Structural and Conformational Changes of Regenerated *Antheraea pernyi* Silk Fibroin Films Treated with Methanol Solution

HAE YONG KWEON, YOUNG HWAN PARK

Department of Natural Fiber Sciences, Seoul National University, Suwon 441-744, Korea

Received 16 November 1998; accepted 3 February 1999

ABSTRACT: The regenerated *Antheraea pernyi* silk fibroin films were prepared from calcium nitrate solution and crystallized with aqueous methanol solution. The structural and conformational changes were investigated by the X-ray diffraction method and infrared spectroscopy upon methanol treatment. The concentration and treatment time of aqueous methanol solution have a great influence on the conformation of regenerated films. The conformational change from a random coil to β-sheet structure was occurred within 5 min by the treatment of 40–60% methanol solution. As the methanol concentration increases up to 100%, the transition time of conformational change is delayed, and the structural change is not occurred in the case of 100% methanol used. The content of a α-helix, β-sheet, and random coil conformation was calculated and examined to find out the effect of methanol treatment on the conformation changes. The β-sheet structure can be transformed from a random coil, while the content of α-helix structure is not changed, regardless of methanol treatment. © 1999 John Wiley & Sons, Inc. J Appl Polym Sci 73: 2887–2894, 1999

Key words: *Antheraea pernyi* silk fibroin; calcium nitrate; methanol; conformation

INTRODUCTION

A silk protein is the product of silkworms, of which there are two general groups, domestic (*Bombyx mori*) and the wild type (*Antheraea pernyi,* etc.). The former has been widely studied as the source of textile fibers and biomaterials such as oxygen-permeable membranes, biosensor, and so on.[1–4] On the other hand, little works have been done for the wild silks due to a wide variety of their species and low productions. The structure and properties of wild silks are now well known; however, their applications are somewhat restricted because of the lack of dissolutions.

The native and regenerated *Bombyx mori* fibroin films consist of a random coil structure and, therefore, is soluble in water. As the conformation of *Bombyx mori* fibroin film changes to β-sheet crystalline structure by the methanol treatment, the film becomes water-insoluble. The treatment of aqueous methanol solution effectively promotes the crystallization of *Bombyx mori* fibroin film by a conformational change within 30 s.[5] The effective concentration, to induce the conformational transition of *Bombyx mori* fibroin film, is above 40% methanol solution. Therefore, many researchers have used pure or aqueous methanol to modify the conformation of *Bombyx mori* fibroin.

The molecular weight and crystallinity of *Antheraea pernyi* silk fibroin are known to be higher than those of *Bombyx mori* silk and the supermolecular structure, as well as amino acid composition, is different from each other. Therefore, the

Correspondence to: H. Y. Kweon, Department of Sericulture & Entomology, National Institute of Agriculture Science & Technology, Suwon, 441-100, Korea.

Journal of Applied Polymer Science, Vol. 73, 2887–2894 (1999)
 CCC 0021-8995/99/142887-08

behavior of conformational changes, due to the crystallization by methanol treatment, is expected to be different between regenerated *Bombyx mori* and *Antheraea pernyi* silk fibroin film.

The dissolution of *Antheraea pernyi* silk fibroin is hardly difficult, and the structural characteristics of regenerated forms have hardly been investigated. Tsukada et al.[6] reported that *Antheraea pernyi* silk fibroin films with α-helix crystalline structure could be prepared by casting an aqueous solution of native silk fibroin taken from the silk gland of mature larvae. They also investigated the structure and thermal properties of *Antheraea pernyi* silk fibroin film regenerated from lithium thiocyanate solution. The effects of casting conditions on the molecular conformation of native *Antheraea pernyi* fibroin films were studied[7,8] and the influence of heat and methanol treatment on the molecular conformation was also investigated.[9,10]

The aim of the present work is to prepare the regenerated *Antheraea pernyi* silk fibroin films from calcium nitrate solution and to investigate the structural and conformational changes of these films induced by immersion in methanol solution with different concentration and time. The X-ray diffraction (XRD) and infrared (IR) spectroscopic method were used for the analysis of the crystalline structure and molecular conformation. The contents of secondary structure of regenerated *Antheraea pernyi* silk fibroin films are also determined.

