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FTIR and WAXD Study of Regenerated Silk Fibroin

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Abstract. In this study, regenerated *Bombyx Mori* (*B. Mori*) silk fibroin from two aqueous solvents was analyzed for structural deviations. Results from Fourier transform infrared spectroscopy (FTIR) and Wide angle x-ray diffraction (WAXD) implied great alteration in the secondary structure, crystallinity and molecular weight due to the regeneration process.

Introduction

Silk I, silk II, and silk III are the suggested structures of fibroin [1-3]. There are also three silk fibroin secondary structures; a random structure, an α-structure in absence of shear force, and a β-structure induced under shear force or action of solvents, such as methanol [4,5] **Table 1** shows *B.mori* fibroin conformations within FTIR amide absorption bands, and their vibrations. The amount of β-sheet absorptions and the intensity of peaks, give a relative estimate of the amount of fibroin crystallinity [6-8]. WAXD diffraction crystallography gives a quantitative analysis of crystallinity and the secondary structure[9,10].

Table 1 Amide wave numbers and protein secondary structure [11-17].

Conformation	Amides and Wavelengths (cm^{-1})		
	I (CO stretch)	II (NH deformation)	III (CN stretch, NH bends)
α-helix	1648-1660	1540-1550	1304-1313
β-sheet	1625-1640, 1690 weak	1520-1530	1219-1245
β-turns	1660-1685		1265-1291
Random coil	1625-1660, 1640-1648	1520-1545	1257-1258
310-helix	1660-1670		1265-1291

The main aim of this study was to establish the conformational changes and the effect on the secondary and crystalline structure due to the regeneration process of fibroin.

Experimental

Silk Dissolution and Preparation SF Films

Bombyx mori silk fibers were degummed by boiling in 0.5% (w/w)Na_2CO_3 at 100^oC followed by rinsing in distilled water, and drying at room temperature. In one experiment, degummed silk fibers (DS) were put in 9.5M LiBr to yield 15% (w/v), and then heated for 4 hours, at 60^oC, with constant agitation. Dialysis of the cooled solution was done against pure water, using cellulose dialysis tubing (MWCO; nominal: 14000) for three days. Filtration was then done for the dialyzed product and let to evaporation (at room temperature), to give a dry silk fibroin film-SFLiBr. A ternary system of solvent; $CaCl_2$:methanol:water, in the ratio of 1:2:8 was used in another experiment, to dissolve degummed silk. The same procedure with LiBr applied to give dry silk fibroin films, $SFCaCl_2$.

Characterization of Silk Fibroin

FTIR spectroscopy (Nicolet NEXUS670; Thermo Nicolet Co., USA) was used to elucidate the fibroin amide absorption bands, and subsequently, the secondary structure. Specimens studied included; degummed *B.mori* fibers (DS) and SF films. WAXD assessment was also done with diffraction angles; 2θ: 5°- 60° (D/max-2550PC diffractometer; Rigaku Co., Japan) at 40 kV and 2000 mA using Cu/K-α1 radiation.

Results and Discussion

FTIR Spectroscopy

DS was used as our reference, with assumption that degumming does not alter the secondary and crystalline structure of fibroin [10,18]. Structural conformations of the specimens were evaluated and represented on FTIR (**Fig.1a-d).** Without figures, the main amide absorption bands appear the same for all specimens. A closer observation with figures and shapes reveals that major absorption bands for the silk films exhibited similar shapes and close intensities, different from the degummed fibers. Also the peaks for films are broader and shorter compared to degummed fibers.

Degummed Silk Fibroin (DS)

Amide I absorption was between 1698 cm^{-1} and 1622 cm^{-1} (C=O stretching, β-sheet). Amide II was exhibited at 1514 cm^{-1}(CN stretching and NH bending, β-sheet). Amide III bands were between 1258 cm^{-1}(α-helix/random coils) and 1229 cm^{-1}(β-sheet). A strong and intense amide A band was observed at 3282 cm^{-1}(β-sheet) indicating NH bending.

