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Research Article

MECHANICAL STRESS AND THERMAL TREATMENTS INDUCED ALPHA-HELIX TO BETA-SHEET TRANSITION IN SILK FIBROIN FILMS

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ABSTRACT

Bombyx mori consisting of the heavy and light chains and forming the filaments of the silk fibre is the interior structural protein which gives silk its mechanical strength, softness, and elasticity. Silk fibroin (SF) finds application in the fields of the biomedical and biotechnological engineering. Silk fibroin stands as an exemplar of fibrous proteins containing crystalline beta-sheets (silk-II) and β-turns (silk-II). Exposure to organic solvents, mechanical stressor, and thermal treatments can induce non-reversible crystallization. This study compares the structural and thermal properties of silk fibroin films obtained from different raw materials: silk fibroin processed (annealing treatment, freezing, and freeze-drying) and not processed. The presence of crystalline and amorphous structures was evaluated using ATR/FTIR spectroscopy and thermal properties of SF films were studied by DSC measurements. Accordingly, to ATR/FTIR results, the presence of beta-sheet (silk-II) structure is present in the samples that were processed by annealing treatment, freezing, and freeze-drying as well as *Bombyx mori* cocoon. Difference in exothermic peaks (around 200°C) in DSC curves reveals that samples not processed present a higher content of amorphous structures than samples processed. The degradation peak around 300°C was observed for all samples. The analysis also points out that water annealing treatment, freezing, and freeze-drying induce conformational changes in secondary structures of SF films. It can be concluded that the presence of crystalline structures in SF films varies with the process. This fact represents a progress in the development of processing alternatives to control SF materials structure and physio mechanical properties.

Keywords: Silk fibroin; Beta-sheet; Crystalline structures; Biomedical engineering

INTRODUCTION

Silk, popularly known in the textile industry for its lustre and mechanical properties, is produced by cultured silkworms. They are commonly defined as protein polymers, which are present in the glands of arthropods such as silkworms, spiders, scorpions, mites, and bees, and then spun into fibres during their metamorphosis. However, the Bombyx mori has been extensively studied due to high biocompatibility, robust mechanical performance, tunable degradation, ease of processing, sufficient supply, and ease of acquisition from the mature sericulture industry (Vepari and Kaplan, 2007; Qi *et al*., 2017).

Fibroin films and matrices can encapsulate and stabilize proteins and enzymes through intermolecular forces and release the proteins undenatured and the enzymes with full activities. SF biopolymer can be transformed into different forms, including scaffolds, hydrogels, microspheres, and nanoparticles for controlled release of a variety of drugs (Huang *et al*., 2017).

B. mori silk fibres consist primarily of two components, SF, and sericin. Sericin is a glue-like amorphous protein that acts as an adhesive binder to keep the structural integrity of the fibres. Sericin is soluble and can be removed by a thermochemical process known as degumming (Motta, *et al*., 2002; Qi *et al*., 2017). SF is a fibrous protein (water-insoluble) and it has hydrophobic domains compost by repeated amino acid (Gly-Ser-Gly-Ala-Gly-Ala) sequence which is assembled into Nano-crystals (β-sheet), and hydrophilic links between the hydrophobic domains that consist of bulky and polar side chains that form the amorphous part of the secondary structure. The chain conformation in amorphous blocks is a random coil, which gives elasticity to silk (Kundu *et al*., 2013; Huang *et al*., 2017; Qi *et al*., 2017). Thus, the SF can have three conformations named silk-I, silk-II and silk-III (Motta, *et al*., 2002; Hu, 2006). Silk-I as a metastable form of SF that is soluble in water and non-crystalline; random coil and α-helix conformations are usually called silk-I. Silk-II has been vice-versa long regarded as a prime example of the well-oriented β-sheet conformation. Silk-III is an unstable structure observed at the water-air interface (Chen *et al*., 2001; Motta, *et al*., 2002; Qi *et al*., 2017).

