

# Effect of Heat Treatment on the Structural and Conformational Changes of Regenerated *Antheraea pernyi* Silk Fibroin Films

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**ABSTRACT:** Regenerated *Antheraea pernyi* silk fibroin films prepared from calcium nitrate solution were treated with heat for crystallization. The structural and conformational changes caused by heat treatment were investigated with X-ray diffraction, infrared spectroscopy, and differential scanning calorimetry. The temperature and treatment time of heat greatly influenced the conformation of the regenerated films. The conformational change was caused by heat treatment over 230°C, and the transition was enhanced by increasing time and temperature. The contents of  $\alpha$ -helix,  $\beta$ -sheet, and random-coil conformations were calculated and examined to investigate the effect of heat treatment on the conformational changes. The  $\beta$ -sheet structure could be transformed from a random-coil conformation on heat treatment, whereas the content of  $\alpha$ -helix structure was nearly unchanged, regardless of heat-treatment conditions. © 2001 John Wiley & Sons, Inc. *J Appl Polym Sci* 81: 2271–2276, 2001

**Key words:** *Antheraea pernyi* silk fibroin; heat treatment; conformation

## INTRODUCTION

Several researchers have reported on the molecular conformations of native *Antheraea pernyi* (*A. pernyi*) fibroin film, which is taken from the posterior silk gland of full-grown silkworm larvae. The influences of casting temperature,<sup>1</sup> drying rate,<sup>2</sup> solvent,<sup>3</sup> and heat treatment<sup>4</sup> on the molecular conformation have also been extensively studied. However, the structural characteristics of the regenerated form have not been intensively investigated<sup>5–7</sup> because the dissolution of *A. pernyi* silk fibroin (SF) is difficult to perform because of the strong intermolecular and intramolecular

interactions between fibroin molecules and chains.

The dissolution of SF is often required when nontextile applications are demanded in the forms of film, porous membrane, powder, and gel, among others. *A. pernyi* SF film, containing several basic amino acids and tripeptides sequence of arginine–glycine–aspartic acid, can be very effective for several biotechnological and biomedical applications not only for enzyme immobilization and matrixes for mammalian cell cultures but also for wound covering and artificial skin.

In recent articles,<sup>6–8</sup> the effects of methanol and ethanol treatment on the structural and thermal characteristics of regenerated *A. pernyi* SF have been reported for films regenerated from calcium nitrate.

The objective of this work was to study the conformational changes of regenerated *A. pernyi*

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SF treated with heat. The molecular conformation and crystalline structure of regenerated *A. pernyi* SF films induced by heat treatment were investigated through X-ray diffraction (XRD), infrared (IR) spectroscopy, and differential scanning calorimetry (DSC). The contents of the secondary structure of regenerated *A. pernyi* SF films were also investigated to study the effects of heat treatment.

## EXPERIMENTAL

### Materials

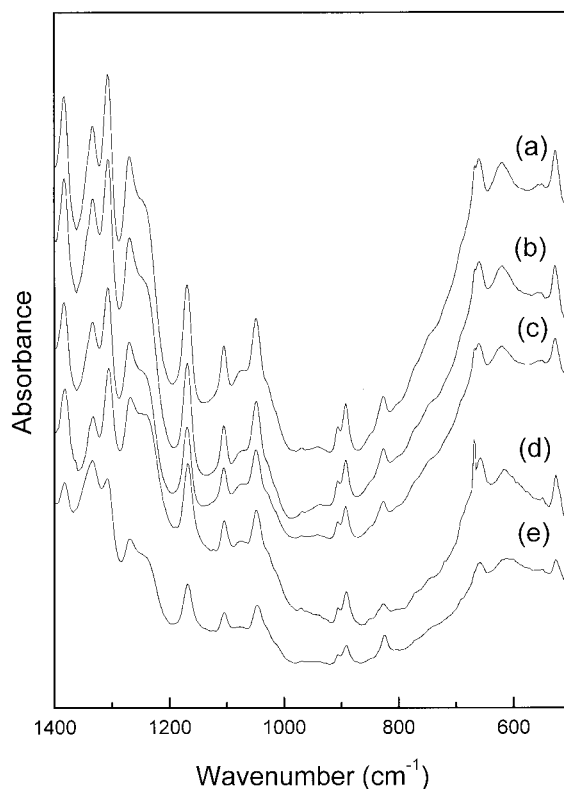
*A. pernyi* silk fibers were degummed with the enzymatic degumming method and dissolved in calcium nitrate solution. They were first treated with degumming solution [Alcalase (2.5 L) from Novo Nordisk (Bagsvaerd, Denmark) (1 g/L), sodium bicarbonate (5 g/L), and nonionic surfactant (1 g/L)] at 55°C for 60 min. The degummed fibers were washed in a mixture solution of nonionic surfactant (2 g/L) and sodium hydrosulfite (5% on the weight of fiber) and thoroughly rinsed in warm distilled water. They were then dried at room temperature and stored in a desiccator prior to use.

