CHAPTER III
EXPERIMENTAL

3.1 Material

The shells of *Penaeus merguiensis* shrimps were kindly provided by Surapon Foods public Co., Ltd., Thailand. Sodium alginate was purchased from Carlo Erba Co., Ltd., in the form of white powder. Maleic anhydride (Fluka Co., Ltd., Switzerland) was analytical grade. Sodium hydroxide 50% w/w aqueous solution was kindly supplied by KTP Cooperation Co., Ltd., Thailand. Sodium hydroxide anhydrous pellets, sodium borohydride, monochloroacetic acid, glacial acetic acid 99.7% w/w, nitric acid 65% w/w and hydrochloric acid 37% w/w were analytical grade purchased from Carlo Erba Co., Ltd. Calcium chloride dehydrated stated as edible grade was supplied from Asia Drug & Chemical Co., Ltd., Thailand. Sodium chloride (Univar) was analytical grade purchased from Asia Pacific Specialty Chemical 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 chitosan powder was sieved by using Restch Sieving Machine type Vibro and chitosan with the size of 63 μm was collected for using in the experiment.

3.2.2 Capillary Viscometer

Cannon Ubbelodhe-type number 50 capillary viscometer was used for the determination of the viscosity-average molecular weight of alginate, chitosan and *O*-CM chitosan.

3.2.3 Fourier Transform Infrared (FTIR) Spectrophotometer

The FTIR spectra of chitosan, *O*-CM chitosan, *N*-(carboxyacyl) chitosan, *O*-CM chitosan/alginate blend films, and *N*-(carboxyacyl) chitosan/alginate blend films 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 1×10^{9} $cmHz^{1/2}w^{-1}$.

3.2.4 Elemental Analysis (EA)

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

3.2.5 Wide-angle X-ray analysis (WAXs)

WAXs experiments were performed for the fibers by X-ray diffractometor (Rigaku model). The X-rays source was Ni-filtered Cu Kα radiation (40 kV, 30 mA). Samples were scanned from 5 to 35° 2θ at a scanning rate of 4° 2θ/min.

3.2.6 Differential Scanning Calorimeter (DSC)

Differential scanning calorimetry was carried out using a thermal analyzer (Perkin Elmer DSC 7). The fiber was about 5 mg, then analyzed under nitrogen atmosphere from 30 to 380°C at the heat rate of 20 °C min^{-1}.

3.2.7 Scanning Electron Micrscope (SEM)

SEM micrographs of fibers were taken on a JEOL JSM-5200 scanning electron microscope operating at 10 kV and a magnification of ×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, Chitosan, *O*-CM chitosan, and *N*-(carboxyacyl) chitosan

3.3.1.1 *Preparation of Chitin*

Chitin can be prepared by demineralization and deproteinization to remove calcium carbonate and protein from shrimp shells. The shrimp shells are cleaned, dried under sunlight 2 days before grinding into small pieces and immersed in 1 N HCl solution for 2 days. Calcium carbonate will be changed into calcium chloride which can be dissolved in the water. After that decalcified product is washed with distilled water until neutral. Deproteinization is performed in 4% w/w of NaOH solution at 80-90 °C for 4 h and the deacetylated product is washed with distilled water until neutral. The product obtained is finally dried at 60 °C for 24 h.

3.3.1.2 *Preparation of Chitosan*

Chitosan will be obtained by deacetylation of chitin in 50% w/w NaOH solution containing 0.5% w/w sodium borohydride ($NaBH_4$) acting as a reducing agent to prevent depolymerization of chitosan. The ratio of chitin to NaOH solution is 1 g of chitin in 10 ml of NaOH solution. The deacetylation will be performed at 110 °C for 1 h. The product obtained is washed

with deionized water until neutral. The resulting chitosan is dried at 60 °C for 24 h. The process is repeated about four times to achieve chitosan with high degree of deacetylation.

3.3.1.3 *Preparation of O-CM chitosan*

Chitosan (10 g), sodium hydroxide (13.5 g) and solvent (100 ml) are added into a flask to swell and alkalize at a 50°C for 1 h. The temperature is maintained in a water bath. The monochloroacetic acid (15 g) is dissolved in isopropanol (20 ml) and added into the reaction mixture dropwise for 30 min and reacted for 4 h at 50°C, then stopped by adding 70% ethyl alcohol (200ml). The solid is filtered and rinsed in 70–90% ethyl alcohol to desalt, dewater, and vacuum dried at room temperature. The products will be Na salt CM-chitosans.

3.3.1.4 *Preparation of N-*(carboxyacyl) *chitosan*

A solution of chitosan (0.16 g) in 2% acetic acid (5 ml) was diluted with methanol (30 ml). To the solution was added the corresponding anhydride (3-5 mol/GlcN) dissolved in methanol (10 ml), and the mixture was allowed to stand at room temperature overnight, to afford gels or viscous solutions. The products were suspended in ethanol (150 ml) several times at room temperature, in order to extract excess of reagents. The products were collected by filtration or centrifugation, washed with ethanol and then ether, and dried at 80°C for 5 h, to afford precipitate (esters or acids). The products were treated in 0.1 M NaOH (25 ml) at room temperature overnight, ethanol (3 vol.) was added, and the precipitates were collected by centrifugation, washed with ethanol, and dried, to afford sodium salts.

