CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Waste of raw silk from cocoons of *Bombyx mori*, reared locally in Mahasarakham, Thailand, was used as the raw materials. The standards of amino acids (L-Glycine, L-Serine, L-Asparagine, L-Aspartic Acid, L-Alanine, L-Glutamic Acid, L-Arginine, L-Valine, L-Tyrosine) are obtained from Wako (Japan) and L-Threonine, L-Leucine are obtained from Himedia (Mumbai, India). Bovine serum albumin (BSA) was purchased from Wako (Japan) and Ninhydrin was purchased from APS (NSW, Australia). All chemicals (Lowry's method and Ninhydrin's assay reagent) were of analytical grade and obtained from Ajax (NSW, Australia). Protein molecular weight marker was purchased from Promega (USA). All chemicals for SDS-PAGE analytical were purchased from Bio-Rad (Hercules, CA, USA). HPLC-grade of acetonitrile was purchased from Fisher Scientific (UK) and Heptafluorobutryic Acid was purchased form Fluka (Buchs, Switzerland) and Trifluoroacetic acids was purchased from Sigma (St. Lous, MO, USA).

3.2 Experimental

3.2.1 Preparation of raw silk

In preparing the waste silk for the hydrolysis experiment, any visible contaminating residue or dust was pick out manually, and the silk waste was rinsed again in cold water to get rid of the remaining contaminants and was then dried in a vacuum oven at 60-70 ^{o}C. The raw material was then cut with a pair of scissors into very short fragments whose average length was 100 mm. The silk sample was then soaked in distilled water for 5 min prior to the reaction

3.2.2 Subcritical water hydrolysis of sericin

As shown in Figure 3.1, the reaction system used in this experiment consists of a 100 cm^3 reactor, a heater, and a temperature controller. Removal of sericin by subcritical water hydrolysis was carried out with in the reactor, which was a SUS- 316 stainless steel pressure vessel (AKICO, Japan). The sample and deionized water were charged into the reaction vessel. The vessel was then tightened and heated to a desired temperature by means of a heating jacket around it. The pressure in the reactor was estimated from a steam table as shown in Table 3.1.

Table 3.1 Saturated steam table [Saad, 1997]

Temperature (°C)	Pressure (MPa)
120	0.1985
130	0.2701
140	0.3613
150	0.4758
160	0.6178
170	0.7917
180	1.0021
190	1.2544
200	1.5538
220	2.7950
230	2.3180
240	3.3440
250	3.9730

The effects of ratio of sample/deionized water, temperature, and times of hydrolysis on the product yield and quality were determined. The ranges of conditions tested are summarized in Table 3.2.

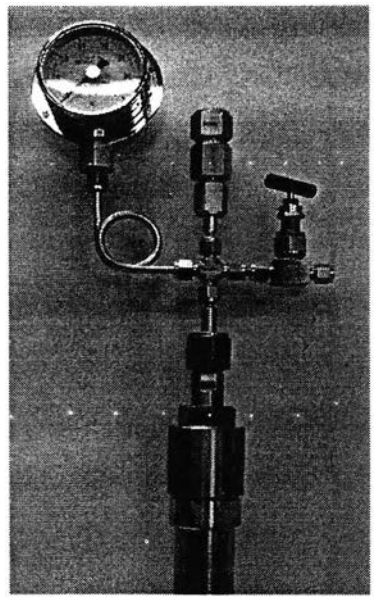

Figure 3.1 Batch system for subcritical water hydrolysis.

Table 3.2 Ranges of variables for sericin hydrolysis.

Variables	Ranges
Temperature (°C)	120-160
Silk sample to water ratio	1:20 -1:100
Time of hydrolysis (min)	10-60

After each reaction, the reactor was immediately cooled to room temperature by submerging it into a water bath. The remaining silk fibre was separated from the soluble product using a filter paper (Watman no. 1, Maidstone, England). The residue was then dried in a vacuum oven at 60-70 °C for about 6-8 h, and dried weight was measured.

