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Isolation and processing of silk proteins for biomedical applications

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ABSTRACT

Silk proteins of silkworms are chiefly composed of core fibroin protein and glycoprotein sericin that glues fibroin. Unique mechanical properties, cyto-compatibility and controllable biodegradability facilitate the use of fibroin in biomedical applications. Sericin serves as additive in cosmetic and food industries, as mitotic factor in cell culture media, anti-cancerous drug, anticoagulant and as biocompatible coating. For all these uses; aqueous solutions of silk proteins are preferred. Therefore, an accurate understanding of extraction procedure of silk proteins from their sources is critical. A number of protocols exist, amongst which it is required to settle a precise and easy one with desired yield and least down-stream processing. Here, we report extraction of proteins employing methods mentioned in literature using cocoons of mulberry and nonmulberry silks. This study reveals sodium carbonate salt-boiling system is the most efficient sericin extraction procedure for all silk variants. Lithium bromide is observed as the effective fibroin dissolution system for mulberry silk cocoons; whereas heterogeneous species-dependent result is obtained in case of nonmulberry species. We further show the effect of common post processing on nanoscale morphology of mulberry silk fibroin films. This knowledge eases the adoption and fabrication of silk biomaterials in devices and therapeutic delivery systems.

1. Introduction

Silk biomaterials are widely envisioned and adopted for applications including tissue modelling (bone, cartilage, ligament, nerve etc.) [1,2], flexible electronic diagnostic devices and implantable optical systems such as eco-resorbable transient electronics [3] and bio-memristors [4]. The large scale cultivation of silkworms through sericulture serves as rational cost-effective source for the silk biopolymer. Additionally, in countries like India, the second largest silk producer in the global market [5], sericulture also supports a large micro-economy in rural areas. Silk from domesticated sources like *Bombyx mori* belongs to mulberry silks, cultivated in plantations. In contrast, temperate and tropical tasar silks from species including *Antheraea pernyi* [6] and *Antheraea mylitta* [7] belong to the nonmulberry silk category. These silk fibroins possess the Arg-Gly-Asp (RGD) recognition sequences, which are known to facilitate cell–biomaterial interaction [8], placing nonmulberry silk biomaterials in an advantageous position for biomedical applications over mulberry silks.

Building off the two chief proteins constituting silks–fibroin and sericin. In silk cocoon, two fibroin brins are conglutinated by the sericin glue resulting in silk fibres [9]. The adhesive protein sericin is amorphous in nature and can be extracted by a thermo-chemical process, known as degumming [10]. Post-extraction, the fibroin protein is the more commonly used and reported silk protein, having been purposed for a wide range of applications. Interestingly, the properties of the regenerated fibroin, including its shelf-life tend to be greatly influenced by the regeneration process and extent of sericin removal [11]. Therefore, the choice of degumming process is a crucial step in the determination of the final fate of regenerated fibroin. Following the removal of sericin, the degummed fibres are further dissolved in aqueous or organic solvents to regenerate fibroin protein solutions [9]. The dissolution of fibroin is itself a complex task, depending on the source of the cocoon. For instance, the presence of hydrophobic poly-Ala sequences in nonmulberry fibroin contributes to a highly ordered crystalline structure, in comparison to the corresponding poly-Gly-Ala sequences of mulberry silk [12]. This makes the dissolution of nonmulberry fibroin a formidable task. Solvent systems like $CaCl_2$/ethanol/water [13], LiBr [14], LiSCN [15], NaOH [16] and $Ca(NO_3)_2$-MeOH-H_2O [17] have been used for dissolution of fibroin

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fibres. Such solutions are subsequently dialysed before processing into diverse biomaterial morphologies.

For all forms of cell culture and biomedical applications involving silk proteins, processing of the cocoons to extract the relevant proteins – fibroin and/or sericin is required [18]. This necessitates a systematic study involving the quantitative assessment of various processing conditions for the cocoon. In this study, we specifically focus on evaluating processes for the extraction-dissolution of silk proteins to obtain the highest yields for fabricating different matrices for tissue regeneration. A library of nonmulberry cocoons consisting of the silk cocoons of *A. mylitta*, *A. assamensis*, *A. proylei* and *Philosamia ricini* is considered. Different degumming procedures (extraction of sericin) such as urea, NaCl, Na_2CO_3, NaOH and regeneration systems including LiBr, $Ca(NO_3)_2$-MeOH-H_2O, LiSCN, $CaCl_2$/ethanol/water and NaOH are compared. The extent of sericin removal is quantified by measuring the fibre diameters and observing the cocoon microstructures using scanning electron microscopy (SEM). While the processing conditions are focused on nonmulberry silkworm species, the silk cocoon of the most widely used mulberry silkworm, *B. mori* is used as reference for this study. Due to its widespread usage and ready availability, we show how *post-processing* of *B. mori* fibroin, after regeneration has the potential to create a diversity of properties, specifically in terms of nanoscale roughness. We anticipate that these studies will provide a valuable reference for researchers in the field as they harness the cocoon and its constituent proteins for increasingly sophisticated applications.

2. Materials and methods

2.1. Materials

Cocoons: Cocoons of *Antheraea mylitta* and *Bombyx mori* were collected from West Midnapore district of West Bengal state of India. *A. assamensis/assama* and *Philosamia/Samia ricini* silk cocoons were obtained from Guwahati, Assam State of India and *A. proylei* from Imphal, Manipur State of India respectively.

