

CHAPTER 2

EXPERIMENTAL

In this study, the experiments were divided into three parts. Firstly, preparation of treated silk fibroin (SF) films was done by dissolving SF powder in distilled water. After that the SF solutions were dried to obtain the SF films and then treated with EtOH. Secondly, in order to improve the properties of SF film, the SF solution was mixed with various gel formers, i.e. Carbopol, Poloxamer and Hydroxypropyl methylcellulose to obtain SF hydrogel films then treated with EtOH. Finally, the properties of the SF films and SF hydrogel films were characterized including the swelling ratio determination, protein loss detection, morphology investigation, cross-linking investigation and thermal analysis.

2.1 Chemicals, Materials and Equipment

2.1.1 Chemicals

Chemicals	MW (g/mol)	Grade	Manufacturers
Calcium chloride dihydrate (CaCl ₂ ·2H ₂ O)	147.02	AR	Ajax Finechem, Auckland, New Zealand
Carbopol 940 (C ₃ H ₄ O ₂)	72.06	AR	Lubrizol Advance Materials, Ohio, USA

Chemicals	MW (g/mol)	Grade	Manufacturers
Ethanol (C ₂ H ₅ OH)	46.07	AR	Merck, Darmstadt, Germany
Hydrochloric acid (HCl)	36.46	AR	Merck, Darmstadt, Germany
Hydroxypropyl methylcellulose (C ₃₂ H ₆₀ O ₁₉)	748.81	AR	Samsung Fine Chemicals, Seoul, Korea
Poloxamer 188 (C ₂ H ₁₀ O ₂)	102.13	AR	BASF AG, Ludwigshafen, Germany
Sodium carbonate (Na ₂ CO ₃)	105.99	AR	Carlo Erba, Milan, Italy
Triethanolamine (C ₆ H ₁₅ NO ₃)	149.19	AR	Carlo Erba, Milan, Italy

2.1.2 Materials

Silk waste was obtained from Jun Mai Thai Company, Phetchabun. Gel formers, i.e. Carbopol 940 (CP), Poloxamer 188 (PL) and Hydroxypropyl methylcellulose (HPMC) were kindly supplied by Bangkok Lab and Cosmetic Ltd, Ratchaburi. All gel formers were used without further purification. Deionized reversed osmosis (DI-RO) water was used in all sample preparations.

2.1.3 Equipment

Equipment	Model	Manufacturers
Differential scanning calorimeter	Pyris Diamon 7	Perkin Elmer, U.S.A
Freeze dryer	LY5FM-ULE	Snijder Scientific, Netherland
Fourier transform infrared spectrometer	SPECTRUM	Perkin Elmer, U.S.A
Hot air oven	UM500	Memmert, Germany
Hotplate & Stirrer	4658	Cole-Parmer, U.S.A.
pH meter	744	Metrohm, Switzerland
Scanning electron microscope	JSM-6335F	JEOL, Japan
SnakeSkin™ dialysis tubing	10000 MWCO membrane	Fisher, U.S.A.
UV-VIS spectrophotometer	UV-1700	Shimadzu, Japan

2.2 Preparation of the Solutions

1) CaCl_2 : $\text{C}_2\text{H}_5\text{OH}$: H_2O solution

The ternary solvent (CaCl_2 : $\text{C}_2\text{H}_5\text{OH}$: H_2O), a mole ratio of 1: 8: 2 was prepared by adding 50 g of CaCl_2 and 30 mL of $\text{C}_2\text{H}_5\text{OH}$ into 50 mL of DI-RO water. Then the mixture was stirred by magnetic stirrer at room temperature for 30 min.

2) 1-6 g/L Carbopol (CP) solutions

The CP mixtures at various concentrations were prepared by dispersing each of 0.1, 0.3, 0.5 and 0.6 g of CP powder separately in 100 mL DI-RO water and stirring with magnetic stirrer for 2 hrs. After complete dispersion, the CP mixture was kept in a refrigerator to obtain complete swelling. Then its volume was adjusted with DI-RO water to 100 mL in a volumetric flask.

3) 10-100 g/L Hydroxypropyl methylcellulose solutions

Each of 1, 3, 5 and 10 g of HPMC powder was dispersed in hot 30 mL DI-RO water and stirred for 1 hr to obtain HPMC mixture at various concentrations. After complete dispersion, the HPMC was cooled, then added 70 mL cold DI-RO water due to forming a viscous colloidal solution and stirred for 1 hr. The HPMC gel was also prepared in the same fashion as done with CP mixture and kept gel solution.

4) 5 g/L Na_2CO_3 solution

A 10 g anhydrous Na_2CO_3 was dissolved in 500 mL distilled water and adjusted the volume to 2000 mL in a volumetric flask.

5) 10-200 g/L Poloxamer (PL) solutions

The PL mixtures at various concentrations, each of 1, 3, 5, 8, 10 and 20 g of PL was prepared by dissolving PL powder separately in 100 mL of cold DI-RO water and stirring for 1 hr. For preparing PL solutions, the steps involved are the same as those used in preparing CP solutions.

6) 0.1-10 g/L SF solution

A 10 g/L SF standard solution was prepared by dissolving 2.5 g of SF powder in DI-RO water and then adjusting to the volume of 250 mL in a volumetric flask. The SF solutions at concentrations of 0.1, 0.5, 1.0 and 2.0 g/L were prepared by diluting 1, 5, 10 and 20 mL of the 10 g/L solution with DI-RO water in each 100 mL volumetric flask, respectively.

7) 10-50 g/L SF solution

Each of 1, 2, 3, 4 and 5 g of SF powder was dissolved separately in a beaker with 50 mL of DI-RO water and stirred using a magnetic stirrer for 1 hr at room temperature. Then the volume was adjusted with DI-RO water to 100 mL in a volumetric flask.