The dissolution of degummed fibers were carried out in the melted calcium nitrate · 4 hydrate for 5 h at 105°C. The solution was then dialyzed in a cellulose tube (molecular cutoff = 3500) against distilled water for 4 days at room temperature. The regenerated *A. pernyi* SF solution (0.3%) was cast on a polystyrene plate with the substrate at 20°C and 40% relative humidity. Film samples of around 30 μm were prepared. The films were treated with heat in an oven adjusted to the desired temperature for various times.

Based on our preparatory experiment, the dissolution of *A. pernyi* SF required a high concentration of chaotropic salts, a high temperature, and a somewhat long treatment time. The silk fiber could not be dissolved in less than 6M calcium nitrate solution at 100°C for prolonged times. When dissolution conditions are severe, decomposition occurs with a decrease in molecular weight, and the regenerated film cannot be formed. Therefore, the preparation conditions of the film were fixed to avoid a variance of sample.

### Measurements

Fourier transform infrared (FTIR) spectra were obtained with a Midac M series spectrometer in



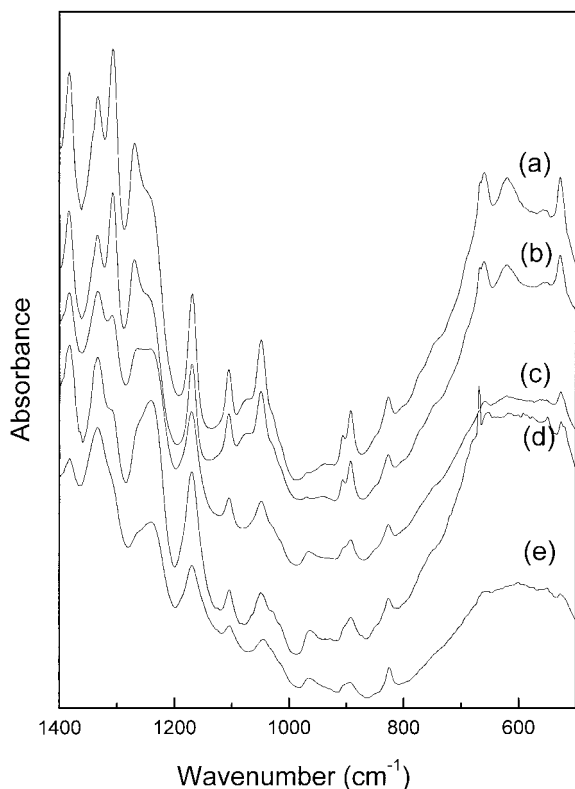
**Figure 1** FTIR spectra of regenerated *A. pernyi* SF film treated with heat at 150°C for various times: (a) 0, (b) 5, (c) 30, (d) 60, and (e) 90 min.

the spectral region of 1400–500  $\text{cm}^{-1}$ . The XRD curve was obtained with a D-MAX-3 diffractometer (Rigaku Co., Tokyo, Japan) and  $\text{CuK}\alpha$  radiation at a wavelength of 1.5418 Å. The scan speed was 0.5°/min, and the range was  $2\theta = 5\text{--}35^\circ$  under 30 kV and 20 mA. DSC curves were obtained with a thermal analysis instrument (TA 2910, New Castle, DE) at a heating rate of 10°C/min and a nitrogen gas flow rate of 50 mL/min.

