Preparation and Characterization of Silk Fibroin Powder and Its Application to Enzyme Immobilization

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Synopsis

The powders of *Bombyx mori* silk fibroin were prepared from aqueous solutions of silk fibroin by several kinds of the insolubilization methods, some of which used methanol. The insolubilization appeared to be effected by the conformational transition of silk fibroin from the random coil to antiparallel β-sheet form, which was monitored with IR spectroscopy. In order to characterize the structure of the powder in the swollen state in water, a spin-labeled silk fibroin powder was prepared and the ESR spectra were observed, as well as ^{13}C-NMR. The heterogeneous structure was analyzed quantitatively in terms of the fraction of fast, slow, and very slow components in the ESR spectra. Finally, an enzyme, invertase, was immobilized with the silk fibroin powder. The thermal stability of the enzyme was much improved by the immobilization.

INTRODUCTION

The conformational transition of *Bombyx mori* silk fibroin membrane occurs easily from random coil form (water-soluble) to silk II, antiparallel β-sheet or silk I forms (water-insoluble) by only physical treatment such as stretching. The transition can be monitored with IR,[1-6] X-ray diffraction,[1-3,6,7] ^{13}C CP/MAS NMR,[4-9] and spin-labeling ESR methods.[10-12] On the basis of such a conformational transition, the enzyme immobilization using the silk fibroin membrane has been tried by us.[13-17] One of the merits for the purpose is simultaneous insolubilization of the membrane and immobilization of enzyme without using any chemical reagents. The properties of enzyme-immobilized silk fibroin membrane were excellent, as reported previously.[13-18] It is possible to form silk fibroin as fiber, gel, or powder as well as membrane depending on the purpose of use. Thus, to use silk is inherent merit in development of biomaterials.[13]

In this paper, the silk fibroin powders were prepared from the aqueous solution of the silk fibroin[19] by several kinds of the insolubilization methods. This is essentially based on the conformational transition of the silk fibroin.[1-13] The characteristics of the silk fibroin powders in the dry state after insolubilization were studied with IR spectroscopy. In addition, the spin-labeled silk fibroin powder was prepared in a similar manner for ESR study. The ESR spectra were observed in the swollen state and simulated by assuming the typical spectra for the fast, slow, and very slow motions of the spin-labeled site (the OH group of the tyrosine side chain).[20,21] The heterogeneous structure of the silk fibroin

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powder in the swollen state was analyzed quantitatively through the determination of the fraction of such three components. The immobilization of an enzyme, invertase, in the silk fibroin powder was tried and the activity of the immobilized enzyme was determined.

EXPERIMENTAL

Preparation of Silk Fibroin Powder

Preparations of the aqueous solution of silk fibroin and the membrane were described in the preceeding papers.[13-19] The silk fibroin powders were obtained as follows: The silk fibroin hydrogel can be obtained by standing the silk fibroin aqueous solution in a refrigerator for long times (about few weeks).[10] The silk fibroin powder (type I) was prepared by lyophilizing and crushing them. On the other hand, the gelation of the silk fibroin in aqueous solution can be also induced rapidly by adding 70% methanol aqueous solution to the silk fibroin aqueous solution.[13,14] The silk fibroin powder (type II) was also prepared from such hydrogel induced by methanol. Moreover, another methods were tried. The precipitate with bubble foam was prepared by stirring the silk fibroin aqueous solution vigorously with a hand mixer. Thus, the silk fibroin powder (type III) was obtained. The type III powder was then immersed into 80% methanol aqueous solution for 3 min and the powder (type IV) was obtained. The methods of the preparation were summarized in Table I together with the amounts of the elution of silk fibroin from the powders in water. The amounts were determined according to Lowry method.[22]

Enzyme Immobilization in the Silk Fibroin Powder

Invertase (from Bakers Yeast, Grade V) was purchased from Sigma Chemical Co. Ltd. Only supernatant solution of the enzyme after centrifugation (5000 rpm, 10 min) was used. The aqueous solution of the invertase was added to the silk fibroin aqueous solution and then the invertase-immobilized silk fibroin powders were obtained as described above.