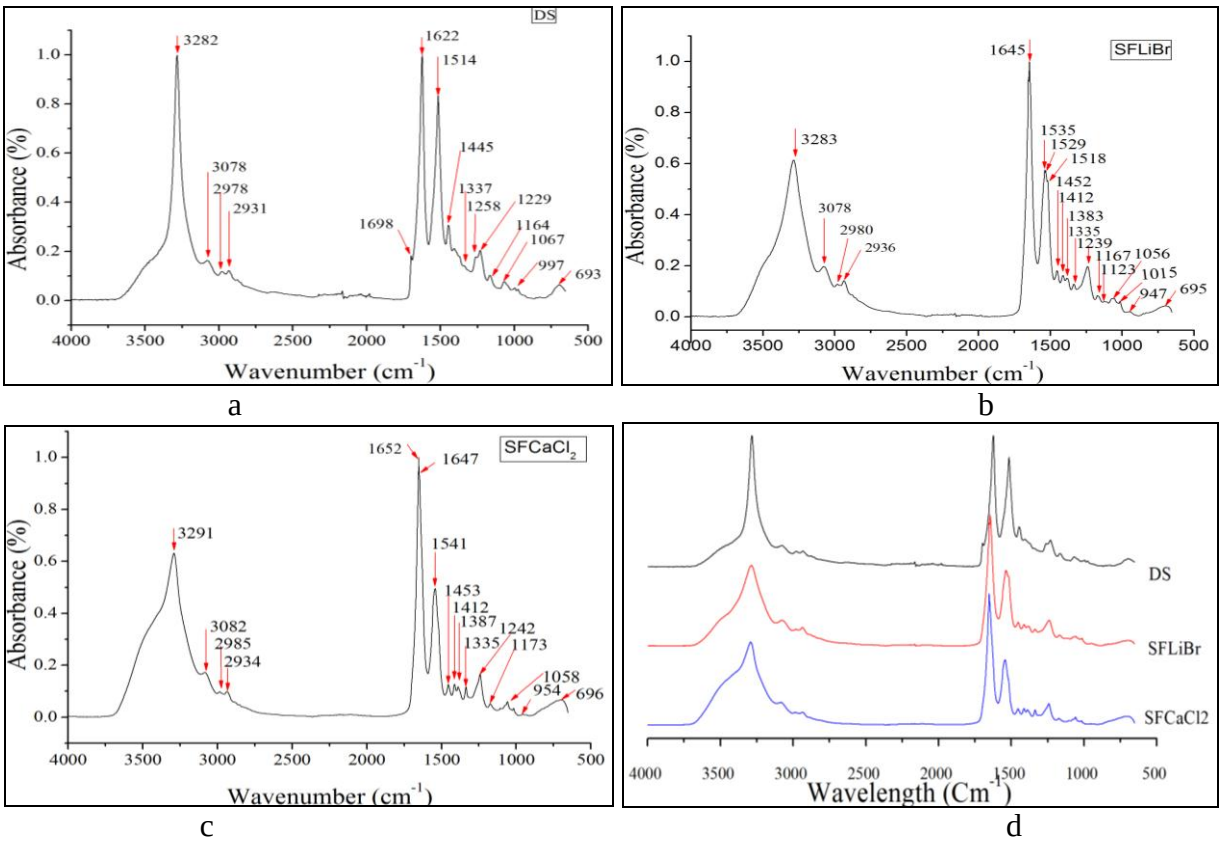


Figure 1. Extract of FTIR spectra showing amide absorption bands: **a.** Degummed silk (DS) **b**. LiBr derived fibroin film (SFLiBr); **c**. CaCl2 derived fibroin film (SFCaCl2); **d**. a combined FTIR spectral analysis for the three specimens

LiBr Derived Silk Fibroin Film (SFLiBr)

FTIR spectra showed a shift in amide I, from β-sheet (1622 cm^{-1} originally in DS, to 1645 cm^{-1}) to random coil structure. Amide II bands shifted from those originally in DS, to between 1535 cm^{-1} (random coil/α-helix) and 1518 cm^{-1}(β-sheet). Shifts in amide III were more pronounced from 1229

cm^{-1} in DS, to another β-sheet position at1239 cm^{-1} in SFLiBr. The amide A band initially at 3281 cm^{-1} shifted a little to 3283 cm^{-1} (β-sheet) but lowered in intensity.

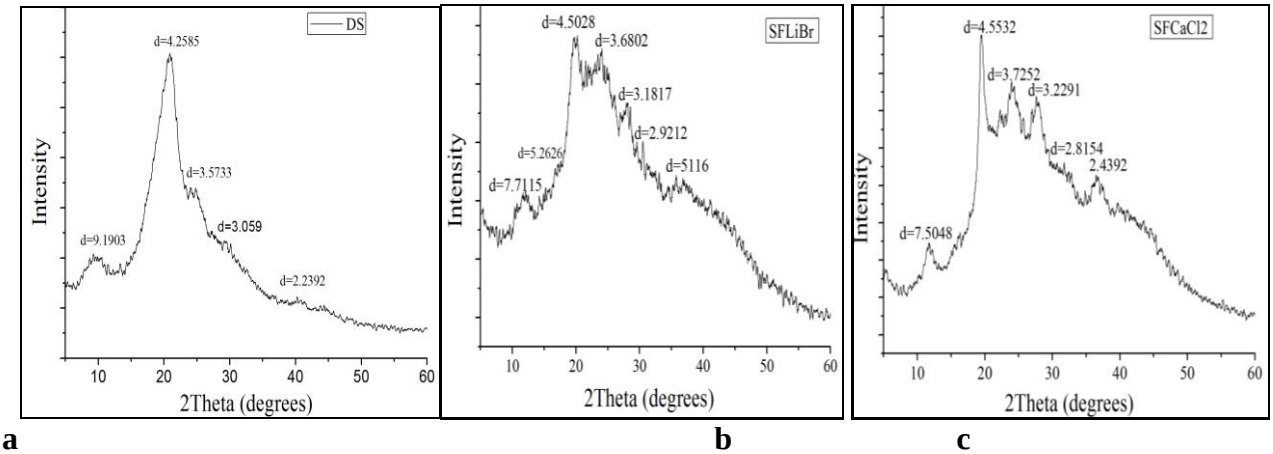
$CaCl_2$ Derived Silk Fibroin Film ($SFCaCl_2$)

