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 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 < 63 μm was collected for using in the experiment.

3.2.2 Capillary Viscometer

The viscosity-average molecular weight of chitosan, and CM-chitin were determined by using Cannon Ubbelohde-type viscometer number 50.

3.2.3 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.

3.2.4 FTIR Spectrophotometer

The FTIR spectrum of chitin, chitosan, CM-chitin, and CM-chitosan were recorded with a Thermo Nicolet Nexus 671 FT-IR Spectrophotometer, with 32 scans at a resolution of 4 cm^{-1}. The film 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 $cm.Hz^{1/2}.W^{-1}$.

3.2.5 Scanning Electron Microscope (SEM)

The microstructure of the as-prepared CM-chitin, CM-chitosan, and the resulting microwave-treated film products was examined on a JEOL JSM-5200 scanning electron microscope (SEM) at a magnification of 2000. The tilt angle of each sample was 30 deg.

3.2.6 Wide-angle X-ray Diffractometer (WAXD)

The WAXD used in this study was a D/MAX2000 series of Rigaku X-ray Diffractometer system. The X-ray source was Ni-filtered CuK-alpha radiation (40 kV/30 mA). Divergence slit and scattering slit at 1 degree together with 0.3 mm of receiving slit were set on the instrument. The dried films were attached on the sample holders and scanned from 5 to 50 degree 2θ at a speed 5 degree/min and 0.02 degree of scan step.

3.2.7 Differential Scanning Calorimeter (DSC)

A Mettler-Toledo DSC 822e/400 differential scanning calorimeter (DSC) was used to evaluate the thermal properties of the as-synthesized CM-chitin and CM-chitosan. About 3 mg of each sample was used and the measurements were carried out under N2 atmosphere at a heating rate of $10^{\circ}C \cdot min^{-1}$ from 50 to 550°C.

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-chitosan was prepared following the method of Liu *et al.* First, 10 g of chitosan powder, 13.5 g of sodium hydroxide, 20 ml of distilled water, 80 ml of isopropanol were mixed together at 50°C for 1 hour. Monochloroacetic acid solution, prepared by dissolving 15 g of monochloroacetic acid in 20 ml of isopropanol, was added into the reaction mixture dropwise over a period of 30 min. The mixture was allowed to react for another 4 hours. To stop the reaction, 70% ethanol was added into 200 ml of the mixture. The solid product was filtered and rinsed in 70 to 90% ethanol and later dried *in vacuo* at room temperature. Finally, CM-chitosan-Na salt was obtained. Prior to further use, 10 g of CM-chitosan was further purified by dissolving in 200 ml of distilled water. CM-chitosan solution was centrifuged at 10,000 rpm for 10 min to remove insoluble components and later dialized in distilled water. After being dialized in fresh distilled water for 4 days, the CM-chitosan solution was evaporated until its concentration was 2% w/w.

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

3.3.2.1 Degree of Deacetylation of Chitin and Chitosan

Degree of deacetylation (DD) of chitin and chitosan was determined by FTIR method (Baxter *et al.*, 1998, Miya *et al.*, 1980). An infrared spectrum was recorded in a range from 4000 to 400 cm^{-1}. The absorbencies of peak

at wavenumber 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.

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 Chitosan, and CM-chitin

The different concentrations (0.00625, 0.0125, 0.025, 0.05, and 0.10 g/100 ml) of chitosan, and CM-chitin solutions dissolved in suitable solvents were prepared. Solvents of chitosan, and CM-chitin are 0.2 M acetic acid/0.1M sodium acetate, and 0.1 M NaCl, 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:

Relative viscosity (η_{rel}) $= (t/t_s)$ (3.1)

Specific viscosity (η_{sp}) $= (t/_{ts})-1$ (3.2)

Reduced viscosity (η_{red}) $= \eta_{sp}/C$ (3.3)

Intrinsic viscosity [η] $= (\eta_{sp})_{C\rightarrow 0}$ (3.4)

