CHAPTER 2

EXPERIMENTAL

In this research, the extraction and determination of the total pigments, sericin and fibroin in silk cocoon were studied. Details of the chemicals, materials, equipment, and procedures are described as follows.

2.1 Chemicals, Materials and Equipment

2.1.1 Chemicals

1) Acetic acid (CH_3COOH), MW 60.00, AR, Merck, Darmstadt, Germany.
2) Acetone $(CH_3)_2CO$, MW 58.08, AR, Merck, Darmstadt, Germany.
3) Aluminium chloride; $AlCl_3.6H_2O$, MW 241.43 AR, Ajax Finechem, Auckland, New Zealand.
4) Calcium chloride dihydrate ($CaCl_2.2H_2O$), MW 147.02, AR, Ajax Finechem, Sydney, Australia.
5) Ethanol (C_2H_5OH), MW 46.07, AR, Merck, Darmstadt, Germany.
6) Hexane (C_6H_{14}) MW 86.18, AR, Fisher Chemicals, Leicestershire, England.
7) Methanol (CH_3OH) MW 32.04, AR, Mallinckrodt Baker, Inc., Phillipsburg, New Jersey, USA.

8) Magnesium chloride hexahydrate ($MgCl_2.6H_2O$) MW 203.30, AR, Carlo Erba, Milan, Italy.
9) Potassium Chloride (KCl) MW 74.56, AR, BDH, Poole, England.
10) Sodium Carbonate (Na_2CO_3) MW 105.99, AR, BDH, Poole, England.
11) Sodium Hydrogen Carbonate ($NaHCO_3$) MW 84.01, AR, Fisher Chemicals, Leicestershire, England.
12) Sodium Dodecyl Sulfate, SDS ($C_{12}H_{25}NaO_4S$) MW 288.38, AR, Sigma, St. Louis, USA.
13) Sodium Hydroxide (NaOH) MW 40.00, AR, Merck, Darmstadt, Germany.
14) Sodium acetate ($CH_3COONa.3H_2O$) MW 136.08, AR, Merck, Darmstadt, Germany.

2.1.2 Materials

Silk waste samples from raw yellow silk cocoon of a polyvoltinerace (called locally as *Nangnoi Si Sa Ket*) of Thai silkworms, *Bombyx mori* were obtained from The Queen Sirikit Sericulture Centre, Nan and Chiang Mai Provinces, Thailand. Raw samples were stored at room temperature until analysis.

2.1.3 Equipment

1) Freeze dryer, model LY5FM-ULE, Snijders Scientific, Tilburg, Netherland.

2) FTIR spectrophotometer, model SPECTRUM 2000, Perkin Elmer, Waltham, USA.

3) Hot air oven, model UM500, Memmert, Schwabach, Germany.

4) Hotplate & Stirrer, model 4658, Cole-Parmer, Illinois, USA.

5) pH meter, model 744, Metrohm, Herisau, Switzerland.

6) Refrigerator, model GR-S32 KT, Toshiba, Tokyo, Japan.

7) Rotary evaporator, model Rotavapor 114, Buchi, Flawil, Switzerland.

8) SnakeSkin™ dialysis tubing, model 10000 MWCO, Fisher Chemicals, Leicestershire, England.

9) Temperature controller, model HMFT, Whatman, Maidstone, England.

10) UV-VIS Spectrophotometer, model 6400, Jenway, Chelmsford, England.

2.2. Preparation of solutions

Preparations of the solutions and reagents used for pigment, sericin and fibroin extraction are described as follows:

1) 5%v/v Acetic acid solution

A 5.0 mL glacial acetic acid was pipetted into 50 mL distilled water. Then the volume was adjusted with distilled water to 100 mL in a volumetric flask.

2) Acetic acid and EtOH mixed solution

The 5 and 10 %v/v of acetic acid in 80 %v/v EtOH solutions were prepared by pipetting 5 and 10 mL of acetic acid into 50 mL of 80 %v/v EtOH then the volume was adjusted with 80 %v/v ethanol to 100 mL in each volumetric flask.

3) Acetone and EtOH mixed solution

The 5 and 10 %v/v of acetone in 80 %v/v EtOH solutions were prepared by pipetting 5 and 10 mL of acetone into 50 mL of 80 %v/v EtOH then the volume was adjusted with 80 %v/v ethanol to 100 mL in each volumetric flask.

4) 1.00 M $AlCl_3$ solution

A 3.33 g of $AlCl_3$ was dissolved in 10 mL distilled water and adjusted the volume to 25 mL in a volumetric flask.

