CHAPTER III
EXPERIMENTAL

3.1 Materials

Chitin was prepared from shrimp shells kindly supplied by the Suraphol Food Public Co., Ltd. Silk fibroin was obtained by degumming of raw silk fiber. Glacial acetic acid purchased from J. T. Baker was analytical grade of 99.9% w/w, and 50% w/w glutaraldehyde was purchased from Fluka Co., Ltd.

3.2 Equipment

3.2.1 Restch Seiving Machine

The chitosan powder with the size of 38 to 75 μm was sieved and collected separately by using Restch Sieving Machine type Vibro.

3.2.2 Capillary Viscometer

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

3.2.3 FTIR Spectrophotometer

The FTIR spectrum of chitosan/silk fibroin blend films were recorded with Vector 3.0 Bruker FTIR Spectrophotometer with 16 scans at a resolution of 4 cm^{-1}. A frequency of 4000-400 cm^{-1} was observed by using deuterated triglycinesulfate detector (DTGS) with specific detectivity of $1x10^{9}$ $cm.Hz^{1/2}.w^{-1}$.

3.2.4 Wide-Angle X-Ray Diffractometer (WAXD)

The wide-angle X-ray diffractometer used in this study was D/MAX-2000 series of Rigaku X-ray Diffratometer system. X-ray of Cu k-alpha at 40

kV/30 mA were used as a source. The k-beta filter was used to eliminate interference peak. Divergence slit and scattering slit at 1 deg together with 0.3 kV/30 mA were used as a source. The k-beta filter was used to eliminate interference peak. Divergence slit and scattering slit at 1 deg together with 0.3 mm of receiving slit were set on the instrument. The experiment was performed in the range of 5-30 degree with scan speed 5 deg/min and 0.02 deg of scan step.

3.2.5 Thermogravimetric Analyzer (TGA)

The thermogravimetric analyzer used to evaluate the thermal stability of the blend films was TGA 5.1 Dupont Instrument model 2950.

3.2.6 Lloyd Tensile Tester

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

3.2.7 Gas Permeability Tester

The Brugger gas permeability tester type GDP/E as shown in Figure 3.1 was used to detect the permeability of oxygen gas through the blend films. The flow rate of oxygen was controlled at 100 cm^3/min at ambient temperature.

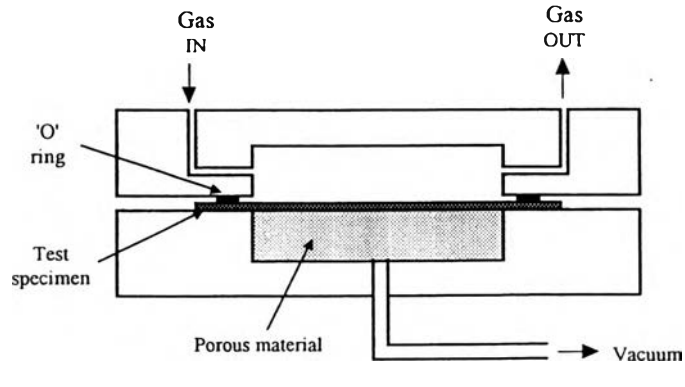

Figure 3.1 Schematic drawing of a Brugger gas permeability tester.

3.3 Methodology

3.3.1 Preparation of Chitin

Chitin was prepared by the method of Shimahara *et al.* (1988). The shrimp shell was cleaned and dried before grinding into small pieces. The decalcification of shrimp shell was performed by immersing in 1 N HCl solution for 2 days with occasional stirring, and the decalcified product was washed until neutral. Protein removal was performed in 4% w/w of NaOH solution by boiling further at 80-90°C for 4 h. The deproteinized portion was washed with deionized water until neutral. The product obtained was chitin.

3.3.2 Preparation of Chitosan

For chitosan preparation, chitin flakes were deacetylated by heating in 50% by weight of NaOH solution containing 0.5% by weight of $NaBH_4$ added based on the weight of chitin to prevent depolymerization. The ratio of chitin to NaOH solution was 1 g of chitin in 10 ml of NaOH solution. The deacetylation was performed in an autoclave at 110°C for 1 h. The deacetylated product obtained was washed exhaustedly with deionized water until neutral. The resulting chitosan flakes were dried in an oven at 60°C for 24 h.

3.3.3 Degree of Deacetylation of Chitosan

The method used to determine the degree of deacetylation of chitosan is based on infrared spectroscopic measurement by Sannan (1978). About 3 mg of chitosan 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 cm^{-1} 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 was calculated from equation 3.1.

$$D = 98.03-34.68(A_{1550}/A_{2878}) \quad (3.1)$$

where D = degree of deacetylation (%)

A_{1550} = absorbance at 1550 cm^{-1} (the C-H band)

A_{2878} = absorbance at 2878 cm^{-1} (the amide II band).

