The Structure of *Bombyx mori* Silk Fibroin Membrane Swollen by Water Studied with ESR, ^{13}C-NMR, and FT-IR Spectroscopies

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Synopsis

Structure of *Bombyx mori* silk fibroin membrane insolubilized with methanol, especially in the swollen state, is studied by means of spin-label ESR, ^{13}C-NMR, and FT-IR (ATR method) spectroscopies. FT-IR data indicate that the conformational transition from random coil to antiparallel β-sheet occurs at the surface of the membrane upon immersion into 80% aqueous methanol. High resolution ^{13}C-NMR observation of the membrane swollen in water shows that, the random coil portion whose segmental motion is very fast remains in the inner part of the swollen membrane. The fraction of this portion decreases with increasing methanol treatment time in the sample preparation. The heterogeneous structure of the swollen membrane was clarified from the complicated ESR spectra of the spin-labeled silk fibroin membranes. The ESR spectra were analyzed quantitatively and the fractions of the fast, slow, and very slow motions of the spin-label site (tyrosine side chain) were determined. A model is proposed for the heterogeneous structure of the swollen silk fibroin membrane.

INTRODUCTION

Recently, it has been proved that *Bombyx mori* silk fibroin protein with a molecular weight of approximately 360,000 is an excellent biomaterial in applications such as enzyme-immobilized membranes for biosensors.[1-5] One of the merits of silk fibroin for this purpose is simultaneous insolubilization of the silk and immobilization of enzyme without using any chemical reagents. This is based on conformational transition of the silk fibroin membrane from water-soluble random coil to water-insoluble silk II (anti parallel β-sheet) induced by methanol immersion or stretching. The random coil structure of *B. mori* silk fibroin including the dynamic character in aqueous solution has been studied by the observation of the long range NMR spin coupling constant $^{3}J_{C'-N-C-H}$ and ^{13}C-NMR relaxation parameters etc., in detail.[6-11] Moreover, the characterization of silk II and silk I structures in solid state has been reported on the basis of the conformational-dependent ^{13}C-NMR chemical shift of the amino acid carbons in ^{13}C CP/MAS NMR spectroscopy.[10-15] However, in general, the enzyme-immobilized membrane has been used in water and, thus, to clarify the structure of the silk fibroin membrane swollen by water is important in connection with the development of enzyme-immobilized membranes.

Spin-label ESR methods are useful for this purpose because of the inherent high sensitivity of ESR observation and the wide detectable range of the motion

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of the spin-labeled site from 10^{-10} to 10^{-4} s. The main amino acids of *B. mori* silk fibroin are glycine, alanine, serine, and tyrosine.[16] The sum of these amino acids is over 85%. Among them, the hydroxy group of the tyrosine side chain is active and thus it can be labeled with nitroxide radical compounds. In our previous paper,[17] the ESR spectrum of *B. mori* silk fibroin spin-labeled at the tyrosine side chain group with the nitroxide radical compound was observed. The activation energy of the spin-labeled *B. mori* silk fibroin in aqueous solution obtained from the plot of the rotational correlation times determined from the ESR spectrum, against temperature^{-1}, is 4.46 kcal mol^{-1} in the temperature range of 20–90°C, which is comparable with the activation energy, 5.4 kcal mol^{-1}, of the segmental motion of *B. mori* silk fibroin determined from the ^{13}C-NMR spin–lattice relaxation time.[18] In addition, the structure and thermal properties of the membrane were essentially unchanged by the spin-labeling treatment.

In this paper, such a spin-labeled silk fibroin was used to study the structure of the swollen membrane. The ESR spectrum was complex, indicating heterogeneous structure of the swollen membrane. The detailed structure of the membrane was proposed by adding the high resolution ^{13}C-NMR data of the swollen membrane and FT-IR (ATR method) observation of the dried membrane to the ESR spectrum simulation[19,20] data, assuming three kinds of the states as viewed from the motion of the spin-labeled site.