5) 1.00 M $CaCl_2$ solution

A 2.77 g of $CaCl_2$ was dissolved in 10 mL distilled water and adjusted the volume to 25 mL in a volumetric flask.

6) $CaCl_2$: C_2H_5OH: H_2O solution

The ternary solvent, $CaCl_2$: C_2H_5OH: H_2O (mole ratio = 1:2:8), called the Ajisawa reagent [114] was prepared by adding 111 g of $CaCl_2$ and 92 ml of C_2H_5OH into144 ml of deionized water. Then the mixed solution was stirred by a magnetic stirrer at room temperature for 30 min.

7) 1.00 M CH_3COONa solution

A 6.80 g of $CH_3COONa.3H_2O$ was dissolved in 25 mL distilled water and adjusted the volume to 50 mL in a volumetric flask.

8) 60 - 90% v/v EtOH solution

The EtOH solution with various concentrations of 60%, 70%, 80 %, and 90% v/v were prepared by pipetting 60, 70, 80, and 90 mL of absolute EtOH respectively into 100 mL volumetric flask then the volume was adjusted with distilled water.

9) Hexane and EtOH mixed solution

The 5 and 10 %v/v of hexane in 80 %v/v EtOH solutions were prepared by pipetting 5 and 10 mL of hexane into 100 mL volumetric flask then the volume was adjusted with 80 %v/v ethanol.

10) 1.0 M KCl solution

A 1.86 g of KCl was dissolved in 10 mL distilled water and adjusted the volume to 25 mL in a volumetric flask.

11) 60 – 100 %v/v MeOH solution

The MeOH solution with various concentrations of 60, 70, 80, 90 and 100 %v/v were prepared by pipetting 60, 70, 80, 90 and 100 mL of absolute MeOH respectively into 100 mL in volumetric flask then the volume was adjusted with distilled water.

12) 0.10 M Na_2CO_3 solution

A 2.60 g of Na_2CO_3 was dissolved in 100 mL distilled water and adjusted the volume to 250 mL in a volumetric flask.

13) 0.10 M $NaHCO_3$ solution

A 2.11 g of $NaHCO_3$ was dissolved in 100 mL distilled water and adjusted the volume to 250 mL in a volumetric flask.

14) 0.50 M NaOH solution

A 2.00 g of NaOH was dissolved in 50 mL distilled water and adjusted the volume to 100 mL in a volumetric flask.

15) 2% w/v Sodium dodecyl sulfate (SDS) solution

A 2% w/v SDS solution was prepared by dissolving 2.00 g of SDS in 50 mL distilled water and then adjusting the volume to 100 mL in a volumetric flask.

2.3 Procedures

The following section will explain the details of experiment on the extraction and determination of total pigment, sericin and fibroin in silk cocoons. In addition, structural characterizations of pigments, sericin and fibroin in silk cocoon are also included.

2.3.1 Pigment Extraction and Determination of total pigments

Pigments in yellow Thai silk cocoons (var. *Nangnoi Si Sa Ket*) were exhaustively extracted by optimizing the extraction conditions. The optimal

condition was investigated on the type of solvent, temperature and time of extraction. Then total amount of pigments in silk cocoons collected from two different sources (Chiang Mai and Nan Provinces, Thailand) were quantified gravimetrically.

2.3.1.1 Solvent selection

The silk cocoon sample was cut into small pieces then dried at 60 °C for 24 h. Approximately 3.0 g of dried silk cocoon was placed in a 250 mL round bottom flask and then 100 mL each of various pure solvents such as water, acetone, hexane methanol ethanol and dichloromethane was added, then the sample was refluxed at 80 °C for 2 h. The pigments extracted was cooled immediately down to room temperature by immersing the flask into a water bath and then filtered by filter paper (Whatman no. 1). The color of the pigments extracted was observed to estimate the efficiency of extraction of each solvent. The reflux extraction system is shown in Fig. 2.1.