EXPERIMENTAL

Materials

The *Antheraea pernyi* silk fibers were degummed with enzymatic degumming method followed by dissolving in a 7*M* calcium nitrate solution for 3 h at 100°C.[11] The *Antheraea pernyi* silk fibers were treated with degummed solution (Alcalase 2.5 L from Novo Industri Co. 1 g/L, sodium bicarbonate 5 g/L, and nonionic surfactant 1 g/L) at 55°C for 60 min. The degummed fibers were rinsed in the mixture solution of nonionic surfactant 2 g/L and sodium hydrosulfite 5% on the weight of fiber (owf) and then throughly rinsed in warm distilled water. The degummed fibers were dried at room temperature and stored in desiccator prior to use.

According to our preparatory experiment, the dissolution of *Antheraea pernyi* silk fiber was required a high concentration of chaotropic salts, treatment temperature, and time. The *Antheraea pernyi* silk fiber could be hardly dissolved in the condition of less than 6*M* concentration and 100°C for prolonged times. The solubility of silk fiber was nearly 100% under the treatment of 7*M* calcium nitrate solution for 3 h at 100°C. If the dissolution conditions are severe, the molecular weight of silk fibroin is lessened; therefore, the film cannot be formed.

The solution was then dialyzed in cellulose tube (molecular cutoff = 3500) against distilled water for 5 days, with the dialysate replaced every 12 h. The solution was filtered through Whatman filter paper (Cat No 1002 150). The regenerated *Antheraea pernyi* silk fibroin films were prepared by casting the 0.3% solution on polystyrene plate as the substrate at 20°C and 40% relative humidity. The regenerated fibroin films were immersed in different concentrations of aqueous methanol solution in the range of 40–100% (v/v).

Measurements

A Fourier transform infrared (FTIR) spectrometer (M series, Midac Co., USA) was used for the qualitative and quantitative analysis of amide bands. The IR spectrum ranges examined in this study were 1400–500 cm^{-1}.

X-ray diffraction curves were obtained by using a D-MAX-3 Diffractometer (Rigaku Co.), with CuKα radiation (λ = 1.54 Å). The scanning speed is 0.5°/min and voltage and current of X-ray sources was 30 kV and 20 mA, respectively.

RESULTS AND DISCUSSION

FTIR Spectra

The IR spectroscopy is a powerful technique to study the molecular conformation of silk proteins. The IR spectra represent typical absorption bands sensitive to the molecular conformation of silk fibroin. The effect of aqueous methanol treatment could be examined for a conformational changes of regenerated *Antheraea pernyi* silk fibroin films by FTIR spectroscopy. Figure 1 shows the IR spectra of silk fibroin films treated with 40% aqueous methanol solution. The strong absorption bands, attributed to the α-helix structure, were obtained for a methanol–untreated film along with a characteristic band of the random coil conformation. In addition, the absorption band at 1240 cm^{-1}, assigned to the β-sheet and/or