Chemicals: LiSCN from Sigma (USA), all other extraction solutions including urea, NaCl, Na_2CO_3, NaOH and dissolution solutions such as $CaCl_2$/ethanol/water, LiBr, $Ca(NO_3)_2$ and ethanol were of analytical grade, procured from Merck, India and used without any further purification. Cellulose dialysis tubing of cut off 12 kDa (Pierce, USA) was used for dialysis against de-ionized water.

2.2. Methods

2.2.1. Degumming of silk cocoons

Initially, four degumming methods were investigated: (i) Degumming with urea buffer was performed in 8 M aqueous urea solution containing 0.04 M Tris–SO_4 (pH 7) and 0.5 M β-mercaptoethanol for 2 h at 80 °C [19]. (ii) Degumming with NaCl (sodium chloride) solution was done by treating the cocoon pieces with 1 wt% NaCl at room temperature overnight under continuous shaking at 100 rpm [20]. (iii) Degumming via Na_2CO_3 method using a 0.02 M aqueous solution of Na_2CO_3 and boiling for an hour [21]. (iv) A modified NaOH based extraction, wherein cut cocoon pieces were treated with 0.5 N sodium hydroxide for 12 h at room temperature under rigorous shaking at 100 rpm [16].

Following each degumming treatment, fibres were pelleted down by centrifuging at 10,000 rpm for 10 min. The supernatant contains the water soluble sericin, which was then dialyzed against de-ionized water for 24 h using cellulose tubing (12 kDa, MWCO) to extract the protein. Sericin is, in its own right, a highly biocompatible protein with a wide variety of uses in tissue engineering and drug delivery. Our group has reported extensively on the novel properties of sericin and its use [20,22,23].

2.2.2. Determination of degumming ratio

The degumming ratio (D_r) to obtain the efficiency of the above processes was calculated as described elsewhere [19]. Briefly, the weight of cocoon pieces before and after the degumming treatment was measured and the degumming ratio was confirmed using the following equation:

$$D_r = \frac{Wt_{initial} - Wt_{degummed}}{Wt_{initial}} \times 100$$

where $Wt_{degummed}$ = weight of the degummed cocoon pieces/fibres and $Wt_{initial}$ = initial weight of the cocoon pieces respectively.

2.2.3. Scanning electron microscopy (SEM) and atomic force microscopy (AFM)

SEM was used to study the morphology of degummed silk fibres and also native silk cocoons. Cocoons from different sources were cut into small pieces and directly used for SEM analysis after gold coating. The degummed fibres were dried under laminar flow overnight and assembled on SEM sample holders via carbon tape. Samples were gold-coated by sputtering at room temperature and images were taken using JEOL JSM (5800) scanning electron microscope at an operating voltage of 20 kV. The diameter of each fibre was calculated using the Image J software (v1.45). Atomic force microscopy (AFM) was used to study the surface morphology of cast fibroin films to investigate the effect of post-processing on fibroin from the mulberry silkworm *B. mori*. All AFM imaging experiments were performed using an Asylum MFP-3D instrument (Asylum Research, Santa Barbara, CA).

2.2.4. Dissolution of degummed silk fibres for fibroin protein

Following degumming, the fibres were pelleted down and thoroughly rinsed in warm distilled water followed by drying at room temperature. Five fibroin fibre dissolution methods were used: (i) LiBr method: The degummed silk fibres were added to 9.3 M LiBr solution at 60 °C for 4 h, followed by dialysis against de-ionized water for 48 h [8]. (ii) $Ca(NO_3)_2$-MeOH method: Fibroin fibres were added to a 75% (w/v) $Ca(NO_3)_2$-MeOH clear solvent system at 65–70 °C for 2 h with vigorous stirring, followed by prolonged dialysis for 96 h [17]. (iii) Lithium thiocyanate (LiSCN) method: Degummed silk fibres were dissolved in LiSCN solution with continuous stirring and gentle heating for 2 h, followed by 24 h dialysis (slight modification of the protocol earlier reported in [15]). (iv) Ajisawa's method ($CaCl_2$/ethanol/water, 1:2:8 molar ratio): Silk fibres were immersed in 20 times (v/w) of Ajisawa's reagent ($CaCl_2$/ethanol/water, 1:2:8 molar ratio) with continuous stirring at 70 °C and dialyzed for 72 h [19]. (5) NaOH method: Silk fibres were dissolved in 1 N NaOH at 4 °C with continuous stirring for 24 h and 96 h dialysis [16].

All silk hydrolysates after dissolution were centrifuged at 8000 rpm for 20 min. The precipitated remnant was taken out and air dried. The percentage of dissolution of silk was the measured as follows:

$$\text{Percentage (\%) of dissolution of silk fibroin} = \left[1 - \frac{\text{Weight of remnants after dissolution}}{\text{Initial weight of degummed fibre}}\right] \times 100$$

2.2.5. Shelf-life of silk protein solution (sol–gel transformation)

Regenerated silk fibroin solutions after dialysis were kept in V-bottomed vials at room temperature and sealed. Gelation was confirmed by change in the transparency of the solution from clear to opaque and sticking to the bottom of the vials when inverted.