CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Materials and Chemicals

Silk *Bombyx Mori* cocoons, *Musa sapientum Linn.* banana trunk and *Penaeus merguiensis* shrimp shell were supplied by Queen Sirikit Sericulture center (Nakorn Ratchasima), bangkok (thailand) and Surapon Food Co. Ltd., respectively. AR grade 30% hydrogen peroxide solution (Fisher Scientific, UK), sodium hydroxide pellets (Ajax Finechem, Australia), 37% hydrochloric acid (RCI labscan, Thailand), 98% sulfuric acid (J.T. baker, Thailand), 25% glutaraldehyde aqueous solution (Sigma-Aldrich) were used.

3.2 Methodology

3.2.1 Preparation of Sericin Solution

The *Bombyx Mori* silk cocoons were washed with distilled water and were dried in an oven at 65°C overnight. Then, cut the cocoons into 1-2 cm pieces and boiled in distilled water, which the ratio of cocoons: water was 1g: 30mL, at 121°C for 30 minutes by using autoclave. After that, the sericin solution was filtered to remove insoluble materials and was stored in refrigerator before used.

3.2.2 Preparation of Chitin Whisker Suspension

α- chitin whisker suspension was prepared by acid hydrolysis method (Nair and Dufrense, 2003; Wattanapanit, 2008). α- chitin flakes were hydrolyzed with 3N HCl, which the ratio of chitin: HCl was 1g: 30mL, under vigorous stirring at 104°C for 6 hours. After hydrolysis, the suspension was diluted with distilled water, followed by centrifugation at 10,000 rpm for 10 minutes and repeated for three times. Then, the suspension was transfer to a dialysis bag and dialyzed in distilled water until neutral. The suspension was stored in refrigerator before used.

3.2.3 Preparation of Cellulose Whisker Suspension

The cellulose whisker suspension was prepared based on the method which reported by de Rodriguez, *et.al.* (2006) with some modifications. Briefly, the *Musa sapientum Linn.* banana trunk or pseudostem was washed with distilled water, followed by cutting into 2-4 cm pieces and dried in oven overnight. The dried banana pieces were soaked in 2%NaOH at 80°C for 2 hours to remove non-cellulosic materials to increase susceptibility to hydrolysis of banana (Dufrense and Vignon, 1998) and rinsed with distilled water between each step. A subsequent bleaching by soaked in 1.5 wt%H_2O_2 at 70°C for 2 hours. This step removed most of the residual lignin and protein. After the chemical treatments, the bleached banana fibers were hydrolyzed by 65wt% H_2SO_4 at 60°C for 1.5 hours under vigorous stirring. After that, centrifuged for three times and dialyzed until neutral.

3.2.4 Preparation of Bionanocomposite Sponges

The bionanocomposite sponges (15 mm-diameter) were fabricated by freeze-dry method. The ratios of cellulose whisker:chitin whisker were ranged from 100:0, 75:25, 50:50, 25:75, and 0:100w/w. The content of sericin was varied from 25%, 50%, and 100%wt. of weight of whiskers. Three compositions were mixed by used water as medium solution, and stirred until got homogeneous solution, and subsequently freeze-dried. Various compositions of cellulose whisker: chitin whisker: sericin was selected to investigate the effect of individual composites. After that, these sponges were cross-linked with 25%w/v glutaraldehyde vapor for 24h, and subsequently placed in air flow to evaporate the excess glutaraldehyde.

3.3 Characterizations

3.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra of sericin, chitin whiskers, cellulose whiskers and cellulose whisker/chitin whisker/sericin nanocomposite sponges were characterized with KBr method, and recorded by a Thermo Nicolet Nexus 670 spectrophotometer with a total 64 scans at a resolution of 4 cm^{-1} for each sample. A frequency range of 4000-400 cm^{-1} was observed.

3.3.2 Scanning Electron Microscopy (SEM)

For the morphological study, samples were placed onto stubs, coated with gold using a JEOL JFC-1100 sputtering device, and observed for their microscopic morphology using JEOL JSM-5200 scanning electron microscopy (SEM).

3.3.3 Transmission Electron Microscopy (TEM)

The transmission electron microscope observations were observed by JEOL model JEM 2100 operating voltage of 200 kV. The samples were prepared by air-drying the particles onto a carbon-coated copper grid and air-dried.

3.3.4 X-Ray Diffraction (XRD)

The x-ray diffraction was used to investigate the relative crystallinity of samples after hydrolysis. The samples were observed by using Bruker AXS, Germany Model D8 Advance at 40 kV and 30 mA with the WAXD mode. An x-ray source was Ni-filtered CuKα-radiation (α = 1.542 A□) and the 2θ of each peak were ranged between 10 - 50° at a scan speed 1° /min.

3.3.5 Thermogravimetric Analysis (TGA)

The samples were analyzed by TGA using a Perkin Elmer (TG-DTA) instrument under N_2 flow of 10 ml/min. The heating process was conducted from 30-900°C at a rate of 20°C/min.

3.4 Water Absorption Properties

The water absorption of the bionanocomposite sponges were carried out by the following method. The sponges (15 mm in diameter) were measured the weight (W_0). Then, the samples were immersed in distilled water at room temperature for 1h. Until the certain time, the samples were subsequently stood on sieve at room temperature to remove excess water for 30 minutes, and measured the weight (W_1). The percentage of water absorption of the sponges was calculated as the following equation (2):

$$water\ absorption\ (\%) = \frac{(W_1 - W_0)}{W_0} \times 100 \qquad (2).$$

3.5 Sericin Releasing Test

The bionanocomposite sponges (15 mm–diameter) were placed in 5 ml of 0.01M 2-Amino-2-hydroxymethyl-1,3-propanediol hydrochloride or Tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) solution at pH 7.4 with shaking rate 100 rpm at 37°C, and compared condition with and without lysozyme. The sericin release test was carried out by sampling the released media solution at the various times until 72h. The concentration of protein release was evaluated following BCA protein assay kit (Pierce, product number 23225). This assay is colorimetric method by measured the absorbance at 562 nm to determine the protein concentration, compared standard curve.

3.6 Cytotoxicity Test

3.6.1 Direct Cytotoxicity

This test was used to evaluate the potential for use of protein directly to the cell. 4×10^4 human skin fibroblast cells/well were cultured in 24-well plate to allow cell attachment on the plate. After incubation under 5 % CO_2 at 37 °C at least 4 hours, the cells were starved with a 2 % culture media (2 % MEM; containing MEM, 2 % FBS, 1 % L-glutamine, 1 % lactabumin, and 1 % antibiotic and antimycotic formulation.) for 24 hours. After starvation, the culture medium was removed and replaced with the as-prepared extraction media, and later incubated for 1, and 7 days. The number of living cells was finally quantified with MTT assay.

3.6.2 MTT Assay

The MTT assay was tested to determine cytotoxicity of neat sponges and bionanocomposite sponges. This assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. First, each culture medium are aspirated and replaced with 300 µl per well of MTT solution — (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) — 5 mg/ml for a 24-well

culture plate. Secondly, the plate was incubated for 30 min at 37°C. The solution is then aspired and 900 µl per well of dimethylsulfoxide (DMSO) containing 125 µl per well of glycine buffer (pH=10) is added to dissolve the formazan crystals. Finally, after 10 min of rotary agitation, the absorbance of the DMSO solution at 570 nm is measured using a UV/Visible spectrophotometer. The results of toxic compounds are expressed as the concentration of sample required to kill 50% (IC_{50}) of the cells, compared to that of the controls.