Preparation of Regenerated *Antheraea yamamai* Silk Fibroin Film and Controlled-Molecular Conformation Changes by Aqueous Ethanol Treatment

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ABSTRACT: The methods of dissolving *Antheraea yamamai* (*A. yamamai*) silk fibroin fibers and preparing the regenerated silk fibroin were studied. The molecular conformations of *A. yamamai* silk fibroin film treated by different concentration ethanol solution were investigated by XRD, FTIR, and Raman spectra. The results indicated that *A. yamamai* silk fibroin fibers could be completely dissolved in dense lithium thiocyanate solution at about 35°C for 1 h. The initial regenerated film consisted of α-helix and random coil components. The aqueous ethanol treatment of the film resulted in significant increase in β-sheet component and improvement of water resistance of the film. This effect was strongly dependent on ethanol concentration, and 60–80% (w/w) ethanol was most effective.
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Key words: proteins; films; conformational analysis; biopolymers

INTRODUCTION

Silkworm silk fibroins are natural structural proteins excreted by two species of silkworms, domestic (*Bombyx mori*) and wild, and have been widely used in textile industry for thousands of years due to their superior properties. In nontextile field, *Bombyx mori* (*B. mori*) silk fibroin has been extensively characterized and used commercially as biomedical sutures, cosmetic, and food additives for decades.[1–3] *B. mori* silk fibroin, nontoxic and nonirritating, has good biocompatibility and is beneficial for cells of human and mammals to adhere and proliferate.[4–8] So in recent years, *B. mori* silk fibroin has been widely studied as biomedical materials.

Among many wild silkworms, *Antheraea yamamai* (*A. yamamai*) is a fairly common species and been produced in East Asia, such as China, Japan, and Korea. The *A. yamamai* silkworm, same as *Antheraea pernyi* (*A. pernyi*) silkworm, belongs to the family *Saturniidae*, species of *Lepidoptera* and *Arthropoda*. As showed in Figure 1, the light green cocoon is ellipse and its size is very large (45–53 mm in length and 23–27 mm in width). The *A. yamamai* silk fibers are basically used as high-quality clothing up to now, such as the raw materials of Japanese kimono. However, no reports concerning the use of *A. yamamai* silk fibers beyond the textile field had been published, especially in the biomedical field.

In contrast to *B. mori* silk fibroin, the *A. yamamai* silk fibroin shows the H—H structure, which indicates that the main component of *A. yamamai* silk fibroin is made with only one type of fibroin heavy chain.[9–12] The gene of *A. yamamai* silk fibroin consists of an initial exon encoding 14 amino acids, an intron (150 bp), and a long second exon coding for 2641 amino acids.[13] The sequence similarity between the intron of *A. yamamai* fibroin gene and that of *A. pernyi* fibroin gene was quite high showing 72% identity. However, in the comparison with the intron sequence of *B. mori* fibroin gene, no similarity was detected. The exon 2 of *A. yamamai* fibroin gene is composed of 80 repetitives with each pair composed of one polyalanine motif and one nonpolyalanine motif which is similar to that of *A. pernyi* silk fibroin. In contrast to that of *B. mori* silk fibroin, the amino acid composition of *A. yamamai* silk fibroin is characterized by more Ala, Asp, and Arg but less Gly. The abundance of alkaline amino acids (Arg and His)

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Figure 1 The morphology of *A. yamamai* silk cocoon. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

and the presence of tripeptide sequence Arg-Gly-Asp (RGD), which is known to exhibit special interactions with mammal cells, are favorable for cells to adhere.[14,15] Based on these features, *A. yamamai* silk fibroin may have excellent biocompatibility if used as biomedical materials, such as tissue engineering scaffolds, cell culture substrate, and tissue engineering matrix.

