Study on Porous Silk Fibroin Materials. I. Fine Structure of Freeze Dried Silk Fibroin

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ABSTRACT: Silk fibroin solution was prepared by dissolving the silk fibroin in triad solvent $CaCl_2 \cdot CH_3CH_2OH \cdot H_2O$. In this article we tested and analyzed the state of frozen silk fibroin solution and fine structure of freeze dried porous silk fibroin materials. The results indicated that the glass transition temperature of frozen silk fibroin solution ranges from −34 to −20°C, and the initial melting temperature of ice in frozen solution is about −8.5°C. When porous silk fibroin materials are prepared by means of freeze drying, if freezing temperature is below −20°C, the structure of silk fibroin is mainly amorphous with a little silk II crystal structure, and if freezing temperature is above −20°C, quite a lot of silk I crystal structure forms. © 2001 John Wiley & Sons, Inc. J Appl Polym Sci 79: 2185–2191, 2001

Key words: silk fibroin; porous materials; freeze drying; fine structure; glass transition

INTRODUCTION

Silk fibroin is a kind of native fibrous polymer produced by domestic silk worms (*Bombyx mori*) and chiefly consists of repeated polypeptide sequence Gly–Ala–Gly–Ala–Ser.[1] It has many unique physical and chemical properties and good biological compatibility that is especially attractive. In nontextile fields, in addition to being used as surgical sutures, food additives, and cosmetics industries,[2–4] silk fibroin has been studied in recent years as enzyme-immobilization materials,[5] wound covering materials,[6] antithromboplastic materials,[7] dialysis membranes,[8] and soft contact lenses.[9] Meanwhile, the practical application of silk fibroin gel and porous materials in such biomedical fields as controlled drug-delivery carriers, cell culture substrates, and artificial skins has also been considered.[10–12] The results of various animal and clinical experiments with fibroin membrane used as wound protective materials indicated that silk fibroin has no toxicity or irritation, and is of good biocompatibility.[13] Based on the good physical and chemical properties of silk fibroin, it is possible to prepare porous silk fibroin materials with required fine structure, morphological structure, physical, and chemical properties through controlling the preparation conditions. Maybe such materials will be applied in biomedical fields more and more.

As a kind of biopolymer, high-purity silk fibroin fiber can be obtained easily from degummed silk. It can be dissolved with neutral salt solution such as LiSCN, LiBr, and $CaCl_2$.[14,15] High-purity silk fibroin solution is prepared through dialyzation. It can be used to prepare different kinds of

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silk fibroin-based materials such as gel, powder, and membranes. Freeze drying is a feasible method to prepare porous silk fibroin materials.

This series of studies aims to apply silk fibroin in biomedical fields such as porous drug-delivery device, cell culture substrate, and artificial skin. What these studies deal with include the relationship between preparing conditions and fine structure; morphological structure; physical and chemical properties of porous silk fibroin materials prepared by means of freeze drying; methods of preparing different kinds of porous silk fibroin materials with different structures and characteristics; the possibility of applying them in biomedical fields.

Shimizu[16] and Kratky[17] discovered the crystal form of silk fibroin and called them α-form (silkI) and β-form (silkII), respectively. Following them, some researchers studied the crystal structure and characteristics of silk I and silk II in more detail.[18,19] It is inevitable that the fine structure of freeze-dried silk fibroin should affect its physical and chemical properties. Magoshi[20] and Kataoka[21] have studied the change of crystalline polymorph of freeze dried fibroin, but the fibroin are collected from posterior division of the silk gland in full-grown larvae of silkworm. What is the crystalline polymorph of freeze dried silk fibroin like, which is obtained by dissolving silk fibroin in triad solvent $CaCl_2 \cdot CH_3CH_2OH \cdot H_2O$? This is a very important problem for preparing porous silk fibroin materials by way of freeze drying. In this article, as one of the serial studies, silk fibroin solution was prepared by dissolving silk fibroin in triad solvent $CaCl_2 \cdot CH_3CH_2OH \cdot H_2O$. Then it was frozen quickly and its state change during increasing of the temperature was determined. The change of fine structure of silk fibroin freeze dried in different conditions was studied by means of X-ray diffractometry, differential scanning calorimetry, density method, etc.

