CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Shrimp Shells

The shells of *Penaeus merguiensis* shrimps were kindly provided by Surapon Foods Public Co., Ltd., Thailand.

3.1.2 Squid Pens

The pens of *Loligo* sp.

3.1.3 Poly (vinyl alcohol)

Poly (vinyl alcohol) was purchased from Fluka Chemie GmbH Co., Ltd. The material was in the form of white small pellets. Molecular weight of the material is 72,000 and has hydrolysation of 97.5 – 99.5%.

3.1.4 Other Chemicals

Sodium hydroxide (NaOH) 50% w/w aqueous solution was kindly supplied by KPT Cooperation Co., Ltd., Thailand. Sodium hydroxide anhydrous pellets (NaOH), sodium borohydride ($NaBH_4$), glacial acetic acid 99.8% w/w and hydrochloric acid (HCl) 37% w/w were of analytical grade and purchased from Carlo Erba Co., Ltd. Sodium azide was purchased from Labchem Asia Pacific Chemical Limited.

3.2 Equipment

3.2.1 Capillary Viscometer

The viscosity-average molecular weights of chitin and chitosan were determined by using Cannon Ubbelohde-type viscometer number 75 and 50 respectively.

3.2.2 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 use in the experiments.

3.2.3 FTIR Spectrophotometer

The FTIR spectra of chitin and chitosan films were recorded with Bruker FTIR Spectrophotometer model Vector 3.0 with 32 scans at a resolution of 4 cm^{-1}. A wavenumber range of 4000-400 cm^{-1} was observed by using deuterated triglycerinesulfate detector (DTGS) with specific detectivity of $1x10^{9}$ $cm.Hz^{1/2}$ w^{-1}.

3.2.4 Transmission Electron Microscopy (TEM)

Transmission electron micrograph was taken by using a transmission electron microscope (JEOL model, JEM-200CX).

3.2.5 Wide-Angle X-Ray Diffractometer

Wide-angle X-ray diffractograms (WAXD) of chitin whisker-reinforced films were recorded by a Rigaku Rint 2000 diffractometer. High intensity monochromatic Ni-Filtered CuKα radiation was generated at 40 kv and 30 mA. The analysis was performed at room temperature in the range of 5-40 degree 2Θ with a scan speed of 5 degree/min and 0.02 degree of scan step.

3.2.6 Tensile Tester

Mechanical properties of PVA/chitin whisker blend films were evaluated on a Lloyd Instrument LRX series of Lloyd tensile tester with the maximum load of 500 N.

3.2.7 Thermogravimetric Analyzer (TGA)

The thermogravimetric analyzer (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 Pt pan. The thermogravimetric analysis (TGA) of the blend films was carried out under N_2 atmosphere at a heating rate of $10^{o}C$/min from room temperature to $700^{o}C$.

3.3 Methodology

3.3.1 Preparation of Chitin

Chitin was prepared from shrimp shells and squid pens by decalcification and deproteinization to remove calcium carbonate and protein, respectively. The shrimp shells and squid pens were cleaned and dried under sunlight before grinding into small pieces. The shrimp shell chips and squid pen chips were treated by immersion in 1 N HCl solution for 2 days with occasional stirring. The decalcified product was washed with distilled water until neutral. Deproteinization was followed by boiling the decalcified product in 4% w/w of NaOH solution at 80-90°C for 4 h. After NaOH solution was decanted, the product was washed with deionized water until neutral and dried at 60°C in a convective oven for 24 h.

3.3.2 Preparation of Chitosan from Squid Pen

Chitosan was obtained from deacetylation of chitin by refluxing the chitin flakes in 50% w/w NaOH solution containing 0.5% w/w $NaBH_4$ 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 by heating in an oil bath at 100°C for 2 h. The resulting chitosan flakes were dried in an oven at 60°C for 24 h. The process was repeated about three times to achieve chitosan with high degree of deacetylation.

3.3.3 Preparation of Chitin Whisker Suspension

Chitin whisker suspensions were prepared by hydrolyzing chitin sample with 3 N HCl at 104°C for 90 min under stirring. The ratio of 3 N HCl to chitin was 30mL/g. After acid hydrolysis, the suspension was diluted with distilled water followed by centrifugation at 10000 rpm for 5 min. This process was repeated three times. Next, the suspension was transferred to a dialysis bag and dialyzed in running water for 2 h and then overnight in distilled water. The pH was subsequently adjusted to 3.5 by adding HCl. The dispersion of whiskers was completed by 5-min ultrasonic treatment for every 40-cm^3 aliquot. Sodium azide

was subsequently added to avoid bacterial growth and the suspension was stored in a refrigerator until used.

