NMR of Silk Fibroin. 8. ^{13}C NMR Analysis of the Conformation and the Conformational Transition of *Philosamia cynthia ricini* Silk Fibroin Protein on the Basis of Bixon-Scheraga-Lifson Theory

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ABSTRACT: The helicity of each residue for the sequence of the alanine residues in *Philosamia cynthia ricini* silk fibroin protein, where the number of the alanine residues were determined as 22, was calculated by using the Bixon-Scheraga-Lifson theory for the helix-coil transition of poly(L-alanine) including the hydrophobic side-chain interactions. The NMR line shape of the carbonyl carbon of the alanine residue observed in aqueous solution was simulated on the basis of the helicity of the alanine sequence determined here. In addition, the change in the NMR spectra of the alanine carbonyl region due to the temperature-induced helix-coil transition was also interpreted in terms of the change in the statistical weight parameter w, where w is related to the formation of an intramolecular hydrogen bond. From this theoretical analysis and CD observations, the structure of *P. c. ricini* silk fibroin in aqueous solution was clarified.

Introduction

It has been recently shown that the silk fibroin is an excellent material for immobilization of several kinds of enzymes.[1,2] These advantages of silk are considered to be due to the unique amino acid compositions and also the primary and higher order structures of silk proteins.[3]

The silk fibroin from *P. c. ricini* is a protein whose major amino acid residues are alanine and glycine, as listed in Table I.;[4] the sum of these two amino acid residues comprises 80% of this silk fibroin protein as well as that of the more famous *Bombyx mori* silk fibroin. However, the relative content of alanine is larger than that of glycine, in contrast to the case of *B. mori* silk fibroin where the reverse is the case. As a result, the most striking conformational characteristic of *P. c. ricini* silk fibroin in aqueous solution is the presence of α-helical portions which consist of alanine residues, -(Ala)$_n$-. Poly(L-alanine) is insoluble in water. However, the liquid silk obtained from the silk gland of *P. c. ricini* silkworm or the silk dried gently is soluble in water, and we can observe the ^{13}C NMR spectra. The silk fibroin is considered to be soluble in water because there are several kinds of hydrophilic amino acid residues as well as alanine residues, as shown in Table I. On the basis of our ^{13}C NMR analysis, the solution structure of *P. c. ricini* silk fibroin is found to be as schematically summarized in Figure 1.[5-9] With increasing temperature or urea content in aqueous solution, a conformational transition, α-helix-random coil, of the -(Ala)$_n$- sequence occurs.[5,7]

In this paper, the number of residues, n, in the alanine sequence, -(Ala)$_n$-, of *P. c. ricini* silk fibroin will be first determined. Then, the helicity for each residue of the sequence will be calculated with the statistical thermodynamic treatment for the helix-coil transition of polypeptides by Bixon, Scheraga, and Lifson[10] in order to simulate the ^{13}C NMR spectra of *P. c. ricini* silk fibroin and also the spectral change through the helix-coil transition. The Bixon-Scheraga-Lifson theory, which involves the hydrophobic side-chain interactions as well as intramolecular hydrogen bonding, has been successfully applied to the thermodynamic analysis of the helix-coil transition of poly(L-alanine).[10] The solution conformation of *P. c. ricini* silk fibroin and the conformational change under the influence of external forces such as temperature will be clarified in detail from this statistical thermodynamic analysis of the ^{13}C NMR data, which are expected to be basic data in the development of excellent biomaterials using silk.

Table I
Amino Acid Composition of Silk Fibroins from *B. mori* and *P. c. ricini* (mol %)[4]

amino acid	*B. mori*	*P. c. ricini*	amino acid	*B. mori*	*P. c. ricini*
Ala	30.0	48.4	Val	2.5	0.4
Gly	42.9	33.2	Leu	0.6	0.3
Ser	12.2	5.5	Ile	0.6	0.4
Tyr	4.8	4.5	Phe	0.7	0.2
Asp	1.9	2.7	Pro	0.5	0.4
Arg	0.5	1.7	Thr	0.9	0.5
His	0.2	1.0	Met	0.1	0.01
Glu	1.4	0.7	Cys	0.03	0.01
Lys	0.4	0.2	Trp		0.3

Materials and Methods

The insoluble fractions of *P. c. ricini* silk fibroin in 20% hydrochloric acid solution after the hydrolysis treatment were obtained as follows.[11] The cocoons from *P. c. ricini* that were reared with *Ailanthus glandulosa* leaves in our laboratory[7] were degummed twice with 0.1% sodium peroxide in order to remove sericine protein.