EXPERIMENTAL

Preparation of Silk Fibroin Solution

The domestic (*Bombyx mori*) silks were treated three times with 0.05 wt % Na_2CO_3 solution at 98 $\sim$ 100°C for 30 min respectively to remove sericin. Then they were rinsed and air dried. The pure silk fibroin fibers were dissolved with triad solvent $CaCl_2 \cdot CH_3CH_2OH \cdot H_2O$ (mole ratio = 1 : 2 : 8) at 78 $\pm$ 2°C through stirring. The prepared solution was purified by dialyzed against water for three days, to obtain 2.7 wt % silk fibroin solution. Then it was stirred slowly at 37 $\pm$ 2°C to make it evaporate and concentrate up to 7.4 $\sim$ 16.7 wt %.

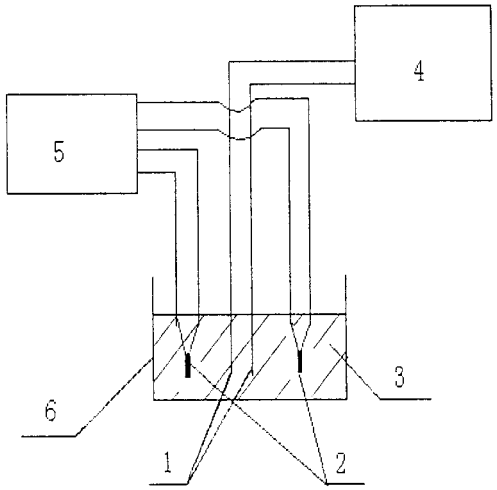

Figure 1 Electric resistance determining set of frozen silk fibroin solution. (1) electric resistance sensor, (2) temperature sensor, (3) silk fibroin solution, (4) electric resistance detector, (5) temperature detector, and (6) vessel.

State Testing of Frozen Silk Fibroin Solution

Differential scanning calorimetry (DSC) measurements were performed on a Perkin-Elmer-DSC-7 analysis system. The silk fibroin solution was frozen quickly in liquid nitrogen, then its DSC curve was determined and recorded in the course of increasing temperature at a speed of 10°C/min in a nitrogen atmosphere.

Electric resistance measurements were carried out by the set shown in Figure 1. Silk fibroin solution was poured into vessel 6 for quick freezing. Its temperature was increased gradually, and the electric resistance value displayed on electric resistance detector 4 recorded.

Preparation of Porous Silk Fibroin Materials (Freeze Drying)

Silk fibroin solution was poured into aluminum vessels and frozen for 6 h at different temperatures (−80, −60, −40, −20, −16, −12, −8, and −4°C). Then they were vacuum dried for 24 $\sim$ 48 h in VIRTIS GENNSIS 25-LE freeze dryer. Finally, spongy porous silk fibroin materials were obtained.

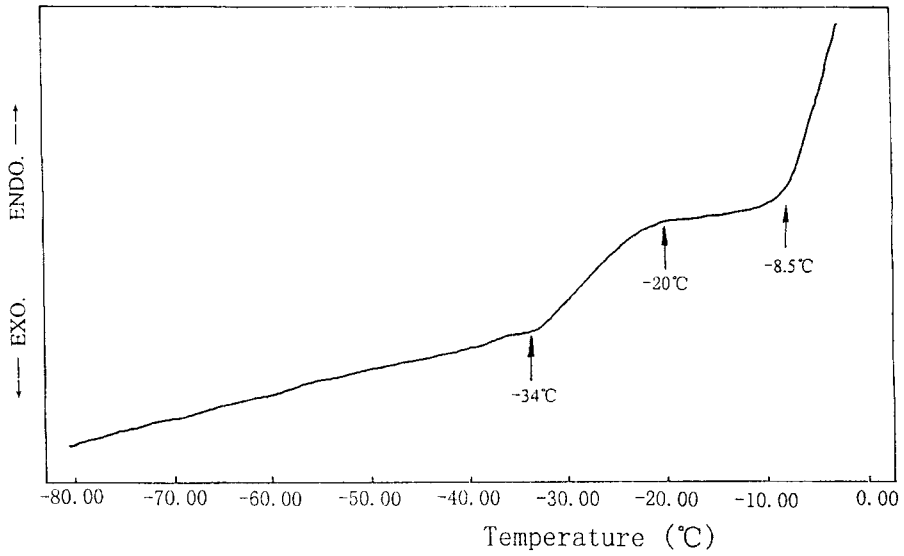

Figure 2 DSC thermogram of quick-frozen aqueous solution of silk fibroin.