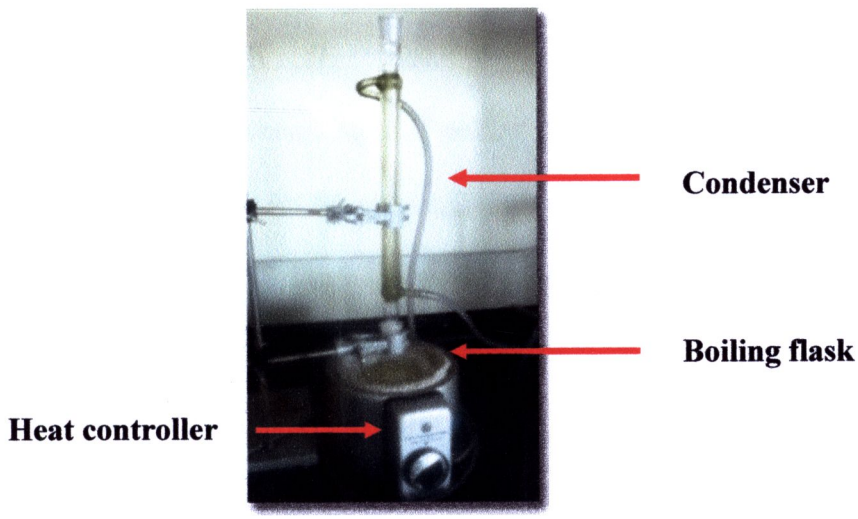

Fig. 2.1 Reflux extraction apparatus.

2.3.1.2 Factors affecting pigments extraction

In the following experiments, the effect of various factors such as effect of methanol and ethanol concentration, extraction time, temperature, solvent polarity, acidity/alkalinity and ionic strength of the extracting solvent were studied. Moreover, the investigation was performed by measuring the absorption of the pigments extracted. A 3.0 g of dried silk cocoon and 100 mL of 80% ethanol was placed in a 250 mL round bottom flask, then the sample was refluxed at 80 °C for 2 h. Absorption measurement was examined spectrophotometrically in the range of 200 - 550 nm.

1) Effect of methanol and ethanol concentrations

Various concentrations of methanol and ethanol were selected for using in pigments extraction. A 3.0 g of dried silk cocoon was placed in a 250 mL round bottom flask and then extracted with 100 mL of various concentrations of aqueous MeOH and EtOH solutions, the experiments were done the same fashion as in the preliminary study. In order to obtain the quantity of the pigments extracted, first, the optimal absorption wavelength of yellow pigments was then carried out as done in 2.3.1 part a of 2). For EtOH extract, 2 mL was pipetted, then diluted to 5 mL in a volumetric flask before measuring the absorbance, whereas the absorbances of MeOH extracts were measured directly. All absorption measurement was examined spectrophotometrically in the range of 320 – 550 nm.

2) Effect of temperature and extraction time

The effect of extraction temperature (50-80°C) and the extraction time (15-360 min) were studied for pigments extraction using a reflux extraction system by heating in a water bath (Fig 2.2). 1.5 g of dried silk cocoon and 50 mL of 80% ethanol was placed in a 250 mL round bottom flask, which was then heated to desire temperatures. The extracts were evaluated with respect to their yield of the extract. Then the absorption of the pigment extracted was measured at 444 nm. The optimal conditions of extraction time and temperature were used throughout further study.

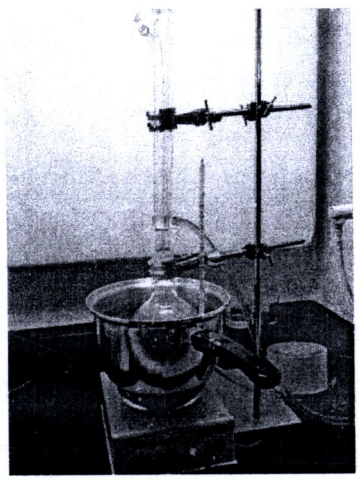

Fig. 2.2 Reflux extraction apparatus by heating in a water bath.

3) Effect of solvent polarity

Each mixed solution of hexane, acetone and acetic acid (5 and 10 % of hexane or acetone or acetic acid in 80 %v/v EtOH solution) were used to study the effect of solvent polarity. The pigment extraction was then carried out as done in 2.3.1 part b of 2).

4) Effect of acidity/alkalinity

A set of acid treatment was done by extracting 1.5 g of yellow silk cocoon with 50 mL mixed solvents of CH_3COOH and 80 %v/v EtOH. The CH_3COOH was also varied in its concentration of 1.75 M, 3.50 M, 5.25 M and 7.00 M in 80 %v/v EtOH. They were prepared by adding each volume of 5, 10, 15 and 20 mL of CH_3COOH (for 1.75 M, 3.50 M, 5.25 M and 7.00 M CH_3COOH, respectively) into each volumetric flask (50 mL). The volume was then adjusted with 80 %v/v EtOH.

