CHAPTER III
EXPERIMENTAL

3.1 Materials

The shells of *Penaeus merguiensis* shrimps were kindly provided by Surapon Foods Public Co., Ltd., Thailand. Chitosan was purchased from Seafresh Chitosan (Lab) Co., Ltd., Thailand, in the form of pale yellow flakes. It was reported that the material had a degree of deacetylation of 95% and molecular weight of about 1×10^5. Sodium alginate was purchased from Carlo Erba Co., Ltd., in the form of white powder. Sodium hydroxide 50% w/w aqueous solution was kindly supplied by KPT Cooperation Co., Ltd., Thailand. Sodium hydroxide anhydrous pellets, sodium borohydride, monochloroacetic acid, glacial acetic acid 99.7% w/w, and hydrochloric acid 37% w/w were analytical grade purchased from Carlo Erba Co., Ltd. Calcium chloride dihydrate stated as edible grade was supplied from Asia Drug & Chemical Co., Ltd., Thailand. Isopropanol was analytical grade purchased from Labscan Asia Co., Ltd., Thailand. Sodium chloride (Univar) was analytical grade purchased from Asia Pacific Specialty Chemicals Limited. Agar powder (bacteriological), peptone water, and beef extract (protose BE) were analytical grade purchased from HiMedia Laboratories Limited. Methanol, ethanol, and acetone were commercial grade and used without further purification. The other chemicals of reagent grade were used as supplied.

3.2 Equipment

3.2.1 Restch Sieving Machine

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

3.2.2 Capillary Viscometer

Cannon Ubbelodhe-type number 75 capillary viscometer was used for the determination of the viscosity-average molecular weight of chitin. The viscosity-average molecular weights of CM-chitin, CM-chitosan and alginate were determined using Cannon Ubbelodhe-type viscometer number 50.

3.2.3 Fourier Transform Infrared (FTIR) Spectrophotometer

The FTIR spectra of chitin, CM-chitin, CM-chitosan, alginate and CM-chitosan/alginate blends were recorded with Vector 3.0 Bruker FTIR Spectrophotometer with 32 scans at a resolution of 4 cm^{-1}. A frequency of 4000-400 cm^{-1} was observed by using deuterated triglycinesulfate detector (DTGS) with a specific detectivity of 1 x 10^9 cm $Hz^{1/2}$ w^{-1}.

3.2.4 UV/Visible Spectrophotometer

The chitosan coating was confirmed by UV/Visible spectrum of ninhydrin-treated chitosan solution extracted from fiber. The UV/Visible spectrum was recorded with Perkin Elmer UV/Visible Spectrophotometer model Lambda 10.

3.2.5 Nuclear Magnetic Resonance Spectrometry (NMR)

^{1}H-NMR spectra of CM-chitin and CM-chitosan were recorded using FT-NMR 500 MHz. spectrometer (JEOL, JNM-A500). CM-chitin and CM-chitosan were both dissolved in D_2O and tetramethylsilane (TMS) was used as reference for chemical shift measurement.

3.2.6 Elemental Analysis (EA)

Elemental analysis results were obtained from a CHNS/O analyzer (Perkin Elmer PE2400 Series II: option CHN). Degree of substitutions of CM-chitin and CM-chitosan were determined based on the C/N ratios of CM-chitin and CM-chitosan from elemental analysis. The C/N

ratio values obtained from elemental analysis were compared with the calculated values.

3.2.7 Scanning Electron Microscope (SEM)

SEM micrographs of fibers were taken on a JEOL JSM-5200 scanning electron microscope operating at 10 kV and a magnification of x 1500.

3.2.8 Atomic Absorption Spectrophotometer (AAS)

The atomic absorption spectrophotometer used in this study was Variance SpectrAA 300P. The measurement was performed at the wavelength of 422.7 nm to determine calcium (Ca) concentration and nitrous oxide-acetylene flame was used.

3.2.9 Universal Testing Machine

The mechanical properties of the fibers were measured according to ISO 2062:1993(E) using Lloyd LR 100K apparatus.