3.3.2 Characterization of Chitosan

3.3.2.1 *Degree of Deacetylation of Chitosan*

The degree of deacetylation of chitosan is determined by the infrared spectroscopic measurement reported by Baxter *et al.*, (1992). The degree of deacetylation can be calculated from the following equation.

$$\%DD = 100 - 115\,(A_{1655}/A_{3450})$$

where

%DD = degree of deacetylation (%)

A_{1655} = absorbance at 1655 cm^{-1} (the C=O stretching)

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

3.3.2.2 *Viscosity-Average Molecular Weight of Chitosan*

Molecular weight of chitosan is determined by dilute solution viscosity measurement. Chitosan solitions of different concentrations in 0.2 M acetic acid/0.1 M sodium acetate are prepared. An Ubbelohde viscometer is filled with 10 ml of sample and left to equilibrate for 15-20minutes. Each of sample is measured at least 3 times. The corresponding equations are given by

Relative viscosity (η_{rel}) = t/t_s

Specific viscosity (η_{sp}) = $(t/t_s)-1$

Reduced viscosity (η_{red}) = η_{sp}/C

Inherent viscosity (η_{inh}) = $(\ln \eta_{rel})/C$

Intrinsic viscosity [η] = $(\eta_{sp}/C)_{c=o}$ = $(\ln \eta_{rel})/C_{c=o}$

where

t = flow time of the polymer solution

t_s = flow time of the solvent

c = concentration of the polymer solution in g/dl

The value of reduced viscosity and inherent viscosity were plotted against the concentration. The value of intrinsic viscosity was obtained from the intercept of graph plotted from the following equations.

$$\eta_{red} = \eta_{sp}/C = [\eta] + k' [\eta]^2 C$$

$$\eta_{inh} = (\ln \eta_{rel})/C = [\eta] - k'' [\eta]^2 C$$

where k' and k" are a constant values.

Molecular weight can be calculated by the Mark-Houwink equation

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

where

[η] is the intrinsic viscosity

M is Viscosity-average molecular weight

K and a are a constant values determined base on the degree of deacetylation

$$K = 1.64 * 10^{-30} * DD^{14.0}$$

$$a = (-1.02 * 10^{-2} * DD) + 1.82$$

3.3.3 Characterization of *O*-CM chitosan

3.3.3.1 *The Structure of O-CM chitosan*

Na salt CM-chitosan 1 g is suspended in 80% ethyl alcohol aqueous solution (100 ml), hydrochloric acid (100 ml, 37%) is added and stirred for

30 min. The solid is filtered and rinsed in 70–90% ethyl alcohol to neutral, vacuum dried. The products will be the H-form of CM chitosans. The structure of *O*-CM chitosan was characterized by FTIR and NMR.

3.3.3.2 *Degree of Substitution of O-CM chitosan*

The degree of substitution of *O*-CM chitosan was estimated by elemental analysis.

3.3.4 Characterization of *N*-(carboxyacyl) chitosan

3.3.4.1 *The Structure of N-(carboxyacyl) chitosan*

The structure of *N*-(carboxyacyl) chitosan was characterized by FTIR and NMR.

3.3.4.2 *Degree of Substitution of N-(carboxyacyl) chitosan*

The degree of substitution of *N*-(carboxyacyl) chitosan was estimated by elemental analysis.

3.3.5 Characterization of Sodium Alginate

3.3.5.1 *The Structure of Alginate*

The structure of alginate was characterized by FTIR.

3.3.5.2 *Viscosity-Average Molecular Weight of Sodium Alginate*

Viscosity-Average Molecular Weight of Sodium Alginate was determined by using the same method as that described for O-CM chitosan. The K and a constant 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. *For O-CM chitosan/Alginate Blend Fiber*

6% of sodium alginate powder and the different concentrations of O-CM chitosan powders (0.5%, 1.0%, and 1.5%) were gradually added into water with vigorous stirring. The mixture was aged for 1 day to ensure complete 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. *For N-(carboxyacyl) chitosan/Alginate Blend Fiber*

The spinning solution of *N*-(carboxyacyl) chitosan and alginate was determined by using the same method as that described for *O*-CM chitosan/alginate blend fiber except the concentration of *N*-(carboxyacyl) chitosan (0.25%, 0.50%, 0.75%).

3.3.6.2 *Spinning Process*

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) viscosity-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 bobbin, the fiber was washed several times with methanol and dried in air at room temperature. After drying, the fiber was cut perpendicular to its alignment to remove from the spool, and stored in a sealed plastic bag.

3.3.7 Fiber Analysis

3.3.7.1 *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 100-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 0.5, 1 and 2 ppm standard Ca solutions.

3.3.7.2 *Miscibility*

Miscibility was characterized by XRD and DSC by using the method as described in the 3.2.6 and 3.2.7

3.3.7.3 *SEM Micrographs*

A small segment of fiber sample was attached to 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 ×1500.

3.3.7.4 *Linear Density*

A specimen of yarn was taken from the package, cut into 15-cm length and weight using a digital electron 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 *Mechanical Properties*

The tenacity and 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 3.3.7.4, 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.

3.3.7.6 *Antimicrobial Property*

Antimicrobial activity of the blend fibers against *E.coli*, *P. aureginosa*, *S.aureus*, *S.mutans*, *S. cerevisiae* and *Candida albicans* was evaluated by parallel streak method to determine antibacterial activity of diffusable antimicrobial agents according to AATCC Test Method 147-1998 . The average width of clear zone of inhibition along a streak on either side can be calculated by the following equation

$$W = (T - D)/2$$

Where

W is a width of clear zone of inhibition.

T is the total diameter of test specimen and clear zone in mm.

D is the diameter of the test specimen in mm.