A strong amide I absorption band showed between 1652 cm^{-1} and 1647cm^{-1} (random coils) almost similar to that in SFLiBr spectra. There was one visible amide II absorption band at 1541 cm^{-1}, corresponding to α-helix. Compared to DS and SFLiBr, this was a strong shift towards a random coil structure. Amide III was indicated at only one band; 1242 cm^{-1}(β-sheet). A much shift in absorption was observed in amide A absorption band (from 3282 cm^{-1}, initially in DS, to 3291 cm^{-1}-α-helix), with a reduced intensity though slightly higher than was observed in SFLiBr.

The shifts from reference (DS) values were higher in $SFCaCl_2$ than in SFLiBr. Also, intensity values of peaks were generally lower in $SFCaCl_2$ than those for SFLiBr and DS. Moreover, relative to DS, the changes in absorbed wavelengths tended more to the random coil structure in $SFCaCl_2$ than it was in SFLiBr. This is an indication of higher reduction in the crystalline secondary structure and the concentration of fibroin in $CaCl_2$ than it was in SFLiBr. FTIR spectroscopy was insufficient for conclusive assertions on the crystalline and secondary structural evaluation. Hence, WAXD analysis was used for quantitative analysis draw better conclusions.

Wide Angle X-ray Diffraction (WAXD)

WAXD was used to predict the crystalline nature and corresponding secondary conformations for each specimen **(Fig.2**). A peak search and analysis were performed, including calculation of d-spacings (Å) and the percentage crystallinity. Using the method described by Hermans and Weidinger [19], and Weidinger and Hermans [20], it was established that; DS, SFLiBr and $SFCaCl_2$ had crystallinity of 46.47%, 19.26% and 16.26% respectively. These results imply a pronounced reduction in the crystallinity of fibroin with regeneration. As earlier observed in the FTIR analysis, this is characteristic of reduced molecular weight during dissolution and dialysis of fibroin. Also, SFLiBr slightly had higher crystalline structure compared to $SFCaCl_2$, which could be explained by separation of several amide bonds in the silk molecular chains during protein solubilization in the $CaCl_2$ ternary system. With lack of control of the dialysis process, differences in quantity of salt residues after dialysis may also account for the slight observed difference in the crystallinity of fibroin films. Again, lithium is particularly hasty to be dialyzed due to its high affinity for silk fibroin [21,22], hence its derivatives may show more crystallinity than $CaCl_2$ derived fibroin.



a **b** **c**

Figure 2. Comparison of the WAXD patterns for the five specimens: **a**- Degummed silk (DS); **b**-LiBr-derived fibroin film (SFLiBr); **c**- CaCl2-derived fibroin film (SFCaCl2)

The diffraction graphs for the SF films are quite different from the reference(DS). The strongest β-sheet crystalline peak, usually at 21.0^o, was only observed in DS. Most observed 2Theta values in SF films and corresponding d-spacings were characteristic of silk I, which implied less β-sheets, hence an amorphous structural dominance. In general, the diffraction patterns of SF films were similar, except for the slight difference in the crystallinity.

Conclusion

Silk fibroin was successfully regenerated using concentrated aqueous lithium bromide solution and a neutral $CaCl_2$:Water:Ethanol system. Collaboration of FTIR and X-ray results revealed that there is an appreciable amount of fibroin that is contained in regenerated silk. This fibroin, without treatment, is mainly silk I, characterized by mainly random coils and α-helix structure. Our findings suggest that the type of solvent used to dissolve silk has insignificant effect on the structure of regenerated fibroin. Results also imply that fibroin undergoes loss of molecular weight and loss of secondary structure crystallinity during regeneration. Hence, an aqueous environment also facilitates the transition to a random structure. Lastly, it was found that untreated regenerated fibroin is largely amorphous. Appropriate treatment is necessary to restore regenerated fibroin to the original or a better level of conformation and improve the cyrtallinity. Also, other dissolution parameters like temperature and time need to be studied deeply.

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