3.3 Methodology

3.3.1 Preparation of Chitin, CM-chitin, and CM-chitosan

3.3.1.1 Preparation of chitin

Chitin was prepared using the method adopted from Shimahara and Takigusshi (1988). Shrimp shells were cleaned and dried before grinding into chips. Demineralization was performed by immersing chips in 1 N HCl solution for 2 days at room temperature with occasional stirring. The demineralized product was neutralized by washing with deionized water and protein removal was performed in 4% w/w of NaOH solution by boiling at 80-90°C for 4 h. The deproteinized portion was washed with deionized water until neutral. Chitin obtained was dried at 60°C for 24 h.

3.3.1.2 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 twice at 10000 rpm for 10 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 sodium salt was obtained (Tokura *et al.*, 1983a).

3.3.1.3 Preparation of CM-chitosan

CM-chitin 3g was *N*-deacetylated by heating in 50% NaOH solution 30 mL containing sodium borohydride 0.015g and isopropanol 30 mL for 5 h at 60°C. Then the product was washed with MeOH and dried at room temperature to give CM-chitosan (Hirano *et al.*, 1992).

3.3.2 Characterization of Chitin

3.3.2.1 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:

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

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)

3.3.2.2 Viscosity-average molecular weight of chitin

The different concentrations (0.001, 0.002, 0.003, 0.004, and 0.005 g/100mL) of chitin solutions dissolved in 5% LiCl/*N,N*-dimethylacetamide 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 30°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, reduced viscosity, and inherent viscosity by the following equations:

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

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

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

$$\text{Inherent viscosity } (\eta_{inh}) = (\ln \eta_{rel})/C \qquad (3.5)$$

$$\text{Intrinsic viscosity } [\eta] = (\eta_{red})_{C\rightarrow 0} \qquad (3.6)$$

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

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 weight of chitin was determined based on Mark-Houwink equation:

$$[\eta] = KM^a \qquad (3.7)$$

where $[\eta]$ is the intrinsic viscosity and M is viscosity-average molecular weight. The *K* and *a* values according to Lee *et al.*, (1974) are 8.93×10^{-4} and 0.71, respectively.

3.3.3 Characterization of CM-chitin

3.3.3.1 Degree of substitution of CM-chitin

The degree of substitution was estimated by elemental analysis.

3.3.3.2 Viscosity-average molecular weight of CM-chitin

The different concentrations (0.01, 0.02, 0.03, 0.04, and 0.05 g/100mL) 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, reduced viscosity, and inherent viscosity by the following equations:

$$\text{Relative viscosity } (\eta_{rel}) = (t/t_s) \qquad (3.8)$$

$$\text{Specific viscosity } (\eta_{sp}) = (t/t_s)-1 \qquad (3.9)$$

$$\text{Reduced viscosity } (\eta_{red}) = \eta_{sp}/C \qquad (3.10)$$

$$\text{Inherent viscosity } (\eta_{inh}) = (\ln \eta_{rel})/C \qquad (3.11)$$

$$\text{Intrinsic viscosity } [\eta] = (\eta_{red})_{C \to 0} \qquad (3.12)$$

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

$$[\eta] = 7.92 \times 10^{-5} M^{1} \qquad (3.13)$$

where $[\eta]$ = Intrinsic viscosity

M = Viscosity-average molecular weight

3.3.4 Characterization of CM-chitosan

3.3.4.1 Degree of substitution of CM-chitosan

The degree of substitution was estimated by elemental analysis.

3.3.4.2 Viscosity-average molecular weight of CM-chitosan

The viscosity-average molecular weight of CM-chitosan was determined by using the same method as that described for CM-chitin in the section *3.3.3.2*.

3.3.5 Characterization of Sodium Alginate

The viscosity-average molecular weight of sodium alginate was determined using the same method as that described for CM-chitin in the section *3.3.3.2.* The K and a constants are 6.9×10^{-6} and 1.13, respectively (Yan *et al.*, 2000).