Fine Structure Analysis of Porous Silk Fibroin Materials

The X-ray diffractometric measurement was performed by a diffractometer (Rigaku Denki Co., Ltd D/max-3C) with CuKα radiation. The voltage and current of the X-ray source were 40 kV and 40 mA, respectively. The diffraction intensity curves were measured at a scanning rate of 2°/min, and within the scanning region of $2\theta = 5 \sim 40°$.

DSC measurements were performed on a Perkin-Elmer-DSC-7 analyzing system. Porous silk fibroin materials were cut into microparticles with radius less than 40 μm. The fibroin particles compressed in sealed aluminum cell were swept with N_2 gas during the course of the analysis. The scanning rate, heating rate, and sample weight were 20.0°C/min, 20°C/min, and 2 mg, respectively.

The density of porous silk fibroin materials were measured with the column in a xylene/carbon tetrachloride mixture system at 25 ± 1°C. First, cut a piece of porous silk fibroin materials with the size of 3 × 3 mm and put it in the xylene to get out of the gas centrifugally at a speed of 2000 rpm for 30 min. Then carefully put it into the density-gradient tube. Finally calculated its density according to the position of the sample in the density-gradient tube after 24 h.

RESULTS AND DISCUSSION

State Change of Frozen Silk Fibroin Solution

Freeze drying is used as a gentle dehydration method to dry materials sensitive to heat especially in food and pharmaceutical industries. Most materials, including dissolved sugars in water, biopolymers, etc., do not crystallize during freezing. In the course of freezing, water is taken out in the form of ice, and the freeze-concentrated solution is amorphous. When the temperature is decreased to below the glass transition temperature T'_g, the solution solidifies into a glassy state, due to which the formation of ice ceases.[22] Because the viscosity of freeze-concentrated solution increases and it delays the formation of ice, there is still quite a part of water not taken out of the solution in the form of ice during the transition into glassy state.[23] DSC curve of the freeze-concentrated sugar solution during heating shows a glass transition temperature T'_g, which is followed by ice melting endotherm T'_m.[24] High molecular weight materials, such as carbohydrate polymer, exhibit improved dehydration characteristics, and they have T'_g and T'_m close to the melting point of pure ice.[25] The amorphous, glassy structure typical of freeze dried materials is formed during prefreezing, and retained until the removal of ice and the unfrozen water from the freeze-concentrated material.

Figure 2 shows the DSC curves of the silk fibroin solution quick frozen in liquid nitrogen during heating. Within the range of $-80 \sim 0$°C, there appears twice fairly quick changes toward the endotherm. At the first time, the changing range is $-34 \sim -20$°C, and at the second time, the initial temperature of change is -8.5°C.

Figure 3 shows the change of electric resistance of frozen silk fibroin solution during heat-

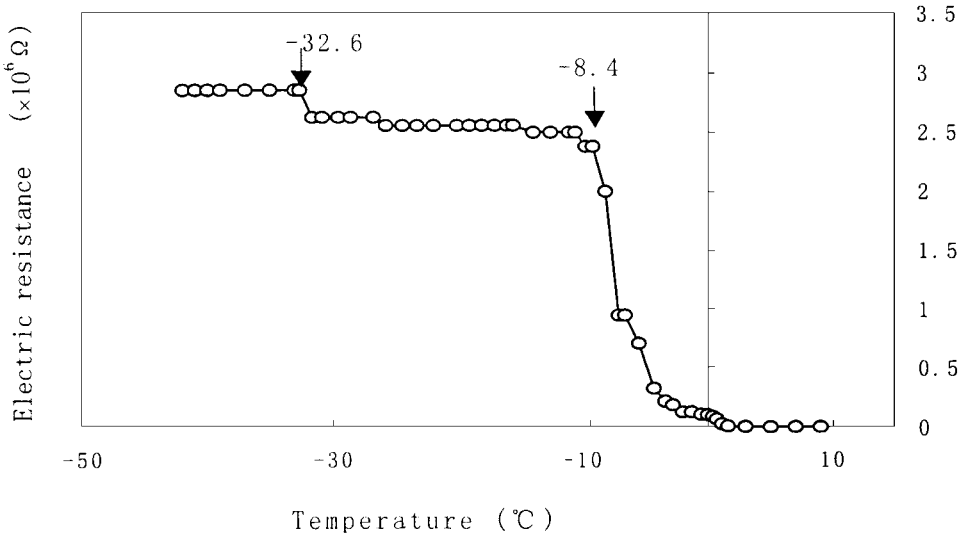

Figure 3 Variation of electric resistance of frozen silk fibroin solution during heating.