8) 1 % v/v Triethanolamine stock solution

A stock solution of triethanolamine (1 % v/v) was prepared by diluting 1 mL triethanolamine with DI-RO water to 100 mL in a volumetric flask.

2.3 Procedures

The processes for preparation and characterization of SF hydrogels with different gel formers comprises the following steps: (i) preparation of the SF films, (ii) preparation of the SF hydrogel films with various gel formers, i.e. Carbopol 940 (CP), poloxamer 188 (PL) and Hydroxypropyl methylcellulose (HPMC), (iii) characterization of the SF films and SF hydrogels films. The procedures are described as follows:

2.3.1 Preparation of the SF films

1) A 15 g *Bombyx mori* silk waste was degummed using 500 mL of 5 g/L Na_2CO_3 solution at 98-100°C for 30 min, in order to remove the sericin. Degummed silk waste was then washed several times with DI-RO water, dried at 60°C for 6 hrs and kept in desiccator. The boiling process was triplicately repeated.

2) A 10 g degummed SF was dissolved in 100 mL of a ternary solvent system of calcium chloride, water and ethanol at mole ratio (CaCl_2 : $\text{C}_2\text{H}_5\text{OH}$: H_2O) of 1: 8: 2 at 110-115 °C for 2 hrs and filtered to eliminate impurities. Then CaCl_2 was removed by dialysis against DI-RO water for three days at room temperature using a cellulose membrane tubing (molecular weight cutoff 10,000). After dialysis, the solution was filtered, then, the SF solution was frozen and freeze-dried to obtain SF powder. The SF powder was stored in desiccator at room temperature for extended period of time.

3) The SF films were prepared by pouring 30 mL of each of 10-50 g/L SF solution into polyethylene (PE) plate and dried in a vacuum oven at 50 °C for 12 hrs. Then, the films were treated with 80% v/v ethanol for 15 min at room temperature,

the solvent (EtOH) was then slowly evaporated. After solvent evaporation, the treated SF films were stored in a desiccator until analysis.

2.3.2 Preparation of the SF based hydrogel

A SF hydrogel films were prepared by mixing 20 mL of 30 g/L SF solution with 10 mL each of gel former solution at various concentrations (1-6 g/L CP, 10-200 g/L PL and 10-100 g/L HPMC) to obtain blends with different compositions. The mixture were stirred for 2 hrs and stored for 24 hrs to promote full swelling. The preparation steps of SF hydrogels are similar in detail to the steps used for the preparation of SF “only” films. The physical appearances of the prepared SF hydrogel films at various concentrations were then observed throughout the preparation and characterization steps.

2.3.3 Characterization of the SF films and SF hydrogels films

Many techniques were used to characterize the properties and structure of the SF films and SF hydrogels films. The characterization includes cross-linking investigation, thermal analysis, morphology investigation, swelling test and protein loss detection. The procedure of each characterization described as follows:

1) Cross-linking investigation

FT-IR spectrometry was used to characterize the secondary structure of the treated SF and SF hydrogel films (30 g/L of SF/ 1 g/L of CP, 30 g/L of SF/ 50 g/L of PL and 30 g/L of SF/ 30 g/L of HPMC). The operation was carried out at room temperature after the sample had been prepared using the KBr disk method. The

FTIR spectrum of each sample was obtained in the region of 4000-400 cm^{-1} . The SF cross-linkage between SF and each gel former in the SF hydrogels structures were investigated in terms of amide peaks obtained from the IR spectra.

2) Thermal analysis

Thermal analyses of the SF powder, treated SF films and SF hydrogel films, and also gel formers were performed using Differential Scanning Calorimeter (DSC). About 8 mg of each sample was weighed in aluminum crucible, placed in the automatic thermal analyzer system, the analysis was done by heating at a scanning rate of 10 $^{\circ}\text{C}/\text{min}$ from 37-300 $^{\circ}\text{C}$ under nitrogen environment.

3) Morphology investigation

The cross-sectional morphologies of the treated SF and SF hydrogel films (30 g/L of SF/ 1 g/L of CP, 30 g/L of SF/ 50 g/L of PL and 30 g/L of SF/ 30 g/L of HPMC) were observed using SEM with a JEOL microscope operating at 5 keV with magnification of $\times 5000$ at 5 μm . Each sample was cut into a small piece (0.3 cm \times 0.3 cm) and sputter-coated with gold in the vacuum chamber to provide a conductive surface. The SEM image was taken in the air, with flattened and plane fitted as required.

4) Swelling test

The 1x1 cm^2 of the films were dried under vacuum for 1 day and weighed. Swelling test was done by immersing dried films in 30 mL deionized water at room temperature for 15 min. Periodically, the swollen films were withdrawn from the solution and weighed after removal of the excess surface water by lightly blotting

with filter paper. Swelling ratio is conventionally used to investigate the property of hydrogels that can be calculated by the following equation.

$$\%SR = \frac{W_s - W_d}{W_d} \times 100$$

where W_s and W_d are the weights of swollen films and dried films, respectively. It is important to note that all the experiments were carried out at room temperature and the equilibrium degree of swelling films was also determined after immersion in water for 24 hrs [43].

5) Protein loss detection

The protein loss from the treated SF and SF hydrogel films with various concentrations of gel formers were determined by measuring their absorbancies of the solutions after swelling test by using a UV-VIS spectrophotometer at 275 nm. Then, the amounts of SF release were determined from the absorbance of the equilibrated solution (see **Appendix B**). The percentage of SF loss from the treated SF and SF hydrogel films were compared.