To obtain water-soluble SF is necessary a degumming process to remove the sericin followed by dissolution using concentrated salt solutions, resulting in a non-β-sheet (less ordered) state fibroin solution which can be dried to form solid less ordered, non-crystalline, SF films.

Mechanism of the exposure to organic solvents and another polymer, application of mechanical stress, and thermal treatments can induce non-reversible crystallization, that is, induce the conformation change of fibroin from silk-I resulting in the formation of self-assembled β-sheets (Hirabayashi and Liang, 1937; Ishida *et al*., 1990; Chen, Li and Yu, 1997; Tretinnikov and Tamada, 2001; Motta, *et al*., 2002; Gil, *et al*., 2005; Hu, *et al*., 2006). The conformational transition of α-helix and random coil to highly stable β-sheets is required in silk products to provide good physiomechanical properties and resistance to dissolution, thermal, and enzymatic degradation (Kundu *et al*., 2013).

In the present study, was compare the structural and thermal properties of silk fibroin (SF) films obtained from the different process: silk fibroin processed (annealing treatment, freezing, and freeze-drying) and not processed. The presence of crystalline (silk-I and silk-II) and amorphous structures were evaluated using Attenuated Total Reflectance-Fourier Transform Infrared (ATR/FTIR) spectroscopy and thermal properties of SF films were studied by DSC measurements.

MATERIALS AND METHODS

Bombyx mori silk fibroin was prepared adapted from (Komatsu *et al*., 2017). Briefly, silk sericin was extracted by treating silk cocoons in an aqueous solution of 0.5 wt% Na_2CO_3 (120°C) for 15 min using an autoclave. The silk fibroin was rinsed thoroughly with water to extract the sericin proteins. The degummed silk fibroin was dissolved in CaCl2.2H20/CH3CH2OH/H2O solution (mole ratio, 1:2:6) at 85°C. Then the fibroin solution (FS) was filtered and dialyzed against distilled water for 3 days to yield SF. The final fibroin concentration was about 2-3 wt%, which was determined by weighing the remaining solid after drying.

Preparation of silk fibroin film

The blend films were prepared by casting the SF solutions onto polystyrene plates and allowing the (A1) solvent to evaporate at room temperature; (A2) annealing treatment; (A3) freezing; and (A4) freeze-drying. In this study, the films were not treated or neutralized in order to evaluate the β-sheet conformation of the membranes in their non-treated state.

Fourier transform infrared spectroscopy (FTIR)

FTIR analysis (Shimadzu, IRAffinity-1, Kyoto, Japan) was used to collect FTIR spectra via Labs Solutions Software v.2.10. The chemical functionalities of the samples were determined by an attenuated total reflectance (ATR) cell on the FTIR spectrophotometer over the range between 4000 and 600 cm^{-1} at 4 cm^{-1} resolutions, averaging 128 scans.

Differential scanning calorimetry (DSC)

DSC was performed on a Shimadzu, TA-60, Kyoto, Japan, calibrated using indium as the reference material. A sample of 2 mg was packed in a hermetically crimped aluminium pan and heated under dry nitrogen purged at 50 mL per min^{-1}. The samples were heated from 25 to 350°C at a rate of 10°C min^{-1}.

RESULTS

Fourier transform infrared spectroscopy (FTIR)

Structural changes, attributable to (A1) solvent to evaporate at room temperature; (A2) annealing treatment; (A3) freezing; and (A4) freeze-drying were initially studied by qualitative analysis of IR spectra analysis. By analysing the infrared spectrum, the structural conformation of SF can be determined, depending on the wavenumber location of the absorption bands of amides I, II and III.

The ATR/ FTIR results obtained for silk fibroin samples (A1-A4 and cocoon) are shown in Figure 1 (A-B). Figure 1A is showed FTIR spectrophotometer over the range between 4000 and 600 cm^{-1} of the samples. All samples showed broad peaks from 3600 to 3100 cm^{-1} which correspond to the -OH stretching and bending vibration mode.