EXPERIMENTAL

Materials

Bombyx mori silk fibroin aqueous solution was prepared as reported elsewhere in detail.[8] Thin (30–50 μm) and thick (100–250 μm) membranes were prepared from the solution.[1,2] The spin-labeled silk fibroin was prepared according to the following process.[17] The spin-label reagent, *N*-(2,2,5,5-tetramethyl-3-carbonyl-pyrroline-1-oxyl)-imidazole, was synthesized according to the method by Barrat et al.[21] The reagent was coupled with the hydroxy group of the tyrosine side chain of *B. mori* silk fibroin in aqueous solution at 0 for 3 h. Then, this solution was dialyzed against distilled water at 4°C. Figure 1 shows the scheme of this labeling briefly. The spin-labeled silk fibroin membrane was obtained

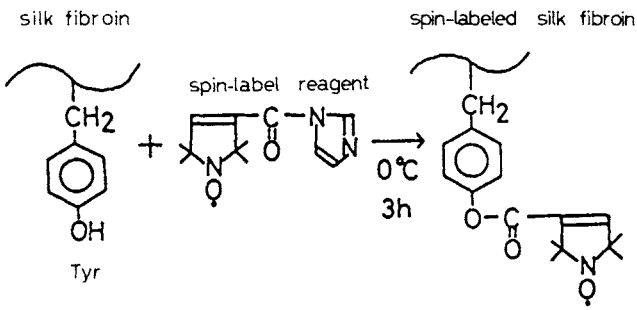

Fig. 1. Scheme of the synthesis of the spin-labeled silk fibroin.

by casting this solution. All membranes were insolubilized by immersing them into 80% methanol aqueous solution and then immediately washed by water repeatedly. The time of the immersing treatment was changed.

Measurements

The degree of swelling of the silk fibroin membrane treated with methanol in water at 20°C was estimated by

$$\text{Degree of swelling (\%)} = (W_{wet}/W_{dry} - 1) \times 100$$

where W_{wet} is the weight of the membrane in the swollen state and W_{dry} is the weight of the membrane after drying at vacuum.

Silk protein eluted from the membranes after soaking them into water at 20°C for 1 month was estimated by the relation given below:

$$\text{protein elution (\%)} = W_{protein}/W_{dry} \times 100$$

where $W_{protein}$ is the weight of the eluted silk protein, which is determined by Lowry method.[22] In this elution experiment, the membrane was cut off to small pieces (2 × 10 mm) with scissor before and after methanol treatment, in order to determine the amount of outflow of silk protein from the cross-section part of the membrane.

FT-IR spectra of the silk fibroin membranes in the dry state were measured with DIGILAB FTS-80 infrared spectrometer by the ATR method at room temperature.

The ^{13}C-NMR spectra of the silk fibroin membranes swollen in water sufficiently were obtained at 25°C with JEOL-FX90Q NMR spectrometer operating at 22.5 MHz after soaking them into water for more than 1 day. The ^{13}C-NMR spin–lattice relaxation times T_1, measured for the protonated carbons, were made by the inversion-recovery method. The values of nuclear Overhauser enhancement (NOE) were determined by direct comparison of the peak areas obtained from complete ^1H decoupling observation with those obtained from the gated ^1H decoupling observation. The total volume of sum of the membrane and water in NMR sample tube was set within the inner space of the NMR probe coil and the capillary of dioxane was used for the reference of the peak intensity.

The ESR spectra of the membranes in the swollen state by water were measured with a JEOL FE-3AX ESR spectrometer at room temperature after soaking them into water for more than 1 day.

RESULTS AND DISCUSSION

Degree of Swelling of the Membrane and Silk Protein Elution from the Membrane in Water

Figure 2 shows the degree of swelling of the silk fibroin membranes treated with methanol in water at 20°C (the thickness of the membrane is 30 μm)

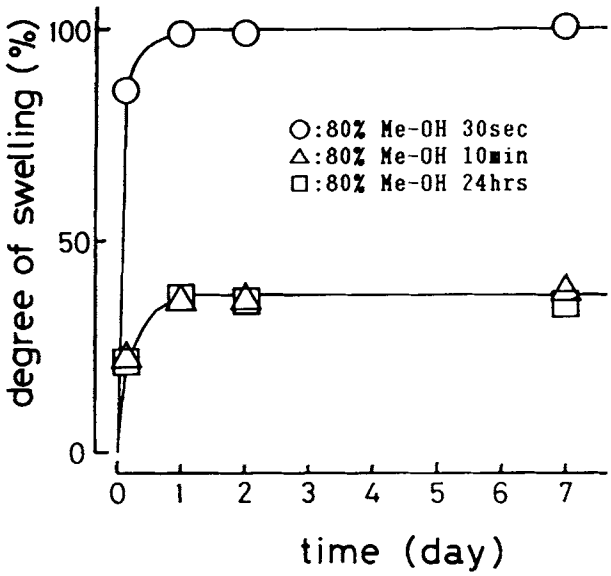

Fig. 2. Degree of swelling of the silk fibroin membranes treated with methanol vs. the soaking time.