The degummed silk was boiled with 20% hydrochloric acid solution for 5 h. The insoluble fraction was collected and washed with distilled water. It was then dialyzed against distilled water for 24 h.

NMR Measurement. ^{13}C NMR spectra of *P. c. ricini* silk fibroin in aqueous solution reported elsewhere were used here.[7] The *P. c. ricini* silk fibroin and the insoluble fraction in 20% hydrochloric acid solution were soluble in trifluoroacetic acid. Thus, the ^{1}H NMR spectra were observed with trifluoroacetic acid as a solvent at 25 °C with a JEOL FX-90Q (90 MHz) NMR spectrometer in order to determine the number of the residues of the alanine sequence in the protein.

CD Measurement. The liquid silk of *P. c. ricini* was collected from the middle silk gland of the mature larvae.[7] It was dried gently at room temperature. The CD spectra of the silk fibroin (0.2 w/v %, pH 7.5) in aqueous solution were observed with a JASCO-40S spectrometer with a DP-600 data processor at 4, 15, and 20 °C. The α-helix content, $[\theta]_H$, was calculated by using the following equation:[12]

$$[\theta]_H = \frac{-[\theta]_{222}}{40000}$$

where $[\theta]_{222}$ means the molar ellipticity at 222 nm.

Calculation. 1. Helicity of Each Residue of the -(Ala)$_n$- Sequence. It has been well-known that the stability of the α-helix

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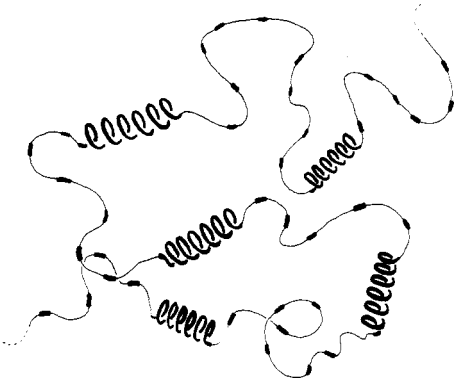

Figure 1. Model of *P. c. ricini* silk fibroin protein in aqueous solution. The broad lines mean alanine residues, and the α-helix portion consists of the alanine sequence, -(Ala)$_n$-

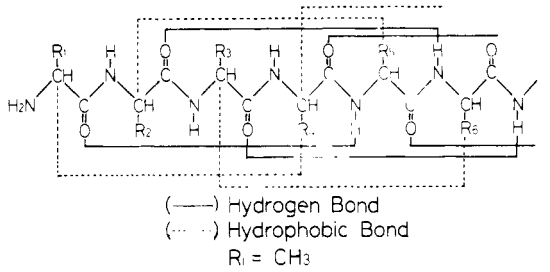

Figure 2. Schematic representation of a part of an alanine sequence.

of a poly(L-alanine) chain depends on the hydrophobic side-chain interaction as well as the intramolecular hydrogen bonding (Figure 2). The intramolecular hydrogen bonding is formed between the NH hydrogen atom of an $i + 4$-th residue and the C=O oxygen atom of the i-th residue. On the other hand, the hydrophobic interaction exists between the β-methyl group of an $i + 3$-rd residue and the α-carbon of the i-th residue. The Bixon–Scheraga–Lifson thermodynamic model, which takes into account both interactions, has been successfully applied to the thermodynamic analysis of the helix–coil transition occurring in a poly(L-alanine) chain;[10] thus, we will use this model in order to calculate the helicity of each residue of the insoluble fraction, the -(Ala)$_n$- sequence. Following the notation of Lifson and Roig,[13] the contribution to the partition function made by an alanine residue in a helical state, stabilized by a hydrogen bond, is denoted by the statistical weight, w. The much smaller contribution, due to a helical state that is not stabilized by a hydrogen bond, is denoted by v. σ is a statistical weight corresponding to the hydrophobic bond. Thus, each of the last two residues in a helical sequence contributes a factor v; any other alanine residue in a helical sequence contributes a factor $w\sigma$. A random coil residue that precedes a helical sequence of two or more units contributes a factor αu, where α is a factor indicating the departure from u when a hydrophobic bond can be present in the random coil state ($\alpha = 1 + v^{1/2}(\sigma - 1)$); any other random coil residue contributes a factor u (= 1). With these assignments of the statistical weights to the different states of the alanine residue in the chain, it is possible to formulate the partition function. The matrix **W**, which gives the statistical weight of the k-th unit when the states of the $k + 2$ and $k + 1$ units are known, is