The silk solution was filtered again with a filter paper (Whatman no. 1, Maidstone, England) to separate any remaining solids from the protein solution. The solution was then assayed to determine the molecular weight range using SDS-PAGE. The amount of total protein was determined using Lowry's method, and the amount of total amino acids by Ninhydrin's assay.

3.2.3 Subcritical hydrolysis of silk fibroin

The silk fibroin fibre obtained after degumming the silk waste at the optimal conditions was used for the fibroin hydrolysis study. This condition was found to be at 120 °C, and 10-30 min, the condition that could be achieved using a general laboratory autoclave. Because the laboratory autoclave allows preparation of a larger amount of fibroin raw material, fibroin was prepared by degumming the silk sericin from the raw fiber in this way. The study of subcritical water hydrolysis of the fibroin sample was carried out in the same manner as that described earlier for sericin hydrolysis in the same reaction system. Again, the effects of temperature, sample to water ratio, and times of hydrolysis on protein and amino acids production yield and quality were determined. The conditions tested are summarized in Table 3.3. After a desired reaction period, the residual fibre was separated, dried, and weighed, and the molecular weight of soluble portion was measured for molecular size range with SDS-PAGE, protein yield and total amino acid yield using Lowry's assay and Ninhydrin assay, respectively.

Table 3.3 Ranges of experimental variables for fibroin hydrolysis

Variables	Ranges
Temperature (°C)	160-220
Silk sample to water ratio	1:20 -1:100
Time of hydrolysis (min)	10-60

3.2.4 Formation of particles from sericin and fibroin solutions

Sericin and fibroin solutions obtained from subcritical water hydrolysis of the silk samples were formed into particles by means of freeze-drying at -40 °C for 24 h. The particle size and size distribution of particles from a selected condition (optimal conditions) were measured using a particle sizer (Malvern Mastersizer S, UK). The

morphology of the particles was observed under SEM and general characteristics of the particles were measured by FTIR, XRD, and DSC.

3.3 Analytical methods

3.3.1 Scanning electron microscopy (SEM)

Morphology of silk samples after hydrolysis reactions and of the sericin and fibroin particles was examined under a scanning electron microscope (JEOL, JSM-5400, Japan)_at an acceleration voltage of 10 kV. The samples were coated with thin film of gold using ion sputtering device (JEOL, JFC-1100E, Japan) before obtaining the micrograph.

3.3.2 SDS-PAGE

The molecular weight distribution of the constituents of silk sericin and fibroin solution was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to the method described previously by Laemmli, (1970) with 12% acrylamide separating gel and 5% stacking gel. The separating gel was first cast into gel plate, which was then overlaid with the stacking gel. Preparation of the gel and sample separtin procedures are described as follows:

1. The separating gels solution (12 % T, see solution in SDS-PAGE method) to adding between the gel plates around 5 cm height. When pouring separating gels, the acrylamide solution was overlaid gently with water and allowed to stand at least 3 h to ensure complete polymerization (polymerize for 30 minutes to 3 hours). Thoroughly rinse the top of the separating gel with water and dry the area above it with filter paper. The remainder of the gel mould was filled with the stacking gel solution 5 ml (5 %T, see solution in SDS-PAGE method) on top of the separating gel. Immediately place a well-forming comb between the gel plates. Align the comb in its proper position, being careful not to trap bubbles under the teeth and allowed it to polymerize for at least 1 h. No overlay was required, because the comb excludes oxygen from the surfaces of the wells. It may be necessary or convenient to let the gel stand overnight before it is used.

2. The gel was then set in the electrophoresis chamber with electrode buffer (1xRunning buffer, see the preparation method given below) in both upper and lower reservoirs as shown in Figure 3.2.