plotted against the soaking time. The degree of swelling of the membranes increases rapidly with increasing soaking time and reaches the equilibrium value within 1 day. This tendency is independent of the methanol treatment time in the sample preparation. The degree of swelling and the protein elution from the silk fibroin membranes treated with methanol at 20°C after soaking them into water for 1 month are summarized in Table I. In this experiment, the methanol treatment time of the membranes changed finely. The degree of swelling depends on the methanol treatment time remarkably in the range of 30 s to 3 min. At more than 3 min, however, the value decreases only slightly. The protein elution from the silk fibroin membranes treated with methanol was scarcely recognized except for the membranes treated for 30 s. When the methanol treatment time is 30 s, the degree of the elution from the membrane cut after the methanol treatment is larger than that cut before the methanol treatment. This indicates that the silk protein elution from noninsolubilized cross section of the membrane occurs for the former case. Therefore, this is part of the evidence for the fact that the conformational transition of the silk fibroin membrane starts from the surface of the membrane by immersing it into 80% methanol aqueous solution.

Conformational Transition of the Surface of the Membrane Observed with FT-IR

Figure 3 shows the FT-IR spectra of the silk fibroin membranes treated with and without methanol (the thickness of the membrane is 100–250 μm). The absorption bands characteristic to the antiparallel β-sheet form,[14] 1625 (amide I), 1528 (amide II) and 1260 cm^{-1} (amide III), were clearly observed for the

TABLE I
Degree of Swelling of the Silk Fibroin Membrane and the Amount of Elution of the Silk Protein from the Membrane Soaked in Water at 20°C after 1 Month

Methanol treatment time	Degree of swelling[a]	Protein elution[b]	
		Experiment A[c]	Experiment B[d]
30 s	103	4.1	1.0
1 min	86		
3 min	56	0.1	0.1
10 min	40	0.0	0.0
1 h	39	0.0	0.0
8 h	35		
24 h	34	0.0	0.0
48 h	33		

[a] Degree of swelling (%) = $(W_{wet}/W_{dry} - 1) \times 100$, where W_{wet} is the weight of the membrane in the swollen state and W_{dry} is the weight of the membrane after drying at vacuum.

[b] Protein elution (%) = $W_{protein}/W_{dry} \times 100$, where $W_{protein}$ is the weight of the eluted silk protein which is determined by Lowry method.[22]

[c] Experiment A: The samples were cut after the methanol treatment.

[d] Experiment B: The samples were cut before the methanol treatment.

membrane treated with methanol. The membrane without methanol treatment gave the absorption bands at 1650 (amide I), 1535 (amide II), and 1235 cm^{-1} (amide III), which were assigned to random coil conformation.[14] These data show that the conformational transition from random coil to antiparallel β-sheet occurred partly by such a methanol treatment, which is essentially in agreement with the previous investigation with ^{13}C CP/MAS NMR.[14] In the ATR method, especially, the structural information with respect to only the surface of the membrane (the depth from surface is about 2 μm in this experimental condition) can be obtained. There is no difference between the spectra of the two insolubilized membranes whose immersing times was changed. This indicates that the degree of the conformational transition occurred sufficiently at the surface of the membrane even if the methanol treatment time was 30 s.

Detection of the Mobile Portion in the Swollen Membrane by High Resolution ^{13}C-NMR

^{13}C-NMR spectra of the silk fibroin membrane treated with methanol in water (the thickness of the membrane is 100–250 μm) are shown in Figure 4 together with the spectrum of the silk fibroin aqueous solution (ca. 2.5 wt %). The existence of a mobile portion in the inner part of the swollen membrane can be detected from the ^{13}C-NMR spectrum of the membrane in water. Previously, the mean correlation time for the segmental motion of the silk fibroin in aqueous solution was determined as 1×10^{-10} s, from NT_1 and NOE values averaged over Gly C_α, Ala C_α, and Ser C_α carbons assuming the log χ^2 distribution model.[7] Every high resolution NMR peak of the membrane in water became broader than those of aqueous solution and the mean correlation time of the mobile portion in the swollen membrane was determined to be about 2×10^{-10} s in the same manner.