$$\mathbf{W}k = \begin{array}{cc|c|cc} k+2 & & h & c & c \cup h \\ k & k+1 & h & h & c \\ \hline h & h & w\sigma & v & 0 \\ h & c & 0 & 0 & v \\ c & c \cup h & \alpha u & u & u \end{array} \quad (1)$$

where the notation $c \cup h$ means c or h.

The partition function $Z(n)$ for the alanine sequence containing $n + 2$ identical alanine residues is given by

$$Z(n) = e\mathbf{W}^n e^+ \quad (2)$$

$$e = (1, 1, 1) \quad (3)$$

$$e^+ = \begin{bmatrix} 0 \\ 0 \\ 1 \end{bmatrix} \quad (4)$$

$$\mathbf{W} = \begin{bmatrix} w\sigma & v & 0 \\ 0 & 0 & v \\ \alpha u & u & u \end{bmatrix} \quad (5)$$

The conditional partition function for the k-th residue in the helical state is given by

$$F(n,k) = e\mathbf{W}^{k-1}\mathbf{H}\mathbf{W}^{n-k}e^+ \quad (6)$$

$$\mathbf{H} = \begin{bmatrix} w\sigma & v & 0 \\ 0 & 0 & v \\ 0 & 0 & 0 \end{bmatrix} \quad (7)$$

The probability that the k-th segment is in a helical state is given by

$$P(n,k) = F(n,k)/Z(n) \quad (8)$$

2. NMR Peak Simulation. The ^{13}C NMR peak simulation of the alanine sequence of *P. c. ricini* silk fibroin is performed by reference to Ullman's method,[14] with the helicity calculated for each residue. The chemical shift of the Ala carbonyl peak attributable to the -(Ala)$_n$- sequence of *P. c. ricini* silk fibroin shows the mean helicity because of the rapid interconversion between the helix and coil conformations on the NMR time scale.[7] Thus, the chemical shift of the k-th residue, δnk, in the sequence is given by

$$\delta nk = \delta hP(n,k) + \delta c(1 - P(n,k)) \quad (9)$$

The intensity arising from the k-th residue at a resonance frequency δ is given by

$$I(n,k)\delta = A^{-1} \exp(-(\delta - \delta nk)^2/2A^2) \quad (10)$$

where a Gaussian line shape was assumed. The value of A corresponds to the peak width. If the value of A is larger, the peak is broader. The intensity for a chain of length n is given by

$$I(n)\delta = \sum_k I(n,k)\delta \quad (11)$$

If the alanine sequence is polydisperse, the total intensity is given by

$$I\delta = \sum_n G(n)I(n)\delta \quad (12)$$

where $G(n)$ is the weight fraction of the n-mer. With these formulas, the line shape of the -(Ala)$_n$- resonance region is simulated.

Results and Discussion

Determination of the Number of Residues in the Alanine Sequences of *P. c. ricini* Silk Fibroin. Figure 3 shows ^1H NMR spectra of L-alanine, *P. c. ricini* silk fibroin, and the insoluble fractions obtained from the hydrolysis treatment. The solvent is trifluoroacetic acid, TFA. The peaks assigned to only alanine residue were observed in the spectra of the insoluble fractions. Since the N-terminal NH_3^+ peak in TFA shifts to higher field than the position of the NH peaks in the internal residues, it is possible to determine the averaged number, n, of the alanine residues in the -(Ala)$_n$- sequence from the relative intensities of these peaks. The value n was determined as 22. The same value was obtained from the CH and CH_3 resonance regions.