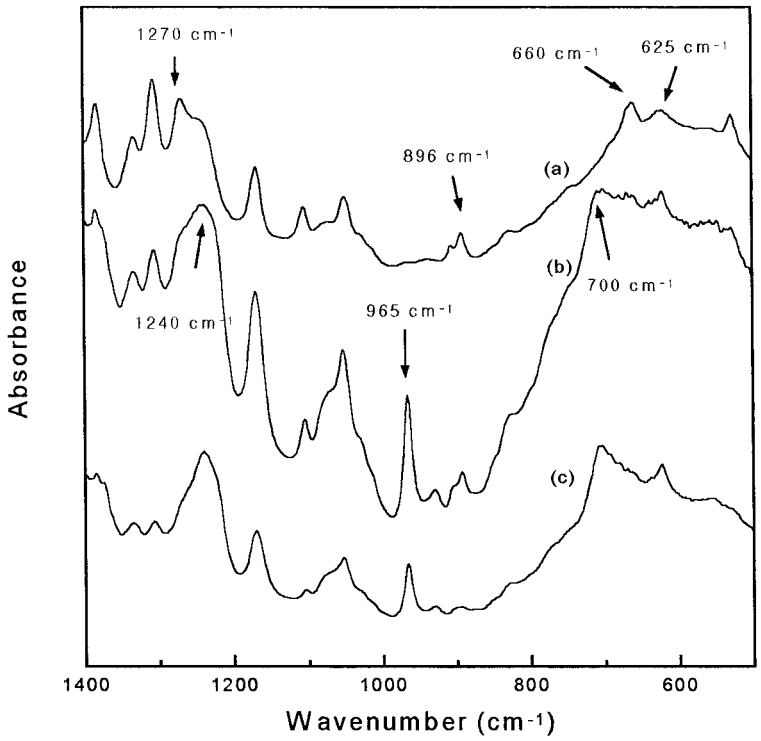

Figure 1 FTIR spectra of regenerated *Antheraea pernyi* silk fibroin film treated with 40% aqueous methanol solution: (a) untreated and (b) 5 and (c) 30 min.

random coil conformation, was also observed as a shoulder. It is reported[8,12] that the characteristic bands of a α-helix conformation appear at 1270 (amide III), 896 (amide IV), and 625 cm^{-1} (amide V), while that of a random coil conformation at 660 cm^{-1}.

After immersed in 40% aqueous methanol solution for 5 and 30 min, however, the films exhibited characteristic absorption bands of the β-structure at 1240 (amide III), 965 (amide IV), and 700 cm^{-1} (amide V), while the band of α-helix structure at 625 cm^{-1} (amide V) remained intact. Therefore, it seems that the structure of regenerated films is transformed from an α-helix and random coil conformation to a β-sheet structure within 5 min after being treated in 40% methanol solution. When 60% methanol solution was used, the IR spectra were very similar to those of 40% solution. The conformational changes did not occur in this range of methanol treatment conditions.

The IR spectra of the regenerated *Antheraea pernyi* silk fibroin films, treated with 70% aqueous methanol solution, are shown in Figure 2. As the immersion time is increased up to 120 min, the strength of absorption band at 660 cm^{-1} (attributed to random coil) and 896 cm^{-1} (attributed to α-helix conformation) was markedly decreased after 30 min of treatment time, while the strength of absorption band at 700 and 965 cm^{-1} (attributed to β-sheet structure) increased.

Contrary to the case of 40 and 60% methanol treatment, the random coil structure was maintained as a main conformation for the regenerated film immersed in 70% methanol solution for 5 min. Although a very small band appears at 965 cm^{-1}, it can be said that the transition to β-sheet conformation is occurring only partially as a initial stage. The sharp absorption bands at 965 and 700 cm^{-1} were appeared when the film immersed in 70% methanol solution for 30 min, indicating that the transformation occurs possibly from a random coil and α-helix to a β-sheet structure. Since the film immersed for 10 min shows an intermediate spectrum, having both α-helix and β-sheet characteristic bands, the conformational changes are significantly affected by the treatment time, dependent on a specific concentration of methanol solution.

The IR spectra of regenerated fibroin films treated with 80% aqueous methanol solution were similar to those for 70% solution. The only differ-