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 chitosan, and CM-chitin was determined based on Mark-Houwink equation. The K and a values were dependent on kind of solvent and measured temperature (Wang et al., 1991; Kaneko, 1982).

$$[\eta] = 6.5 \times 10^{-5} M^{0.88} \text{ for chitosan} \quad (3.5)$$

$$[\eta] = 7.92 \times 10^{-5} M^{1} \text{ for CM-chitin} \quad (3.6)$$

where $[\eta]$ = Intrinsic viscosity

M = Viscosity-average molecular weight

3.3.3 Sample Preparation and Microwave Treatment

A weighed amount of 2% w/w CM-chitin or CM-chitosan solution was pour very slowly onto a polystyrene petri dish (diameter = 18 mm) and was let dried at 40°C. After 4 hours, either CM-chitin or CM-chitosan film was obtained. To prevent either CM-chitin or CM-chitosan film from dissolving during microwave treatment (viz. the microwave vessel contained distilled water), the polymer film was first sandwiched between cover glasses and later wrapped by a piece of polypropylene film. The sample was put into a reacting vessel of a CEM Mars5 laboratory microwave oven. The temperature of the reacting vessel was varied between 80 and 140°C and the reaction time was varied between 5 and 70 min.

3.3.4 Characterization of Microwave-Treated Films

3.3.4.1 Weight Loss and Degree of Swelling

To evaluate the efficiency of microwave treatment technique for crosslinking of CM-chitin and CM-chitosan, an exactly weighed amount of microwave treated films was suspended and shaked in distilled water for 48 h at room temperature. The %WL of a sample at a particular condition was calculated using the following equation (Zhao *et al.*, 2003):

$$\% \text{ WL} = \frac{W_b - W_d}{W_b} \times 100 \quad (3.7)$$

Where W_b is the weight of dried film before suspension in distilled water and W_d is the weight of the dried film after shaking.

The swelling of crosslinked CM-chitin and CM-chitosan was evaluated by a gravimetric method. The dried film was immersed in distilled water. After 48 h, the sample was quickly blotted with tissue paper to remove excess water on the surface and weighed in an electronic balance. The DS was calculated using the following equation:

$$DS = \frac{W_s - W_d}{W_d} \quad (3.8)$$

Where W_s is the weight of hydrogel in swollen state.

3.3.5 Cell Culture Studies

3.3.5.1 Preparation of Chitin, Chitosan, and Microwave-Treated CM-chitin and Microwave-Treated CM-chitosan Films for Cell Culture Studies

1% w/w chitin solution was prepared by dissolving chitin powder in formic acid by repeated freezing (at -20°C) and thawing method until the solution was clear. 1% w/w chitosan solution was prepared by dissolving chitosan powder in 0.2 M acetic acid. Both solutions were centrifuged at 10,000 rpm for 10 min to remove insoluble materials. The solutions were poured onto a polystyrene petri dish (diameter = 18 mm) and let dry at 40°C. After 4 hours, chitin and chitosan films were neutralized with 1 M NaOH solution, excessively washed with distilled water, and dried *in vacuo* at room temperature. Both CM-chitin and CM-chitosan films were prepared following the same procedure described in Section 2.4. The microwave treatment of these films was done at 120°C for 30 min, after which time the films were washed in distilled water at room temperature for 24 hours to remove insoluble materials and finally dried *in vacuo* at room temperature.

These films were later cut into specimens of a circular shape (diameter = 14 mm). Prior to related cell culture studies, all of the film samples were sterilized in 70% ethanol for 30 min, rinsed in sterile deionised water, and kept in a culture medium prior to further use.