TABLE I
Preparation of the Silk Fibroin Powders and the Amounts (wt %) of the Elution of Silk Fibroin from the Powders in Water during 3 Weeks at 4°C

Sample	Preparation	Amounts of elution (wt %)
Type I	Lyophilizing and crushing the *hydrogel* induced by *standing for long times*	0.6
Type II	Lyophilizing and crushing the *hydrogel* induced by *methanol*	0.6
Type III	Lyophilizing and crushing the *precipitate* induced by *stirring vigorously*	33.2
Type IV	*Methanol treatment* of Type III	0.7

Enzyme Activity Measurement

The activity of invertase was determined by the evaluation of the amounts of the product, glucose after the enzyme reaction with sucrose as substrate.[23] For the purpose, the glucose sensor with the glucose oxidase-immobilized silk fibroin membrane built up in our laboratory[13-17] was used. The invertase activity was determined at 40°C and pH 4.6 (0.2 *M* citrate buffer) after 30 min enzyme reaction.

Characterization of the Silk Fibroin Powder

The IR spectra of the silk fibroin powders were observed with a Shimazu IR-435 spectrometer. The ^{13}C-NMR spectra were obtained in water with a JEOL FX90Q NMR spectrometer at 22.5 MHz. The ESR spectra of the silk fibroin powders which labeled the tyrosine side chain groups with a nitroxide radical compound[10] were measured in water with a JEOL FE-3AX ESR spectrometer at X-band. Details of the preparation of the spin-labeled silk fibroin (the spin-labeled site; the OH group of the tyrosine side chain) were described elsewhere.[10]

RESULTS AND DISCUSSION

The Structure of the Silk Fibroin Powders in Solid State

The amounts of the elution of silk fibroin from four kinds of silk fibroin powders (types I–IV) in water after standing for 3 weeks at 4°C were listed in Table I. Among the silk fibroin powders, 33 wt % of the type III powder was dissolved in water. On the other hand, other three kinds of powders were insoluble (the amounts of the elution were less than 1%). This means that the insolubility of the powder cannot be obtained only by stirring the silk fibroin aqueous solution vigorously.

The IR spectra (amide V band) of four kinds of silk fibroin powders, B–E are shown in Figure 1. The IR spectrum of the silk fibroin membrane which is soluble in water is also shown [Fig. 1(A)]. The conformation of the water-soluble membrane is essentially a random coil[4-7] as well as that of the silk fibroin in aqueous solution.[24,25] In the IR spectra of these samples, the absorptions at 650 and 700 cm^{-1} have been assigned to the random coil form and the antiparallel β-sheet form, respectively.[6] The intensities of the absorptions at 650 cm^{-1} in the spectra B, C, D, and E were relatively weaker than that in the spectrum A, indicating that the conformational transition from the random coil to antiparallel β-sheet form partly occurred in the powders (types I–IV). The appearance of the absorption at 700 cm^{-1} confirms the transition.[1-6] However, the relative intensity of 700 cm^{-1} absorption is weak in the spectrum D compared with other three spectra, B, C, and E. In the amide I, II, and III bands, similar tendencies were obtained among four kinds of powders. Thus, it is concluded that the conformational transition (random coil $\rightarrow$ β-sheet) occurring in the type III powder was insufficient to cause insolubility of the powder against water. This is in agreement with the data of the elution (Table I).

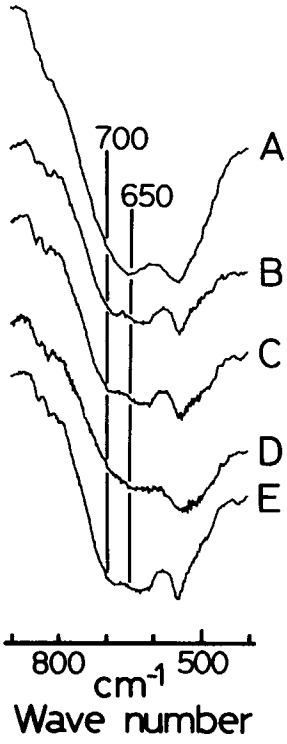

Fig. 1. IR spectra of the water-soluble silk fibroin membrane (A) and the silk fibroin powders [(B) type I; (C) Type II; (D) type III; (E) type IV). The preparation of the powders was described in the text.