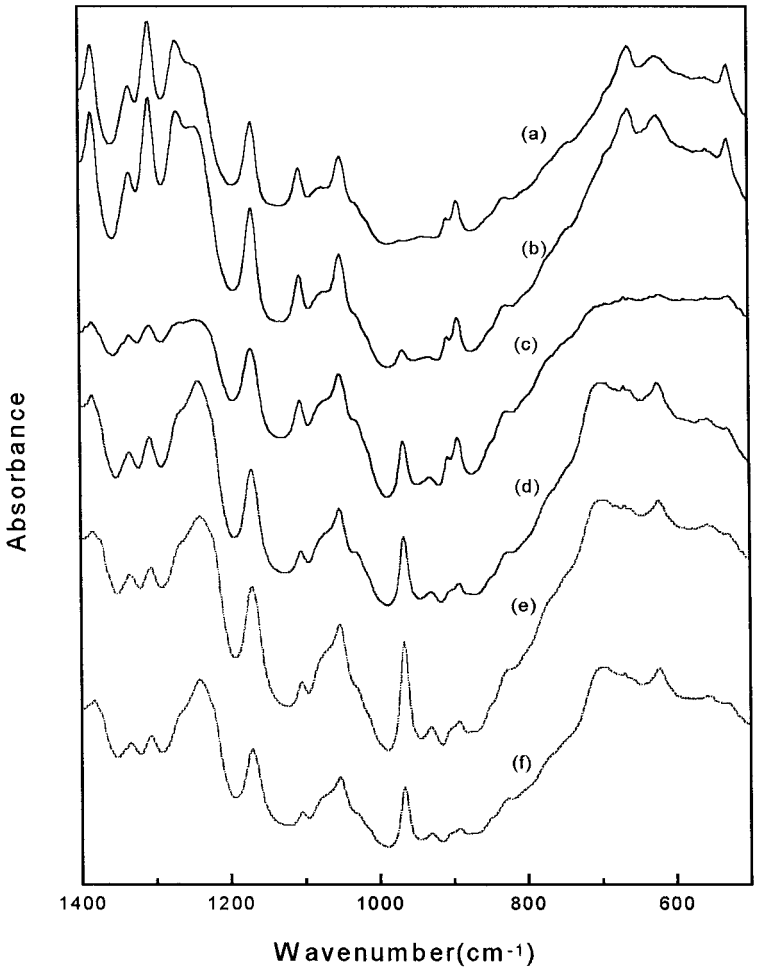

Figure 2 FTIR spectra of regenerated *Antheraea pernyi* silk fibroin film treated with 70% aqueous methanol solution: (a) untreated and (b) 5, (c) 10, (d) 30, (e) 60, and (f) 120 min.

ence is that the structural transition time was somewhat delayed compared with the case of 70% methanol solution.

As shown in Figure 3, however, the IR spectra of regenerated films treated with 100% methanol were not changed with time up to 120 min immersion time. It is concluded that as the treatment concentration of methanol solution increases, the transition or crystallization time of regenerated silk fibroin film is delayed. Moreover, the conformational changes did not occur interestingly in the case of 100% methanol treatment for the regenerated *Antheraea pernyi* silk fibroin films. This result is totally different from the case of methanol treatment of regenerated *Bombyx mori* silk fibroin films.

The regenerated *Antheraea pernyi* silk fibroin film was mainly composed of a α-helix and random coil conformation before crystallization. Considering the conformational structures of a native *Antheraea pernyi* fibroin film and regenerated fibroin film from lithium thiocyanate solution reported by others,[6,10] it can be concluded that the conformational structure of fibroin film was independent on the dissolution and preparation method and only dependent on the chemical composition of materials.

According to our results, the conformation of regenerated *Antheraea pernyi* silk fibroin film was significantly affected by the concentration as well as the treatment time of aqueous methanol solution. The random coil conformation was transferred to β-sheet structure within 5 min by the treatment of 40–60% methanol solution.

As a certain concentration of aqueous methanol solution is used for the crystallization, the