## RESULTS AND DISCUSSION

### FTIR Spectra

The effect of heat treatment was examined for conformational changes of regenerated *A. pernyi* SF films through FTIR spectroscopy. Figure 1 shows the IR spectra of SF films treated with heat at 150°C. The strong absorption bands at 1270 (amide III), 895 (amide IV), and 625  $\text{cm}^{-1}$  (amide V), attributed to the  $\alpha$ -helix structure, and the band at 660  $\text{cm}^{-1}$  (amide V), attributed to the random-coil conformation, were observed for un-



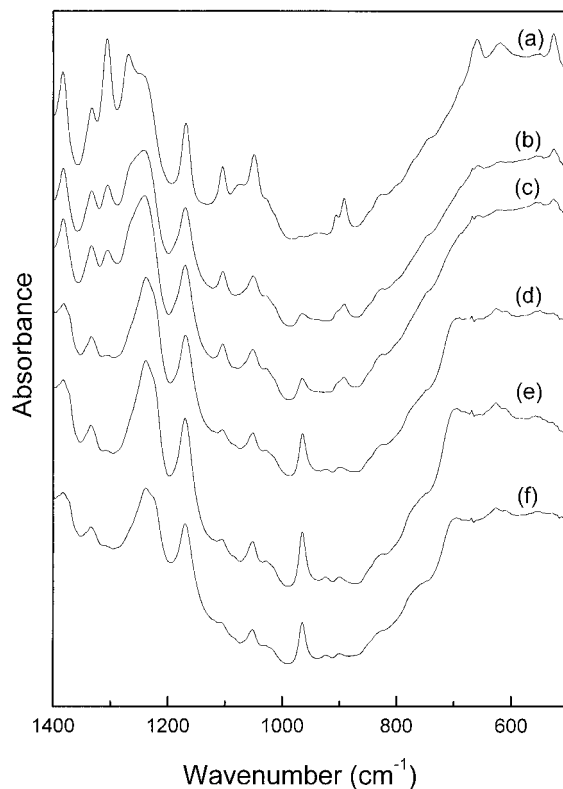
**Figure 2** FTIR spectra of regenerated *A. pernyi* SF film treated with heat at 230°C for various times: (a) 0, (b) 5, (c) 30, (d) 60, and (e) 90 min.

treated film. In addition, the absorption band at 1240  $\text{cm}^{-1}$ , assigned to the  $\beta$ -sheet and/or random-coil conformation, was also observed as a shoulder. The IR spectra of regenerated film treated at 150°C did not change with time in the range of 0–90 min. Although the heat-treatment temperature increased to around 200°C, the IR spectra were very similar to those at 150°C. Considering that the glass-transition temperature ( $T_g$ ) of *A. pernyi* SF is about 200–220°C, it is expected that the conformational change would not occur in the heat-treatment conditions at lower than 200°C (below  $T_g$ ) for a prolonged time. Therefore, the temperature of heat treatment was raised to over 230°C, at which the degradation might not occur, and the conformational change was examined.

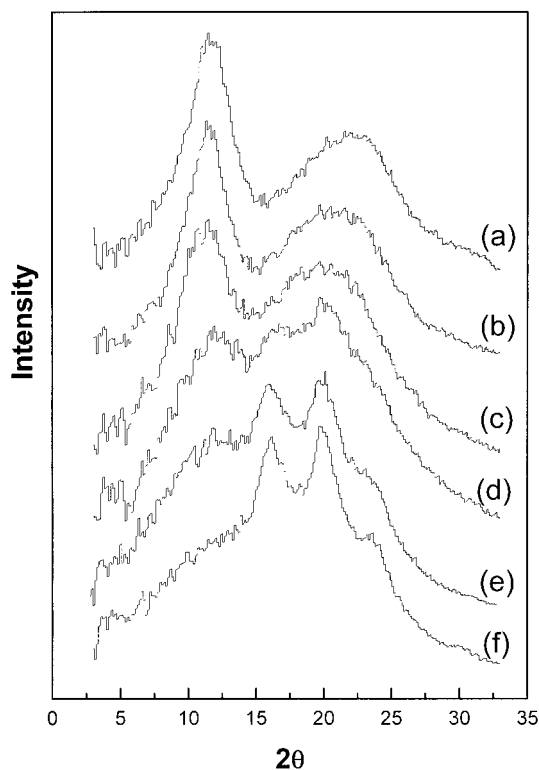
The IR spectra of SF films treated at 230°C are shown in Figure 2. As the heat-treatment time increased up to 60 min, the strength of the absorption bands at 1270 and 660  $\text{cm}^{-1}$  decreased remarkably, whereas the characteristic absorption bands of the  $\beta$ -sheet structure at 1240 and 965  $\text{cm}^{-1}$  increased. When *A. pernyi* SF was

treated for 90 min at 230°C, characteristic absorption bands at 965 and 895  $\text{cm}^{-1}$ , which represent the  $\beta$ -sheet and  $\alpha$ -helix conformations, were observed. This means that the conformational transition of *A. pernyi* SF occurred in the process at those conditions of heat treatment.