However, from only these IR data, it is difficult to evaluate the fraction of random coil and β-sheet forms in the samples. We tried here to evaluate the fraction on the basis of the correlation between IR and ^{13}C CP/MAS NMR data of the silk fibroin as reported previously[6] and also the ^{13}C CP/MAS NMR spectra of the lyophilized fibroin and those immersed in methanol observed recently.[9] Especially, in the ^{13}C CP/MAS NMR spectra, the chemical shift of the C_β carbon peak of the alanine residue is different between these two forms and, thus, the fraction can be evaluated from the relative intensities of two peaks.[4-9] Finally, the fraction of β-sheet was 50–60% for the powders, types I, II, and IV, but 25% for the type III powder.

The Structure of the Silk Fibroin Powders in Swollen State

Since the silk fibroin powder is used in water as an immobilized enzyme support as mentioned below, it is important to clarify the structure of the powder swollen in water. Figure 2 shows ^{13}C-NMR spectra of the silk fibroin in aqueous solution[19] and type I powder swollen in water. The silk fibroin powder gives high resolution NMR spectrum, although the observable peak intensities decrease considerably compared with the case of the aqueous solution. Among the peaks, the peak intensity of the alanine C_β carbon is relatively strong, indicating that there still remains rapid threefold rotation around the

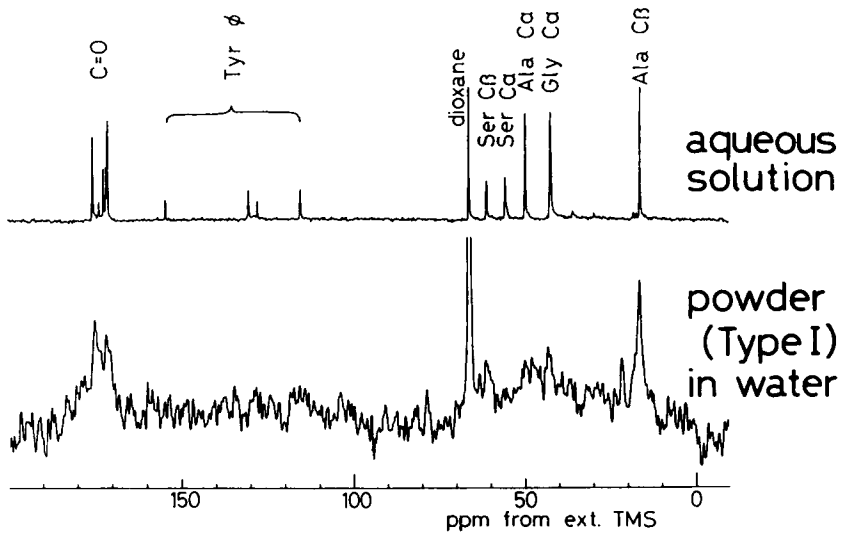

Fig. 2. ^{13}C-NMR spectra of the silk fibroin in aqueous solution and the silk fibroin powder (type I) swollen in water.

C_α—C_β bond of the Ala side chain in the silk fibroin powder swollen in water. This is in agreement with the conclusion obtained from the ^{2}H quadrupole splitting of [Ala-3,3,3-$^2H_3$]-fibroins in the solid state.[26] Namely, such a rapid threefold rotation occurs even in the solid state. The peaks of the carbonyl carbons were also observed. Among them, the mobility of the Ala carbonyl group was also relatively high. Thus, there is high-mobile domain in the swollen silk fibroin powder which gives high resolution ^{13}C-NMR peaks.

Quantification of the Heterogeneous Structure of the Swollen Silk Fibroin Powder by Spin-Labeling ESR

For the purpose, the ESR spectra of the spin-labeled silk fibroin powders (types I, II, and IV) swollen in water were measured. The side chain of the tyrosine residue was spin-labeled with a nitroxide radical compound.[10] These ESR spectra were complex (Fig. 3), indicating the presence of several kinds of components in the powders as viewed from mobility of the tyrosine side chain (the spin-labeled site). The mobility decreases qualitatively in the order of types I, II, and IV powders. Assuming that the ESR spectrum consists of three components, i.e., fast, slow, and very slow ones from viewpoints of the motion in the ESR time scale, the fraction of each component was determined from the computer spectrum simulation[20,21] as shown in Figure 4. The results were listed in Table II. The fraction of the fast component which corresponded to the rotational correlation time τ_c, of the order of 10^{-10} s, decreases in the order of types I, II, and IV powders. Conversely, the fraction of very slow component which corresponded to $\tau_c > 10^{-8}$ s. increases in that order. These data indicate that the immersion of the silk fibroin powder in methanol promotes extremely creation of the immobile domain in the powder. Especially, the conformational transition from water-soluble random coil form to water-insoluble