3.3.5.2 Cells and Cell Culture

Two types of cells were used in this study: 1) mouse connective tissue, fibroblast-like L929 cells and 2) primary normal human gingival fibroblast cells (NHGF) (these cells were established from the explant cultures of gingival connective tissue that was excised from a patient undergoing an oral surgery). When the cells reached 80% confluence, they were serially subcultured. Only cells from the 3^{rd} to 6^{th} subcultured passage were used in the described experiments. Both cell types were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, USA), supplemented with 10% fetal bovine serum, 100 units·ml^{-1} penicillin, and 100 μg·ml^{-1} streptomycin. The cells were incubated at 37°C in a wet atmosphere containing 5% CO_2.

3.3.5.3 Cytotoxicity Tests

The direct evaluation of cytotoxicity of culture films was conducted in 24-well culture plates, using NHGF as reference cells. Cells were seeded within a metallic ring of 12 mm in diameter at 2.5 × 10_4 cells per well and incubated at 37°C. After 24 hours, the metallic ring was removed and the old culture medium was replaced by a serum-free medium (SFM). After 3 hours, the old SFM was replaced again and the culture cells were then covered with the culture films of 14 mm in diameter. To ensure a complete contact between the culture films and the cells, the films were pressed with a metallic ring. The number of living cells and the total amount of proteins produced were determined with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) and bicinchoninic acid (BCA) assays, respectively. Bare culture wells were used as controls. The measurements were conducted after 24 or 48 hours.

The indirect evaluation of cytotoxicity of the culture films was conducted in adaptation from the ISO10993-5 standard test method, using cell line L929 as reference cells. The culture films were submerged in fresh culture medium and were incubated for 24 hours. The extraction ratio was 10 $mg \cdot ml^{-1}$. After 24 hours, the fluid extract was subsequently diluted to obtain extraction medium with concentrations of 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, and 0.0156 $mg \cdot ml^{-1}$ (relative to the original extraction ratio). In the preparation of reference cells, the cells were seeded onto a 96-well plate at a density of 10_3 cells per well. After incubation for 48 hours, the culture medium was removed and the as-prepared extraction media were added to the culture wells (i.e. each sample solution was tested in 4 wells). The setup was incubated further for 24 hours, after which time the extraction media were removed and the cells were re-incubated for another 24 hours in fresh culture medium. The number of living cells was determined with MTT assay.

3.3.5.4 Cell Adhesion Tests

The assay for cell adhesion tests was modified from the method of Chatelet *et al.* Briefly, cell cultures were conducted in 24-well plates. NHGF cells were seeded on the culture films within a metallic ring at 5×10^4 cells per well. After the removal of the metallic ring and the culture medium, the seeded culture films were washed twice with a sterile phosphate-buffer saline (PBS) solution to eliminate free cells. The number of living cells was determined with MTT assay and the measurements were conducted after 1 or 2 hours.

3.3.5.5 MTT and BCA Assays

The3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay is based on the reduction of the yellow tetrazolium salt to purple formazan crystals by dehydrogenase enzymes secreted from the mitochondria of metabolically active cells. The amount of purple formazan crystals formed is proportional to the number of viable cells. First, the culture medium was aspirated and replaced with 250 (50) µl of MTT

solution at 0.5 (5) $mg \cdot ml^{-1}$ for a 24-well culture plates (96-well culture plate). Secondly, the plate was incubated for 1 hour (4 hour) at 37°C. The solution was then aspirated and 900 µl of DMSO containing 125 µl of glycine buffer (pH = 10) was added to dissolve the formazan crystals. Finally, after 10 min of rotary agitation, the absorbance of the DMSO solution at 540 nm was measured using a Thermospectronic Genesis10 UV-visible spectrophotometer.

In the BCA assay, the cells were solubilized in 100 µl of radioimmunoprecipitation (RIPA) buffer. Then, the solution was centrifuged at 4,000 rpm for 5 min to remove insoluble materials. 10 µl of the centrifuged solution was added into 1 ml of bicinchoninic acid (BCA) solution. The mixed BCA solution was then incubated. After 15 min, the absorbance of the mixed solution at 562 nm was measured using the Thermospectronic Genesis10 UV-visible spectrophotometer.