It is difficult to collect silk fibroin from the full-grown *A. yamamai* silk worm's posterior silk gland. Natural *A. yamamai* silk fibroin fibers, with high crystallinity, high orientation, and hard to be biodegraded, cannot be directly used to prepare the biodegradable materials. Therefore, it is necessary to dissolve *A. yamamai* fibers firstly to obtain regenerated *A. yamamai* silk fibroin aqueous solution. After that we can use the solution to prepare the biomaterials with required configuration, structure, and function with the aqueous solution, such as porous material, gel, film, fiber, powder, etc. However, an important problem need to be solved is that how to make these materials become water insoluble. The studies on *B. mori* silk fibroin have proved that exposing the silk fibroin to organic solvent such as aqueous methanol or ethanol is an effective method.[16–19] The reason is that dehydration of *B. mori* silk fibroin in aqueous methanol or ethanol promotes solvent-induced silk fibroin crystallization as the silk fibroin chains transform from random coil to β-sheet conformation.[20–22] If the conformational transition could also be induced by exposing *A. yamamai* silk fibroin to aqueous ethanol solution, a new kind of silk fibroin-based biomaterial with water insolubility could be prepared.

In this study, *A. yamamai* silk fibroin fibers were dissolved by aqueous lithium thiocyanate to obtain regenerated *A. yamamai* silk fibroin solution and the film was prepared by casting the solution on a polyethylene dish then air-dried at 25°C. The molecular conformation of regenerated *A. yamamai* silk fibroin films treated by ethanol with different concentration was also investigated.

MATERIALS AND METHODS

Preparation of regenerated *A. yamamai* silk fibroin solution and film

The *A. yamamai* cocoons, purchased from Hunan Province (China), were treated three times with 2.5 wt ‰ Na_2CO_3 solution at 98–100°C for 30 min, respectively, to remove sericin. Degummed *A. yamamai* silk fibroin fibers (10 g) were dissolved in 200 mL of 10*M* aqueous lithium thiocyanate solutions for 2 h at 35°C. The solution was dialyzed, and then the regenerated *A. yamamai* silk fibroin solution with concentration of about 2.2 wt % was obtained. The regenerated silk fibroin film was prepared by casting the silk fibroin solution on a polyethylene dish and air-dried at 25°C. The silk fibroin film was immersed in ethanol solution (40, 60, 80, and 99.7% v/v ethanol) for 2 h at room temperature and then air-dried.

Molecular conformation analyses of regenerated *A. yamamai* silk fibroin solution

Circular dichroism spectrum of the regenerated *A. yamamai* silk fibroin in aqueous solution was collected on a Jasco spectropolarimeter, model 715, with a quartz cell of 1 mm path length for far-UV, at protein concentrations of 0.1 mg/mL. The bandwidth was 1.0 nm, and the CD spectrum was obtained at a scan speed of 100 nm/min with a response time of 0.25 s.

Amino acid composition analyses of regenerated *A. yamamai* silk fibroin film

The regenerated *A. yamamai* silk fibroin films were hydrolyzed with 6*N* HCl at 110°C for 24 h. The hydrolyzate was diluted with water to 25 mg/mL and the diluted solution subjected to a HITACHI-835-50 Amino Acid Analyzer for composition analyses.

Molecular conformation analyses of regenerated *A. yamamai* silk fibroin film

X-ray diffraction of regenerated *A. yamamai* silk fibroin film was performed by a Rigaku D/Max-3C diffractometer with Cu Kα radiation from a source operated at 40 kV and 40 mA. Diffraction was measured in reflection mode at scanning rate of 2°/min.

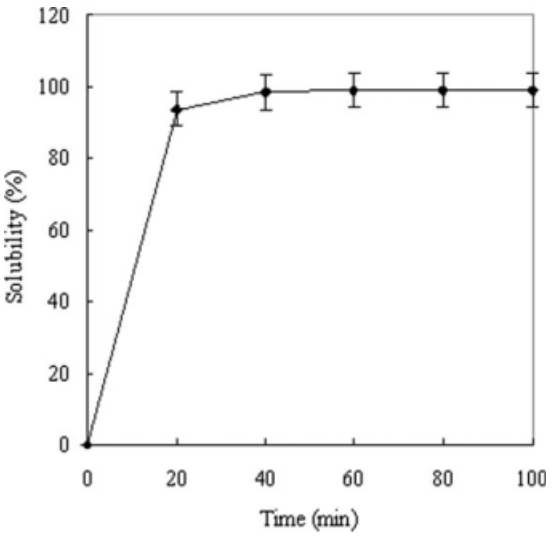

Figure 2 Solubility of *A. yamamai* silk fibroin fibers by 10*M* aqueous lithium thiocyanate solution at different dissolving time (35°C).

