CHAPTER III
EXPERIMENTAL

3.1 Materials

A. xylinum TISTR 975 was purchased from Microbiological Resources Centre, Thailand Institute of Scientific and Technological Research (TISTR). D-glucose anhydrous (analytical grade) was purchased from Ajax Finechem. Yeast extract powder (bacteriological grade) was purchased from Himedia. Sodium hydroxide anhydrous pellets (analytical grade) were purchased from Ajax Finechem. Glacial acetic acid (analytical grade) was purchased from Merck. *B. mori* silk cocoons were obtained from Queen Sirikit Sericulture Center (Thailand).

3.2 Methodology

3.2.1 Preparation of Bacterial Cellulose Pellicle

3.2.1.1 Culture Medium

The culture medium used for bacterial cellulose synthesis of *A. xylinum* contains 4.0 %(w/v) D-glucose and 1.0 %(w/v) Yeast extract in distilled water. The prepared culture medium was sterilized in an autoclave at 115 °C for 15 min.

3.2.1.2 Cultivation of Bacterial Cellulose-Producing Bacteria

Pre-inoculum was prepared by adding *A. xylinum* TISTR 975 in a 100 mL Erlenmeyer flask containing 20 mL of culture medium. After a static incubation at 30°C for 2 days, the bacterial cellulose pellicle appeared on the surface of culture medium. After that, 10 mL of stock culture was transferred to a 500 mL Erlenmayer flask containing 100 mL of culture medium, followed by a static incubation at 30°C for 4 days (Maneerung *et al.*, 2007). The bacterial cellulose pellicle with an appropriate thickness was obtained.

3.2.1.3 Purification of Bacterial Cellulose

The obtained bacterial cellulose pellicle was purified by boiling in a 1.0 %(w/v) sodium hydroxide solution at 90 °C for 2 h (2 times) to remove bacterial cells and culture medium, followed by a neutralization with 1.5 %(v/v) acetic acid solution at room temperature for 30 min and repeated washing with distilled water until pH become neutral. The bacterial cellulose pellicles were kept in distilled water prior to use.

3.2.2 Preparation of Sericin Solution

The preparation of sericin solution was done according to the work of Ang-atikarnkul (2011). Briefly, silk cocoons were cut into small pieces (around 1 cm to 2 cm). Then, silk cocoons were washed in distilled water and dried in an oven at 65 °C for 24 h. Sericin solution was extracted from silk cocoons by boiling in distilled water in an autoclave at 121 °C for 30 min. After that, silk cocoons were filtered out to obtain sericin solution. Finally, sericin solution was dried by freeze drying process at -40°C for 24 hours.

3.2.3 Preparation of Bacterial Cellulose Containing Sericin

Sericin concentration varied from 10, 20, 30, 40, 50 mg/ml. Freeze-dried sericin was dissolved in distilled water by autoclaving for 10 min to obtain sericin solution. After that, bacterial cellulose was immersed in sericin solution and heated in an autoclave for 2 hours to obtain bacterial cellulose containing silk sericin. Finally, it was dried by freeze drying process for 48 hours.

3.3 Characterization and Testing

3.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

The chemical structures of bacterial cellulose, sericin, and bacterial cellulose containing sericin were characterized by using the KBr method and analyzed by a Nicolet/Nexus 670 FT-IR spectrometer at a wavenumber range of 4000 cm^{-1} to 400 cm^{-1}.

3.3.2 Scanning Electron Microscope (SEM)

The surface and cross section of pure bacterial cellulose and bacterial cellulose containing sericin were observed under a JEOL JSM-5200 SEM microscope.

3.3.3 X-ray Diffraction (XRD)

The x-ray diffraction was used to identify the crystalline structure of samples. The x-ray diffraction was performed with Bruker AXS, Germany Model D8 Advance by using Ni-filtered CuKα-radiation at 40 kV and 30 mA. Diffraction curves were measured at scanning rate of 2°/min and the 2θ ranged from 5° to 50°.

3.3.4 Kjeldahl Analysis

The amount of sericin incorporated into bacterial cellulose pellicle was determined by using Kjeldahl analysis. The sample was digested by using concentrated sulfuric acid (10 mL) and copper sulfate (0.1 g) as a catalyst which accelerated the conversion of amine nitrogen to ammonium sulfate solution, followed by heating the mixture by using heating mantle at 400 °C for 1 h. Then, 35 %(v/v) hydrogen peroxide solution was dropped to the mixture until getting a clear solution. After that, the digested sample was subjected to distillation unit. A 150 mL Erlenmeyer flask containing 30 mL of 0.1 M hydrochloric acid and 3 drops of phenolphthalein, used as an indicator, was set to the end of the unit. Consequently, a 40 %(v/v) sodium hydroxide solution was dropped to the digested sample to convert the ammonium sulfate solution to ammonia gas. Then, the ammonia gas was trapped with hydrochloric receiving solution by boiling and condensation in distillation step. The amount of ammonia entrapped in hydrochloric solution was determined by back titration with a 0.1 M sodium hydroxide solution. The sericin content was calculated based on nitrogen percentage by using the following equation (1).