The Figure 1B is showed FTIR spectrophotometer over the range between 2000 and 1000 cm^{-1} that permit a better resolution for amide groups presence. The stretching vibration referred to amide II and amide III was observed for all samples. Our results (Figure 1B) showed that the samples cocoon, A2, and A3 have stretching vibration at 1624, 1635 and 1624 cm^{-1}, respectively. These results indicate that annealing treatment (A2) and freezing (A3) process permitted conformational transition of α-helix and random coil to highly stable β-sheets (amide I), the same form with cocoon. The samples A1 (to evaporate at room temperature) and A4 (freeze-drying) showed stretching vibration at 1645 cm^{-1}, thus the evaporate of the

solvent and freeze-drying technique not permitted conformational transition of α-helix and random coil to highly stable β-sheets (amide I), the same form with cocoon (Figure 1).

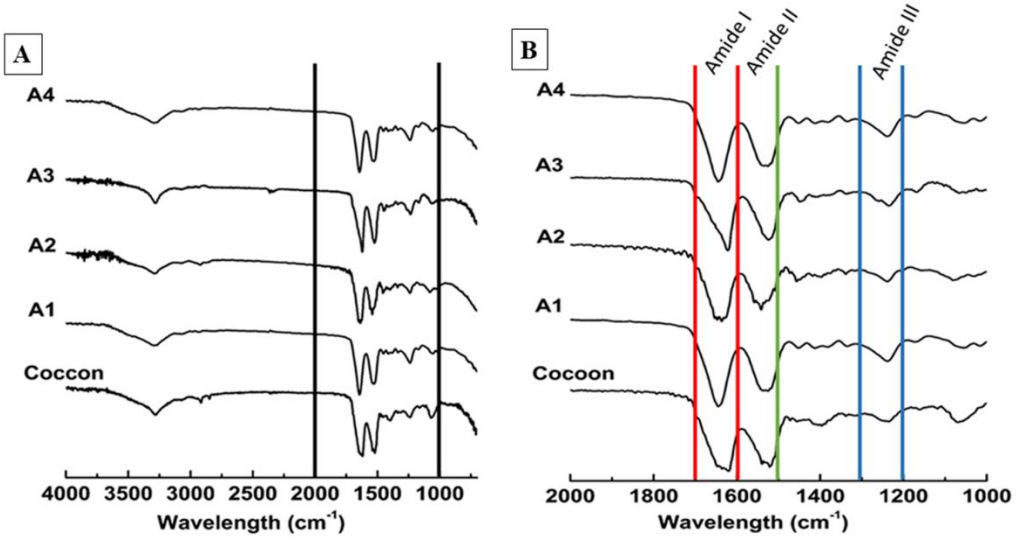

Figure 1: ATR/FTIR spectra of silk fibroin samples: cocoon, (A1) solvent to evaporate at room temperature, (A2) annealing treatment, (A3) freezing, and (A4) freeze-drying. A: spectrum 4000-600 cm^{-1}, B: spectrum 2000-1000 cm^{-1}.

Differential scanning calorimetry (DSC)

Figure 2 shows the DSC thermograms for fibroin samples (cocoon and A1-A4). Endothermic peaks below 75°C were observed, related to the evaporation of the adsorbed water in the films. SF film was not submitted to any kind of physical or chemical treatment (Figure 2-A1), presented an endothermic peak related to its thermal degradation at 286°C and a small exothermic peak/step at 213°C. The same behaviour was observed in samples passed by the freezing process (A4). The cocoon shows endothermic peaks at 318°C and a small exothermic peak at 180°C. The A2 and A3 samples show the endothermic peaks at 290 and 291°C but did not show the exothermic peak (Figure 2).