Fourier transform infrared (FTIR) spectra were obtained with a Nicolet Avatar-IR360.

Raman spectra were recorded using a Dilor LabRam-1B spectrometer, operating at a resolution of 1 cm^{-1}. The Spectra Physics Model 164 argon ion laser was operated at 632.8 nm with about 6 mW of power.

Water solubility of *A. yamamai* silk fibroin film

Two hundred fifty milligrams of film was shaken in 25 mL of water at 37°C for 24 h, then the undissolved film was filtrated and dried at 140°C to obtain the remained weight. The solubility could be calculated by eq. (1).

$$\text{water solubility(wt \%)} = \frac{\text{original weight (g)} - \text{remaining weight(g)}}{\text{orginal weight(g)}} \times 100\% \quad (1)$$

RESULTS AND DISCUSSION

Dissolution of *A. yamamai* silk fibroin fiber

Dissolving temperature can affected the solubility of *A. yamamai* silk fibroin fibers. When the temperature is below 30°C or the concentration of lithium thiocyanate solution is less than 10*M*, it is difficult to dissolve *A. yamamai* silk fibroin fibers completely. Figure 2 shows the solubility of *A. yamamai* silk fibroin fibers dissolved in 10*M* lithium thiocyanate solution at 35°C. The *A. yamamai* silk fibroin fibers could be totally dissolved 60 min later, whereas the solubility is 93% when dissolved for 20 min. It showed that lithium thiocyanate solution had strong ability to dissolve *A. yamamai* silk fibroin fibers.

Molecular conformation of regenerated *A. yamamai* silk fibroin solution

As we know, all kinds of secondary structures (α-helix, β-sheet, β-turn, and random coil) of protein have characteristic peaks in far-UV area of circular dichroism spectroscopy (178–250 nm). For example, the negative strong peak (negative cotton effect) around 195 nm is the characteristic peak of random coil structure, the negative wide peak at 217 nm and the positive strong peak (positive cotton effect) at 198 nm are the characteristic peaks of β-sheet structure, and the peaks at 192 nm (positive), 209 nm (negative), and 222 nm (negative) are the characteristic peaks of α-helix structure.[23] It can be seen from Figure 3 measured by us that the CD spectrum of regenerated *A. yamamai* silk fibroin in aqueous solution, obtained by dissolving *A. yamamai* silk fibers with lithium thiocyanate solution, have strong negative peaks around 205 nm and 222 nm, indicating that the molecular conformations of regenerated *A. yamamai* silk fibroin in aqueous solution are mainly α-helix and random coil structures.

Amino acid composition of regenerated *A. yamamai* silk fibroin film

It is shown in Table I that the total content of glycine, alanine, and serine in *A. yamamai* silk fibroin is

