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 Chitosan (II)

Chitosan (II) was manufactured by Seafresh Chitosan (Lab) Company Limited with the molecular weight $4x10^4$ and the degree of deacetylation 90%.

3.1.3 Other Chemicals

Sodium hydroxide (NaOH) 50% w/w aqueous solution was kindly supplied by KPT Cooperation Co., Ltd., Thailand. Sodium acetate (CH_3COONa), sodium hydroxide anhydrous pellets (NaOH), potassium chloride (KCl), sodium borohydride ($NaBH_4$), monochloroacetic acid and hydrochloric acid (HCl) 37% w/w were analytical grade purchased from Carlo Erba Co., Ltd. Glacial acetic acid 99.7% w/w was analytical grade purchased from Labscan Asia Co., Ltd., Thailand.

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 chitin, chitosan, CM-chitin, and CM-chitosan were determined by using Cannon Ubbelohde-type viscometer number 75, 50, 50, and 50, respectively.

3.2.3 Digital Rheometer

The apparent viscosities of the solutions were measured using a Brookfield DV III digital rheometer with a small sample adapter and a # 21 spindle.

3.2.4 Elemental Analysis

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

3.2.5 FTIR Spectrophotometer

The FTIR spectrum of chitin, chitosan, CM-chitin, and CM-chitosan were recorded with a Bruker FTIR 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. A frequency of 4000-400 cm^{-1} was observed by using deuterated triglycinesulfate detector (DTGS) with specific detectivity of 1 x 10^9 $cm.Hz^{1/2}.W^{-1}$.

3.2.6 Nuclear Magnetic Resonance Spectrometry

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

3.2.7 Freeze Dryer

The porous scaffolds are obtained by using E-C Apparatus Modulyo Freeze Dryer

3.2.8 Scanning Electron Microscope (SEM)

SEM micrographs of scaffolds were taken on a JEOL JSM-5200 scanning electron microscope.

3.2.9 Universal Testing Machine

The mechanical properties of the films were measured by using Lloyd Instrument LRX series of Lloyd tensile tester with the maximum load of 500 N.

3.3 Methodology

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

3.3.1.1 Preparation of chitin

Chitin was prepared from shrimp shell by decalcification and deproteinization to remove calcium carbonate and protein, respectively. The shrimp shells were cleaned and dried under sunlight before grinding into small pieces. Shrimp shell 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 to pH paper. 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 chips were washed with deionized water until neutral to pH paper. The product obtained was dried at 60°C in a convective oven for 24 h.

3.3.1.2 Preparation of chitosan

Chitin was deacetylated by heating in 50% w/w NaOH solution containing 0.5% w/w sodium borohydride ($NaBH_4$) 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 in an autoclave at 110°C for 1 h. The deacetylated product obtained was thoroughly washed with deionized water until neutral to pH paper. The resulting chitosan (I) flakes was dried in an oven at 60°C for 24 h.

3.3.1.3 Preparation of CM-chitin

Alkaline chitin was prepared by suspending chitin power (4 g) in 42% NaOH solution (80 ml). After the suspension was allowed in desiccator for 30 min under reduced pressure, crush ice (160 g) was added and the mixture was mechanically stirred for 30 min in an ice bath to dissolve chitin. A viscous alkaline

chitin solution was obtained. For successful synthesis of CM-chitin, the concentration of NaOH solution should not less than 14 %. Monochloroacetic acid solution was prepared by dissolving in 14% NaOH solution in an ice bath and 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 to 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.

3.3.1.4 Preparation of CM-chitosan

CM-chitin was further N-deacetylated by refluxing in the solution of 40% NaOH and isopropanol (1:1) for 5 h at 60^0C to obtain CM-chitosan Na salt.

3.3.2 Characterization of Chitin, Chitosan, CM-chitin and CM-chitosan

3.3.2.1 Degree of deacetylation of chitin and chitosan

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 absorbencies of peak at wave number of 3450cm^{-1} (the hydroxyl band), 2878 cm^{-1} (the C-H stretching), 1655 cm^{-1} (the amide I 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:

$$\text{DD of chitin} = 98.03 - \left[34.68 \times \left(A_{1550} / A_{2878} \right) \right] \qquad (3.1)$$

$$\text{DD of chitosan} = 97.67 - \left[26.486 \times \left(A_{1655} / A_{3450} \right) \right] \quad (3.2)$$

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)

A_{1655} = Absorbance at 1655 cm^{-1} (the amide I band)

A_{3450} = Absorbance at 3450cm^{-1} (the hydroxyl band)

3.3.2.2 Degree of substitution of CM-chitin and CM-chitosan

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).

3.3.2.3 Viscosity-average molecular weight of chitin, chitosan, CM-chitin, and CM-chitosan

The different concentrations (0.01, 0.02, 0.03, 0.04 and 0.05 g/100 ml) of chitin, chitosan, CM-chitin, and CM-chitosan solutions dissolved in suitable solvents were prepared. Solvents of chitin, chitosan, CM-chitin, CM-chitosan are 5% LiCl/N,N-dimethylacetamide, 0.2 M acetic acid/0.1M sodium acetate, and 0.1 M NaCl (CM-chitin, CM-chitosan), respectively. All of samples were passed through filter papers before using. The Ubbelohde viscometer was filled with 10 ml of sample solution and then equilibrated in water bath at desired temperature. The sample solution was passed through the capillary once before the running time was measured. Each sample was measured five times. The running times of solvent and solutions were used to calculate the relative viscosity, specific viscosity, and reduced viscosity, by the following equations:

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

Specific viscosity (η_{sp}) $= (t/t_s)-1$ (3.4)

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

Intrinsic viscosity [η] $= (\eta_{sp})_{C\rightarrow 0}$ (3.6)

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

The viscosity-average molecular weight of chitin, chitosan, CM-chitin, and CM-chitosan was determined based on Mark-Houwink equation (3.7, 3.8, 3.9, 3.10). The K and a values were dependent on kind of solvent and measured temperature (Lee, 1974: Kaneko, 1982).