3.3.4 Preparation of Chitin Whisker-Reinforced PVA Films

The PVA solution was prepared by dissolving PVA in distilled water at 90°C and stirred for 3 h. Then chitin whiskers solution was poured into the PVA solution. The solutions of whiskers and PVA were mixed in various proportions in order to obtain composite films ranging between 0%-20% weights of chitin whisker solution. The PVA solution/chitin whisker solutions were stirred mechanically at room temperature for 12 h. The films were prepared by solution casting into plastic mould. The solvent was evaporated at 40°C for 12 h under convective oven.

3.3.5 Preparation of Chitin Whisker-Reinforced Chitosan Films

The chitosan solution was prepared by dissolving chitosan in a 2%v/v acetic acid solution at a concentration of 1% w/v. Then chitin whiskers solution was poured into chitosan solution. The solutions of whiskers and chitosan were mixed in various proportions in order to obtain composite films ranging between 0%-20% weights of chitin whisker solution. The chitosan/chitin whisker solutions were stirred mechanically at room temperature for 24 h. The films were prepared by solution casting into plastic mould. The solvent was evaporated at 40°C for 24 h under convective oven.

3.3.6 Fourier Transform Infrared Spectroscopy (FT-IR)

The FTIR spectra of chitin and chitosan were obtained from a vector 3.0 Bruker Spectrophotometer with a resolution of 4 cm^{-1}. A frequency range of 4000 to 400 cm^{-1} was observed using a deuterated triglycinesulfate detector (DTGS) with a specific detectivity of $1x10^{9}$ $cmHz^{1/2}$ w^{-1}. The chitin and chitosan powder was attached to the sample holder and scanned with 32 scans.

3.3.7 Degree of Deacetylation of Chitin

The degree of deacetylation of chitin was measured based on infrared spectroscopic measurement. Chitin powder (~1 mg), which was passed through 75

μm sieve, was mixed and ground with 60 mg of potassium bromide powder and pressed to give a KBr disk. FTIR spectra of chitin were recorded in the range between 4000 and 400 cm^{-1}. Absorbance of peaks at wave was number of 2878 cm^{-1} (the C-H stretching) and 1550 cm^{-1} (the amide II band) was evaluated by the base line method (Sannan et al., 1978). The degree of deacetylation was calculated from equation (3.1):

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

where DD = degree of deacetylation (%)

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

A_{2878} = absorbance at 2878 cm^{-1} (C-H stretching)

3.3.8 Degree of Deacetylation of Chitosan

The degree of deacetylation of chitosan was measured based on infrared spectroscopic measurement. Chitosan powder (~1 mg), which was passed through 75 μm sieve, was mixed and ground with 60 mg of potassium bromide powder and pressed to give a KBr disk. FTIR spectra of chitin were recorded in the range between 4000 and 400 cm^{-1}. Absorbance of peaks at wave number of 3450 cm^{-1} (the C-H stretching) and 1655 cm^{-1} (the amide II band) was evaluated by the base line method (Sabnis and Block, 1997). The degree of deacetylation was calculated from equation (3.2):

$$DD = 97.67 - 26.48(A_{1655}/A_{3450}) \quad (3.2)$$

where DD = degree of deacetylation (%)

A_{1655} = absorbance at 1655 cm^{-1} (amide II band)

A_{3450} = absorbance at 3450 cm^{-1} (C-H stretching)

3.3.9 Viscosity-Average Molecular Weight of Chitosan

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

different concentrations (0.01, 0.02, 0.03, 0.04, and 0.05g/100 mL) in 0.2 M acetic acid/0.1M sodium acetate were prepared. An Ubbelohde viscometer was filled with 10 mL of sample, and was then mounted vertically in a water bath regulated to 30 ± 0.1°C and left to equilibrate for 15-20 minutes. The sample was passed through the capillary once before the running times were measured. Each sample was measured at least 3 times. The running times of solvent and solution were used to calculate the relative viscosity, specific viscosity, inherent viscosity, and reduced viscosity. The corresponding equations are:

$$\text{Relative viscosity } (\eta_{rel}) = t/t_s \tag{3.3}$$

$$\text{Specific viscosity } (\eta_{sp}) = (t/t_s)-1 \tag{3.4}$$

$$\text{Reduced viscosity } (\eta_{red}) = \eta_{sp}/C \tag{3.5}$$

$$\text{Inherent viscosity } (\eta_{inh}) = (\ln \eta_{rel})/C \tag{3.6}$$

$$\text{Intrinsic viscosity } [\eta] = (\eta_{red})_{C\rightarrow 0} \tag{3.7}$$

where:

t = flow time of chitin or chitosan solution (sec)

t_s = flow time of solvent (sec)

c = concentration of polymer solution in g/dL.