3.3.6 Fiber Spinning

3.3.6.1 Preparation of spinning solution

A. CM-chitosan/alginate blend fiber

12 g of sodium alginate powder and the different amounts (1 and 2 g) of CM-chitosan flakes were gradually added into different amounts (187 and 186 g) of water with vigorous stirring to make final CM-chitosan concentrations of 0.5% and 1.0% w/w. The mixture was aged for 1 day to ensure complete dissolution and blending. Next, the solution was filtered through a layer of cloth under an applied N_2 pressure of about 0.5 kg/cm^2 to remove insoluble material and then directly poured into the glass column of the spinning apparatus. After that, the solution was left to stand at room temperature for 2 days to remove any trapped air bubbles.

B. Chitosan-coated alginate fiber

12 g of sodium alginate powder was dissolved in 188 g of water with vigorous stirring. Next, small insoluble particles were removed by using a layer of cloth under an applied N_2 pressure of about 0.5 kg/cm^2 and then the solution was directly poured into the glass column of the spinning apparatus. After that, the solution was left to stand at room temperature for 3 days to remove any trapped air bubbles. 2 g of chitosan flakes were dissolved in 100 mL of 2% v/v aqueous acetic acid solution as reserved chitosan solution. Each 10, 30 and 50 mL of reserved chitosan solution and $CaCl_2$ 27 g were added into different amounts (890, 870 and 850 mL) of water to make final $CaCl_2$ concentration of 3% w/v and chitosan concentrations of 2.2×10^{-2}%, 6.7×10^{-2}% and 11.1×10^{-2}% w/v.

3.3.6.2 Spinning process

A. CM-chitosan/alginate blend fiber

A nitrogen pressure of about 0.5 kg/cm^2 was applied to push the spinning solution through a 30-hole (0.2-mm diameter) viscose-type spinneret into the first coagulation bath (100 cm in length, 1800 mL in volume) containing 5% w/v $CaCl_2$ in 50% v/v MeOH solution in which the spinneret was submerged. Following the coagulation, the coagulated filaments were subsequently passed through another 100-cm long bath containing methanol. Next, the filaments were brought forward to a set of two rollers and then to a winder. After winding up the filaments on a bobbin, the fibers were washed several times with methanol and dried in air at room temperature. After drying, the fibers were cut perpendicular to its alignment to remove from the spool, and stored in a sealed plastic bag.

B. Chitosan-coated alginate fiber

A nitrogen pressure of about 0.5 kg/cm^2 was applied to push the spinning solution through a 30-hole (0.2-mm diameter) viscose-type spinneret into the first coagulation bath (50 cm in length, 900 mL in volume) containing different concentrations (2.2 x 10^{-2}%, 6.7 x 10^{-2}%, and 11.1 x 10^{-2}% w/v) of chitosan solution in which the spinneret was submerged. Following the coagulation, the coagulated filaments were subsequently passed through another 100-cm long bath containing 50% v/v MeOH solution. Next, the filaments were brought forward to a set of two rollers and then to a winder. After winding up the filaments on a bobbin, the fibers were washed several times with methanol and dried in air at room temperature. After drying, the fibers were cut perpendicular to its alignment to remove from the spool, and stored in a sealed plastic bag.

The spinning apparatus is shown in Figure 3.1.

