



CHAPTER III METHODOLOGY

3.1 Materials and Chemicals

Shells of *Metapenaeus dobsoni* shrimps were kindly provided by Surapon Foods Public Co., Ltd. (Thailand). Natural rubber latex was obtained from *Hevea brasiliensis* trees (Chantaburi province, Thailand). Anhydrous sodium hydroxide (NaOH) pellets (Labsan analytical science, analytical grade), 50 % (w/w) NaOH solution (Chemical enterprise, commercial grade), hydrochloric acid (HCl, Labsan analytical science, analytical grade), glacial acetic acid (CH₃COOH, J.T.Baker, analytical grade), acetone (Chemical enterprise, commercial grade), monochloroacetic acid (≥ 97 % purity, Fluka, analytical grade), tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl, Scharlau chemie S.A. , molecular biology grade), modified Lowry protein assay kit (Thermo Scientific), 28.0-30.0 wt.% ammonium hydroxide (NH₃) solution (J.T. Baker, analytical grade), and toluene (Labsan analytical science, analytical grade) were used in this study.

3.2 Methodology

3.2.1 Preparation of Chitin

To prepare chitin, shrimp shells were first dried under sunlight for a few days. Then, 1 kg of dried shrimp shells was immersed in 10 L of a 1 N HCl solution with occasional stirring at room temperature for 2 days. The acidic solution was changed daily. The demineralized shrimp shells were subsequently neutralized by extensive rinse with deionized water and dried at 60 °C for 48 hr. The demineralized shrimp shells were further deproteinized in a 4 % w/v NaOH solution at a ratio of NaOH (solution:shrimp shells of 10:1) with continuous stirring at 80 °C for 4 hr. The obtained chitin was filtrated, neutralized by distilled water, and dried at 60 °C for 24 hr.

3.2.2 Preparation of CM-Chitin

CM-chitin was synthesized via alkaline chitin solution route. Firstly, 20 g of chitin was added to a 400 ml of 42 % (w/w) NaOH solution. The mixture was kept under reduced pressure in a vacuum oven at room temperature for 30 min. Then, 640 g of crushed ice was added to the solution in order to get a 4 % (w/w) alkaline chitin solution in a 14 % (w/w) NaOH solution. The solution was stirred for 30 min at low temperature in ice bath. After that, 388 g of a 25 % (w/w) monochloroacetic acid in a 14 % (w/w) NaOH solution was slowly added to the chitin mixture with stirring for 30 min. The mixture solution was settled at room temperature overnight and neutralized by glacial CH_3COOH . The mixture was subsequently dialyzed with running water for 2 days and with deionized water for 2 days. Then, the mixture was centrifuged at 12000 rpm for 10 min at room temperature to remove insoluble materials. The supernatant was added to acetone and centrifuged at 12000 rpm for 10 min at 10 °C to remove the precipitates. The obtained CM-chitin product was reprecipitated in ethanol. Finally, CM-chitin was removed from ethanol by centrifugation and dried at 60 °C for 24 hr.

3.2.3 Preparation of Deproteinized Natural Nubber Latex

The commercial natural rubber latex was centrifuged at 12000 rpm for 5 min at room temperature to remove protein aqueous materials in serum. Then, a 4 % (v/v) NH_4OH solution was added to the supernatant. To further remove the protein residues, a 0.04 % (w/v) protease was also added. The solution was shaken in a shaking incubator at 100 rpm for 48 hr at 37 °C. After that, the solution was centrifuged at 12000 rpm for 5 min to remove protease and the remaining proteins. The deproteinized natural rubber latex was resuspended and preserved in a 4 % (v/v) NH_4OH solution.

3.2.4 Preparation of CM-Chitin/Natural Nubber Blend Films

The CM-chitin/natural rubber blend films were prepared by solution-casting method. Firstly, a desired amount of CM-chitin was dissolved in a 4 % (v/v) NH_4OH solution to get a 2 % (w/v) CM-chitin solution. The obtained CM-chitin solution was mixed with deproteinized natural rubber latex at five different CM-chitin:natural

rubber ratios (100/0, 90/10, 80/20, 70/30, and 60/40). These mixtures were casted on the Teflon mold at room temperature before drying at 35 °C.

3.2.5 Preparation of Plasticized CM-Chitin/Natural Rubber Blend Films

The plasticized CM-chitin/natural rubber blend films were prepared by starting with dissolve CM-chitin in 4% w/w NH₄OH to get 2 %w/v CM-chitin solution. And CM-chitin solution was mixed with glycerol to get homogeneous with different weight ratio. (CM-chitin/natural rubber/glycerol, 80/20/10, 80/20/20, 80/20/30). After that the mixture was blended with deproteinized natural rubber latex. Then these mixtures were casted on the Teflon mold at room temperature before drying at 35 °C.