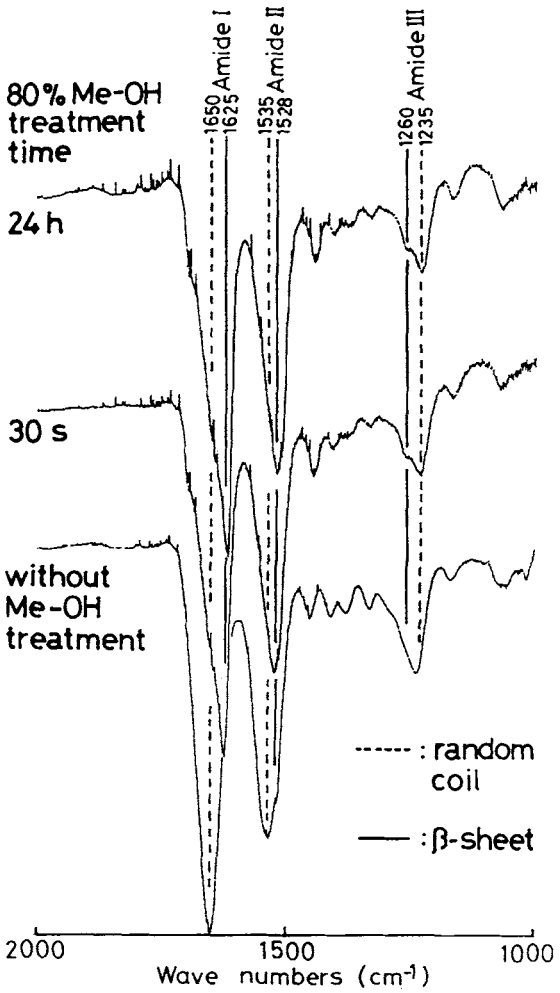

Fig. 3. FT-IR spectra of silk fibroin membranes treated with or without methanol at room temperature.

The intensity of the ^{13}C-NMR peak of the membrane decreases with increasing the methanol treatment time as shown in Figure 4. Thus, the fraction of the portion observed by ^{13}C-NMR of the silk fibroin membrane swollen in water, F_{NMR} (%), was estimated by reference to the peak intensity of dioxane as mentioned in the Experimental section. The F_{NMR} values of the silk fibroin membranes treated with methanol for 30 s, 10 min, and 24 h (the thickness of the membrane was changed; 50 and 100–250 μm) are summarized in Table II together with the degree of swelling of each membrane. Both the F_{NMR} and degree of swelling of the membrane decrease with increasing the methanol treatment time. When the methanol treatment time is 30 s, the F_{NMR} and degree of swelling of the thick membrane are smaller than those of the thin membrane. Since longer time is required in preparation of the thick membrane than the case of the thin membrane, more fine structure might be formed in

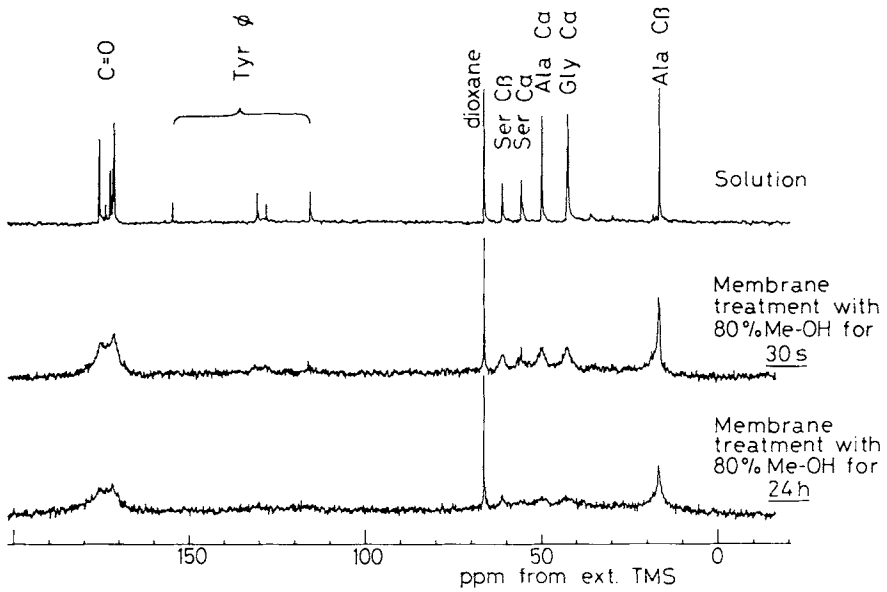

Fig. 4. ^{13}C-NMR spectra of the silk fibroin in aqueous solution and the silk fibroin membrane treated with methanol in water at 25°C.