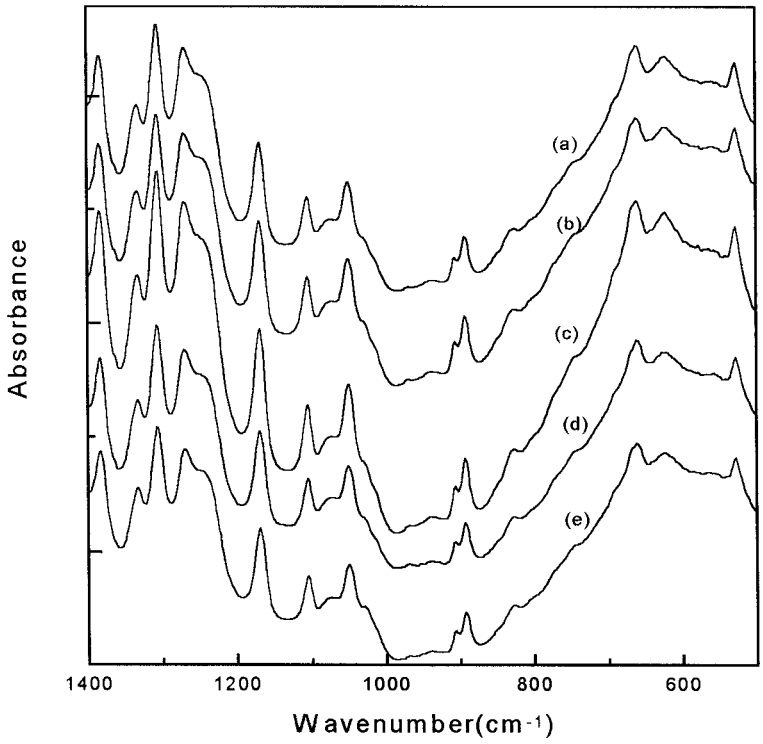

Figure 3 FTIR spectra of regenerated *Antheraea pernyi* silk fibroin film treated with 100% aqueous methanol solution: (a) untreated and (b) 5, (c) 30, (d) 60, and (e) 120 min.

action of two components, water and methanol, should be considered to explain the behavior of conformational changes. When the fibroin film was immersed in the aqueous methanol solution, both water and methanol diffuse into the fibroin film. The concentration gradient was expected to take place in the contact area between methanol solution and film. Since the water is known as a powerful swelling agent and methanol is a poor solvent for the silk fibroin, the water might be swelling the fibroin film first and then promoting the penetration of methanol inside. The rearrangement of the inter/intramolecular hydrogen bonds occurred, and this causes a conformational change from the random coil structure to the β-sheet structure.

X-ray Diffraction Curves

X-ray diffraction curves of regenerated *Antheraea pernyi* silk fibroin films, immersed in 80% aqueous methanol solution, are shown in Figure 4. The methanol–untreated film [Fig. 4(a)] is characterized by the presence of two peaks at 11.5 and 22.0°, corresponding to the α-helix crystalline spacing of 7.69 and 4.03 Å, respectively. These two peaks exhibit the characteristics of native *Antheraea pernyi* fibroin film, collected from the posterior division of the silk gland in full-grown larvae of *Antheraea pernyi,* with an α-helix crystalline structure.[8,10] The similar results were reported for *Antheraea pernyi* silk fibroin film regenerated from lithium thiocyanate solution.[6]

When the regenerated films were immersed in methanol solution for enough times [Fig. 4(c) and (d)], XRD curves of fibroin films immersed in 80% aqueous methanol solution exhibited different profiles from those of untreated one, with a major peak at about 20.0° and two minor peaks at about 16° and 24°. The crystalline spacing of 4.3 Å corresponding to the major peak is the characteristic of *Antheraea pernyi* silk films with typical β-sheet structure.[10] On the other hand, the transition state was observed for the specimen immersed in methanol for 10 min, showing major peak at 11.5° and two shoulder peaks at 20° and 22.0°. This indicates the simultaneous existence of both α-helix and β-sheet structure. The results obtained from X-ray diffractogram are consistant with those from FTIR spectra.

Analysis of Secondary Structures

The rate of structural transition of *Antheraea pernyi* fibroin film, regenerated from calcium nitrate solution, depends on the concentration of methanol solution and immersion time. The effect of methanol treatment on the structural transition of regenerated film was examined by FTIR spectroscopy. According to the IR spectroscopic analysis, the characteristic IR bands at 965 and 700 cm^{-1} were appeared, while the 1270 cm^{-1} band disappeared upon methanol treatment. The appearance or disappearance time of these characteristic bands can be measured to determine the rate of structural transition from α-helix and random coil to β-sheet form. Figure 5 shows the relationship between the transition time and methanol concentration for the regenerated *Antheraea pernyi* silk fibroin film. The transition was occurred within 5 min for the film immersed in 40–60% methanol solution. On the other hand, this occurred within 30 min in 70% and 60 min in 80% methanol solution.