ing. When heated from −42 to 10°C, the solution also exhibited twice fairly quick change to lower electric resistance and their initial temperatures are −32.6 and −8.4°C respectively.

The silk fibroin solution is principally in random coil structure.[26] As will be discussed later, when it is frozen at low temperature, its structure is mainly amorphous. There are only local vibrations of small mobile cell such as side radicals, chain elements, and short side chains, alterations of bond length and bond angle in silk fibroin at glassy state, for heat kinetics energy is low and chain segments are at "frozen" state. However, in the glass transition zone, chain segments begin to "thaw," or the movement of chain segment is stimulated. As a result, heat capacity increases, DSC curves shift to endotherm, electric conductivity increases, and electric resistance decreases. Similarly, refractive index, modulus, dielectric constant, and heat of swelling index should all change. Nagura[27] determined the NMR absorption spectra of amorphous silk fibroin. The result showed that in the first derivation of Broad Line NMR spectra, there appeared a wide peak below −30°C and a narrow peak at about −30°C, and the higher the temperature the more sharp the narrow peak. It can be observed in the experiments of this article that the appearance of the materials prepared from silk fibroin solution frozen below −20°C was white and their soft handle improved. However, when the fibroin solution was freeze dried above −20°C, they took on light yellow and the softness decreased. We also found in our subsequent studies that there was a big difference in morphological structure and physical characteristics between porous silk fibroin materials vacuum dried above −20°C and those below −20°C. Therefore it can be inferred that −34°C in Figure 2 and −32.6°C in Figure 3 correspond to the glass temperature T'_g of frozen silk fibroin solution, its glass transition zone is about −34 to −20°C, and the initial melting temperature T'_m of the ice in frozen solution is about −8.5°C.

There are quite a number of amino acids with polar side groups in silk fibroin such as Ser, Tyr, Glu, and Asp that have strong affinity to water, accordingly have much lower steam pressure compared with that of pure ice. As a result, the initial melting temperature of ice in frozen silk fibroin solution is about 8.5°C lower than that of pure ice.

Fine Structure of Freeze-Dried Silk Fibroin

Figure 4 and Figure 5 are the X-ray diffraction intensity curves of porous silk fibroin materials prepared with silk fibroin solutions of concentration 2.7 and 16.7 wt % respectively, and were freeze dried at different temperatures. The principal diffraction peaks of silkI crystal structure are 7.25 Å (12.2°, ms), 4.5 Å (19.7°, s), 3.60 Å (24.7°, m), 3.16 Å (28.2°, m),[18] and that of silk II are 9.7 Å (9.1°, ms), 4.69 Å (18.9°, ms), 4.30 Å (20.7°, vs).[16] As shown in Figure 4, the major structure of silk fibroin freeze dried at −80 ~ −20°C is amorphous with a little silkII, and when the silk fibroin is freeze dried at −16

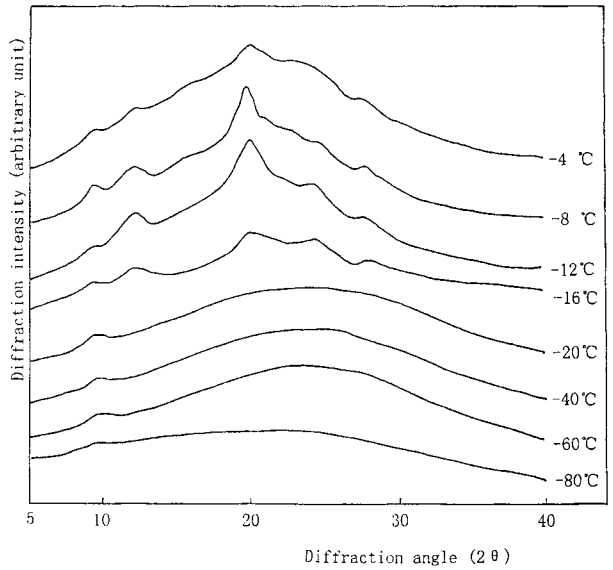

Figure 4 X-ray diffraction intensity curves of silk fibroin freeze dried at different temperature (concentration of silk fibroin solution is 2.7 wt %).