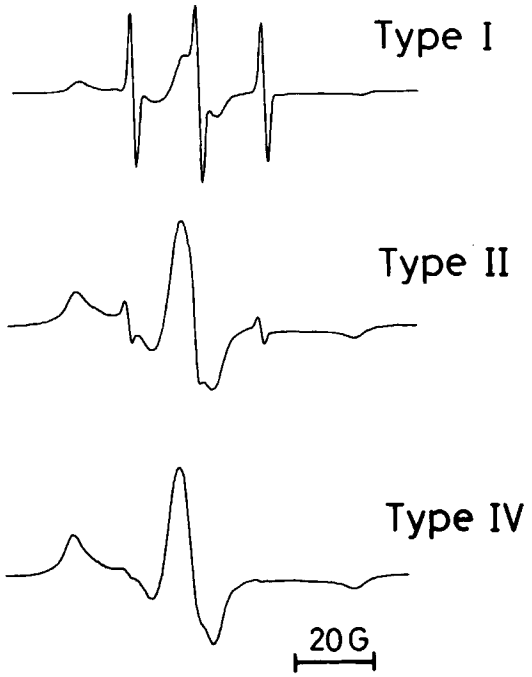

Fig. 3. ESR spectra of the spin-labeled silk fibroin powders (types I, II, and IV) swollen in water. The tyrosine side chain groups are spin-labeled with a nitroxide radical compound.

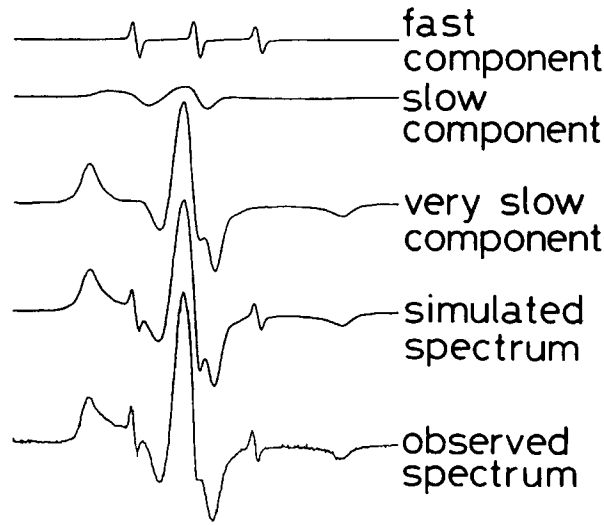

Fig. 4. An example of the ESR spectrum simulation. The ESR spectrum of the spin-labeled silk fibroin powder (type II) was simulated by assuming the spectra of fast, slow, and very slow components which correspond to the rotational correlation times of 10^{-10}, 10^{-9}, and 10^{-8} s, respectively. The fraction of the fast, slow, and very slow components was determined as 0.01, 0.28, and 0.71, respectively.

TABLE II
The Fraction of Fast, Slow, and Very Slow Components Determined from the ESR Spectrum Simulation of the Spin-Labeled Silk Fibroin Powders Swollen in Water[a]

Sample	Component		
	Fast	Slow	Very slow
Type I	0.32	0.12	0.57
Type II	0.01	0.28	0.71
Type IV	0.00	0.00	1.00

[a] The ESR spectra are shown in Figure 3.

β-sheet form also occurs for the case of type III → IV. Concerning the heterogeneous structure of the silk fibroin membrane insolublized with methanol in the swollen state, we will report elsewhere in detail.[11,12] It is likely that a similar heterogeneous structure is essentially held for the case of the powder; the surface is the immobilized domain with β-sheet structure and the mobile domain is present in the inner part.

Immobilization of Invertase by Silk Fibroin Powder

An enzyme, invertase, was immobilized in silk fibroin powder in order to obtain the improvement of the enzyme properties.[13] The method of immobilization was described in the Experimental section. The leakage of invertase from the invertase-immobilized silk fibroin powders (types II and IV) was determined by evaluation the invertase activity of the supernatant after immersing them into the citrate buffer at 4°C and pH 4.6 for 1 week. The amount of the leakage was 50% for the type II powder and 16% for the type IV powder. Thus, type IV powder was used for the examination of the thermal stability of immobilized invertase. In this case, the soluble enzyme eluted from the powder was removed by careful washing with water.