The content of secondary structure of regenerated *Antheraea pernyi* fibroin films was calculated to estimate the conformational changes quantitatively on the methanol treatment. Bhat and Nadiger[13] reported the method for calculating the crystallinity index of silk fibroin by using the intensity ratio of the IR absorption bands at

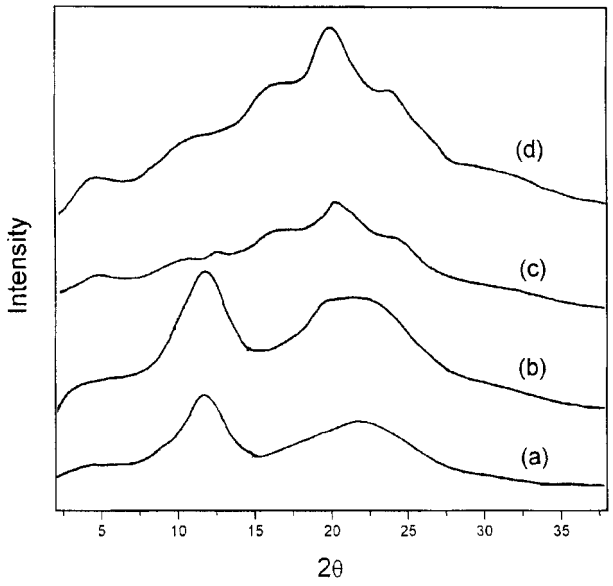

Figure 4 X-ray diffractograms of regenerated *Antheraea pernyi* silk fibroin film treated in 80% aqueous methanol solution: (a) untreated and (b) 10, (c) 60, and (d) 120 min.

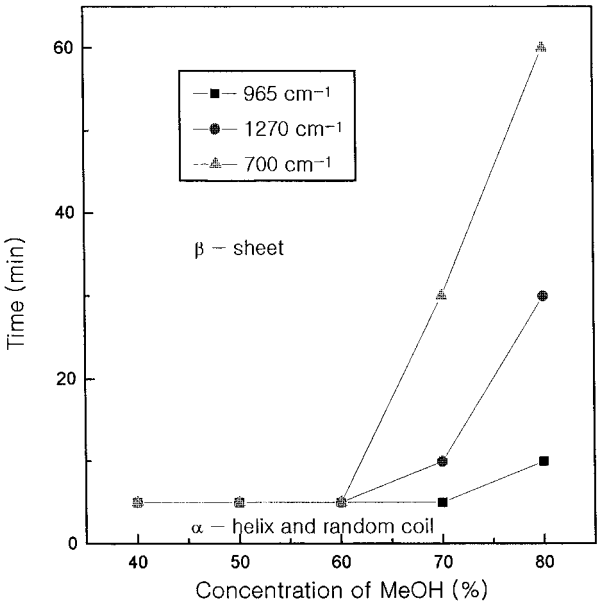

Figure 5 Relations between the transition time to β-sheet structure and methanol concentration for the regenerated *Antheraea pernyi* fibroin film.

1265 and 1235 cm^{-1}. However, in the case of *Antheraea pernyi* fibroin film, this method could not be used due to the uncertain calculated value of crystallinity index.[11]

The amide V frequencies of *Antheraea pernyi* silk fibroin were found to be a particularly sensitive absorption band for the conformational changes. Several researchers have used the amide V region to estimate the content of secondary structure of poly(L-alanine)[14] and poly(γ-methyl-L-glutamate).[15] Assuming a Gaussian function, each IR absorption band of amide V region can be separated into the three conformations, α-helix (625 cm^{-1}), β-sheet (700 cm^{-1}), and random coil (660 cm^{-1}) structure. Figure 6 shows a typical example of curve fitting of amide V region observed by using the Gram 386 program. The fitting curve is well consistent with observed one.