Figure 3 shows the IR spectra of SF films treated with heat at 250°C. After 5 min, the characteristic absorption bands at 1270 and 660  $\text{cm}^{-1}$  were lower, and the bands at 1240 and 965  $\text{cm}^{-1}$  appeared. After 30 min, strong absorption bands at 965 and 700  $\text{cm}^{-1}$  appeared and bands at 1270, 895, and 660  $\text{cm}^{-1}$  almost disappeared. Moreover, as the temperature of heat treatment increased, the structural transition occurred within much shorter treatment time. However, the characteristic peak around 620  $\text{cm}^{-1}$ , assigned to the  $\alpha$ -helix conformation, still existed, although regenerated *A. pernyi* SF film was treated with heat at 250°C for 90 min. The results show that the conformation of regenerated *A. pernyi* SF film is significantly affected by heat-treatment temperature and time.



**Figure 3** FTIR spectra of regenerated *A. pernyi* SF film treated with heat at 250°C for various times: (a) 0, (b) 5, (c) 10, (d) 30, (e) 60, and (f) 90 min.



**Figure 4** X-ray diffractograms of regenerated *A. pernyi* SF film treated at 240°C for various times: (a) 0, (b) 10, (c) 20, (d) 30, (e) 60, and (f) 90 min.

#### XRD Curves

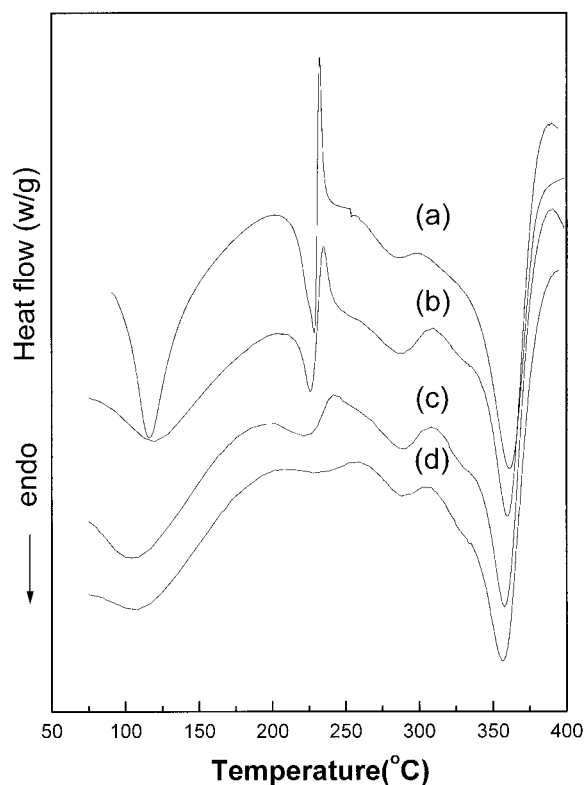
XRD curves of regenerated *A. pernyi* SF films treated with heat at 240°C are shown in Figure 4. The untreated film could be characterized by the presence of two peaks at 11.5° and 22.0°, corresponding to the  $\alpha$ -helix crystalline spacing of 7.69 and 4.03 Å, respectively. These two peaks exhibited the characteristics of native SF, collected from the posterior division of the silk gland in full-grown larvae of the *A. pernyi* silkworm<sup>2</sup> and regenerated *A. pernyi* film prepared from lithium thiocyanate and calcium nitrate solution.<sup>5,6</sup>

When the regenerated films were treated with heat at 240°C for 30 min, new diffraction peaks at about 16.4° and 20.0°, which were assigned to the antiparallel pleated sheet, appeared. With the increase of heat-treatment time, *A. pernyi* SF clearly exhibited two major peaks at about 16.4° and 20.0° and a minor peak at about 23.8°, corresponding to the crystalline spacings of 5.40 [002], 4.43 [201], and 3.73 Å because of the growth of the  $\beta$ -sheet structure. Figure 4 shows that the diffraction intensity of the  $\alpha$ -helix structure gradually decreased with heat treatment in accordance with an intensity increase of the  $\beta$ -sheet structure.

