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Regenerated *Bombyx* silk solutions studied with rheometry and FTIR

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Abstract

Several different solvent systems are commonly used to dissolve *Bombyx mori* silk fibroin to prepare regenerated silk membranes and fibers though differences in the behavior of these solvents have not been fully investigated. Here we compare the effects of four of these on the rheology of silk fibroin solutions and on protein secondary structure as revealed by FTIR spectroscopy of cast membranes. The results demonstrated that $Ca(NO_3)_2$–MeOH–H_2O and LiBr–EtOH–H_2O had the strongest solvation on the silk fibroin chains, which showed an almost constant viscosity (Newtonian behavior) over most of the shear rate range (0.1–$500\ s^{-1}$). In contrast, the 9.5 M aqueous LiBr appeared to have the weakest solvation with similar effects on the silk fibroin molecules to pure water as indicated by rheological behavior. It was also found that the silk fibroin membranes prepared using all four solvent system showed mainly random coil conformation with a small proportion of β-sheet by FTIR spectroscopy. We discuss the implications of these findings for the preparation of regenerated silk for different applications. © 2001 Published by Elsevier Science Ltd.

Keywords: *Bombyx mori* silk; Solvent; Rheometry

1. Introduction

Bombyx mori silk fiber has a long history of use as textile materials. In recent decades, regenerated silk fibroin (RSF) from *B. mori* silk fiber has been used in other areas, such as environmentally sensitive hydrogel [1], chemical valve material [2], pervaporation membrane [3] and enzyme immobilization substrate used for biological sensors [4]. Several special solvents have been used to prepare RSF solutions from *B. mori* cocoon silk. The most common used solvent is aqueous 9.0–9.5 M LiBr [5] (also see the references in Ref. [5]), although mixtures of aqueous calcium chloride and ethanol $CaCl_2$–EtOH–H_2O [6–8], calcium nitrate in methanol $Ca(NO_3)_2$–MeOH [9] or aqueous lithium bromide and ethanol LiBr–EtOH–H_2O [10,11] were also used as well as aqueous lithium thiocyanate LiSCN [12] and aqueous sodium thiocyanate NaSCN [13].

In this research, we chose four common solvent systems: $CaCl_2$–EtOH–H_2O; $Ca(NO_3)_2$–MeOH–H_2O; LiBr–EtOH (with and without water); and LiBr–H_2O. We avoided

some other solvents, such as hexafluoroisopropanol in combination with lithium salt solutions and alkali metal thiocyanate solutions, since the former is protected by patents [9] and in the latter the dissolution of the silk is quite slow. We report differences in the rheological behavior of RSF in the solvents we chose. We correlate these with differences in conformation revealed by FTIR spectroscopy of membranes cast from the RSF solutions prepared using different solvents.

2. Experimental

2.1. Materials

Raw *B. mori* silk consists of fibroin fibers bound together by several hydrophilic coat proteins (sericins), which were removed from the raw silk by boiling in 0.5% (w/w) $NaHCO_3$ solution (two 30 min changes). Silk fibers degummed in this way were washed with copious amounts of distilled water and allowed to air dry at room temperature. Analytical grade reagents were used throughout.

2.2. Preparation of B. mori silk fibroin solution

The different solvents we used are described as follows:

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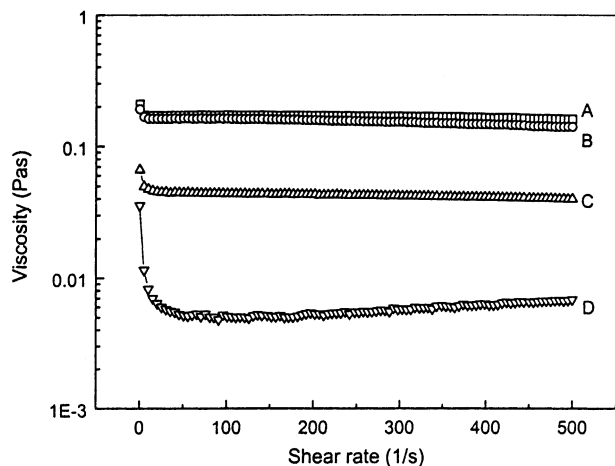*E-mail address:* fritz.vollrath@zoology.oxford.ac.uk (F. Vollrath).