3.3.4 Viscosity-Average Molecular Weight of Chitosan

Different concentration solutions (0.00, 0.00625, 0.0125, 0.025, 0.05, and 0.1 g/ 100 mL) of chitosan in 0.2 M acetic acid-0.1 M NaCl-4.0 M urea were prepared. An Ubbelohde viscometer was filled with 10 mL of sample and then equilibrated in water bath, which maintained the temperature at 25°C. The sample was pass through the capillary once before the running time was measured. Each sample was measured 3 times. The running times of solvent and solutions were used to calculate the relative viscosity, specific viscosity, and reduced viscosity. The reduced viscosity was plotted against the concentration with the intercept being the intrinsic viscosity.

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

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

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

$$\text{Intrinsic viscosity } [\eta] = (\eta_{red})_{c \to 0} \quad (3.5)$$

When t is the running time of chitosan solution, t_s is the running time of solvent and C is the concentration in g/100mL

The viscosity-average molecular weight of chitosan was determined based on the Mark-Houwink equation following

$$[\eta] = 8.93 \times 10^{-4} M^{0.71} \quad (3.6)$$

where [η] is the intrinsic viscosity, M is viscosity-average molecular weight.

3.3.5 Preparation of Chitosan Solution

Chitosan flake was dried at 110°C for 1 h before use. Chitosan solution was prepared by dissolution of chitosan in 1 % by weight acetic acid solution. The chitosan solution was allowed to stand overnight at 4°C in a refrigerator to get rid of air bubbles before use.

3.3.6 Preparation of Silk Fibroin Solution

To obtain silk fibroin, 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 2:2:8 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 sintered 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 Blend Films

The blend films of chitosan and silk fibroin were prepared by mixing various ratios of 1% by weight of silk fibroin solution and 1% by weight of chitosan 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. These films were allowed to dry at ambient temperature for 72 h. When the films were dried completely, they were soaked in 0.5% by weight of NaOH in MeOH for 24 h to neutralize the acid and then washed exhaustedly with MeOH. The blend films were dried at ambient temperature and stored over silica in a desiccator before use. For the crosslinked chitosan/silk fibroin blend films, glutaraldehyde used as crosslinking agent was added into the blend solution at the amount of 0.01 mole/glucosamine unit of chitosan.

3.3.8 FTIR Spectra of Chitosan/Silk Fibroin Blend Films

FTIR spectra of chitosan/silk fibroin were recorded on the Bruker Fourier transform infrared spectrophotometer, Model Vector 3.0 with 16 scans at a resolution of 4 cm^{-1}. The samples with the thickness of 10 μm were attached to the sample frames and scanned from frequency of 4000 to 400 cm^{-1} using a deuterated triglycinesulfate detector (DTGS) with specific detectivity of $1x10^9$ $cm.Hz^{1/2}.w^{-1}$.

3.3.9 Thermogravimetric Analysis of Chitosan/Silk Fibroin Blend Films

Thermogravimetric analysis of the blend films was carried out under N_2 atmosphere at heating rate of 10°C/min from 40°C to 700°C. About 8 mg of sample was used for each measurement.

3.3.10 Crystallinity Determination

The wide-angle X-ray diffractograms of the blend films were recorded at room temperature using Rigaku X-ray Diffractometer system, Model D/Max-2000. The X-ray source was Cu k-alpha (40 kV/30 mA). The k-beta filter was used to eliminate interference peak. The dried films with thickness of 25μm were attached to the sample holders and 2θ scan range was from 5 to 30° at a speed of 5°/min and 0.02 degree for scan step.

3.3.11 Swelling Behavior Determination

The swelling samples were cut into the disk form with diameter of 16 mm and 25-30 μm in thickness. The samples were immersed in pH buffer solutions at various pH values and in various types of salt solutions. The degree of swelling was calculated from equation 3.7.

$$\text{Degree of swelling (\%)} = \frac{W - Wo}{Wo} \qquad (3.7)$$

where W_0 is the weight of dry film and W is the weight of swollen film.

3.3.12 Mechanical Testing

Tensile strength and elongation at break of the blend films were performed with a Lloyd Tensile Tester according to the standard ASTM D882, at a gauge length of 50 mm and 20 mm/min of strain rate. The dimension of samples was 25 mm x 150 mm and the thickness was 35-40 μm. These mechanical properties were determined in both dry and wet states. For the dry state, the blend films were dried at 60°C for 24 h before measurement. For the wet state, the blend films were soaked in distilled water for 2 days to reach equilibrium before testing.

3.3.13 Oxygen Permeability Testing

The measurement of oxygen permeability of the blend films was performed with Brugger gas permeability tester. The blend films were cut into the circular form with the diameter of 110 mm and about 10 μm in thickness. The films were sealed completely with grease on the top of the porous material and the two halves of the permeability cell were clamped together. The 'O' ring ensured an air-tight seal between two halves. The oxygen gas was circulated through the top half of the permeation cell and vacuum applied below the specimen until all the air had been removes from the specimen. Vacuum was then turned off and the rate of oxygen gas permeation through the films was recorded with times. The oxygen permeability rate could be calculated from equation 3.8.

$$G = \frac{1.47 \times 10^{9}}{K N} \qquad (3.8)$$

where G = permeability rate (cm^3/m^2.d.bar)

K = temperature (Kelvin)

N = reciprocal of slope from the plot of the change of the vacuum pressure versus time.