In a previous report,<sup>6</sup> the regenerated *A. pernyi* SF crystallized by methanol showed that 4.43 Å of crystalline spacing was well developed compared to other crystalline spacings. On the other hand, it was observed in this article that the regenerated *A. pernyi* SF crystallized by heat treatment showed sharp diffraction peaks corresponding to 5.40 and 4.43 Å. It seems that the crystalline spacings of *A. pernyi* SF can be affected differently according to the method of crystallization, such as heat and solvent treatment. The difference of crystallization mechanism between heat and methanol treatment is not clearly elucidated; however, heat treatment facilitates the intersheet ordering between molecular chains of regenerated *A. pernyi* SF more than methanol treatment.

#### DSC

Differential scanning calorimetric curves of regenerated *A. pernyi* SF films treated at 240°C are shown in Figure 5. The regenerated SF exhibited a typical DSC thermogram of *A. pernyi* SF with the  $\alpha$ -helix as well as a random-coil conformation,



**Figure 5** DSC thermograms of regenerated *A. pernyi* SF film treated at 240°C for various times: (a) 0, (b) 10, (c) 30, and (d) 90 min.

which showed an endo–exo transition at around 230°C.<sup>4,7</sup> The endothermic peak at 228°C was attributed to the strong molecular motion within the  $\alpha$ -helix crystals, whereas the exothermic peak at 232°C was attributed to the crystallization during heating by the formation of the  $\beta$ -sheet structure from random-coil conformation.<sup>9</sup> Moreover, a major endothermic peak at about 360°C and a minor endothermic peak at about 286°C are known as the decomposition of *A. pernyi* SF with an unoriented  $\beta$ -sheet structure.<sup>1,2,7</sup>

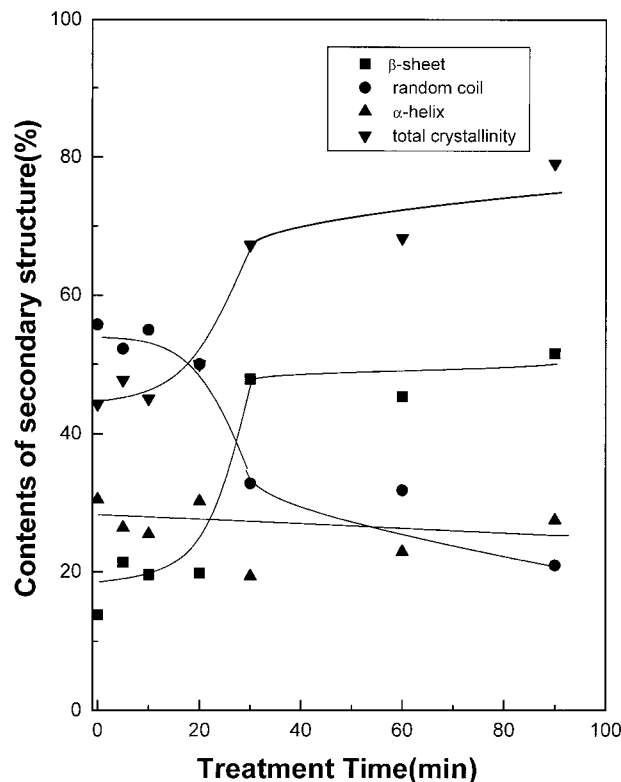
As the treatment time increased, the intensity ratio of the endo–exo transition peak, appearing around 230°C, decreased. Finally, the exothermic peak assigned to the cold crystallization from random coil to  $\beta$ -sheet structure disappeared when the regenerated film was treated with heat at 240°C for 90 min. These results conformed with the results of FTIR and XRD.

#### Analysis of Secondary Structure

The content of the secondary structure of regenerated *A. pernyi* SF films was affected to estimate the conformational changes quantitatively of the heat treatment. Bhat and Nadiger<sup>10</sup> suggested the method for calculating the crystallinity index of SF with the intensity ratio of the IR absorption bands at 1265 and 1235  $\text{cm}^{-1}$ . However, in the case of *A. pernyi* SF, this method could not be used because of an uncertain calculated value of crystallinity index.<sup>11</sup>

The amide V frequencies of *A. pernyi* SF were found to be a particularly sensitive absorption band for the conformational changes. The absorption band of the amide V region was used to estimate the content of the secondary structure of poly(*L*-alanine),<sup>12</sup> poly( $\gamma$ -methyl *L*-glutamate),<sup>13</sup> and regenerated *A. pernyi* SF.<sup>6</sup> When a Gaussian function is assumed, each IR absorption band of the amide V region can be separated into three conformations, the  $\alpha$ -helix (625  $\text{cm}^{-1}$ ), random-coil (660  $\text{cm}^{-1}$ ), and  $\beta$ -sheet (700  $\text{cm}^{-1}$ ) structures. The fitting curve calculated with the Gram 386 program was well consistent with observed one (data not shown).