Next, the determination was also performed from the carbonyl region of the alanine residue in the ^{13}C NMR spectrum of intact *P. c. ricini* silk fibroin observed in aqueous solution at 25 °C, directly.[7] The ratio of the relative intensities of two alanine carbonyl peaks, h/h*, was determined as 10 from the Gaussian peak simulation, as shown in Figure 4. These peaks are broadened with decreasing temperature as mentioned below, reflecting a decrease of the chain mobility. However, the value h/h*

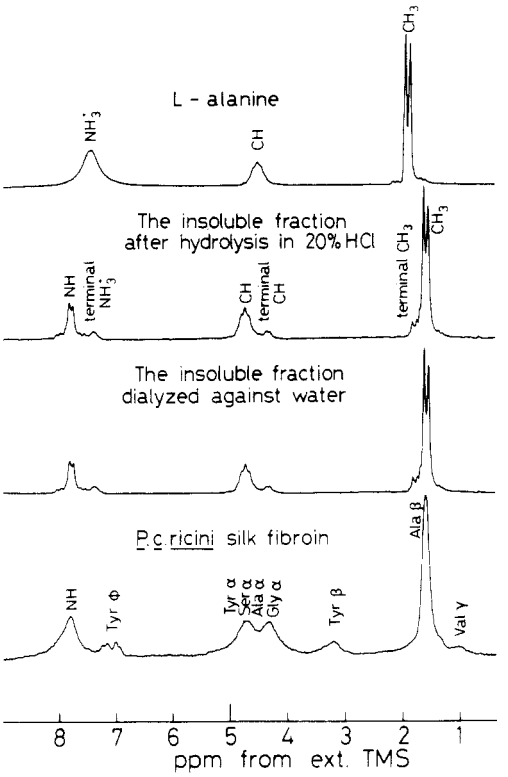

Figure 3. ^{1}H NMR spectra of L-alanine, the insoluble fraction of *P. c. ricini* silk fibroin after hydrolysis treatment and *P. c. ricini* silk fibroin in TFA.

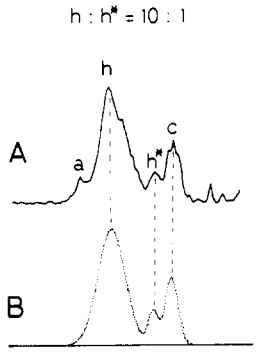

Figure 4. Carbonyl species of the alanine residues in *P. c. ricini* silk fibroin: A, observed spectrum at 25 °C; B, simulated spectrum assuming Gaussian. The peak a has been assigned to the γ-carbon of aspartic acid.[7]

is essentially unchanged within experimental error. If the peak h* is assigned to the alanine carbonyl carbons of the terminal residues in the -(Ala)$_n$- sequence in addition to the peak h assigned to the internal alanine residues, n is determined to be 22. Such an assumption concerning the peak assignment will be supported from the peak simulation based on the Bixon–Scheraga–Lifson theory as mentioned below. Thus, two independent methods yield the same value for n.

Helicity of Every Residue of the -(Ala)$_n$- Sequence Determined by the Bixon–Scheraga–Lifson Theory. The statistical thermodynamic analysis for the helix–coil transition of poly(L-alanine) by the Bixon–Scheraga–Lifson theory is applied to the determination of the helicity of every residue of the -(Ala)$_n$- sequence in *P. c. ricini* silk fibroin. The values of the statistical weights w, v, and σ in the statistical weight matrix used here were 1.0, 0.03, and 1.7, respectively.[10,15,16] The calculated spectrum for the -(Ala)$_{22}$- sequence by assuming the same Gaussian line shape (A = 0.08) among each residue is shown in Figure

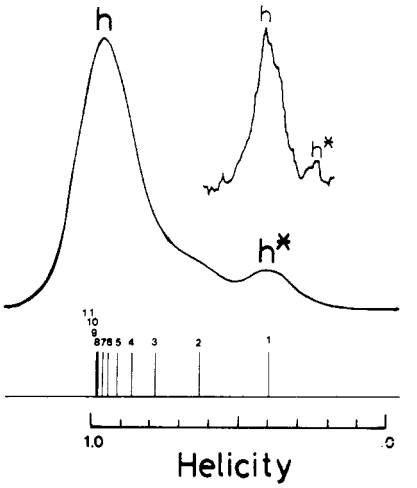

Figure 5. Observed (at 0 °C) and calculated carbonyl spectra, h and h*, of the -(Ala)$_{22}$- sequence in *P. c. ricini* silk fibroin on the basis of Bixon–Scheraga–Lifson theory. The values of w, v, σ, and A were 1.0, 0.03, 1.7, and 0.08, respectively, as described in the text.