Fig. 1. Rheological behavior of *B. mori* silk fibroin (2.0% w/v) in different solvents (with a linear increase of shear rate): (A) Ca(NO₃)₂–MeOH–H₂O; (B) LiBr–EtOH–H₂O; (C) CaCl₂–EtOH–H₂O and (D) LiBr–H₂O.

1. Ca(NO₃)₂–MeOH–H₂O, a 75:25 (weight ratio) mixture of Ca(NO₃)₂·4H₂O and absolute methanol [9];
2. LiBr–EtOH–H₂O, a 45:44:11 (weight ratio) mixture of LiBr, absolute ethanol and de-ionized water [10];
3. LiBr–EtOH, a 40:60 (weight ratio) mixture of LiBr and absolute ethanol;
4. CaCl₂–EtOH–H₂O, a 1:2:8 (mole ratio) mixture of CaCl₂, absolute ethanol and de-ionized water [6];
5. LiBr–H₂O, aqueous 9.5 M LiBr.

Four hundred milligrams of air dried degummed silk fibers were dissolved in 20 ml of each solvent at 80°C on a water bath with vigorous magnetic stirring to give a 2.0% (w/v) RSF solution.

2.3. Regenerated B. mori silk fibroin membrane preparation

The silk fibroin solutions prepared in the different solvents were dialyzed against frequent changes of de-ionized water for at least three days at room temperature (about 20°C). Samples of the silk fibroin solution were poured into a 10 mm diameter PTFE containers and allowed to air dry under ambient conditions (approximately 50% relative humidity; 20°C). Membranes with a thickness of 10–30 μm were removed after about two days.

2.4. Rheometry

Rheological measurements were performed on a Bohlin CVO 120 high resolution rheometer using a 40 mm, 4° cone and plate. The shear rate was linearly or logarithmically increased without oscillation from 0.1 to 500 s^{-1} over 1,000 s. Temperature was controlled at 25.0 ± 0.1°C. The rheological curves were obtained for RSF in the different solvents and in aqueous solutions prepared from these by dialysis.

2.5. FTIR spectroscopy

All infrared spectra were recorded using a Nicolet Magna 550 FTIR spectrometer. To eliminate spectral contributions due to atmospheric water vapor, the instrument was continuously purged by dry air using a JUN-AIR compressor and a Peak Scientific drying unit. Spectra were recorded using a liquid nitrogen cooled MCT-A detector. For each measurement, 256 interferograms were co-added and Fourier-transformed using Genzel–Happ apodization to yield spectra with a nominal resolution of 4 cm^{-1}. All spectra were normalized using the absorption band of C–H stretching at 2935 cm^{-1} as an internal standard.

3. Results and discussion

3.1. Solubility in the different solvents

The silk fibers dissolved readily in all the solvents. In Ca(NO₃)₂–MeOH–H₂O, CaCl₂–EtOH–H₂O and LiBr–H₂O, they usually dissolved within 10 min, while in LiBr–EtOH–H₂O and LiBr–EtOH, dissolution was complete within 30 min. When cooling to the room temperature, the solutions made from Ca(NO₃)₂–MeOH–H₂O, CaCl₂–EtOH–H₂O, LiBr–EtOH–H₂O and LiBr–EtOH were homogeneous, while a small quantity of floc precipitated in the solution made from LiBr–H₂O.

