Tables III and IV). However, the C_α ^{13}C chemical shift of the α-helix is displaced downfield by 1.9 ppm as compared with that of silk I. Unfortunately, the Ala C_α peak of the α-helix form is very close to the Ser C_α peak of the β-sheet form (53.1–54.8 ppm from Table II). Accordingly, it is extremely difficult to distinguish the α-helix form from the silk I type form from NMR data alone when the proportion of the former is very small, unless otherwise [*Ala*-2-^{13}C]fibroin sample is used. For this reason, it is more likely that the peaks previously ascribed to the α-helix should be ascribed to silk I form. The difficulty of the relative proportion of the α-helix form between *B. mori* and *A. mylitta* fibroins described above could be easily resolved by this new assignment of peaks. In supporting this new assignment, Kobayashi et al.[43] showed on the basis of the CD pattern that the conformation of *B. mori* fibroin in solution is a helical form like silk I. Further, no α-helical conformation was seen in the ^{13}C NMR spectra of the silk gland of intact *B. mori* and in regenerated aqueous fibroin solution.[42,44] Thus, it is unlikely that a considerable amount of the

α-helix form is present in the *B. mori* fibroin in the solid state. Accordingly, it is concluded that the minor conformer seen in the silk II sample, especially in the cocoons, is not the α-helix but silk I type.

Concluding Remarks

We find that the three forms of silk fibroins, silk I and II and the α-helix forms, are easily distinguished by the conformation-dependent ^{13}C chemical shifts as determined by the high-frequency CP–MAS NMR method. This approach is very useful for the conformational characterization of less crystalline samples such as silk I. We note that the present NMR approach is much more powerful in analyzing the local conformations of the amino acid residues under consideration than previous conventional techniques such as X-ray diffraction, infrared spectroscopy, etc. Accordingly, conformational characterization as viewed from minor amino acid residues, such as Tyr, Val, etc., is possible with selectively ^{13}C-enriched samples prepared by a similar procedure to that of [*Gly*-1-^{13}C]fibroin.

Note Added in Proof: In Table V, the alternative and energetically more favorable conformation[39] of silk I, if the local conformations of all residues are the same, has the torsion angles (−168°, −60°), which gives the identical ^{13}C shift of Ala C_β carbon on the basis of the calculated ^{13}C chemical shift contour map[12] to that of (−25°, 134°).

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Communications to the Editor

High Molecular Weight Reactive Poly(diphenylsiloxane)

Silicone resins are normally viscous liquids or gums because of their –Si–O– main-chain structure. It is well-known that the thermal stability of silicone resins is improved by substituting methyl groups with phenyl groups, but the glass transition temperature (T_g) is still below room temperature even with a high phenyl content. For example, silicone resins containing 75% phenyl groups have a T_g of −30 °C. When all methyl groups in poly(dimethylsiloxane) are replaced with phenyl groups, the resulting polymer is expected to have a high T_g, because the oligomeric diphenylsiloxane has a T_g of 150 °C. However, conventional polymerization yields only small amounts of high molecular weight poly(diphenylsiloxane).

We have found that the polymerization reaction as well as the chloromethylation proceeds in the chloromethylation reaction of oligomeric diphenylsiloxane with a Friedel–Crafts catalyst. In this communication the synthesis and the properties of novel high molecular weight poly(diphenylsiloxanes) with a reactive group in the side chain are described.

Oligomeric diphenylsiloxane having a weight-average molecular weight of 1400 (DP = 7) and OH groups at both chain ends was obtained from Petrearch Systems. The chloromethylation was carried out in chloromethyl methyl ether solution with $SnCl_4$ as a catalyst. We used chloromethyl methyl ether as the chloromethylation reagent because of its availability. In the case of the mass prep-

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