Figure 7 is the estimated content of secondary structure of regenerated *Antheraea pernyi* silk fibroin film treated with 60% aqueous methanol solution. The content of β-sheet structure increased abruptly when the film was immersed in methanol solution only for 5 min and then gradually increased with treatment time. The opposite trend was observed for the content of random coil structure with the immersion time. On the other hand, the content of α-helix structure was nearly constant at the value of about 30%, regardless of

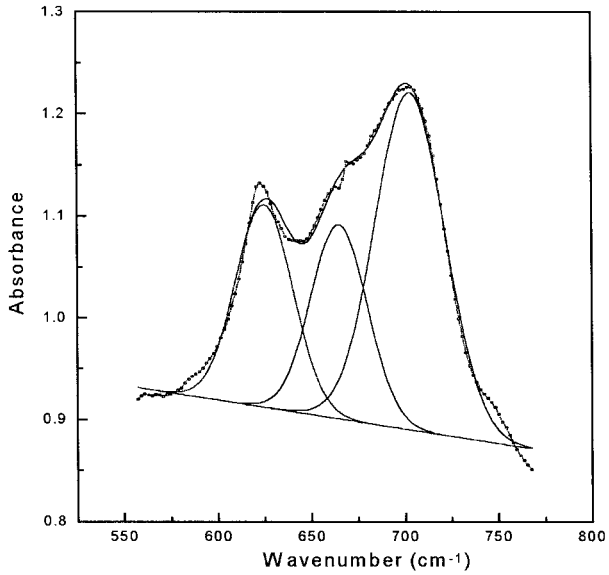

Figure 6 Typical example of the curve fitting of amide V region.

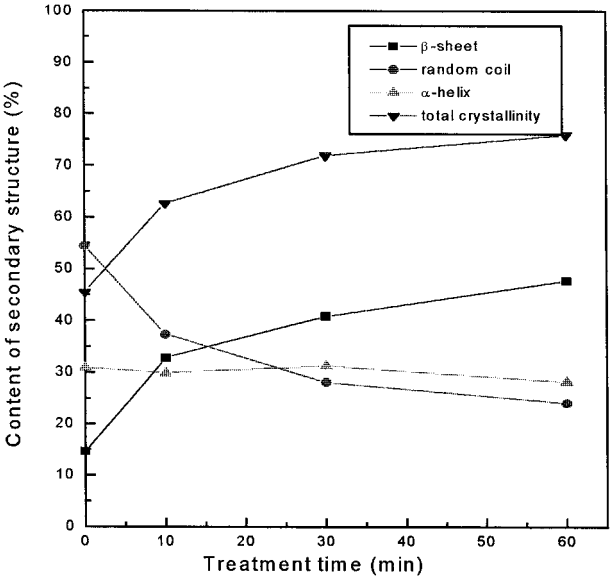

Figure 8 Content of secondary structure of regenerated *Antheraea pernyi* fibroin film treated with 80% aqueous methanol solution.

treatment concentration and time. The total crystallinity, which can be represented as the sum of β-sheet and α-helix content, was as high as 91% for the film immersed for 30 min. The change of content of secondary structure was coincide with the appearance of characteristic peaks of IR spectra.

Figure 8 shows the content of secondary structure of regenerated *Antheraea pernyi* silk fibroin film treated with 80% aqueous methanol solution. The trend of conformational changes was similar; however, the transition from a random coil to β-sheet structure was delayed, compared with the results shown in Figure 7. The total crystallinity was about 76% when the film was immersed in 80% methanol solution for 60 min. The crystallinity values of regenerated films becomes lowered as the concentration of methanol solution. Here, it is noticed that the α-helix content was not significantly changed with treatment time and concentration of methanol solution. This indicates that the β-sheet structure can be transformed from a random coil not a α-helix structure. In other words, the α-helix crystal in *Antheraea pernyi* fibroin film seems to be very stable. The similar results were reported by Tsukada et al., who have studied the annealing temperature dependence of the conformation of poly(L-alanine).[16]