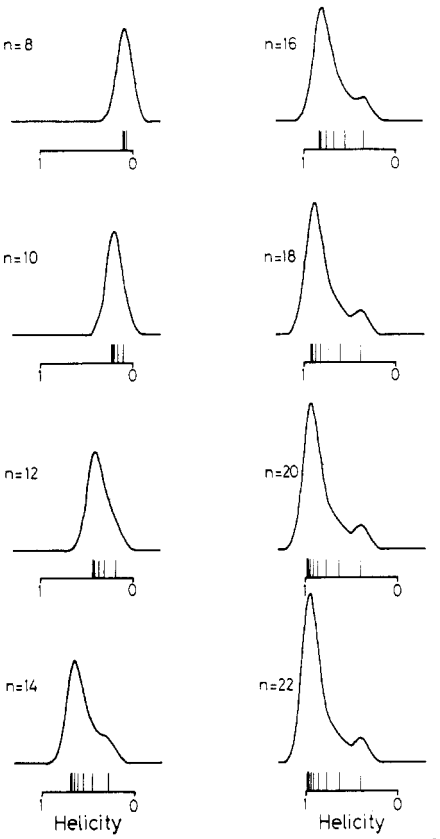

Figure 6. Carbonyl spectra calculated as a function of the number n for the -(Ala)$_n$- sequence.

5 together with the carbonyl spectrum, h + h*, of the alanine residue in *P. c. ricini* silk fibroin observed at 0 °C.[7] The calculated helicities of each residue are shown as sticks for residues 1–11 of the -(Ala)$_{22}$- sequence. The calculated peak positions which correspond to the helicities 1 and 0 are assumed to be those of the peaks h and c in the observed spectrum, respectively. The spectral character is reproduced from the calculation. Especially, by comparing the calculated spectrum with the observed one, we can attribute the h* peak to the alanine carbonyl carbons in the terminal residues.

Figure 6 shows the carbonyl spectra calculated with the Bixon–Scheraga–Lifson treatment as a function of the number n in the -(Ala)$_n$- sequence. With increasing n, the

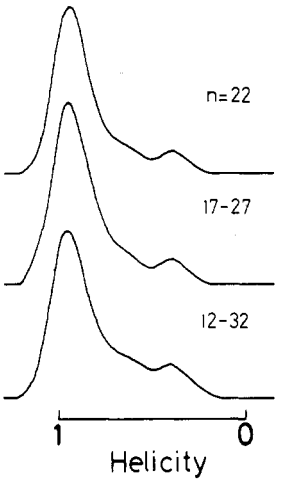

Figure 7. Carbonyl spectra calculated for the -(Ala)$_n$- sequence taking into account the distribution of n. The value of n is 22, averaged over n = 17–27 or over n = 12–32 assuming equal population of each sequence.

calculated main peak shifts to the peak position that is corresponding to 100% helix. In addition, the peak is asymmetrical at n = 12 and another small peak attributable to the terminal groups appears at $n \geq 16$. This tendency is consistent with the conclusion where the number of the residues of the alanine sequence of *P. c. ricini* silk fibroin is 22 obtained from the experimental data.

Next, the theoretical spectra averaged over n = 17–27 and over n = 12–32 assuming equal populations of each sequence are shown in Figure 7. The calculated patterns were essentially the same, indicating that the influence of the distribution of sequence on the carbonyl spectrum of the alanine residues is small when the averaged number of the alanine residues is 22. Of course, if the fraction of the alanine sequences with relatively short lengths is large, the theoretical spectra should differ from the observed spectrum, as is easily expected from Figure 6.

Helix–Coil Transition of *P. c. ricini* Silk Fibroin Monitored by the Carbonyl Spectra of the Alanine Residues. The helix–coil transition of *P. c. ricini* silk fibroin was induced by changes in temperature, and ^{13}C NMR spectroscopy was able to monitor the conformational change as shown in Figure 8.[7] The peaks h and h* in the carbonyl resonance region showed gradual upfield shifts as the temperature was increased. On the other hand, the position of the peak c scarcely changed between −5 and 25 °C. Above 25 °C, the behavior of the peaks h* and c is relatively complex. This may be caused from, in part, the occurrence of the β structure. Thus, a comparison of the calculated and observed temperature-dependent data is performed here between −5 and 25 °C. The peak c was already assigned to the isolated alanine residue and/or alanine sequences whose lengths were too short to form an α-helix; the conformation was random coil.[5,7] Each peak become sharp with increasing temperature, indicating an increase in the segmental motion of the protein.