~ -4°C, quite a lot of silkI forms. In the course of freeze drying, within the range of $-20 \sim -8$°C, the crystallinity increased significantly, but it decreased a little at -4°C compared with $-16 \sim -8$°C. Figure 5 exhibits a similar behavior to Figure 4. But owing to higher concentration of

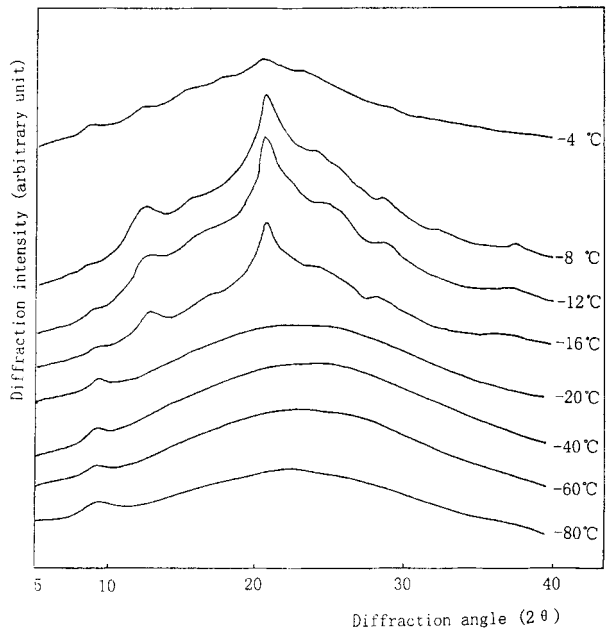

Figure 5 X-ray diffraction intensity curves of silk fibroin freeze dried at different temperature (concentration of silk fibroin solution is 16.7 wt %).

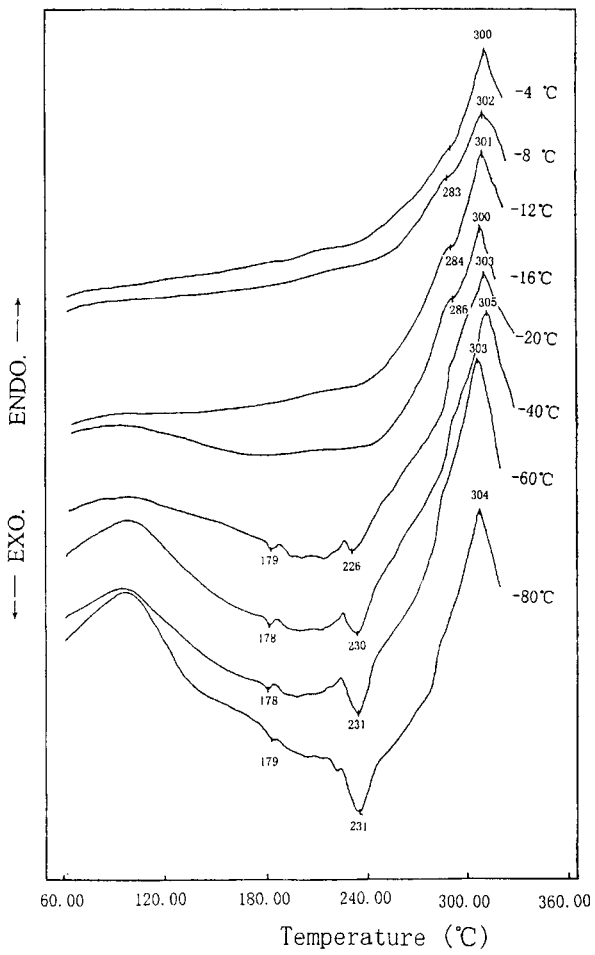

Figure 6 DSC thermogram of porous silk fibroin materials freeze dried at different temperature (concentration of silk fibroin is 4.5 wt %).

silk fibroin solution, the crystallinity in Figure 5 is a little higher than that of silk fibroin solutions with lower concentration in Figure 4, when the freeze drying temperature is from -16 to -4°C.