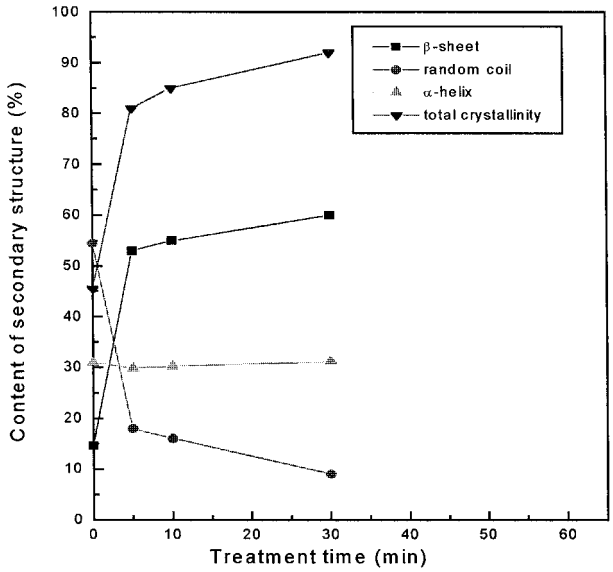

Figure 7 Content of secondary structure of regenerated *Antheraea pernyi* silk fibroin film treated with 60% aqueous methanol solution.

As a result of calculated contents of the α-helix, β-sheet, and random coil conformation, the contents were significantly changed with the concentration of methanol. The α-helix content was constant, regardless of methanol solution treatment, since the α-helix structure could be stabilized by intrahelical hydrogen bonding. However, the interhelical interaction in the α-helix crystal would be somewhat weakened after 60 min, on the basis of decrease in a peak intensity of XRD curves and IR absorption bands.

CONCLUSIONS

The regenerated *Antheraea pernyi* silk fibroin films were prepared from calcium nitrate solution and conformational changes of these films were investigated upon the crystallization with aqueous methanol solution. The crystallization behavior on a conformational transition is strongly affected by the concentration and treatment time of methanol solution, different from the case of *Bombyx mori* silk fibroin films.

The contents of secondary structure, a α-helix, β-sheet, and random coil conformation, were calculated by IR absorption bands of amide V region. The β-sheet structure was transformed from a random coil conformation by the methanol treatment, while a α-helix structure is particularly stable on the crystallization of *Antheraea pernyi* fibroin film.

REFERENCES

1. Minoura, N.; Tsukada, M.; Nagura, M. Biomaterials 1990, 11, 430.
2. Minoura, N.; Tsukada, M.; Nagura, M. Polymer 1990, 31, 265.
3. Kuzuhara, A.; Asakura, T.; Tomoda, R.; Matsunaga, T. J Biotechnol 1986, 5, 199.
4. Asakura, T.; Kitaguchi, M.; Demura, M.; Sakai, H.; Momatsu, K. J Appl Polym Sci 1992, 46, 49.
5. Magoshi, J.; Magoshi, Y. J Polym Sci, Polym Phys Ed 1979, 17, 515.
6. Tsukada, M.; Freddi, G.; Gotoh, Y.; Kasai, N. J Polym Sci, Polym Phys Ed 1994, 32, 1407.
7. Magoshi, J.; Nakamura, S. J Polym Sci, Polym Phys Ed 1985, 23, 227.
8. Tsukada, M. J Polym Sci, Polym Phys Ed 1986, 24, 457.
9. Freddi, G.; Monti, P.; Nagura, M.; Gotoh, Y.; Tsukada, M. J Polym Sci, Polym Phys Ed 1997, 35, 841.
10. Tsukada, M.; Freddi, G.; Monti, P.; Bertoluzza, A.; Kasai, N. J Polym Sci, Polym Phys Ed 1995, 33, 1995.
11. Kweon, H. Y. Ph.D. Thesis, Seoul National University, 1998.
12. Miyazawa, T.; Blout, E. R. J Am Chem Soc 1961, 83, 712.
13. Bhat, N. V.; Nadiger, G. S. J Appl Polym Sci 1980, 25, 921.
14. Tsukada, M.; Freddi, G.; Kasai, N. J Polym Sci, Polym Phys Ed 1994, 32, 1175.
15. Nagura, M.; Miyahara, S.; Ohkoshi, Y.; Ishikawa, H. Sen-i Gakkaishi 1991, 47, 130.
16. Tsukada, M.; Nagura, M.; Ishikawa, H. J Polym Sci, Polym Phys Ed 1987, 25, 1325.