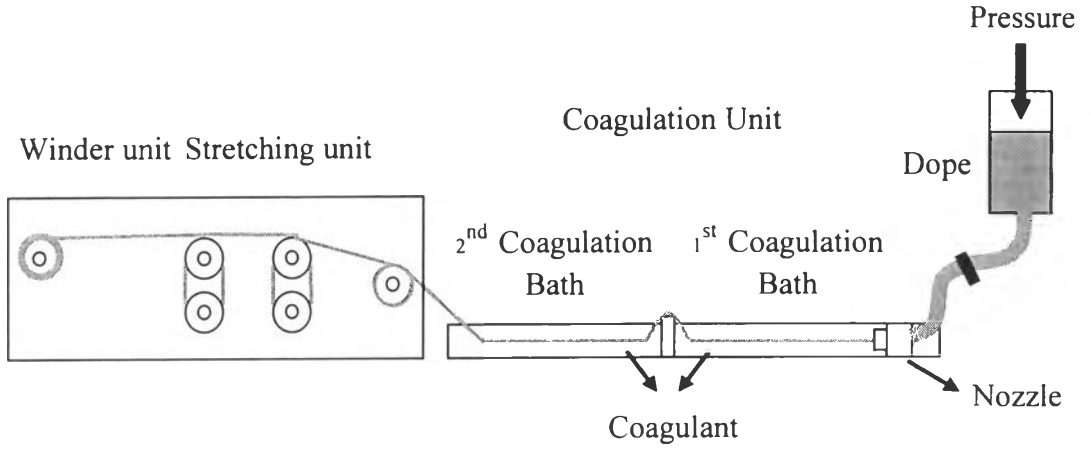

Figure 3.1 Spinning apparatus.

3.3.7 Fiber Analysis

3.3.7.1 Confirmation of chitosan coating

An amount of fiber of exactly known weight was dissolved in 30 mL of 2% v/v acetic acid solution with vigorous stirring for 1 day. After that, the mixture was filtered through a filter paper to remove any insoluble material. The filtered solution was heated at 50°C to concentrate the solution. After the solution cooled down to room temperature, it was diluted with 2% v/v acetic acid solution to make a final volume of 3 mL. 3 mL of 0.4% w/v ninhydrin solution was added into the 3-mL tested solution with soft stirring. Next, the solution was heated at 80°C until it boiled. After the solution cooled down to room temperature, it was analyzed for the presence of chitosan using UV/Visible spectrophotometer with a reference solution. The reference solution, heated at 80°C before analyzing, composed of 3 mL of 2% v/v acetic acid solution and 3 mL of 0.4% w/v ninhydrin solution.

3.3.7.2 Calcium content

An amount of fiber of exactly known weight was digested in 10 mL HNO_3 with heating until the fiber was dissolved completely. After

the solution cooled down to room temperature, it was diluted in a 50-mL volumetric flask with water together with the addition of 5 mL of 4% w/v aqueous potassium chloride. Next, the sample solution was filtered through a filter paper (Whatman no. 42) and kept in a plastic bottle at room temperature. The filtered solution was analyzed for the amount of Ca using AAS with a calibration curve created from 0.4, 1 and 2 ppm standard Ca solutions.

3.3.7.3 SEM micrographs

A small segment of fiber sample was attached to a stub with the help of a piece of adhesive tape and then sputter coated with gold. SEM micrographs were taken on the scanning electron microscope operating at 10 kV and a magnification of x 1500.

3.3.7.4 Linear density

A specimen of yarn was taken from the package, cut into 15-cm length and weighed using a digital electronic balance with five-decimal point. The linear density of the yarn was expressed in tex—the mass, in grams, of one kilometer of yarn. The value quoted for each sample was the average value of 20 specimens.

3.3.7.5 Antibacterial properties

Antibacterial properties of the fibers against *E. coli* and *S. aureus* were evaluated by visual clear zones. A representative bacteria colony was picked, placed in a nutrient broth (peptone 10 g, beef extract 3 g, NaCl 3 g in distilled water 1000 mL; pH 7.0) and incubated at 37°C for 24 h. Then the obtained fresh culture where bacteria cells grew luxuriantly was picked and placed in a nutrient agar (agar 15 g, peptone 10 g, beef extract 3 g, NaCl 3 g in distilled water 1000 mL; pH 7.0). After that, the fibers were placed in the nutrient agar containing the fresh culture and incubated at 37°C for 24 h (Liu *et al.*, 2001 and Li *et al.*, 2002).

3.3.7.6 Mechanical properties

The tensile strength and the elongation at break of fibers were measured in the form of yarn according to ISO 2062:1993(E). After the specimens of yarns were taken from the package and measured for their linear density as mentioned in the section *3 3.7.3*, they were brought to moisture equilibrium under the conditioned atmosphere overnight before testing. The atmosphere for preconditioning, conditioning, and testing were as specified in ISO 139:1973(E)—a relative humidity of 65 ± 2% and a temperature of 27 ± 2°C. The load cell used was 100 N. Gauge length was set at 50 mm with a rate of displacement of 50 mm/min. The value quoted for each sample was the average value of 20 specimens.