CHAPTER III
EXPERIMENTAL

3.1 Materials

Chitin was prepared from shrimp shell that was kindly supplied from Surapon Foods Public Co., Ltd. by the method of Shimahara and Takigushi (1988). Chitin was ground to powder with the size of 71-75 μm before use. Glutaraldehyde was purchased from Fluka. Monochloroacetic acid and sodium hydroxide were analytical grade.

3.2 Equipment

3.2.1 Restch Sieving Machine

The chitin powder was sieved by using Restch Sieving Machine type Vibro and chitin with the size of 71-75 μm was collected for using in the experiment.

3.2.2 Capillary Viscometer

The viscosity-average molecular weight of CM-chitin was determined by using Cannon Ubbelohde-type viscometer number 50.

3.2.3 Elemental Analysis

The degree of substitution of CM-chitin was estimated by elemental analysis using PERKIN ELMER Series II CHNS/O Analyzer 2400.

3.2.4 FTIR Spectrophotometer

The FTIR spectrum of silk fibroin/CM-chitin blend films were recorded with a Bruker FTIR Spectrophotometer, model Vector 3.0, with 32 scans at a resolution of 4 cm^{-1}. The samples with the thickness of 10 μm were attached to the

sample frames. A frequency of 4000-400 cm^{-1} was observed by using deuterated triglycinesulfate detector (DTGS) with specific detectivity of 1 x 10^9 $cm.Hz^{1/2}.W^{-1}$.

3.2.5 Wide-angle X-ray Diffractometer (WAXD)

The wide-angle x-ray diffractometer used in this study was a D/MAX-2000 series of Rigaku X-ray Diffractometer system. The X-ray source was Ni-filtered CuK-alpha radition (40 kV/30 mA). Divergence slit and scattering slit at 1 degree together with 0.3 mm of receiving slit were set on the instrument. The dried films with thickness of 25 μm were attached on the sample holders and scanned from 5 to 30 degree 2θ at a speed 5 degree/min and 0.02 degree of scan step.

3.2.6 Differential Scanning Calorimeter (DSC)

The differential scanning calorimeter (DSC) used to evaluate the thermal properties of the blend films was a NETZCH model TASC 414/3. The sample of 8-15 mg was placed in an aluminum pan. The differential scanning calorimeter (DSC) of the blend films was carried out under N_2 atmosphere at heating rate of 10°C/min from room temperature to 250°C.

3.2.7 Thermogravimetric Analysis (TGA)

The thermogravimetric analysis (TGA) used to evaluate the thermal stability of the blend films was a TGA 5.1 Dupont Instrument model 2950. The sample of 8-15 mg was placed in a Platinum pan. The thermogravimetric analysis of the blend films was carried out under N_2 atmosphere at heating rate of 10°C/min from room temperature to 700°C.

3.2.8 Lloyd Tensile Tester

The strength of the blend films was characterized by a Lloyd Instrument LRX series of Lloyd tensile tester with the maximum load of 2500 N.

3.3 Methodology

3.3.1 Preparation of Chitin

The shrimp shells were washed with water, dried under sunlight and crushed into chips about 0.5 cm^2 in size. The dried shell chips (100 g) were immersed in 1 L of 1 N hydrochloric acid. The mixture was kept for 2 days at room temperature with occasional stirring. The demineralized shell chips (100 g) were collected and washed with distilled water until neutral. The second step was deproteination with sodium hydroxide aqueous solution. The demineralized shell chips were added to 1 L of 4 % NaOH solution and boiled at 70-80°C for 4 h with occasional stirring. An approximate amount of water was added as the vaporization of water proceeded. After boiling, chitin chips were collected and washed with distilled water until neutral. The product was dried in oven at 110°C for 24 h.

3.3.2 Preparation of CM-chitin

Alkaline chitin was prepared by suspending powdered chitin (4g) in 42% NaOH solution. After the suspension was allowed in desiccator for 30 min under reduced pressure, crush ice (160g) was added and the mixture was mechanically stirred for 30 min in an ice bath. Monochloroacetic acid solution was prepared by dissolving in 14% NaOH in an ice bath and was added dropwise into the alkaline chitin solution with stirring over 30 min. After standing overnight at room temperature, the mixture was neutralized with acetic acid under cooling in an ice bath and dialyzed against running water for 2 days, followed by dialysis against distilled water for 1 day. The dialysate was centrifuged at 5000 rpm for 20 min in order to remove insoluble material, and the supernatant was added into 3 volumes of acetone. After standing overnight, the precipitate was collected by centrifugation and washed with acetone. The product was resuspended in ethanol and collected by filtration. After drying at room temperature, CM-chitin Na salt was obtained (Tokura *et al.*, 1983a).

3.3.3 Degree of Deacetylation of Chitin

The method used to determine the degree of deacetylation of chitin is based on quantitative infrared spectroscopic technique (Sannan *et al.*, 1978). About 3 mg of chitin powder, passed through a 200-mesh sieve, was mechanically mixed with 400 mg of potassium bromide powder to prepare a KBr disk. An infrared spectrum was recorded in a range from 4000 to 400 cm^{-1}. The absorbances at 2878 cm^{-1} (the C-H band) and 1550 cm^{-1} (the amide II band) were evaluated by the baseline method. The degree of deacetylation (DD) was calculated from the following equation:

$$DD\ (\%) = 98.03 - 34.68(A_{1550}/A_{2878}) \qquad (3.1)$$

where DD $= $ Degree of deacetylation (%)

A_{1550} = Absorbance at 1550 cm^{-1} (the amide II band)

A_{2878} = Absorbance at 2878 cm^{-1} (the C-H band)

3.3.4 Degree of Substitution of CM-chitin

The degree of substitution was estimated by elemental analysis with combustion at 950°C. The sample (1-2 mg) was filled in tin foil and analyzed under air with oxygen as a combustion gas (flow rate of 20 ml/min) and with He as a carrier gas (flow rate of 200 ml/min).

