Physico-chemical properties of Artificial tear ducts from Fractionated Thai silk fibroin

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Abstract. Artificial tear ducts (ATD) or Jones tubes, have been used in patients with tear ducts obstruction for draining tears into the nose. Commercial ATDs are generally made of glass but they have slippery surfaces causing the ducts pushed out and easily broken. Polymer-based ATDs, are more flexible and adhesive to tissues. However, the users could encounter material-related problems due to allergic reactions, low tissue adhesion and complications. We designed artificial tear ducts from Thai silk fibroin (SF) solution. The SF was fractionated using freeze-thaw cycles (-4°C and 25°C for 5 cycles) into SF-P (precipitates, 63.53±4.58% yields) and SF-S (soluble, 34.05±5.76% yields (w/v)). The concentrated solutions (20%W/W) of SF, SF-P and SF-S were dip-coated into tubes. The leaking test was performed using simulated natural tear flow rate of 0.0022 mL/min for 6 h. SF-S duct lost its shape and leaked. Absorption of the balanced salt solution (BSS) of the SF and SF-P were at 5.67±0.76 % and 8.05±1.28 wt.% respectively, giving their wet inner and outer diameters at 2 and 2.5 mm. and the thickness 500 microns. Crystallinities of SF and SF-P analyzed using ATR-FTIR, were at 42.27 and 44.51% respectively. The thermal decomposition temperature of SF and SF-P analyzed using TGA, were at 277 and 280°C. Degradability in BSS containing lysozyme 1.69 U/mg to mimic tears at 37°C in vitro showing that both ATDs are stabilized for at least 4 weeks. BSS height obtained capillary test that compared with glass ducts, SF and SF-P at 7.0±0.0, 6.9±0.4 and 7.1±0.4 mm, respectively, while glass ducts were 34.81±0.0. The SF and SF-P have flexural stress were 55.24±7.68 MPa and 81.42±2.71 MPa, %flexural strain at max stress was 5.43±0.46% and 2.54±0.17% and flexural Modulus was 1.82±0.29 GPa and 6.13±0.51GPa respectively. The results of all experiments indicate that SF-P had the highest potential for further development into natural polymer-based ATDs.

1. Introduction
Artificial tear ducts (ATD) or Jones tubes, have been used in patients with tear ducts obstruction for draining tears into the nose. Commercial ATDs are generally made of glass but they have slippery surfaces causing the ducts to be pushed out and easily broken [1-2]. Polymer-based ATDs, such as silicone and polyethylene are more flexible and adhesive to tissues [3-4]. However, the users could encounter material-related problems due to allergic reactions, low tissue adhesion and complications. Generally, ATDs are reported to have 1.5 - 3.5 mm. inner diameter, 18-40 mm. length.

Thai silk fibroin is a natural protein derived from domesticated Bombyx mori that is known for its biocompatibility, slow biodegradability and good mechanical properties. Which has been reported silk fibroin scaffold for abdominal wall facial reinforcement proved to be free of complication rate and

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irritation in 93.5% volunteer when applied to abdomen [5], silk fibroin graft tube was implanted into the right carotid artery of a mice was reported to be stabled for 1-3 month [6]. The trilayered silk scaffolds fabricated for vascular tube had ultimate tensile strength of 12 MPa [7]. Therefore, We investigated the feasibility of using fractionated silk fibroin using freeze-thaw cycles to fabricate ATDs.

2. Experimental

2.1 Preparation of SF solution
Silk cocoons (B. mori, Luengpaivorj, produced by The Queen Sirikit Department of Sericulture, Ministry of Agriculture and Cooperatives, Nakhon Ratchasima, Thailand) were degummed with 0.02 M NaCO₃ and dissolved in 9.3 M LiBr at 60°C for 4 h. The dissolving protein was dialized in DI water for 72 h and was centrifuged at 9000 rpm [8]. The finished silk solution was stored at a temperature of 4°C.

2.2 Fraction of SF Solution
The silk fibroin protein solution was diluted with water to 6%W/V (SF), then it was fractionated using freeze-thaw cycles (4°C and 25°C for 5 cycles) into precipitants (SF-P) and soluble (SF-S).