$[\eta] = 8.93\times10^{-4}M^{0.7}$ in 5 % LiCl/DMAc at $30^{O}C$ (3.7)

$[\eta] = 7.92\times10^{-5}M^{1}$ in 0.2MCH_3COOH/0.1M CH_3COONa at $25^{O}C$ (3.8)

$[\eta] = 7.92\times10^{-5}M^{1}$ in 0.1 M NaCl at $25^{O}C$ (3.9)

$[\eta] = 7.92\times10^{-5}M^{1}$ in 0.1 M NaCl at $25^{O}C$ (3.10)

where [η] = Intrinsic viscosity

M = Viscosity-average molecular weight

3.3.3 Preparation of Chitosan, CM-chitin, and CM-chitosan Solutions

Chitosan (I) and chitosan (II) solutions were prepared by dissolution of chitosan (I and II) in 0.2 M acetic acid to achieve the concentration of 2.5 and 6% w/w for chitosan (I) and 2.5% w/w for chitosan (II). CM-chitin and CM-chitosan were dissolved in distilled water to obtain the concentration of 2 and 3 % by weight, respectively. The solutions were stirred continuously until the clear solutions were obtained. For convenience, the scaffolds which prepared from the solutions of 6% chitosan (I), 2.5% chitosan (I), 2.5% chitosan (II), 2% CM-chitin, 3% CM-chitosan, and 2.5% CM-chitosan are defined as chitosan (I-H), chitosan (I-L), chitosan (II),

CM-chitin, CM-chitosan (H), and CM-chitosan (L), respectively as shown in Table 1. The reason which used different polymer concentration was the solubility of each polymer in each solvent. In this study, the solutions of chitosan, CM-chitin, and CM-chitosan were prepared to obtain as high concentration as possible while the complete dissolution could still achieved.

3.3.4 Preparation of Chitosan, CM-chitin, and CM-chitosan Sponge Pads

The aqueous solutions of chitosan (I and II), CM-chitin, and CM-chitosan solution were poured into moulds (5x7x0.2 cm^3). The moulds were placed into a refrigerator to freeze at -80°C. The frozen solutions in moulds were lyophilized to produce sponge like soft pads within a freeze-dryer for 24 hours. Afterwards, the samples were kept in a desiccator for further analyses and uses.

3.3.5 Crosslinking of Chitosan, CM-chitin, CM-chitosan Sponge Pads by Using Steam Technique

Sponge pads of chitosan, CM-chitin, CM-chitosan were exposed to saturated steam in autoclave at 110, 115, and 121°C for 15 minutes.

3.3.6 Characterization and Testing of Scaffolds

3.3.6.1 FT-IR analysis of scaffolds

The scaffolds were dipped into liquid nitrogen and ground to powder. About 3 mg of scaffold powder was mechanically mixed with 400 mg of potassium bromide powder to prepare a KBr disk. A frequency ranging from 4000-400cm^{-1} was observed. FT-IR spectra of scaffolds were obtained by using a Vector 3.0 Bruker Spectrophotometer.

3.3.6.2 Weight loss

The scaffolds were cut into square shape form with 2x2 cm^2 and 2.5 mm in thickness. A preweighted dry scaffold (W_i) was immersed in distilled water or 0.2 M (for chitosan) for 24 h. When the scaffold was removed from the water, excess water at the surface of the scaffold was blotted out with Kimwipes paper, kept it dry in an oven at 110°C for 24 h, and kept in a desiccator for 2 h prior

to weigh. The weight of the dried scaffold was measured (W_f) and the percentage of weight loss was calculated from the following equation (Kim *et al.*, 1996):

$$\text{Weight Loss}(\%) = \frac{(W_i - W_f)}{W_i} \times 100 \qquad (3.11)$$

where W_i and W_f denote the weight of initial and final weight scaffold, respectively.

3.3.6.3 Degree of swelling

The scaffolds were cut into square shape form with 2x2 cm^2 and 2.5 mm in thickness. The weights of the completely dried samples were measured (W_d), and the samples were immersed in water. At the time interval, the samples were wiped off excess surface water using Kimwipes paper and weighed (W_s). The procedure was repeated until there was no further weight change. Water content was determined by gravimetric method and calculated from the following equation (Wang *et al.*, 1997):

$$\text{Degree of swelling}(\%) = \frac{(W_s - W_d)}{W_s} \qquad (3.12)$$

where W_s and W_d denote the weight of swollen and dried weight scaffold, respectively.

3.3.6.4 SEM micrographs

The surface and cross-sectional morphology of non-steamed and steamed scaffolds prepared from chitosan (I-H), CM-chitin, and CM-chitosan (H) solutions were studied by SEM. SEM micrographs were taken on the scanning electron microscope operating at 1 kV. The pore sizes were determined by using "SEMAFORE" software. At least 50 pores were assessed from three samples. The values were expressed as the means±standard errors.

3.3.6.5 Mechanical properties

The tensile strength and elongation at break of chitosan, CM-chitin, and CM-chitosan scaffolds were measured by Llyod Tensile Tester using the gauge length of 20 mm and the extension rate of 100 mm/min at room temperature.

Test scaffold were cut in the dimension of 1.5 x 5 cm^2 and the thickness of the scaffolds were about 2.5 mm.