the former membrane. Actually, Kitamura reported that the ion transmission coefficient of the silk fibroin membrane decreased with increasing the casting time.[23] The ^{13}C-NMR spectrum could not be obtained for the silk fibroin hydrogel,[7,24] which was prepared by standing the aqueous solution of the silk fibroin for long times (about a few weeks) at 4°C. This means that the molecular motion of the silk fibroin hydrogel is slow compared with the case of the fibroin chain, which is in the inner part of the membrane treated with methanol. Thus, structure of the membrane treated with methanol is considerably heterogeneous in swollen state by water as viewed from molecular motion.

Quantitative Evaluation of the Heterogeneous Structure of the Swollen Silk Fibroin Membrane by Spin-Label ESR

Since the structure and thermal properties of the silk fibroin membrane with spin-labeled tyrosine side chain are essentially unchanged by the spin-label

TABLE II
Fraction of the Portion Observed by High Resolution ^{13}C-NMR, F_{NMR}, in the Silk Fibroin Membrane and Degree of Swelling of the Membrane Soaked in Water After a Few Weeks (%)

Thickness	50 μm			100–250 μm		
Methanol treatment time	30 s	10 min	24 h	30 s	10 min	24 h
F_{NMR}	7.5	2.1	0.7	4.9	5.1	2.1
Degree of swelling	180	40	37	68	52	43

modification,[17] the use of the spin-labeled silk fibroin membrane for the structure analysis of the swollen silk fibroin membrane is available. In particular, the information about the silk fibroin membrane can be obtained through the mobility of the tyrosine side chain (the spin-labeling site) with ESR spectroscopy. Figure 5 shows the ESR spectra of the spin-labeled silk fibroin in aqueous solution and several kinds of the spin-labeled silk fibroin membranes treated with methanol in both dry and swollen states (the thickness of the membrane is 100–250 μm). The ESR spectrum of the aqueous solution is composed of three sharp peaks, which means that the correlation time τ_c for the rotational motion of the nitroxide radical group is 6×10^{-10} sec. This is determined from both the peak height and width.[25] On the other hand, the ESR spectrum of the membrane in dry state is very broad and the τ_c value is determined as 2×10^{-8} s from the maximum separation width.[26] The ESR spectra of the membrane in swollen state (right side of Fig. 5) are complex, indicating the presence of several kinds of components from the viewpoint of the motion of the nitroxide radical group in the membrane. Namely, there are several kinds of the microenvironment around the Tyr side chain in the membrane swollen by water.

The relative intensities of three sharp ESR peaks of the membrane in the swollen state decrease with increasing the methanol treatment time. Thus, by assuming that the ESR spectrum consists of three components from viewpoint of the motion in the ESR time scale, the fraction of each component was determined by the nonlinear least squares method from the computer spectrum simulation[19,20] (Fig. 6). Three typical spectra for the fast, slow, and very slow components which are noted as $M_f(g)$, $M_s(g)$, and $M_{vs}(g)$, respectively, are input and these integrated intensities are normalized. The orders of the τ_c of the fast, slow, and very slow components were 10^{-10}, 10^{-9}, and 10^{-8} s, respectively. The simulated spectrum $S_{cal}(g)$ is given by