$$\text{Nitrogen Content (mg of N/g of sample)} = \frac{(M_{HCl} \times V_{HCl}) - (M_{NaOH} \times V_{NaOH})}{\text{weight of sample(g)}} \times 14 \quad (1).$$

3.3.5 Swelling Behavior

The sample was cut in to a disc shape with a diameter of 1.5 cm and dry sample was weighed before immersing the sample in distilled water at room

temperature for 24 h. After that, the sample was blotted with filter paper to remove excess water on the surface and was weighted again. The swelling ratio was calculated by using the following equation (2):

$$\text{Swelling} = (W_s - W_d)/ W_d \qquad (2).$$

where W_s and W_d are the weights of sample in swollen and dry states, respectively.

3.3.6 Water Vapor Transmission Rate

The water vapor transmission rate (WVTR) was determined from the monograph of the European pharmacopiae (Razzak *et al.*, 2007). The bacterial cellulose and bacterial cellulose containing sericin samples were cut into disc shape with diameter of 33 mm. Then, the samples were placed and sealed on a mouth as a cap of bottle with diameter of 33 mm. After that, the bottles containing 25 mL of water were weighted and then placed in incubator at 35 °C for 24 hour.

The water vapor transmission rate was calculated by using the formula (3):

$$\text{WVTR}(g/m^2/h) = \frac{(W_i - W_t) \times 10^6}{(24 \times A)} \qquad (3).$$

where, W_i and W_t are the weight of bottle before and after place in an incubator respectively, A is area of bottle mouth (mm^2)

3.3.7 Antioxidant Activity

The antioxidant activity of sericin was determined based on the 2,2-diphenyl-1-picryhydrazyl (DPPH) free radical scavenging activity method. Each specimen was cut into disc shape shape with a diameter of 1.5 cm and then put sample in 3.5 mL of methanolic solution containing DPPH reagent at a concentration of 1×10^{-4} mol/L, and the mixture was vortexed at a high speed. Then, the mixture was kept in the dark at room temperature for 30 min. After that, the mixture was centrifuged at 6400 rpm for 5 min to remove insoluble residues, and the free radical scavenging activity of the clear supernatant was determined using a Taccan Infinite

M 200 UV/Vis spectrophotometer at a wavelength of 517 nm and compared with a blind control containing DPPH and distilled water instead the presence of sericin was also assayed (Wu, *et al.*, 2007). The scavenging activity of sericin solutions at different concentrations was calculated by using the following equation (4):

$$\text{Scavenging activity (\%)} = \frac{(A_0 - A_x)}{A_0} \times 100 \qquad (4).$$

where A_x and A_0 refer to the absorbance of the DPPH solution with and without sericin, respectively.

3.3.8 Sericin Releasing Behavior

The sample was cut to a circular shape with a diameter of 1.5 cm and the amount of sericin released from bacterial cellulose matrix was determined by immersing the sample in a 0.1 M 2-Amino-2-hydroxy methyl-1,3-propanediol hydrochloride or Tris(hydroxymethyl) aminomethane hydro chloride (Tris-HCl) solution at pH 7.4 and placing the sample in a shaking incubator at 100 rpm at 37°C. Then, the releasing medium was sampling at a specific time interval for 72 hours. The protein releasing concentration in the medium was determined based on the BCA assay reagent (Pierce) with the use of a UV/Vis spectrophotometer at a wavelength of 562 nm. The amount of released sericin was calculated from a calibration curve of bovine serum albulmin standard in the concentration range of 20 μg/mL to 2000 μg/mL.

3.3.9 Cytotoxicity Test

This assay was a modified version of conventional direct and indirect contact tests conformed to the published standard methods (BS-EN30993-5 and ISO10993-5). The MTT assay is a tetrazolium-dye based colorimetric microtitration assay. Metabolism-competent cells are able to metabolize the tetrazolium (yellow) to formazan (blue); this color change is measured spectrophotometrically with a plate reader. It is assumed cells that are metabolically deficient will not survive, thus the MTT assay is also an indirect measurement of cell viability. The cells were seeded in a 96-well plate at a density of 3,000 cells/well, and incubated for 48 hours. The circular pieces of samples with diameter of 1.5 cm. were examined. One piece of

sample was dissolved in 1 ml of medium for 24 hours. The medium with leaching substance of sample was added to the cells and incubated for 24 hours. The test samples were removed from the cell cultures and the cells were reincubated for a further 24 hours in fresh medium and then tested with MTT assay. Briefly, 50 µl of MTT in PBS at 5 mg/ml was added to the medium in each well and the cells were incubated for 4 hours. Medium and MTT were then aspirated from the wells, and formazan solubilized with 200 µL of DMSO and 25 µl of Sorensen's Glycine buffer, pH10.5. The optical density was read with a microplate reader (Molecular Devices) at a wavelength of 570 nm. The average of 4 wells was used to determine the mean of each point. The data were analyzed with the SoftMax Program (Molecular Devices) to determine the % survival and IC_{50} for each toxin sample.