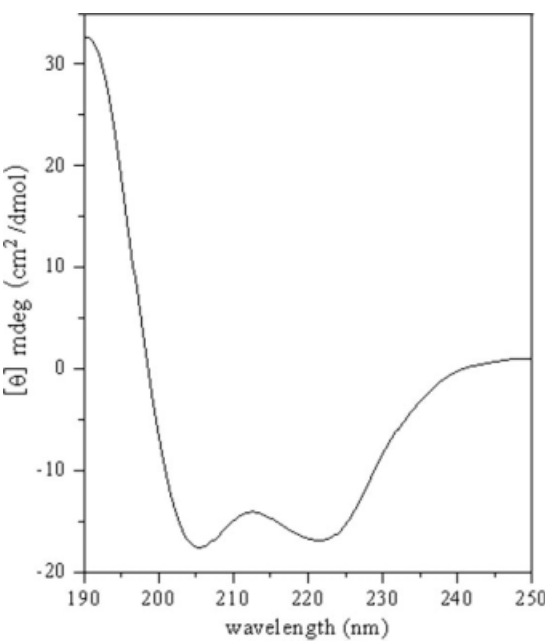

Figure 3 Circular dichroism spectrum of regenerated *A. yamamai* silk fibroin solution obtained by dissolving fibers with lithium thiocyanate aqueous solution.

TABLE I
Amino Acid Composition (mol %) of *B. mori* Silk Fibroin Fiber, *A. yamamai* Silk Fibroin Fiber, and Its Regenerated Silk Fibroin Film

Amino acid	*B. mori* silk fibroin fibers	*A. yamamai* silk fibroin fiber	Regenerated *A. yamamai* silk fibroin film
Asp	1.54	5.53	5.36
Thr	0.88	0.20	0.23
Ser	10.41	9.80	9.63
Glu	1.30	0.90	0.88
Gly	43.30	28.03	27.23
Ala	30.56	43.91	45.00
Val	1.86	0.50	0.56
Met	0.08	0.00	0.03
Ile	0.58	0.22	0.24
Leu	0.38	0.24	0.25
Tyr	5.01	4.90	4.81
Phe	0.55	0.11	0.11
Lys	0.27	0.06	0.06
His	0.19	0.96	0.96
Arg	0.41	2.66	2.61
Pro	0.37	0.23	0.28
NH_3	2.31	1.76	1.76
Total	100.00	100.00	100.00

above 80%. It contains more alanine, aspartic, and arginine than that of *B. mori* silk fibroin. When the *B. mori* silk fibers were dissolved in LiBr aqueous solution, the amount of the acidic and basic amino acids decreased for regenerated silk fibroin.[22] Thus, in this study, the lithium thiocyanate solution was used to dissolve silk fibroin. The amino acid composition of *A. yamamai* silk fibroin film has no obvious difference with that of *A. yamamai* silk fibroin fibers. Acidic or basic amino acids did not reduce obviously.

X-ray diffraction of regenerated *A. yamamai* silk fibroin film

According to the studies on *A. pernyi* silk fibroin molecular conformation,[24–26] with Cu Kα radiation, the X-ray diffraction (XRD) patterns have been determined as follows: 11.8° and 22.0° for α-helix structure, and 16.5°, 20.2°, 24.9°, 30.90°, 34.59°, 40.97°, and 44.12° for β-sheet structure.

There appear strong diffraction peaks around 16.5° and 20.2° in XRD curve of *A. yamamai* silk fibroin fiber [Fig. 4(a)]. Those indicate that the main conformation in native silk fibroin fiber is β-sheet structure. Curve (b) shows that regenerated *A. yamamai* silk fibroin film before ethanol treatment with two characteristic intense peaks of α-helix structure around 11.6° and 22.7°. Various concentrations of ethanol solution cause different change in the diffraction profiles. The 60 and 80% ethanol-treated regenerated film [Fig. 4(c,d)] result in a fundamental change in profile: the peak around 11.6° disappeared, the peak around 22.7° becomes weaker, and two intense peaks appear around 20.2° and 16.5°. All of these indicate that the molecular conformation of *A. yamamai* silk fibroin obviously changes from random coil to β-sheet conformation. Figure 4(e) shows that the essential changes of silk fibroin molecular conformation have not taken place when the film was treated by 99.7% ethanol. When immersed into 40% ethanol solution, the regenerated film will be dissolved; therefore, its structure and properties are not analyzed in this study.