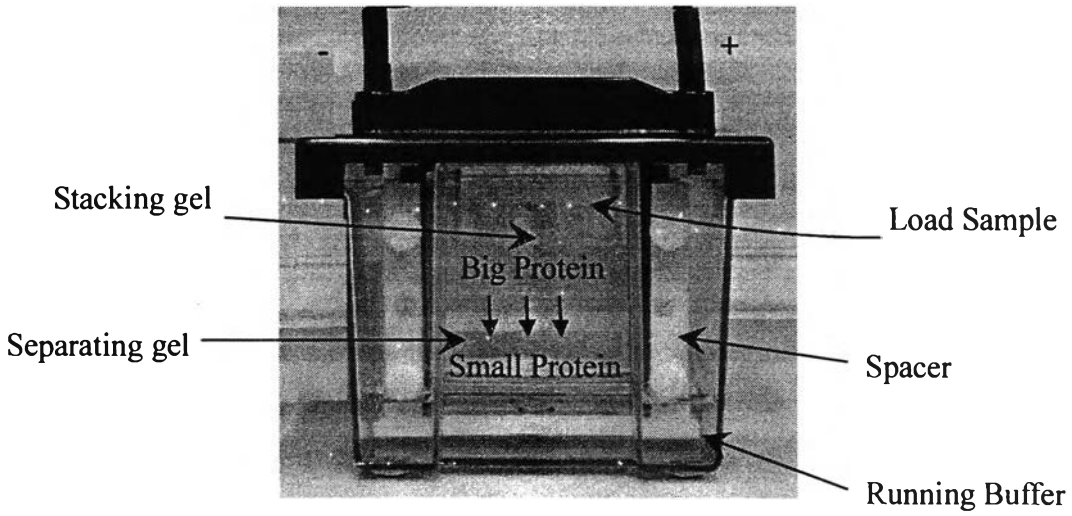

Figure 3.2 An illustration of an apparatus used for SDS-PAGE

3. 5 µl of protein marker and/or 15 µl sericin and fibroin solution (see method for preparation of sample solution below), were loaded onto the gel, which was then subjected to electrophoresis at a 150 voltage until the Bromophenol Blue tracking dye reached the bottom of the gel.

4. After sample separation, the gel was next stained in staining solution with 0.25% Coomasie brilliant blue R-250 (Sigma-Aldrich, Milwaukee, WI, USA) for 30-60 min at room temperature with continuous shaking. After staining gel, the gel was destained several times with a large excess of destaining solution until the background had been satisfactorily removed. The molecular weight size of sericin and fibroin solution at difference conditions were determined by comparison of their relative mobilities (R_f) with those of several marker proteins of known molecular weight.

Preparation of sericin and fibroin solution

Sericin or fibroin solution was diluted with an equal volume (ratio 1:1) of (1x) sample buffer (prepared as described below). After adding the buffer solution, the sample was then heated at 100 °C for 2 min.

Protein molecular weight marker

The broad range protein molecular weight markers consist of nine precisely sized recombinant proteins of molecular weight 225, 150, 100, 75, 50, 35, 25, 15 and 10 kDa. Each protein was present at a concentration of 0.1 µl/ µl, except for the 50 kDa protein, which was present at 0.3 µl/ µl and served as a reference indicator. This protein marker had the intensity triple of that of the other proteins which all appeared in equal intensity on the gel.

Preparation of stock solution

The following stock solutions were required for preparing separating gel, stacking gels, 1x- Sample buffer, and 1-x running buffer in SDS-PAGE.

1. Acrylamide/Bis- Acrylamide concentrate (30 %T, 2.7 %C) in 70 ml of double distilled water. When the acrylamide was completely dissolved, water was added to obtain a final volume of 100 ml. Filter the solution under vaccuum through a 0.45 mm membrane. Store the stock acrylamide concentrate at 4°C in a dark bottle for no more than 1 month.