Magoshi[28] investigated the thermal behavior of amorphous silk fibroin films. The results indicated that the exotherm at $207 \sim 231$°C is attributed to the transition of silk fibroin from amorphous to silk II, and that the endotherm at $290 \sim 300$°C is attributed to the thermal decomposition. When they are treated with methanol, the endothermic and exothermic peaks before thermal decomposition disappear gradually, with an increase of treatment time and crystallinity.[29] According to our experimental results, as showed in DSC curve of Figure 6, for porous silk fibroin materials prepared by freeze drying, the transition from amorphous structure to silk II takes place at $226 \sim 231$°C and the endothermic peak at

300 ~ 305°C attributes to thermal decomposition. There are several endothermic and exothermic peaks for porous silk fibroin materials frozen below −20°C. Especially, the endothermic peak at 226 ~ 231°C is quite obvious, which is the transition temperature from amorphous to crystal structure. This indicates that porous silk fibroin materials are mainly amorphous. However, in the DSC curves of porous silk fibroin materials frozen above −20°C, all the endothermic and exothermic peaks disappeared at 178 ~ 231°C. This indicates that the crystallinity increases significantly.

The endothermic peak of water is about 100°C as shown in DSC curve of porous silk fibroin materials (Fig. 6). Within −80 ~ −4°C, the endothermic peak of water in the DSC curve weakens gradually, with the increase of freezing temperature. This may be caused by the increase of the crystallinity of silk fibroin, which weakens its absorptive capacity of water. When the freezing temperature is within −16 to −4°C, there appears a flat site at 283 ~ 286°C in the DSC curve, which is still kept for further investigation.

Because of the ordered and close packing of the silk fibroin molecule in the crystalline region, the density ρ_c in the crystalline region is higher than ρ_a in the amorphous region. Similar to other polymers, the crystallinity of silk fibroin that is made up of the crystalline region and amorphous region can be reckoned according to its density ρ. As shown in Figure 7, when the freeze drying temperature is at −80 ~ −20°C, the density of porous silk fibroin materials are low (1.297–1.302 g/cm^3), and with the increasing of temperature, the density increases a little. This indicates that when the silk fibroin solutions in random-coil structure are freeze dried quickly at temperature below the glass transition zone (−34 ~ −20°C), the heat

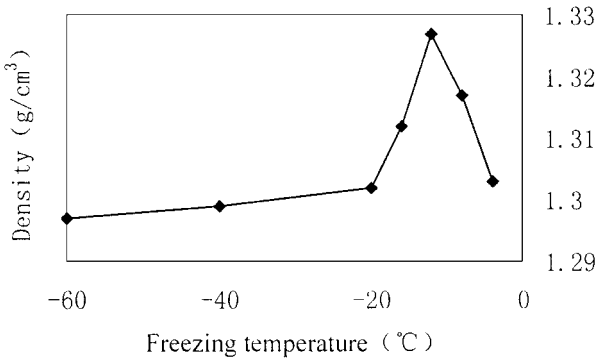

Figure 7 Influence of freezing temperature on density of freeze dried silk fibroin.

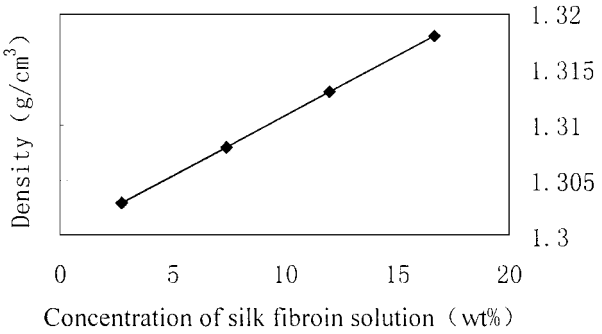

Figure 8 Influence of concentration of silk fibroin on density of freeze dried silk fibroin.

kinetics energy of chain segments is low and thereby it is difficult for silk fibroin to crystallize. Moreover, the lower the freezing temperature is, the more difficulty silk fibroin has crystallizing. Figure 8 also shows that the lower the concentration of silk fibroin solutions are before freeze dried, the more difficulty silk fibroin crystallizes.