3.2. Rheology of the B. mori regenerated silk fibroin solutions

There are only a few reported studies of the rheology of RSF solutions [14,15]. Fig. 1 shows the effect of a linear increase in shear rate on the viscosity of RSF solutions. There was a rapid initial shear thinning at low shear rates (<20 s^{-1}), after which the viscosity in RSF Ca(NO₃)₂–MeOH–H₂O, LiBr–EtOH–H₂O and CaCl₂–EtOH–H₂O solutions remained approximately constant up to 500 s^{-1}. The latter Newtonian behavior might be explained by the strong interaction and aggregation of the silk fibroin chains in these solvents [9] to form compactly coiled structures which remain stable even at high shear rate as a result of strong solvation. This might explain the high solubility of silk, 18 and 20%, respectively, in Ca(NO₃)₂–MeOH [9] and LiBr–EtOH–H₂O [10]. Initial shear thinning has also been seen in dilute solutions of spider dragline dope [16] and in other protein solution and colloids [17]. However, the shape of rheological curve of RSF in LiBr–H₂O is apparently different from those in Ca(NO₃)₂–MeOH–H₂O, LiBr–EtOH–H₂O and CaCl₂–EtOH–H₂O. There was a significant shear thinning at shear rates <100 s^{-1} followed by a shear thickening also seen in aqueous RSF solutions (Fig. 2) and certain spider dragline dope solutions [16]. The shear thickening indicated that the silk fibroin chains in LiBr–H₂O were not as restricted as those in Ca(NO₃)₂–MeOH–H₂O, LiBr–EtOH–H₂O and CaCl₂–EtOH–H₂O but were quite
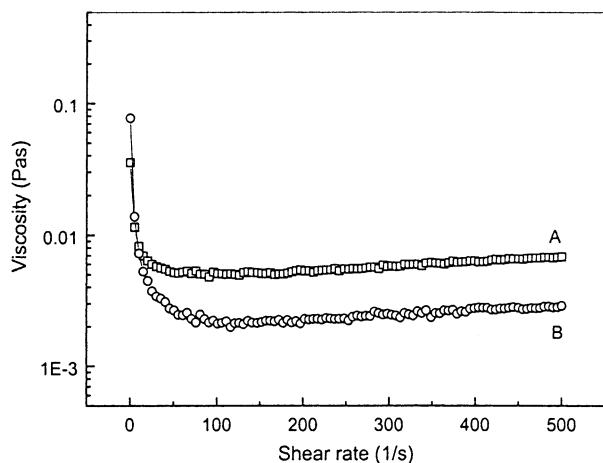
Fig. 2. Rheological behavior of *B. mori* silk fibroin (2.0% w/v) in: (A) LiBr–H$_2$O and (B) water.

Fig. 3. Rheological behavior of *B. mori* silk fibroin (2.0% w/v) in different solvents (with a logarithmic increase): (A) Ca(NO$_3$)$_2$–MeOH–H$_2$O; (B) LiBr–EtOH–H$_2$O; (C) CaCl$_2$–EtOH–H$_2$O; and (D) LiBr–H$_2$O.

free to extend or even form intermolecular interactions [16,18,19].

The *B. mori* silk fibroin molecule is a highly repetitive regular block copolymer in which ten highly hydrophobic blocks are separated by nine less hydrophobic blocks [20,21]. The unique structure of silk fibroin gives it the intrinsic propensity to form a β-sheet structure under favorable conditions [22], therefore the silk fibroin chains may be extended at the high shear rate to form intermolecular interactions [23]. Synthetic amphiphilic block copolymers show an initial shear thinning followed by a shear thickening at higher shear rates as a result of molecular extension and aggregation thought to be dependent on hydrophobic/hydrophilic interactions [18,24]. Some members of this class of polymers also form nanofibrils [25,26] similar to those found in *B. mori* silk [27]. Hence we suggest that the rheology of RSF LiBr–H$_2$O solution and RSF aqueous solution can be explained by the fact that silk fibroin is an amphiphilic block copolymer.

To further investigate the rheological behavior at low shear rates, we recorded the rheological curves with a logarithmic increase of the shear rate instead of a linear increase (Fig. 3). The rheological curves of RSF in Ca(NO$_3$)$_2$–MeOH–H$_2$O, LiBr–EtOH–H$_2$O and CaCl$_2$–EtOH–H$_2$O were not very regular but showed a slight shear thickening followed by a shear thinning at very low shear rates (<1.0 s^{-1}). This might indicate that shear caused a slight extension and interaction of silk fibroin chains even in these solvents. This instability of the rheology at low shear rates may result from an unstable balance between shear tending to unwind the molecules and the effects of the solvent tending to return them to a compactly coiled conformation. This unstable region in the rheology could be followed by a more steady extension leading to the slight shear thickening. At somewhat higher shear rates (>1.0 s^{-1}) the macromolecular chains reached a new equilibrium resulting in an almost constant viscosity. Furthermore, since the RSF CaCl$_2$–EtOH–H$_2$O solution gave more rheological instability
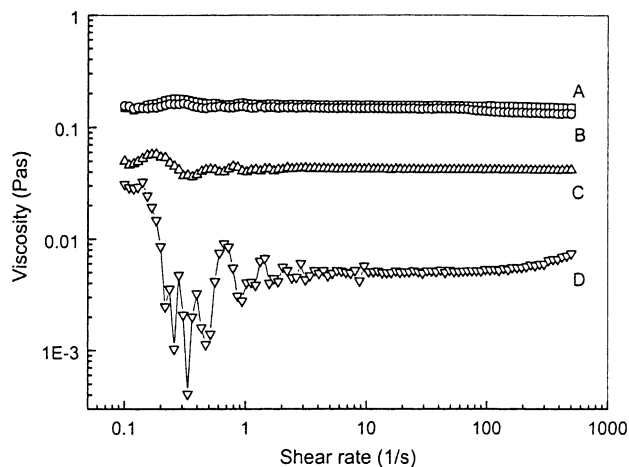
than Ca(NO$_3$)$_2$–MeOH–H$_2$O and LiBr–EtOH–H$_2$O solutions, suggesting that the solvation of CaCl$_2$–EtOH–H$_2$O was not as strong as Ca(NO$_3$)$_2$–MeOH–H$_2$O and LiBr–EtOH–H$_2$O.