For alkaline treatment, NaOH was used in place of CH_3COOH and the experimental procedure was the same as done with CH_3COOH. The NaOH was also varied in its concentration of 0.01 M, 0.05 M and 0.10 M in 80 %v/v EtOH. They were prepared by pipetting each volume of 1, 5, and 10 mL of 0.5 M NaOH into each 50 mL volumetric flask, followed by adding 40 mL of 80 %v/v EtOH. The volume was then adjusted with distilled water.

5) Effect of ionic strength

The ionic strength of the solvent was adjusted using different types of salt, i.e. KCl, $CaCl_2$, $AlCl_3$ and CH_3COONa in 80 % v/v EtOH. The concentration of each salt was maintained at 0.01 M. They were prepared by pipetting 0.50 mL of 1.0 M KCl, 1.0 M $CaCl_2$, 1.0 M $AlCl_3$ and 1.0 M CH_3COONa in each 50 mL volumetric flask, followed by adding 40 mL EtOH (80 %v/v). The volume was then adjusted with distilled water.

The effect of CH_3COONa concentrations was studied by varying the concentrations to be 0.01, 0.02, 0.10, 0.20, 0.40 and 0.80 M in 80 %v/v

EtOH. They were prepared by adding 0.5, 1, 5 and 10 mL of 1.0 M CH_3COONa (for 0.01, 0.02, 0.1, and 0.2 M CH_3COONa, respectively) and 5 mL and 10 mL of 4 M CH_3COONa (for 0.4 and 0.8 M CH_3COONa, respectively) to 40 mL ethanol (80 %v/v) and adjusting the volume with distilled water to 50 mL of volumetric flask.

2.3.1.3 Determination of the total pigments

In order to compare the quantity of total pigments in silk cocoon *(Nangnoi Si Sa Ket)* raised in different areas, the silk cocoon from Nan and Chiang Mai provinces were used for the extraction.

The pigment was removed from the silk cocoon samples by repeating reflux extraction. A 6.0 g of the silk cocoon sample (W_{SC}) was extracted with 0.8 M CH_3COONa in 80 %v/v EtOH at 80 °C for 30 min. The extracted cocoon was then re-extracted several times until a clear extracted solution obtained.

All crude pigments extracted were combined and preconcentrated by using a rotary evaporator equipped with a water bath set at 40 °C. then centrifuged at 6000 rpm for 15 min. The pigments dispersed in the upper phase of supernatant was separated and then transferred for freeze drying. The obtained pigments powder was weighed and the total amount of pigments in silk cocoon was determined gravimetrically (W_1) and the data are shown in Appendix.

2.3.2 Extraction and Determination of the sericin protein

Sericin protein was firstly extracted with the degumming solvent. The remains from pigments extraction were oven dried at 60 °C for 24 h. Then a mixture of 96 mL 0.1 M Na_2CO_3, 84 mL 0.1 M $NaHCO_3$ and 30 mL 2 %w/v SDS was added

into the dried remains and heated at 95 °C for 30 min. After filtration, the degummed solution was adjusted to the pH 4 by 5% acetic acid, then centrifuged. The colloidal lower phase was further purified for 24 h against running deionized water using dialysis tube with molecular cutoff of 10,000 to remove the excess salt. The sericin solution was then frozen prior to freeze-drying. The obtain sericin powder was weighed and the amount of sericin in silk cocoon were determined gravimetrically (W_2) and the data are shown in Appendix.

2.3.3 Extraction and Determination of the fibroin protein

The fibroin extraction was done simultaneously with sericin extraction. After degumming the sericin and filtration, the degummed silk cocoon was oven dried at 60 °C for 24 h. Dried degummed silk cocoons was dissolved in a mixture of 50 mL ternary solvent, of $CaCl_2/C_2H_5OH/H_2O$ (1:2:8) at 100-105 °C for 2 h. The solution was then filtered and dialyzed (molecular cutoff 10,000) for 3 days against running deionized water to remove $CaCl_2$ (the water was changed twice a days), then freeze-dried. The obtained fibroin was weighed and the amount of the fibroin in silk cocoons was determined gravimetrically (W_3) and the data are shown in Appendix.

2.3.4 Structural characterizations of fibroin sericin and pigment powders

After fibroin, sericin and pigment powders were obtained, the structure and functional groups of all components were investigated using FT-IR spectrometry. All spectra were recorded in transmittance mode using KBr pellets in the range of 2000 – 400 cm^{-1} with 2 cm^{-1} resolution.