When silk fibroin solutions are frozen at −20 ~ −8.5°C, the removal of ice makes the silk fibroin in random-coil structure concentrated. In the meantime, the distance between chain segments in different coils shortens, and the spatial density of chain segments increases, so that they can run through each other. At this point, the molecular heat kinetic energy is high and chain segments can rotate around the main axis and make molecules stretch—namely, the variation of conformation make it possible to form a partly ordered structure. Thus the silk fibroin by vacuum drying exhibits large crystallinity and density (−16 and −12°C in Fig. 7).

CONCLUSIONS

Dissolving silk fibroin with triad solvent $CaCl_2 \cdot CH_3CH_2OH \cdot H_2O$, by testing and analyzing the state of frozen silk fibroin solutions and the fine structure of freeze dried silk fibroin, we obtained the following conclusions:

It can be believed that the glass transition zone of frozen silk fibroin solutions is −34 ~ −20°C and the initial melting temperature of ice in frozen fibroin solutions is about −8.5°C.

When porous silk fibroin materials are prepared by means of freeze drying, their fine structure is mainly amorphous with a little silk II if the freeze drying temperature is below −20°C;

and if the freeze drying temperature is above −20°C, quite a lot of silk I forms.

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REFERENCES

1. Kaplan, D.; Adams, W. W.; Farmer, B.; Viney, C. Silk Polymers, Materials Science and Biotechnology, ACS Symposium Series No. 544; American Chemical Society: Washington, DC, 1994.
2. Minoura, N.; Tsukata, M.; Nagura, M. Biomaterials 1990, 11, 430.
3. Asakura, T. Bioindustry 1987, 4, 878.
4. Chen, K.; Iura, K.; Takano, R.; Hirabayashi, K. J Sericuet Sci Jpn 1992, 62, 56.
5. Demura, M.; Xomura, T.; Asakura, T. Sen-i Gakkaishi 1990, 46, 391.
6. Tusbouchi, K. PCT Int. Appl., WO 9726927.
7. Tamada, Y. Jpn kokai Tokkyo koho, JP09227402.
8. Li, M. Z.; Saito, K.; Higa, M.; Tanioka, A. Nippon Kaisui Gakkaishi 1996, 150, 443.
9. Minoura, N. Biomedical Applications of Polymeric Materials; Tsuruta, T., et al., Eds.; CRC Press: Boca Raton, FL, 1993; p 128.
10. Demura, M.; Asakura, T. J Membr Sci 1991, 59, 39.
11. Tsukada, M.; Freddi, G.; Minoura, N.; Allara, G. J Appl Polym Sci 1994, 54, 507.
12. Sijia, M.; Toshinobu, N.; Akira, T.; Koji, A. Sen-i Gakkaishi 1998, 54, 85.
13. Wu, C. Y.; Tian, B.; et al. Properties and Applications of Wound Protective Membrane Made from Fibroin, Third International Silk Conference, China, Suzhou, 1996.
14. Ajisawa, A. Nippon Sanshi Gakkaishi 1969, 38, 340.
15. Ajisawa, A. Nippon Sanshi Gakkaishi 1969, 38, 365.
16. Shimizu, M. Sanshi Shikenjo Houkoku 1941, 10, 475.
17. Kratky, O. Disc Faraday Soc 1951, 11, 171.
18. Hirabayashi, K.; Ishikava, H. Sen-i Gakkaishi 1967, 23, 538.
19. Konishi, T. Sen-i Gakkaishi 1968, 24, 550.
20. Magoshi, J. Kobunshi 1985, 34, 98.
21. Kataoka, K. Nippon Sanshi Gakkaishi 1974, 43, 483.
22. Roos, Y. H. J Therm Anal 1997, 48, 535.
23. Rasmussen, D.; Luyet, B. Biodynamica 1969, 10, 319.
24. Roos, Y. H.; Karel, M. Int J Food Sci Technol 1991, 26, 553.
25. Roos, Y. H.; Karel, M. J Food Sci 1991, 56, 1676.
26. Gotoh, Y.; Tsukada, M.; Minoura, N. Int J Biol Macromol 1996, 19, 41.
27. Nagura, M.; Ishikawa, H. Polym J 1979, 11, 159.
28. Magoshi, J. J Polym Sci Polym Phys Ed 1977, 15, 1675.
29. Magoshi, J. Kobunshi Ronbunshu 1974, 31, 648.