We note that the rheological behavior of RSF in LiBr–H$_2$O differed markedly from those in Ca(NO$_3$)$_2$–MeOH–H$_2$O, LiBr–EtOH–H$_2$O and CaCl$_2$–EtOH–H$_2$O though it was very similiar to an aqueous RSF solution (not shown). In the latter, after an initial shear thinning, there was also a region of unstable rheology with an initial increase followed by a decrease followed by a further increase and decrease until a new stability is established at shear rates >10 s^{-1}. The similarity of the rheology of RSF in LiBr–H$_2$O and pure water demonstrated that the solvation of the former was almost as weak as water, and the RSF chains in LiBr–H$_2$O showed mostly free random coil conformation (not compact coiled state) as it does in water [28].

Solvents Ca(NO$_3$)$_2$–MeOH–H$_2$O, LiBr–EtOH–H$_2$O and CaCl$_2$–EtOH–H$_2$O were ternary systems all containing water. Because the solubility of silk fibroin in CaCl$_2$–EtOH is poor, and anhydrous Ca(NO$_3$)$_2$ is much harder to obtain than Ca(NO$_3$)$_2$·4H$_2$O, we compared only LiBr–EtOH and LiBr–EtOH–H$_2$O in this study (though Mathur et al. used Ca(NO$_3$)$_2$–MeOH as solvent in their report [9]). Fig. 4 shows that the rheological behavior of RSF LiBr–EtOH solution was similiar to LiBr–EtOH–H$_2$O solution. However, it showed a higher viscosity which decreased more markedly at high shear rates in RSF LiBr–EtOH solution than LiBr–EtOH–H$_2$O solution.

3.3. FTIR spectra of B. mori regenerated silk fibroin membranes

One of the most important uses for RSF solution is the manufacture of regenerated membranes or films. It is generally accepted that RSF membranes cast from dilute silk fibroin solution at room temperature show mostly random
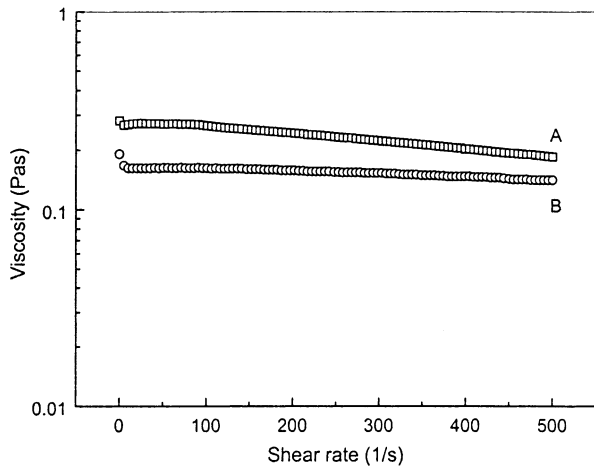
Fig. 4. Rheological behavior of *B. mori* silk fibroin (2.0% w/v) in: (A) LiBr–EtOH and (B)LiBr–EtOH–H$_2$O.

coil conformation [1,5,6,29–32]. Membranes with this conformation are a good starting point for studying changes in secondary structure in silk fibroin [1,29].