The activity of free invertase decreased 8.9% of the original value observed at 40°C after thermal treatment; the enzyme was kept at 75°C for 30 min before the activity observation.[23] A similar experiment was done for invertase immobilized in silk fibroin powder (type IV); 88.5% of the original value was obtained. Thus, the degree of the inactivity of invertase after such a thermal treatment decreased remarkably by immobilization with silk fibroin powder. Such an increase of the thermal stability has already been reported for glucose oxidase immobilized in silk fibroin membrane.[13-17]

In conclusion, the silk fibroin powder, which was prepared by stirring the aqueous solution and then by immersing it into methanol, is useful for the purpose of enzyme immobilization.

References

1. K. Hirabayashi, H. Ishikawa, N. Kasai, and M. Kakudo, *Kogyo Kagaku Zashi*, **73**, 1381 (1970).

2. J. Magoshi, *Kobunshi Ronbunshu*, **31**, 765 (1974).

3. J. Magoshi, Y. Magoshi, and S. Nakamura, *J. Appl. Polym. Sci. Appl. Polym. Symp.*, **41**, 187 (1985).
4. T. Asakura, *Int. Wool. Text. Res. Conf. 7th*, **1**, 354 (1985).
5. H. Saito, R. Tabeta, T. Asakura, Y. Iwanaga, A. Shoji, T. Ozaki, and I. Ando, *Macromolecules*, **17**, 1405 (1984).
6. T. Asakura, A. Kuzuhara, R. Tabeta, and H. Saito, *Macromolecules*, **18**, 1841 (1985).
7. T. Asakura and T. Yamaguchi, *J. Seric. Sci. Jpn.*, **56**, 300 (1987).
8. H. Saito, M. Ishida, M. Yokoi, and T. Asakura, *Macromolecules*, to appear.
9. M. Ishida, T. Asakura, M. Yokoi, and H. Saito, *Macromolecules*, to appear.
10. T. Asakura, H. Yoshimizu, M. Tsukada, K. Setoyama, and K. Mitsuda, *Sen-i Gakkaishi*, **43**, 335 (1987).
11. H. Yoshimizu and T. Asakura, *J. Appl. Polym. Sci.*, to appear.
12. T. Asakura, H. Yoshimizu, and M. Kakizaki, *Biotechnol. Bioeng.*, to appear.
13. T. Asakura, *Bioindustry*, **4**, 36 (1987).
14. A. Kuzuhara, T. Asakura, R. Tomoda, and T. Matsunaga, *J. Biotechnol.*, **5**, 199 (1987).
15. M. Demura and T. Asakura, *Biotechnol. Bioeng.*, **33**, 598 (1989).
16. M. Demura, T. Asakura, and T. Kuroo, *Biosensor*, **4**, 361 (1989).
17. M. Demura, T. Asakura, E. Nakamura, and H. Tamura, *J. Biotechnol.*, **10**, 113 (1989).
18. T. Asakura, H. Yoshimizu, A. Kuzuhara, and T. Matsunaga, *J. Seric. Sci. Jpn.*, **57**, 203 (1988).
19. T. Asakura, Y. Watanabe, A. Uchida, and H. Minagawa, *Macromolecules*, **17**, 1075 (1984).
20. H. Sakai and Y. Imamura, *Bull. Chem. Soc. Jpn.*, **53**, 1749 (1980).
21. T. Asakura, K. Suzuki, K. Horie, H. Sakai, and Y. Imamura, *J. Biomed. Mater. Res.*, **16**, 529 (1982).
22. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **195**, 265 (1953).
23. K. Kawashima and K. Umeda, *Biotechnol. Bioeng.*, **16**, 609 (1974).
24. T. Asakura, *Mackomol. Chem. Rapid Commun.*, **7**, 755 (1986).
25. T. Asakura, Y. Watanabe, and T. Itoh, *Macromolecules*, **17**, 2421 (1984).
26. H. Saito, R. Tabeta, A. Kuzuhara, and T. Asakura, *Bull. Chem. Soc. Jpn.*, **59**, 3383 (1986).

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