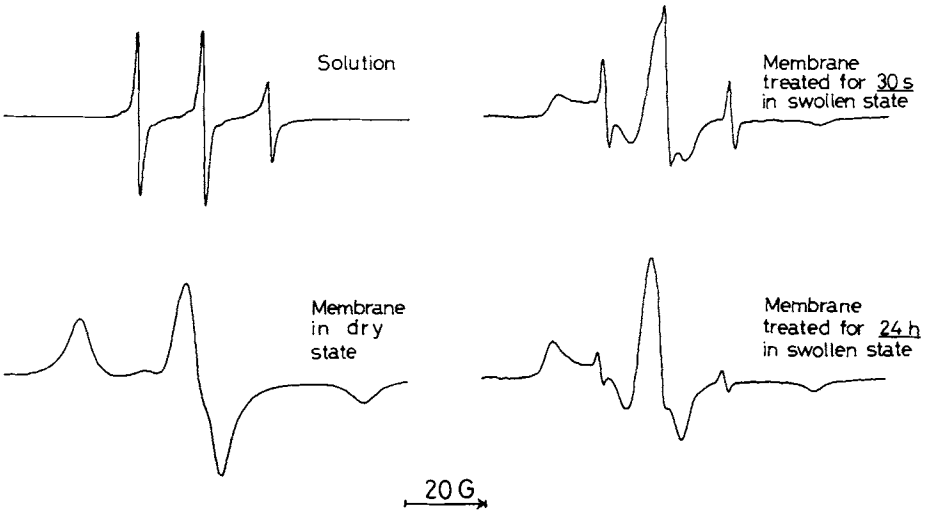

Fig. 5. ESR spectra of the spin-labeled silk fibroin in aqueous solution and the spin-labeled silk fibroin membranes treated with methanol in dry and swollen states at room temperature.

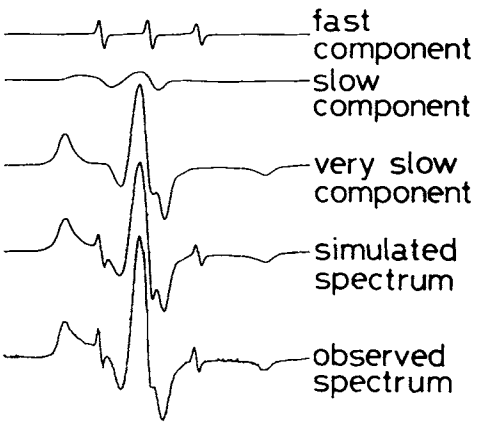

Fig. 6. An example of the ESR spectrum simulation. The ESR spectrum of the spin-labeled silk fibroin membrane treated with methanol for 24 h (the thickness of the membrane is 100–250 μm) was simulated by assuming the spectra of fast, slow, and very slow components which correspond to the rotational correlation times of 10^{-10}, 10^{-9}, and 10^{-8}s, respectively. The fraction of the fast, slow, and very slow components was determined as 0.01, 0.12, and 0.87, respectively.

$$S_{\text{cal}}(g) = F_f \times M_f(g) + F_s \times M_s(g) + F_{\text{vs}} \times M_{\text{vs}}(g)$$

$$F_f + F_s + F_{\text{vs}} = 1$$

where F_f, F_s, and F_{vs} are the fractions of each component. The data points for computer input, n, was 722. The estimation function J is defined by

$$J = \sum_{g}^{n} [S_{\text{obs}}(g) - S_{\text{cal}}(g)]^2$$

where $S_{\text{obs}}(g)$ is the observed and input spectrum whose integrated intensity was normalized. The F_f, F_s, and F_{vs} are determined so that the value of J is minimized. As an example, the output of the spectrum simulation for the swollen membrane treated with methanol for 24 h is shown in Figure 6. The results of the spectrum simulation are summarized in Table III. Although a small amount of the spin-labeled silk protein might be eluted from the membrane treated with methanol for 30 s into water, this eluted protein was not detected in the ESR spectrum because it was removed by wiping before ESR observation. With increasing the methanol treatment time, the F_f decreases and the F_s and F_{vs} increase. When the methanol treatment time is the same, the F_f and F_s of the thick membrane are larger than that of the thin membrane.

Proposal of the Heterogeneous Structure Model of the Swollen Silk Fibroin Membrane

When the silk fibroin membrane with random coil form which was soluble in water was immersed into 80% methanol aqueous solution, the conformational transition from random coil to antiparallel β-sheet form starts from the surface

TABLE III
Fraction of Each Component, F_f, F_s, and F_{vs}, Determined from the ESR Spectrum of the Spin-Labeled Silk Fibroin Membrane Treated with Methanol in Swollen State by Water (%)[a]

Thickness	30 μm		100–250 μm	
Methanol treatment time	30 s	24 h	30 s	24 h
F_f	0.02	0.00	0.04	0.01
F_s	0.16	0.00	0.26	0.12
F_{vs}	0.82	1.00	0.71	0.87

[a] Details of the determination are given in the text.

of the membrane. This was suggested from the FT-IR data. The aggregated portion with the antiparallel β-sheet form plays a role in the crosslinking and, as a result, the membrane becomes insoluble against water. The mobility of the chain with such a β-sheet form must be very low because of the formation of intra- and/or intermolecular hydrogen bonding. On the other hand, the random coil portion whose segmental motion is very fast remains in the inner part of the membrane. The fraction of this portion decreases with increasing the methanol treatment time as observed from ^{13}C-NMR and ESR spectra.