In our study, all the RSF membranes (cast after dialysis of the different RSF solutions against de-ionized water)
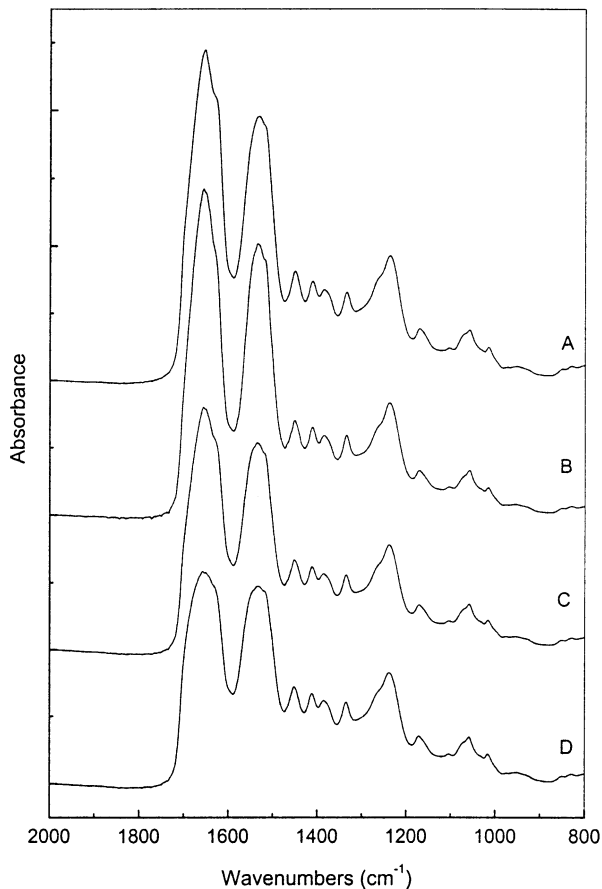

Fig. 5. FTIR spectra of regenerated *B. mori* silk fibroin membranes using: (A) LiBr–H$_2$O; (B) Ca(NO$_3$)$_2$–MeOH–H$_2$O; (C) CaCl$_2$–EtOH–H$_2$O; and (D) LiBr–EtOH–H$_2$O as solvent.
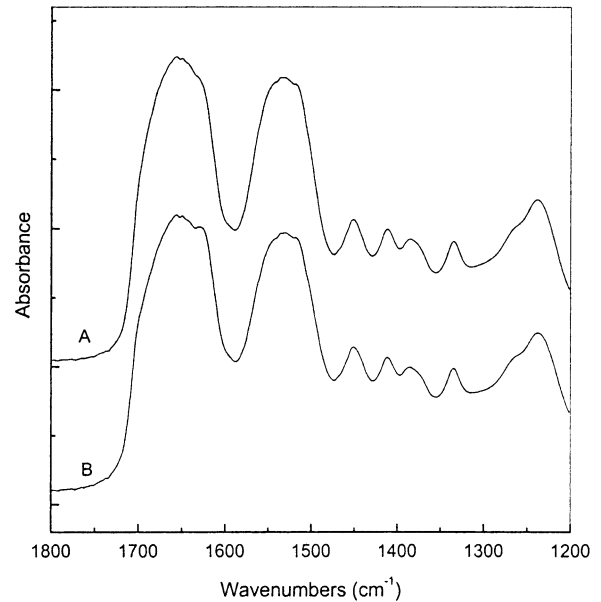

Fig. 6. FTIR spectra of regenerated *B. mori* silk fibroin membranes using: (A) LiBr–EtOH–H$_2$O; (B) LiBr–EtOH as solvent.

showed mainly random coil conformation. In Fig. 5, the RSF membrane cast from dialyzed LiBr–H$_2$O solution showed the characteristic peaks at 1654 cm^{-1} (amide I), 1532 cm^{-1} (amide II) and 1238 cm^{-1} (amide III). By comparison, the RSF membranes cast from dialyzed Ca(NO$_3$)$_2$–MeOH–H$_2$O, LiBr–EtOH–H$_2$O and CaCl$_2$–EtOH–H$_2$O solutions showed the characteristic peaks at 1657 cm^{-1}, 1651 cm^{-1} (amide I) (also see Fig. 6), 1535 cm^{-1} (amide II) and 1238 cm^{-1} (amide III). The single peak of amide I band in membrane cast from LiBr–H$_2$O solution (1654 cm^{-1}) was split into two (1657 cm^{-1} (stronger) and 1651 cm^{-1} (weaker)) in these membranes while the peak of amide III band increases 3 cm^{-1}. Though all the adsorption bands were assigned to random coil [1,5,6,29–32], there seemed a slight difference in conformation of RSF membranes cast from LiBr–H$_2$O solution compared with those membranes cast from Ca(NO$_3$)$_2$–MeOH–H$_2$O, LiBr–EtOH–H$_2$O and CaCl$_2$–EtOH–H$_2$O solutions.