Figure 6 shows the estimated content of the secondary structure of regenerated *A. pernyi* SF film treated at 240°C. The content of the  $\beta$ -sheet structure was markedly changed after 20 min, and about 45% compositional increments of the  $\beta$ -sheet structure could be obtained with heat treatment at 240°C for a time longer than 30 min. The opposite trend was observed for the content of

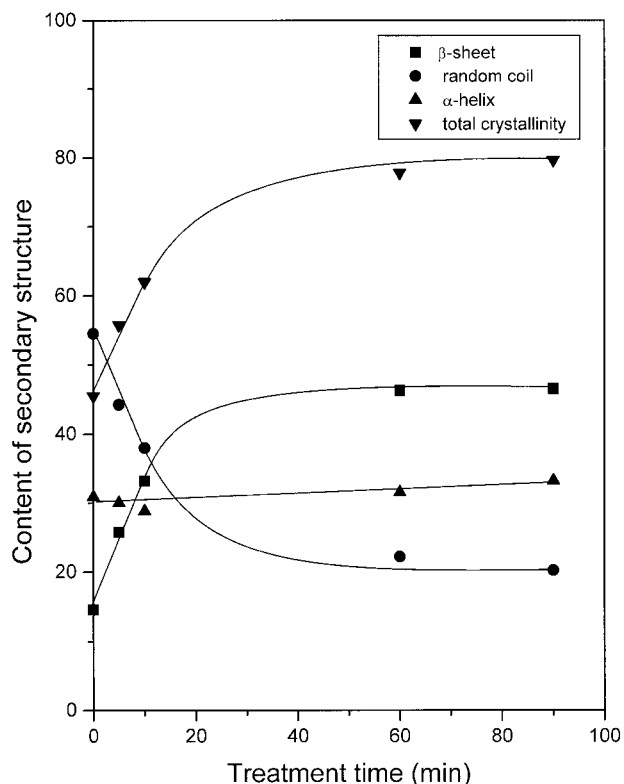


**Figure 6** Content of the secondary structure of regenerated *A. pernyi* SF film treated at 240°C.

the random-coil structure with heat-treatment time. On the other hand, the content of the  $\alpha$ -helix structure was nearly constant at a value of about 30%, regardless of heat-treatment temperature and time.

Figure 7 shows the content of the secondary structure of regenerated SF treated with heat treatment at 250°C. The content of the  $\beta$ -sheet structure increased linearly with time up to 20 min and remained constant afterward. The changes of the content of the secondary structure conformed with the appearance of characteristic peaks of IR spectra. The content of the  $\alpha$ -helix was not significantly changed with treatment time and temperature.

The conformation and crystalline structure of regenerated *A. pernyi* SF are the random-coil conformation and  $\alpha$ -helix structure. When the sample film was treated with heat over 230°C, the crystalline structure of *A. pernyi* SF film could be formed, which consisted of the major  $\beta$ -sheet and minor  $\alpha$ -helix structures. Figures 6 and 7 show that the  $\beta$ -sheet structure was not transformed from the  $\alpha$ -helix conformation but could be grown from random-coil conformation. Similar results



**Figure 7** Content of the secondary structure of regenerated *A. pernyi* SF film treated at 250°C.

were reported on the conformation of heat-treated poly(*L*-alanine) and SF methanol-treated *A. pernyi*.<sup>6,12</sup>

## CONCLUSIONS

Regenerated *A. pernyi* SF films were prepared from calcium nitrate solution, and the conformational changes of heat treatment for these films were analyzed for crystallization. The crystallization behavior on a conformational transition was strongly affected by the temperature and by time of heat treatment. The growth of crystalline spacings was greatly influenced by the method of crys-

tallization, such as heat and solvent treatment. Development of intersheet ordering was facilitated by heat treatment.

The contents of the secondary structure, namely the  $\alpha$ -helix,  $\beta$ -sheet, and random-coil conformation, were calculated by IR absorption bands of the amide V region. The  $\beta$ -sheet structure was transformed from the random-coil conformation by heat treatment, whereas an intrahelical interaction in the  $\alpha$ -helix structure remained stable on the crystallization of *A. pernyi* fibroin film.

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