FTIR spectra of regenerated *A. yamamai* silk fibroin film

FTIR spectra are very sensitive to the molecular conformation of *A. pernyi* silk fibroin. The studies on the molecular conformation of *A. pernyi* silk fibroin by Tsukada et al.[24–28] indicate that *A. pernyi* silk fibroin is characterized by random coil absorption peak around 660 cm^{-1} (amide V), α-helix absorption peaks around 1655 cm^{-1} (amide I), 1546 cm^{-1} (amide II), 1270 cm^{-1} (amide III), 625 cm^{-1} (amide V), and β-sheet absorption around 1630 cm^{-1} (amide I), 1520 cm^{-1} (amide II), 1240 cm^{-1} (amide III), and 695 cm^{-1} (amide V).

As shown in curve (a) of Figure 3, the strong absorption peaks of *A. yamamai* silk fibroin fiber appeared at 1628 cm^{-1} (amide I), 1514 cm^{-1} (amide

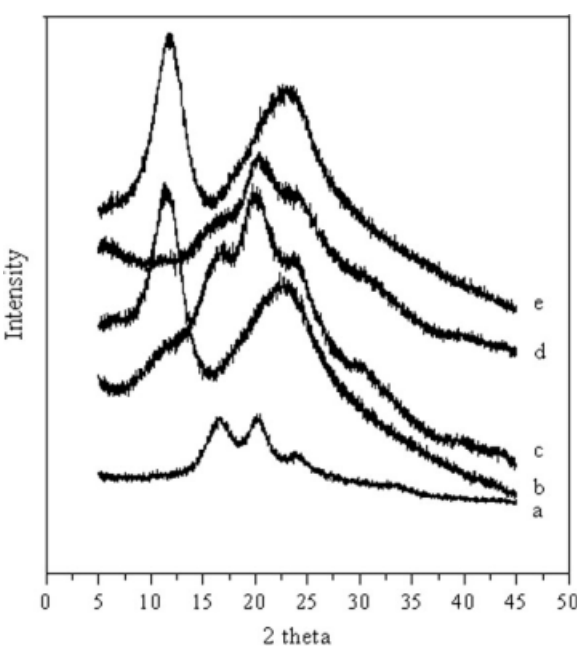

Figure 4 X-ray diffraction curves of *A. yamamai* silk fibroin: (a) native silk fibroin fiber; (b) regenerated silk fibroin film; regenerated silk fibroin films treated with (c) 60%, (d) 80%, and (e) 99.7% ethanol solution.

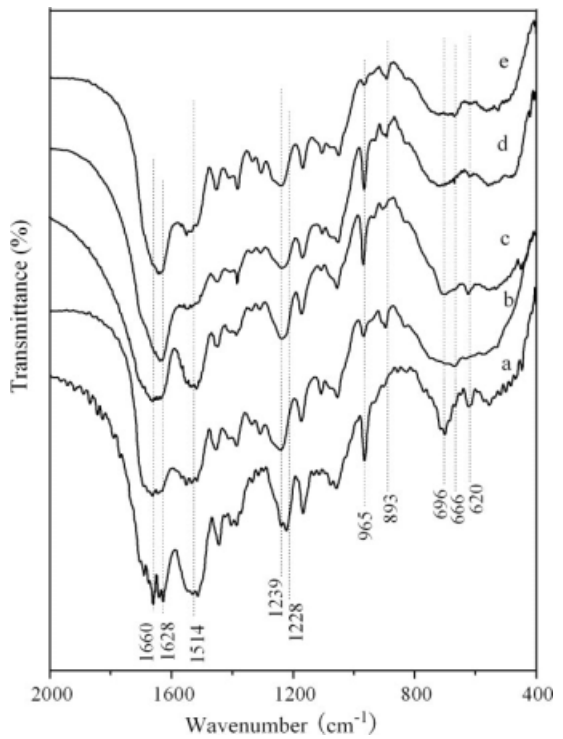

Figure 5 FTIR spectra of *A. yamamai* silk fibroin: (a) native silk fibroin fiber; (b) regenerated silk fibroin film; regenerated silk fibroin films treated with (c) 60%, (d) 80%, and (e) 99.7% ethanol solution.