Figure 9 shows the helicity of each residue calculated for the -(Ala)$_{22}$- sequence when the statistical weight w is changed from 1.0 to 0.78. As is expected, the helicity decreases with decreasing value of w. With these values, change in the spectra was simulated and is shown in Figure 8. Since all peaks become sharp with increasing temperature, the value of A also decreased. Both peaks h and h* in the calculated spectra shift to higher field with decreasing w, which is in agreement with the experimental data. Thus, we could determine the value of w for the

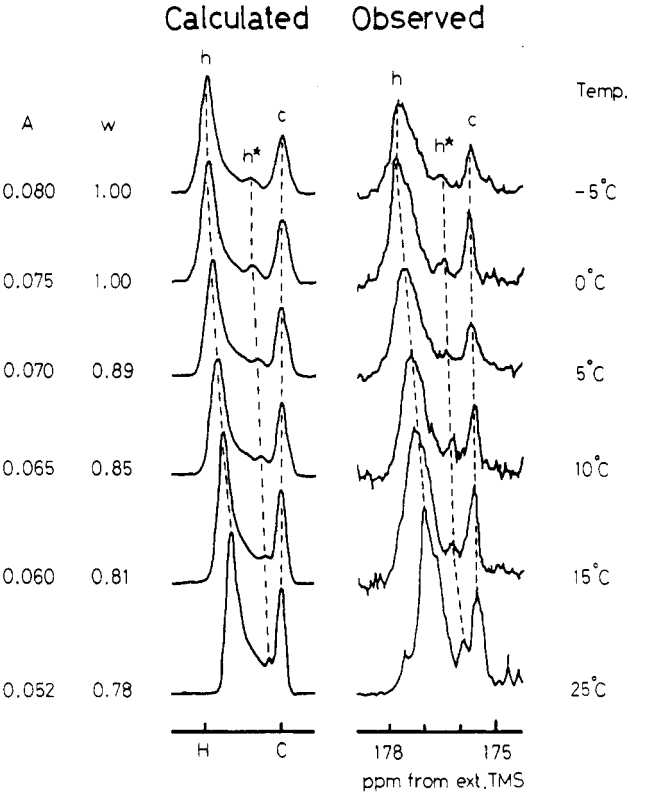

Figure 8. ^{13}C NMR spectra of *P. c. ricini* silk fibroin in the alanine carbonyl region observed as a function of temperature and the calculated spectra for the -(Ala)$_{22}$- sequence as functions of the statistical weight w and the parameter A.

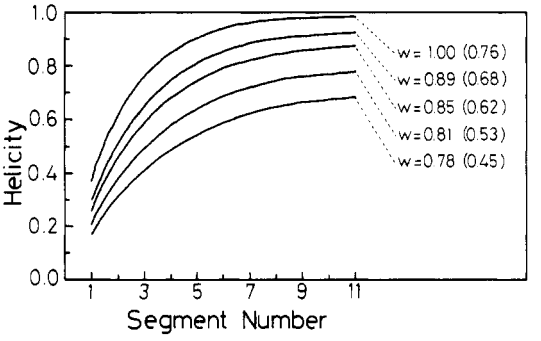

Figure 9. Plots of the fractional helicity versus the number of the residue in the -(Ala)$_{22}$- sequence counting from one end with various values of the statistical weight w. The numbers in the parentheses indicate the mean helicity.

-(Ala)$_{22}$- sequence in *P. c. ricini* silk fibroin at ambient temperature.

Determination of the α-Helix Content of *P. c. ricini* Silk Fibroin by Means of ^{13}C NMR and CD Methods. The α-helix content $[\theta]_H$ of *P. c. ricini* silk fibroin was determined as a function of temperature from both the mean helicity calculated for the -(Ala)$_{22}$- sequence (Figures 8 and 9) and the fraction of the peaks h plus h* (0.341), because the carbonyl peaks which are attributable to other amino acid residues, as well as to the peak c of the alanine residue, show no temperature dependence as described in our previous paper.[7] The results are summarized in Table II. The CD spectra of *P. c. ricini* silk fibroin were typical for the α-helix over the range of temperatures 4, 15, and 20 °C, although the α-helix content decreases with increasing temperature. The $[\theta]_H$ values were determined by using the molar ellipticity at 222 nm and are also listed in Table II. Both methods, NMR and CD, yield the same $[\theta]_H$ value at ambient temperature within experimental error. The values determined here are smaller than those