The values of reduced viscosity and inherent viscosity were plotted against the concentration. Then, the value of intrinsic viscosity was obtained from the intercept of the plot, multiplied by 100 to change the dimensions into mL/g. The viscosity-average molecular weights of chitin and chitosan were determined based on the Mark-Houwink equation:

$$[\eta] = KM^a \tag{3.8}$$

where $[\eta]$ is the intrinsic viscosity, M is viscosity-average molecular weight, K and a values were 6.95×10^{-5} and 0.88, respectively (Wang et al., 1991).

3.3.10 Viscosity-Average Molecular Weight of Chitin

Molecular weight of chitin was determined by using the same method as described for chitosan in section 3.3.9. Chitin solution of different concentrations (0.001, 0.002, 0.003, 0.004, and 0.005g/100mL) in 5% LiCl/N,N-dimethylacetamide were prepared. The experiment was performed by using Cannon Ubbelohde type number 75 of capillary viscometer at 30 ± 0.1°C. The K and a constants are 8.93×10^{-4} and 0.71, respectively (Lee, 1974).

3.3.11 Transmission Electron Micrograph of Chitin Whisker

Transmission electron micrograph was taken by using a transmission electron microscope (JEOL model, JEM-200CX). A drop of a dilute suspension of chitin whiskers was deposited and allowed to dry on a formvar grid.

3.3.12 Mechanical Properties

Tensile strength and the elongation at break of the chitin whisker-reinforced films were measured on a Lloyd Instrument LRX series of Lloyd tensile tester according to ASTM D882 using the gauge length 50 mm and crosshead speed 50 mm/min. The films were cut to the dimension of 15 x 150 mm and the thickness of the films was in the range 30 – 45 μm. The tensile strength values and elongation at break values were averaged from 5 samples.

3.3.13 Wide-Angle X-ray Diffractometer (WAXD)

Wide-angle X-ray diffractograms (WAXD) of chitin whisker-reinforced films were recorded by a Rigaku Rint 2000 diffractometer. High intensity monochromatic Ni-Filtered CuKα radiation was generated at 40 kv and 30 mA. The dried film with thickness of 25 μm was attached to the sample holder. The analysis was performed at room temperature in the range of 5-40 degree 2Θ with scan speed of 5 degree/min and 0.02 degree of scan step.

3.3.14 Thermogravimetric analysis (TGA)

Thermal stability of chitin whisker-reinforced films was evaluated using a TGA 5.1 DuPont Instrument model 2950. Film sample (3-10 mg) was placed

in the Pt pan. Thermogravimetric analysis of blend films was carried out from 30° c to 700°C at a heating rate of 10°C/min under nitrogen atmosphere.

3.3.15 Swelling Behavior Determination

Swelling behavior of the chitin whisker-reinforced films in water was studied. The films with diameter of 17 mm were immersed in water. At predetermined time intervals, the swollen films were weighed after they were wiped with soft paper tissue. The degree of swelling for each sample was calculated by using the following equation:

$$\text{Degree of swelling (\%)} = \frac{W - W_o}{W_o} \times 100 \qquad (3.9)$$

where W and W_o are the weights of the swollen film and the dry film, respectively.

3.3.16 Weight Loss (%) Determination

The films were cut into disk form with diameter of 17 mm and 25-35 μm in thickness. The weights of the completely dried samples before immersion were measured, and the samples were immersed in water at 37°C. The weight loss of these samples was calculated from the following equation:

$$\text{Weight Loss (\%)} = \frac{Wi - Wd}{Wd} \times 100 \qquad (3.10)$$

where Wi is the initial weight before immersion and Wd is the dried weight after immersion at specific time.

3.3.17 Heat Treatment

Triplicate samples of pure polymer and chitin whisker-reinforced films were processed to enhance stability to water by saturated steam heat treatment (autoclave) at 110°C for 5 min. The samples were removed from the heat source at specified time and stored in desiccators at ambient temperature for at least 24 h prior to characterization.