On the basis of these data, the model for the heterogeneous structure of the swollen silk fibroin membrane treated with methanol is proposed as shown in Figure 7. The square symbols in Figure 7 indicate the aggregated portion with antiparallel β-sheet form and its dimension means a measure of the degree of growth of such aggregation. The aggregated portion with the antiparallel β-sheet form tends to concentrate at the surface of the membrane. From Table III, the Tyr residues of the silk fibroin membrane in the swollen state can be classified into three types. The first type is present in the chain at the neighborhood of the antiparallel β-sheet form and its mobility is considerably restricted. Actually, the sequence has been reported for the crystalline fraction (C_p fraction), i.e., the precipitated fraction (ca. 55% of the original silk fibroin) after chymotrypsin hydrolysis of *B. mori* silk fibroin as Gly—Ala—Gly—Ala—Gly—Ser—Gly—Ala—Ala—Gly—[Ser—Gly—(Ala—Gly)$_n$]$_8$—Tyr, where n is usually 2.[27] In addition, 32% of the soluble fraction after chymotrypsin hydrolysis has approximately the same sequence as the C_p fraction.[16] Most of the C-terminal tyrosine residues in this sequence are considered to be very slow component F_{vs} because the sequence, —(Ser—Gly—Ala—Gly—Ala—Gly)—, easily takes antiparallel β-sheet structure by methanol immersion.[1] The second type is present in the chain with random coil form, but its mobility decreases by the influence of the aggregation of the chain. The third type is also present in the chain with the random coil form whose segmental motion is very fast when the motion was scarcely influenced by the aggregation. Except for the thin membrane treated with methanol for 30 s, a good agreement between F_{NMR} and F_f (compare Tables II and III) is obtained, supporting the model proposed here. It is concluded that the silk fibroin membrane in the swollen state by water has heterogeneous structure as proposed in Figure 7 and such a hetero-

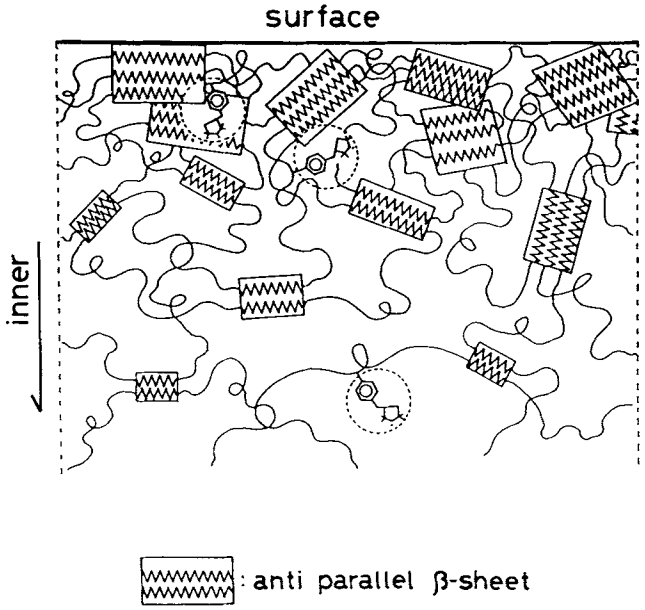

Fig. 7. Model of the heterogeneous structure of the silk fibroin membrane treated with methanol in swollen state. From the FT-IR data, the aggregated portion with the antiparallel β-sheet form tends to concentrate at the surface of the membrane. From the ^{13}C-NMR data, the random coil portion whose segmental motion is very fast remains in the inner part of the membrane. From the ESR data, the Tyr residues (spin-label sites) of the membrane in the swollen state can be classified into three types (see the text).

geneous structure is considered to be one of the causes of excellent biomaterials of silk fibroins.

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