Table II
α-Helix Content $[\theta]_H$ of *P. c. ricini* Silk Fibroin in Aqueous Solution Determined with ^{13}C NMR and CD Methods as a Function of Temperature

temp, °C	mean helicity[a]	$[\theta]_H^{C=O}$ [b]	$[\theta]_H^{CO}$ [c]
-5	0.76	25.9	
0	0.76	25.9	
5	0.68	23.2	22.0[d]
10	0.62	21.1	
15	0.53	18.0	18.8
20			17.6
25	0.45	15.3	

[a] The helicity of the -$(Ala)_{22}$- sequence portion determined by both ^{13}C NMR and thermodynamic analyses (Figures 8 and 9). [b] Determined from the product of the α-helix fraction (h + h*; 0.341) and the mean helicity. [c] Determined from the CD method. [d] At 4 °C.

reported previously although the same NMR data are used. This is due to an assumption that the mean helicity of the alanine sequence at 0 °C is 100% in the previous paper. However, the mean helicity at 0 °C should be 76% when the number of the alanine residues of the sequence is 22 as mentioned above. Thus, it is necessary to know the number of alanine residues in estimating the α-helix content of *P. c. ricini* silk fibroin with the ^{13}C NMR method.

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Synthesis, NMR Characterization, and Electrical Properties of Siloxane-Based Polymer Electrolytes

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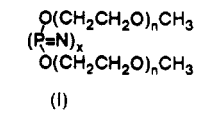
ABSTRACT: A cross-linked siloxane-based polymer has been prepared by the reaction of poly(methylhydrosiloxane), poly(ethylene glycol) monomethyl ether, and poly(ethylene glycol). The polymer, which has been structurally characterized by ^{29}Si NMR, forms complexes with $LiSO_3CF_3$ that exhibit good ionic conductivities (up to 7.3×10^{-5} Ω^{-1} cm^{-1}, 40 °C, for a $LiSO_3CF_3$ 15 wt % complex). The polymer and its salt complexes have been characterized by elemental analysis, ^{29}Si NMR, X-ray powder diffraction, DSC, and ac complex impedance spectroscopy. The dependence of the ionic conductivity was investigated as a function of temperature, salt concentration (1-25 wt %), and alkali-metal cation (Li, Na, K, Rb, and Cs).

Introduction

Solvent-free polymer electrolytes can be formed by the interaction of polar polymers with metal salts.[1,2] Ion transport in these electrolytes has been a topic of considerable research in the last 5 years, and this field has been recently reviewed.[3] The currently accepted model for ionic conductivity has led to the synthesis of network and comb polymers that have a high degree of segmental motion as judged by low values of the glass transition temperature, T_g.[4,5]



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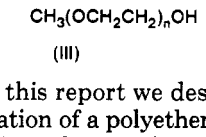

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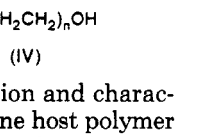

(IV)

In this report we describe the preparation and characterization of a polyether-substituted siloxane host polymer and its salt complexes. We were prompted to prepare these materials because siloxanes typically exhibit low values of T_g. The siloxane polymer was prepared from the reaction of poly(methylhydrosiloxane), PMHS (II); poly(ethylene glycol) monomethyl ether, MePEG(III); and poly(ethylene glycol), PEG (IV). Other groups have investigated siloxane-based electrolytes but in most cases the polymer was a block copolymer of dimethylsiloxane and ethylene oxide repeat units.[6] A urethane network formed from poly(dimethylsiloxane-*graft*-ethylene oxide) has also been studied.[7] While the work presented in this paper was in progress, other groups reported preliminary results on similar polymers and electrical measurements.[8]

Experimental Section

Poly(ethylene glycol) monomethyl ether, MePEG (Aldrich, MW_{av} = 350); poly(ethylene glycol), PEG (Aldrich, MW_{av} = 300); and poly(methylhydrosiloxane), PMHS (Petrarch, MW = 4500-5000), were dried under vacuum at 60 °C for 2 days, no evidence of H_2O in any of the starting materials was noted by IR spectroscopy. A zinc octoate catalyst (Petrarch, 50 wt % PDMS) was used as received. The polymer host that we designate as siloxane(30) was prepared by the reaction of a stoichiometric

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