2.3 Fabrication of artificial tear ducts (ATDs)
The protein solutions were concentrated to 20%W/W using dialysis against 20%W/V PEG (Poly ethylene glycol, MW 35,000 Da, Sigma-aldrich, USA). Then they were dip-coated to form cylindrical ducts with the inner and outer diameters of 2 and 2.5 mm., respectively, with the thickness of 0.5 mm. Beta-sheet structure of the silk protein was induced by submerging the ducts under 70% ethanol solution for 2 h then they were dried in a fume hood [8].

2.4 Characterizations
The silk fibroin solutions were characterized as follows: 1) yield (%dry weight), 2) molecular mass (SDS-PAGE), 3) viscosity (Oscillatory Rheometer (The Haak Mars Rheometer, Thermo Scientific, USA) at the shear rate of 0-100 per second, at 28°C.), 4) hydrophilicity (water contact angles of thin films that analyzed by Drop Shape Analyzer (DSA 10/MK2, Kruss, Germany)). After the protein was fabricated into ATDs. They were characterized on 1) morphology (camera and SEM (JEOL JSM-6610LV, Oxford X-Max 50, Australia) after immersion under balanced saline solution (BSS) for 24 h before freeze-drying), 2) Fourier-transform infrared spectroscopy(FTIR, PERKIN ELMER Spectrum GX, USA) in Attenuated Total Reflectance (ATR) mode for chemical structure and percentage of crystallinity), 3) Thermal properties (thermogravimetric analysis (TGA/DSC1, Mettler Toledo, USA) at 30-800°C, heating rate of 10°C /min, N₂ gas flow rate 50 ml/min), 4) BSS absorption (weight differences after immersing under BSS for 24 h, 37°C), 5) In vitro, degradability (in BSS containing 1.69x10⁴ Unit/mg lysozyme [9] to mimic natural tear at 37°C in vitro for 4 weeks), 6) The leaking (flow simulation of BSS through the ATDs with a flow rate of 0.0022 ml/min and a pressure of 101.52 kPa [10]), 7) Capillary action (height of in the ATDs, compared to glass ducts [11]), 8) Flexural strength (three-point bending mode, universal testing machine (Instron 5567, USA) at 1 mm/min speed, 1 kN force [12]).

All characterizations were done at 4 replications. (Except extraction yield, crystallinity index, T_d and flexural strength). Static analysis was done using Minitab program, the significant difference was determined from the 95% confidence level (P-value > 0.05).

3. Results and discussion
Silk fibroin is composed of three protein subunits: heavy-chain (350 kDa), light-chain (25kDa) and P-25 (30 kDa). SDS-PAGE of pure Thai silk fibroin (Nangnoi Srisaket) SDS-PAGE had only a single clear band at 25kDa and a smear protein at the higher 200 kDa [13]. In this research, the SF sample of Luengpaivorj showed smeared bands ranging from 15 to 250 kDa (Figure 1), and the clear band at 25 kDa. The SF-P and SF-S had similar smeared band. Clearer bands at 25 kDa and lower than 20 kDa can be observed in SF-S. Properties of silk fibroin solutions were shown in Table 1. Appearance viscosities (Pa.s) of SF-P was significantly higher than those of the SF and SF-S indicated that its protein structure was larger and more extensive [14]. We can see the evidence from SDS-PAGE that the SF-S content lower molecular weight protein molecules. Water contact angles of SF films were slightly higher than
those of the SF-P and SF-S. However, all of the samples had water contact angles that indicate hydrophilic materials.

**Figure 1.** Molecular weight of Thai silk fibroin samples (Leung Pairoj) and their distribution compared to the standard protein makers in the SDS-PAGE analysis.