3.3.5 Viscosity-Average Molecular Weight of CM-chitin

The different concentrations (0.01, 0.02, 0.03, 0.04 and 0.05 g/100 ml) of CM-chitin solutions dissolved in 0.1 M NaCl were prepared. The Ubbelohde viscometer was filled with 10 ml of sample solution and then equilibrated in water bath, which was maintained the temperature at 25°C. The sample was passed through the capillary once before the running time was measured. Each sample was measured 5 times. The running times of solvent and solutions were used to calculate

the relative viscosity, specific viscosity, and reduced viscosity, by the following equations:

$$\text{Relative viscosity } (\eta_{rel}) \quad = (t/t_s) \qquad (3.2)$$

$$\text{Specific viscosity } (\eta_{sp}) \quad = (t/t_s)\text{-}1 \qquad (3.3)$$

$$\text{Reduced viscosity } (\eta_{red}) \quad = \eta_{sp}/C \qquad (3.4)$$

$$\text{Intrinsic viscosity } [\eta] \quad = (\eta_{sp})_{C \to 0} \qquad (3.5)$$

where t is the running time of CM-chitin solution, t_s is the running time of solvent and C is the concentration in g/100 ml.

The viscosity-average molecular weight of CM-chitin was determined based on Mark-Houwink equation. The K and a values were calculated according to Kaneko et al., (1982).

$$[\eta] \quad = \quad 7.92 \times 10^{-5} M^{-1} \qquad (3.6)$$

where $[\eta]$ = Intrinsic viscosity

M = Viscosity-average molecular weight

3.3.6 Preparation of Silk Fibroin Solution

Raw silk fiber was degummed by heating in 0.5% Na_2CO_3 solution at 100°C for 1 h followed by washing with boiling water and drying at 60°C for 24 h in an oven. Silk fibroin 6 g was then dissolved in 94 g of 1:2:8 by mole of $CaCl_2$:EtOH:H_2O solvent system at 100°C for 15 minutes (Chen et al., 1994). The resulting silk fibroin solution was filtered through the sinter glass filter and

subsequently dialyzed against distilled water for 7 days. The dialyzed silk fibroin solution was filtered and diluted to achieve a concentration of 1% w/w.

3.3.7 Preparation of CM-chitin Solution

CM-chitin solution was prepared by dissolution of CM-chitin in distilled water to obtain the concentration of 1% by weight. The solution was stirred continuously overnight.

3.3.8 Preparation of Blend Films

The blend films of silk fibroin and CM-chitin were prepared by mixing various ratios of 1% by weight of silk fibroin solution and 1% by weight of CM-chitin solution. The blend solution was stirred slowly for 12 h and left overnight to get rid of air bubbles before casting onto the clean dry petri dishes in a dust-free atmosphere at room temperature. For the crosslinked CM-chitin/ silk fibroin blend films, glutaraldehyde used as crosslink agent was added into the blend solution to achieve the concentration of 0.005%, 0.01% and 0.05%.

3.3.9 Equilibrium Water Content (EWC)

The blend films were cut into the disk form with diameter of 16 mm and 25-30 µm in thickness. A preweighted dry film was immersed in distilled water for 24 h. When the film was removed from the water, excess water at the surface of the film was blotted out with Kimwipes paper and the weight of the swollen film was measured. The procedure was repeated until there was no further weight change. Water content was determined by gravimetric method (Kim *et al.*, 1996) and calculated from the following equation:

$$\text{EWC (\%)} \quad = \quad \frac{Wh - Wd}{Wd} \; \text{x} \; 100 \qquad (3.7)$$

where Wh and Wd denote the weight of hydrated and dried film, respectively.

3.3.10 Swelling Behavior

The blend films were cut into disk form with diameter of 16 mm and 25-30 μm in thickness. The weights of the completely dried samples were measured, and the samples were dipped into a vial filled with different pH buffer solutions and various salt solutions, i.e., LiCl, NaCl, $CaCl_2$ and $FeCl_3$, with the concentration of 0.25 M at room temperature. The degrees of swelling of these samples were calculated from the following equation (Wang *et al.*, 1997):

$$\text{Degree of swelling (\%)} = \frac{Ws - Wd}{Wd} \times 100 \qquad (3.8)$$

where Ws and Wd denote the weight of swollen and dried film, respectively.

3.3.11 Mechanical Properties

The tensile strength and elongation at break of the CM-chitin/silk fibroin blend films were measured by Lloyd Tensile Tester by following the standard ASTM D882 using the gauge length of 50 mm and an extension rate of 20 mm/min at room temperature. Test films were cut in the dimension of 25 mm x 150 mm and the thickness of the films was in the range of 35-45 μm. Then the films were dried at 60°C for 24 h before testing.