2. 1.5 M tris HCl pH 8.8

3. 0.5 M tris HCl pH 6.8

4. 10% (w/v) sodium dodecyl sulfate (SDS)

5. 20% (w/v) sodium dodecyl sulfate (SDS),

6. 0.05% (w/v) Bromophenol

6. 10% (w/v) ammonium persulfate (APS).

The APS solution should be prepared fresh daily.

Procedures for preparation of assay reagent solutions

Separating and Stacking gels

To preparation the monomer solution by combining all reagents shown in Table 3.4, except ammonium persulphate and TEMED. Deaerate the solution under vacuum for at least 15 minutes and the two catalysts, Ammonium persulphate and TEMED, were added just prior to casting the gels.

Table 3.4 Formulations for SDS-PAGE Separating and Stacking gels.

Components	Separating Gel 12.0 % T	Stacking Gel 5.0 %T
Acrylamide/bis (30 %T, 2.67 %C)	4 ml	670 µl
Distilled water	3.4 ml	3.075 ml
1.5 M tris-HCl, pH 8.8	2.5 ml	-
0.5 M tris HCl pH 6.8	-	1.25 ml
20% (w/v) SDS	50 µl	25 µl
10% (w/v) Ammonium persulphate	70 µl	25 µl
TEMED	7 µl	5 µl
Total monomer solution	10.027 ml	5.05 ml

1x- Sample buffer

Prepare this solution by combining the solution with following: 4 ml of Milli Q water, 1 ml of 0.5 M tris/HCl pH 6.8, 1.6 ml of 10% SDS, 800 µl of glycerol, 400 µl of β-mercaptoethanol , and 200 µl of 0.05% (w/v) Bromophenol. Store at room temperature.

1x- Electrode buffer or 1-x running buffer

The electrode buffer can be made as a 5x concentrate consisting of 15 g tris-base, 72 g glycine, and 5 g SDS per liter of Milli Q water. Do not adjust the pH of the electrode buffer, just mix the reagents together and confirm that the pH is near 8.3. 5x electrode buffer concentrated must be stored in glass containers. To use 1x concentrate, dilute it with four parts water.

Staining solutions

To prepare this solution by combining the solution with following: 0.1% w/v Coomassie Brilliant Blue R-250 in 30 % (v/v) methanol and 10 % (v/v) acetic acid (in distilled water).

Destaining solution

To prepare this solution by combining the solution with following: 30% (v/v) methanol and 10 % (v/v) acetic acid (in distilled water).

3.3.3 Protein and amino acids compositions

3.3.3.1 Protein assay

The protein content in the sericin and fibroin solution was assayed by Lowry's method (Lowry, 1951). The procedure described here is suitable for sample containing up to 0.5 mg of protein/ml. To each tube containing 0.5 ml of sample solution, 2.5 ml of assay reagent E (prepared as described below) was added and thoroughly mixed using a vortex. The mixture was incubated for 10 min. To this mixture, 0.25 ml of

diluted Folin-Ciocalteu reagent (Reagent F) was added and vortexed., The mixture was then incubated for 30 min, after which the spectroscopic absorbance was measured at 750 nm, using bovine serum albumin (BSA) solutions (the concentrations range between 0.05-0.3 mg/ml) as reference.

To prepare the assay reagent E, 49 ml of reagent C was mixed with 49 ml of reagent D. Then, 1 ml of reagent A and 1 ml of reagent B were added to this mixture. The new reagent solution must be prepared for the daily use.

Reagent Solution:

Reagent A : 1 % (w/v) of Copper Sulfate

Reagent B : 2 % (w/v) of Sodium Potassium Tartrate

Reagent C : 0.2 M Sodium Hydroxide

Reagent D : 2 % (w/v) of Sodium Carbonate

Reagent F : diluted Folin-Ciocalteu reagent with distillate water ratio 1:1.