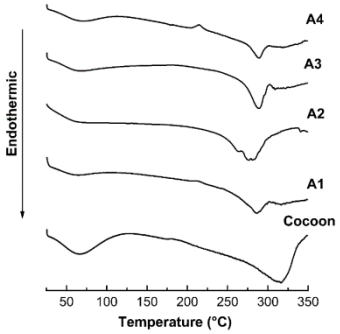

Figure 2: DSC of silk fibroin samples: cocoon, (A1) solvent to evaporate at room temperature, (A2) annealing treatment, (A3) freezing, and (A4) freeze-drying.

DISCUSSION

SF has proven to be an interesting biomaterial for tissue engineering, drug delivery, and other biomedical applications (Kundu *et al*., 2013; Sun *et al*., 2014; Huang *et al*., 2017). The relatively simple process, transparency, mechanical robustness, biocompatibility, easy surface-patterning, and degradability at a controllable rate have promoted the interest in using silk for biocompatible and bio functional optical materials and interfaces. Silk fibroin films have been cast from aqueous systems and submitted to different mechanical stress (Vepari and Kaplan, 2007).

Previous studies have suggested that the β-sheet conformation can be induced in silkworm fibroin by a stretching force, and the formation of spider dragline silk also involves a stress-induced β-sheet formation by the extensional flow. But the mechanism involved in the conversion of a hydrogel of silk fibroin in the silk-I state into the silk-II state remains unresolved (Li *et al*., 2001; Kundu *et al*., 2013). The changes in silk structure can result in better mechanical and degradability properties of the films.

To determine the conformational structure of the fibroin films were observed the amide I region between 1600 and 1700 cm^{-1} which is the most useful for the IR structural analysis of proteins. The amide I band represents primarily the C=O stretching vibration of the amide group. In general, the amide I mode associated with the α-helical conformation occurs at 1650-1660 cm^{-1}, the random coil conformations give bands in the range of 1640-1650 cm^{-1}, and the β-sheet conformation results in IR bands between 1620 and 1640 cm^{-1}. The annealing treatment and freezing process induced conversion of the α-helix and random coil to highly stable β-sheets, but the evaporation of the solvent and freeze-drying process was not efficient.

Several authors have published about silk fibroin and its β-sheet conformation induced by exposure to organic solvents, mechanical stressor and thermal treatments (Ishida *et al*., 1990; Chen, *et al*., 1997; Tretinnikov and Tamada, 2001; Hu, *et al*., 2006; Hu *et al*., 2012;

Jaramillo-Quiceno, *et al*., 2017), but the influence of the freezing and freeze-drying process have not been reported.

The endothermic peak is attributed to the molecular motion within the α-helix crystals and the exothermic peak is attributed to the crystallization during heating from a silk-I to a silk-II structure (Hu *et al*., 2012). Thermal degradation peaks of SF films at temperatures around 270°C are characteristic of amorphous SF (silk-I) (Motta, *et al*., 2002; de Moraes *et al*., 2010; Jaramillo-Quiceno, *et al*., 2017), indicating that did not occur the transition to β-sheet conformation (silk-II). The samples had the highest degradation temperature, are related to its high crystallinity and orientated chain alignment in nature (de Moraes *et al*., 2010). The exothermic event is related to random coil transition to β-sheet, thus the fibroin crystallization and its absence can indicate that the crystallization process is finished during the getting process of the SF film. Thus, as discussed in the FTIR section (Figure 1), the annealing treatment (A2) and freezing (A3) process formed major silk-II in the structure, and for solvent to evaporate at room temperature (A1) and freeze-drying (A4) did not occurs the transition the silk-I to silk-II.

CONCLUSION

The molecular conformation of SF membranes is an important parameter that needs to be controlled since it affects their physical and chemical properties. The annealing treatment and freezing process were able to induce the formation of silk-I and silk-II in SF films. These methods can produce any desired structure (from silk-I to silk-II with controllable beta sheet crystallinity) in silk materials, and thereby provides control over the properties (e.g., mechanical strength, degradation rate, cell response). This control can provide useful options for biomedical applications of silk materials, such as in tissue engineering, drug delivery, or for optical systems based on silk materials.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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