The FTIR literature on RSF membranes from aqueous solutions seldom considers conformations other than random coil. Though Asakura et al. reported the evidence for a silk II (β-sheet) structure in a RSF membrane using ^{13}C CP-MAS NMR, the authors could not verify this using FTIR spectroscopy [31]. We used a highly sensitive MCT-A detector which clearly showed a shoulder in the amide I peak at around 1630 cm^{-1}. In order to examine the multiple components in the amide I peak, a second derivative technique [5] was used. Thus second derivative spectra of a RSF membrane cast from LiBr–H$_2$O solution (Fig. 7) clearly revealed the two components (1624 and 1703 cm^{-1}) characteristic of the β-sheet conformation [5,33] and a third component at 1653 cm^{-1} which was assigned to random
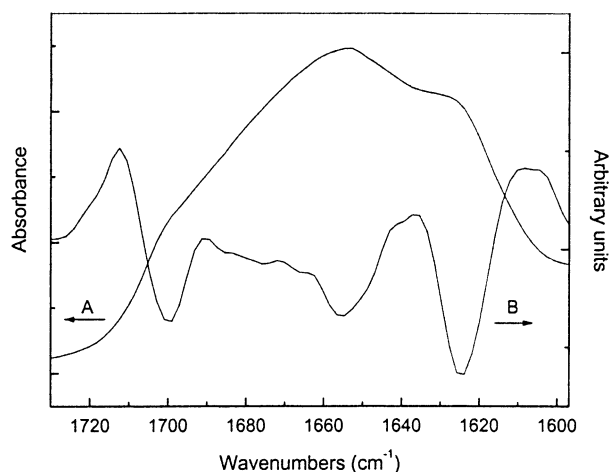
Fig. 7. Normal FTIR spectrum (A) and second derivative spectrum (B) of regenerated *B. mori* silk fibroin using LiBr–H$_2$O as solvent.

coil (as discussed above). Closely similar results were obtained for membranes cast from Ca(NO$_3$)$_2$–MeOH–H$_2$O, LiBr–EtOH–H$_2$O and CaCl$_2$–EtOH–H$_2$O solutions. It is intriguing that the FTIR spectra of RSF membranes cast from LiBr–EtOH solution showed a more significant peak of β-sheet conformation in amide I than the membranes cast from LiBr–EtOH–H$_2$O solution (Fig. 6). This indicated that there was more β-sheet structure in membranes cast from LiBr–EtOH solution. Furthermore, it correlates with our finding that silk fibroin is more viscous in LiBr–EtOH than in LiBr–EtOH–H$_2$O, and showed shear thinning not seen in the latter solvent.

4. Conclusions

We report the effects on rheological behavior and FTIR spectra of four solvents commonly used to dissolve *B. mori* silk fibers. Our rheological results showed that Ca(NO$_3$)$_2$–MeOH–H$_2$O and LiBr–EtOH–H$_2$O had the strongest solvation for the silk fibroin chains, which showed an almost constant viscosity (Newtonian behavior) over most of the shear rate range (0.1–500 s^{-1}). In contrast, the LiBr–H$_2$O (9.5 M LiBr aqueous solution) appeared to have the weakest solvation with similar effects on the fibroin molecules to pure water. Thus the results help to explain why silk is so soluble in the first two reagents while it dissolves less in aqueous LiBr.

FTIR spectra of RSF membranes cast from dialyzed solutions prepared in the different solvents showed mainly random coil conformation with a small proportion of β-sheet. The spectra also indicated that the secondary structure of the silk fibroin in the membrane cast from LiBr–H$_2$O is slightly different from those prepared using the other solvents. Moreover, membranes cast from LiBr–EtOH showed the most β-sheet conformation among all the RSF membranes studied.

These results have the following implications:

1. When RSF membranes are to be used to study the conformation changes in silk fibroin, it is better to use LiBr–H$_2$O as a solvent, because it gives the least conformation presumed to be closest to the original state of fibroin in the silk gland (mainly random coil [34]).
2. If the RSF containing membrane is used after completing the conformation transition from random coil to β-sheet (e.g. for immobilizing an enzyme or to make the silk fibroin insoluble), it is probably better to use CaCl$_2$–EtOH–H$_2$O and Ca(NO$_3$)$_2$–MeOH–H$_2$O because they dissolve silk fibers quickly.
3. If the purpose of dissolution of silk fiber is to make polymer blends (e.g. silk–polyamide blend), it may be advantageous to use Ca(NO$_3$)$_2$–MeOH–H$_2$O and LiBr–EtOH (both with and without water) because they can dissolve up to 20% of silk fibroin.

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