II), 1223 cm^{-1} (amide III), and 701 cm^{-1} (amide V) indicate that the main molecular conformation of *A. yamamai* silk fibroin fiber is β-sheet structure. Curve (b) shows intense bands at 1660 cm^{-1} (amide I), 1549 cm^{-1} (amide II), and 620 cm^{-1} (amide V) which are assigned to α-helix structure, 893 cm^{-1} (amide IV) and 666 cm^{-1} (amide V), attributed to random coil structure. The molecular conformation of regenerated *A. yamamai* silk fibroin film before ethanol treatment shows coexistence of α-helix and random coil. The 60 and 80% ethanol-treated regenerated films [Fig. 5(c,d)] show that amide II band gradually shifted from 1549 to 1514 cm^{-1}, amide III from 1239 to 1233 cm^{-1}, whereas the amide IV band at 893 cm^{-1} is weakened, the band at 965 cm^{-1} is strengthened, and amide V band at 620 cm^{-1} remains unchanged, the band at 666 cm^{-1} disappear, and the band at 696 cm^{-1} is strengthened. These changes closely correspond to X-ray diffraction patterns indicate that ethanol solution treatment can cause the transformation of random coil to β-sheet. The regenerated film treated with 99.7% ethanol [Fig. 5(e)] does have no substantial changes in the FTIR spectra.

Raman spectra of regenerated *A. yamamai* silk fibroin film

Tsukada et al.[29–31] has investigated the effect of methanol and heat treatment by Raman spectroscopy on the structure and molecular conformation of *A. pernyi* silk fibroin films. Raman spectra of *A. pernyi* silk fibroin films exhibit strong bands for α-helix structure at 1657 (amide I), 1263 (amide III), 1106, 908, 530, and 376 cm^{-1}. With the conformational transition of α-helix structure to β-sheet structure, amide III and I bands shift to 1668, 1241, and 1230 cm^{-1}, respectively. The band at 1106 cm^{-1} disappears and new bands appear at 1095 and 1073 cm^{-1}, whereas the intensity of the bands at 530 and 376 cm^{-1} decreases significantly.[29]

The Raman spectrum of *A. yamamai* silk fibroin fiber [Fig. 6(a)] exhibits strongbands near 1667 (amide I), 1224 (amide III), and 1094 cm^{-1} (ν_{cc} skeletal stretching), respectively, which are the characteristic bands of β-sheet structure. The regenerated silk fibroin film before ethanol treatment [Fig. 6(b)] shows amide I bands at 1656 and 1647 cm^{-1}, amide III bands at 1271 and 1257 cm^{-1}, and ν_{cc} skeletal stretching band at 1104 cm^{-1}. All these bands attribute to α-helix and/or random coil. The 60 and 80% ethanol-treated films [Fig. 6(c,d)] show that amide I band shifts to 1667 cm^{-1}, amide III bands at 1271 and 1257 cm^{-1} are weakened, and bands at 1244 and 1223 or 1227 cm^{-1} are strengthened, whereas