3.2.6 Crosslinking of Blend Films

The blend films were crosslinked in a glutaraldehyde vapor system in a seal desiccator. A 10 ml of 25 % glutaraldehyde solution was added into a sealed desiccator. The blend films were placed on a holed shelf in the desiccator at room temperature and the desiccator was closed by the cover. The blend film was crosslinked in glutaraldehyde vapor with different time: 15 min, 30 min, 45 min, and 60 min.

3.3 Characterization

3.3.1 CHNS/O Analyzer

The degree of substitution of the CM-chitin was evaluated by way of an elemental analysis using a CHNS/O analyzer (Perkin Elmer PE2400 Series II) at combustion and reduction temperatures of 950 °C and 500 °C, respectively. Each sample was analyzed in air (60 psi), using oxygen as a combustion gas (15 psi) and helium as a carrier gas (20 psi). The degree of substitution of chitin was evaluated according to the following equation (Huang *et al.*, 2010):

$$DS = DD + 0.5833 \times C/N - 4$$

where C is the percentage of carbon and N is the percentage of nitrogen.

3.3.2 Viscosity Measurement

The molecular weight of CM-chitin which prepared from shrimp shell was estimated by viscosity of the CM-chitin solution (Tokura *et al.*, 1983). It was measured with an Ubbelode type viscometer at 25 °C. The ionic strength was adjusted by the dialysis of CM-chitin with NaCl solution. The molecular weight of CM-chitin was estimated according to the following equation:

$$[\eta] = KM^\alpha$$

where $K = 7.92 \times 10^{-5}$, $\alpha = 1.00$.

3.3.3 Fourier Transform Infrared Spectrometer (FT-IR)

The CM-chitin/natural rubber blend films were characterized for the their functional groups by FT-IR spectrometer (Thermo Nicolet, Nexus 670) in transmittance mode with 64 scans and at a resolution of $\pm 4 \text{ cm}^{-1}$ over the wavenumbers range of 4000 cm^{-1} to 700 cm^{-1} . Moreover, FT-IR spectrum of chitin film was used to estimate the degree of deacetylation of chitin which was evaluated according to the following equation (Sannan *et al.*, 1977):

$$DD = 101 - 35.71(Abs_{1550}/Abs_{2878})$$

where Abs_{1550} is the absorbance at 1550 cm^{-1} and Abs_{2878} is the absorbance at 2878 cm^{-1} .

3.3.4 Scanning Electron Microscope (SEM)

The CM-chitin/natural rubber blend films were examined for the morphological structure by the scanning electron microscope (Hitachi, S-4800). The samples were put on the holder with an adhesive tape and coated with a thin layer of platinum. The scanning electron images were investigated by using an acceleration voltage of 2 kV with a magnification in 3 kX.

3.3.5 Mechanical Testing Machine

The mechanical properties of the CM-chitin/natural rubber blend films were investigated by mechanical testing machine (Lloyd) in the pull with yield setup mode with preload of 0.1 N, speed of 50 mm/min and gage length of 4 cm. The values of stress at maximum load and elongation at break were reported.

3.3.6 Thermogravimetric Analyzer (TGA)

The thermogravimetric analyzer (Perkin Elmer, TGA7) was used to determine the thermal behavior of CM-chitin/natural rubber blend films. The experiment was carried out by weighting a powder sample of 1 mg to 5 mg and loaded into a platinum pan. The temperature range was scanned from 30 °C to 750 °C at a heating rate 20 °C min⁻¹ under nitrogen flow.

3.3.7 UV-VIS Spectrophotometer

The UV-VIS Spectrophotometer (Tecan, Infinite M200) was used to examine the protein content of the deproteinized natural rubber latex. From the Modified Lowry method, both law natural rubber latex and deproteinized natural rubber latex films were soaked in tris-HCl pH 7.4 for 2 hr. Each samples of soaked tris-HCl solution was added into Modified Lowry reagent and Folin-Phenol reagent. The color change of samples was analyzed at 750 nm with UV-VIS spectrophotometer.

3.4 Water Absorption Properties

The crosslinked CM-chitin films were prepared to be 2.5 cm of cyclic shape. Then, the films were immersed a 3 ml of buffer pH 5.5 for 24 hr. The dimension stability of samples in wet state was also estimated in term of degree of swelling and weight loss (Wongpanit *et al.*, 2005). The degree of swelling was evaluated according to the following equation:

$$\text{Degree of swelling (\%)} = \frac{W_s - W_{d1}}{W_{d1}} \times 100$$

where W_{d1} is the weight of the dried film prior to immersion in buffer pH 5.5 and W_s is the weight of the sample in the swollen state.

The samples were then dried in convection oven and measured the weight loss after immersion in buffer 5.5. The weight loss was estimated according to the following equation:

$$\text{Weight loss (\%)} = \frac{W_{d1} - W_{d2}}{W_{d1}} \times 100$$

where W_{d2} is the weight of the dried film after immersion for 24 hr.