**Table 1.** Extracted yield of Thai silk fibroin solutions, appearance viscosity and water contact angle of solutions.

| Samples | Yield of extraction (%(wt./wt.)) (n = 5) | appearance viscosity (η, Pa.s) (n = 4) | Water contact angle (°) (n=4) |
|---------|------------------------------------------|----------------------------------------|-------------------------------|
| SF      | 90.84 ± 0.87%a                            | 0.47 ± 0.03d                           | 67.95±3.32g                  |
| SF-P    | 63.53 ± 4.58%b                            | 1.33 ± 0.42e                           | 65.32±3.41h                  |
| SF-S    | 34.05 ± 5.76%c                            | 0.68 ± 0.08f                           | 62.39±2.17i                  |

a, b, c were showed statistically significant difference at the 95% confidence level (P-value > 0.05).
d, e, f, g, h, i were showed no statistically significant difference at the 95% confidence level (P-value > 0.05).

Figure 2 showed the inner cross-section surface of the ATDs. SEM morphology revealed the SF-P had good polymer-water miscibility observed from porous structure all over the entire surface. This supported by the result from SDS-PAGE and water contact angle that the SF-P has high MW and less hydrophilicity than SF-S. SF and SF-S ATDs had leaf-like structures, showing the interaction of intermolecular polymer chains were higher than their interaction with water.

**Figure 2.** Morphology of ATDs, the appearance of ATDs and the cross-section surface of SF, SF-P and SF-S ATDs.

Typical peaks in ATR-FTIR spectra for silk fibroin are characteristic reported at the of the amide I (C=O stretching, 1700-1600 cm⁻¹), amide II (N–H bending, C–N stretching, 1600-1500 cm⁻¹) and amide III (N–H deformation, C–N stretching, 1350-1200 cm⁻¹) bands of proteins [15]. We found the characteristic peaks of silk fibroin at 1) 1646, 1539 and 1230 cm⁻¹ (silk I, coils), 2) 1620, 1513 and 1260 cm⁻¹ (silk II, β-sheet and β-turn) at the amide I, II and III, respectively (Fig.3). Crystallinity index calculated from Eq. 1 using amide I peak only due to the clear visible differences between silk I and silk II peaks [12].
The results from Table 2 showed that the fractionated silk fibroin had higher crystallinity indices compared to the SF indicating the higher ordered structure. These results in the higher decomposition temperature BSS absorption of SF, SF-P and SF-S ATDs were found at 5.67 ±0.76%, 8.05±1.28 and 13.18±0.33 wt.%, respectively. Their hydrophilicity was in lined with the water contact angle results. All of the ATDs lost 10 wt.% in BSS solution containing lysozyme in 4 weeks. However, SF-S had tendency to degrade faster than the other. The ATDs from SF-S failed the leaking test so those from SF and SF-P were tested further. The capillary tests of glass, SF and SF-P tube were found BSS height at 7.0±0.0, 6.9±0.4 and 7.1±0.4 mm, respectively. The results of three-point bending test showing the superior mechanical strength of the SF-P ATDs compared to those of the SF (Table 2) and the flexibility (flexural strain) of SF was higher.

### Table 2. Crystallinity index (%), Thermal decomposition temperature, and flexural strength of the ATDs.

| Samples  | Crystallinity index (%) | T<sub>D</sub> (onset, °C) | Ultimate flexural stress (MPa) (n=5) | Flexural strain at max stress (%) (n=5) | Flexural Modulus (GPa) (n=5) |
|----------|-------------------------|---------------------------|-------------------------------------|----------------------------------------|-------------------------------|
| SF       | 42.27                   | 277                       | 55.24±7.68<sup>a</sup>              | 5.43±0.46<sup>a</sup>                 | 1.82±0.29<sup>a</sup>        |
| SF-P     | 44.51                   | 280                       | 81.42±2.71<sup>b</sup>              | 2.54±0.17<sup>d</sup>                 | 6.13±0.51<sup>f</sup>        |
| SF-S     | 44.11                   | 280                       | -                                   | -                                      | -                             |

<sup>a, b, c, d, e, f</sup> were showed statistically significant difference at the 95% confidence level (P-value > 0.05).

4. Conclusions

All physico-chemical results, especially ordered structure and mechanical properties indicated that the ATDs fabricated from Thai silk fibroin fractionated protein (SF-P) has high potential to be use as ATDs.
Further intensive studies on biocompatibility and degradation, especially the freeze-thaw induced precipitants, should investigate.

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