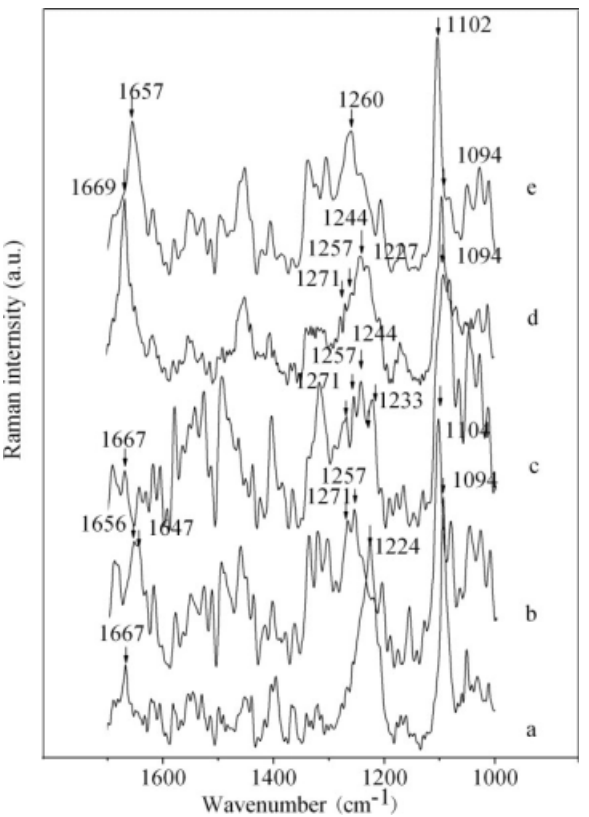

Figure 6 Raman spectra of *A. yamamai* silk fibroin: (a) native silk fibroin fiber; (b) regenerated silk fibroin film; regenerated silk fibroin films treated with (c) 60%, (d) 80%, and (e) 99.7% ethanol solution.

the band at 1104 cm^{-1} disappears and new bands appear at 1094 cm^{-1} These results further confirm that the molecular conformation of *A. yamamai* silk fibroin obviously changed from random coil conformation to β-sheet structure by 60–80% ethanol treatment. The regenerated film treated with 99.7% ethanol [Fig. 6(e)] shows the similar spectrum with that of untreated one.

Water solubility of regenerated *A. yamamai* silk fibroin film

Water solubility of regenerated *A. yamamai* silk fibroin film before ethanol treatment is high up to about 83% and that of silk fibroin film treated by 99.7% ethanol just slightly decrease to 75%. However, water solubility of silk fibroin film treated by 60 and 80% ethanol get down rapidly to about 23%. It is apparent that the treatment by 60–80% aqueous ethanol is highly effective in improving the water resistance of regenerated *A. yamamai* silk fibroin film.

When treated by 60 and 80% ethanol, the molecular conformation of *A. yamamai* silk fibroin film obviously change from random coil to β-sheet conformation and result in remarkably changes of film's physio–chemical properties. However, the structure and properties of silk fibroin film change slightly when treated by 99.7% ethanol solution. Too low ethanol concentration, however, is useless because it cause dissolution and disintegration of the film. Similar behavior has been reported for *B. mori* or *A. pernyi* silk fibroin film treated by methanol or ethanol solution.[28,29] The situation seems to be that the optimal ethanol concentration for film stabilization is determined by balance of actions of ethanol and water. The transformation proceeds presumably as follows: when the silk fibroin film is immersed in ethanol, water firstly forms the hydrogen bonds with the silk fibroin, swells the amorphous region of the protein through the competition with the inter- or intramolecular hydrogen bonds existed in the silk fibroin, then ethanol penetrates the swollen region, generating hydrophobic environment, and making the hydrophobic molecule chain segments in random coils of silk fibroin get close each other and form crystal nucleus. Finally, stable β-sheet conformation is formed by growth of crystal nucleus and rearrangement of hydrogen bonds. In this procedure, water may act as swelling agent toward the compact and dense silk fibroin, thus promoting the loosening of film matrix, penetration of ethanol, and rearrangements of inter- and intramolecular hydrogen bonds leading to the formation of β-sheet. This phenomenon provides a useful method of processing protein-based materials through the hydrophilicity/hydrophobicity controls.

CONCLUSIONS

1. Lithium thiocyanate aqueous solution is a good solvent for *A. yamamai* silk fibroin fibers, the dense lithium thiocyanate solution can completely dissolve *A. yamamai* silk fibroin fibers at about 35°C.
2. In regenerated *A. yamamai* silk fibroin solution, the molecular conformations of silk fibroin are α-helix and random coil structure.
3. The molecular conformation of regenerated *A. yamamai* silk fibroin films cast at 25°C is the coexistence of α-helix and random coil. Ethanol treatment of the film resulted in significant increase in β-sheet component and improvement of water resistance of the film. This effect is strongly dependent on ethanol concentration, and 60–80% (w/w) ethanol was most effective.

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