3.3.3.1 Amino acids assay

Amino acids produced were analyzed using Ninhydrin assays, The assay follows the following standard procedure. First, 1 ml of the ninhydrin solution (Reagent A), 2.4 ml glycerol solution (Reagent B), 0.2 ml citrate buffer solution (Reagent C), and 0.2 ml manganese chloride solution (Reagent D) were added into a test tube containing 0.2 ml of sample solution. The test tube was then covered with a piece of paraffin film or taped to avoid the loss of solvent due to evaporation. A loosely capped test tube can also be used instead. With gentle stirring, allow the mixture to react at 80-100 °C for 12 minutes (a complete reaction). After cooling to room temperature in a cold water bath, record the absorbance with a spectrophotometer at 570 nm. For the analysis of sericin solution, L-Serine solutions whose concentrations range between 0.3-0.9 mg/ml were used as reference, whereas for the fibroin solution, L-Alanine solutions (concentration range between 0.2-0.9 mg/ml) were used.

Reagent Solution:

Reagent A : 1 % (w/w) of ninhydrin solution

Reagent B : 55% (v/v) of glycerol solution

Reagent C : 0.5 M citrate buffer solution pH 5.5

Reagent D : 100 mg/ml of manganese chloride solution

3.3.4 Measurement of particle sizes

Average sizes and size distribution of silk sericin and fibroin particles were measured by using a particle analyzer (Malvern Mastersizer S, UK) with an automatic of particle content counter in suspension at 25 °C. The $10x10^{-3}$ % (w/v) particle suspension was prepared in 100% ethanol.

3.3.5 Fourier Transform Infrared Spectroscopy (FTIR)

Fourier transform infrared spectroscopy is used primarily for qualitative and quantitative analysis of organic compounds, and also for determining the chemical structure of many inorganic. When infrared (IR) radiation reaches a molecule, bonds in the molecule will absorb the IR frequencies that match frequencies of bond motion within the molecule causing an increase in the amplitude of bond motion. The frequency at which this takes place can be detected by an IR instrument and correlated to known molecular motion at that frequency, which indicates what bonds are present in the molecule and thus, what atoms the bond connect. In this work, FTIR spectra of silk fibre and silk particles were obtained using a PerkinElmer, Spectrum One (Illinois, USA), in the spectral region of 4000-500 cm^{-1}. To determine the molecular conformation of silk sericin and fibroin powder, all samples were measured by transmittance method. For the measurement, the thickness of specime was controlled ca. 5 μm

3.3.5 X-ray diffraction (XRD)

XRD was conducted at ambient temperature using a diffractometer (JEOL, JDX-8030, Japan) with Cu$K\alpha$ radiation in wavelength of 1.542 Å was used. The scaning rate was 0.5°/min and range was $2\theta = 5 \sim 35°$ under the acceleration voltage was 30 Kv and 20 mA. The sericin and fibroin powder were studied.

The X-ray diffraction pattern for the silk fiber showed a broad peak which can be generally be related to the lattice spacing through the Bragg's relation:

$$2d\sin\theta = n\lambda$$

where λ is the wavelength of the X-Ray radiation used in the diffraction experiment, which is 1.542 Å , d is the spacing between diffractional lattice planes, θ is the measured diffraction angle, and n is an integer which is an order of the reflection (assigned to 1).

3.3.7 Differential Scanning Calorimetry (DSC)

The thermal behaviors of sericin and fibroin powder were determined endothermic peaks, purity, crystalline and decomposition temperature of each particle. In addition, the exothermic peak was assigned to attributed to the transition molecular of formation sericin and fibroin solution to particle were changed. The thermal properties of sericin and fibroin particles are examined using a differential scanning calorimeter (NETZSCH DSC 204 F1, Germany). Sericin and Fibroin particles were obtained different conditions, each sample 5-10 mg were sealed in aluminum pans. The heating rate used was 10 °C/min from room temperature to 350 °C or until decomposition temperature of particles.