CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Shrimp Shells

The shrimp shells of *Metapenaeus intermedius* shrimp were kindly provided by Surapon Foods Public Co., Ltd., Thailand.

3.1.2 Squid Pens

The squid pens, *Loligo pealei*, were kindly provided from seafood markets.

3.1.3 Other Chemicals

Sodium hydroxide (NaOH), acetic acid (CH_3COOH) and sodium chloride (NaCl) were analytical grade purchased from Carlo Erba Co., Ltd., Thailand. Hydrochloric acid (HCl) and methanol (CH_3OH) were analytical grade purchased from Labscan Asia Co., Ltd., Thailand. Calcium chloride ($CaCl_2$) was analytical grade purchased from Univar Co., Ltd., Thailand. Formic acid (HCOOH) was analytical grade purchased from VWR. International Ltd (BHD). All of solvents were used without further purification.

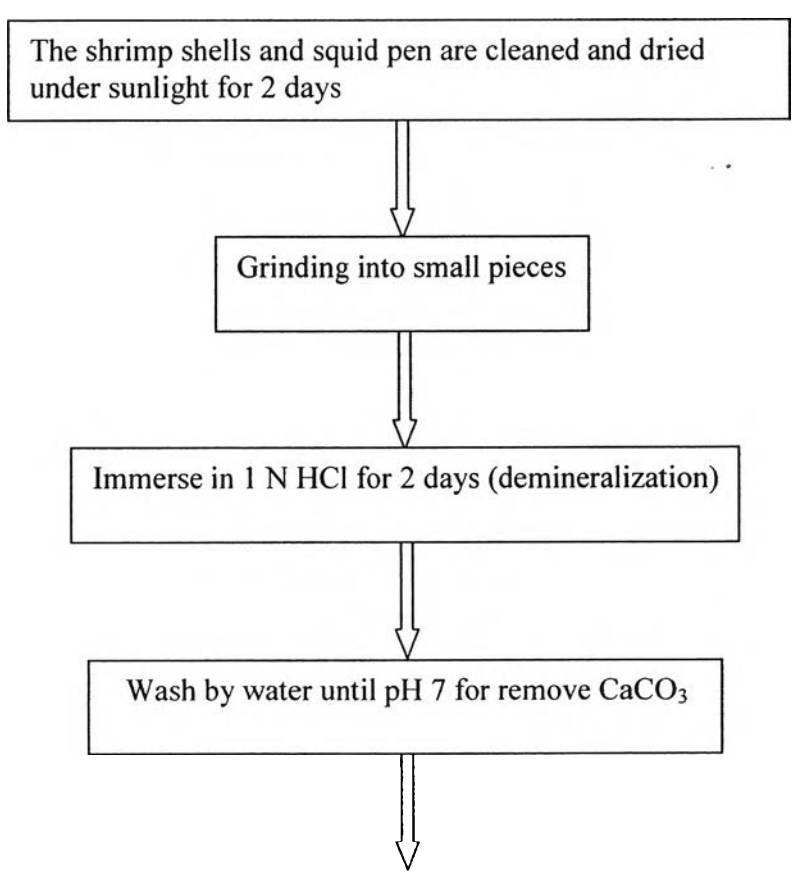
3.2 Equipment

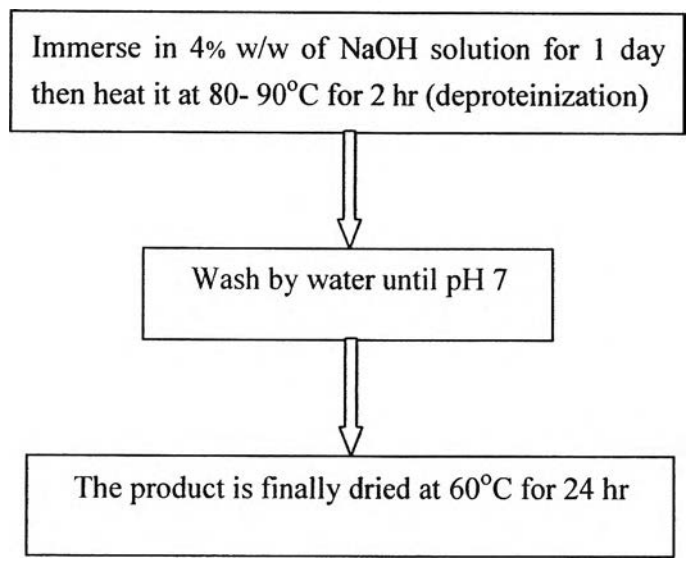
The chitin powder was sieved by using Restch Sieving Machine type Vibro and chitin with the size of 63μm was collected for using in this experiment. The α-chitin whisker morphology was observed by TEM JEOL JEM 1230 with an accelerating voltage of 100 kv. The β-chitin film and α-chitin whisker-reinforced β-chitin nanocomposite films were characterized for various properties. The tensile properties was examined by using A Lloyd model LRX universal testing machine with a 500 N load cell and cross-head speed was set at 50 mm min^{-1}. Thermal stability was evaluated

by A TG-DTA Perkin-Elmer Pyris Diamond instrument. A Thermo Nicolet Nexus 671 FT-IR spectrophotometer was used to determine the degree of deacetylation of β-chitin film and chitin nanocomposite films and to predict the interaction between β-chitin matrix and α-Chitin whisker. The surface morphology of films and the cells morphology were confirmed by a JEOL JSM- 5200 scanning electron microscope (SEM) with an acceleration voltage of 15 kv. A RIGAKU RINT 2000 as the mode of Wide Angel X-ray Diffraction (WAXD) was applied to detect the crystallinity of chitin and chitin nanocomposite films. In addition, the oxygen permeability rate and the oxygen permeation of chitin and chitin nanocomposite films were investigated by A GDP/E Brugger Munchen gas permeation tester.

3.3 Methodology

3.3.1 Preparation of Chitin





3.3.2 Characterization of Chitin

3.3.2.1 Degree of Deacetylation of Chitin

The degree of deacetylation (DD) of chitin is determined by FTIR by the infrared spectroscopic measurement by Khan, T.A. *et al.* (2002). The degree of degree of deacetylation can be calculated from the following equation.

$$\%\,DD = 100 - [(A_{1655}/A_{3450}) \times 100] / 1.33 \qquad (1)$$

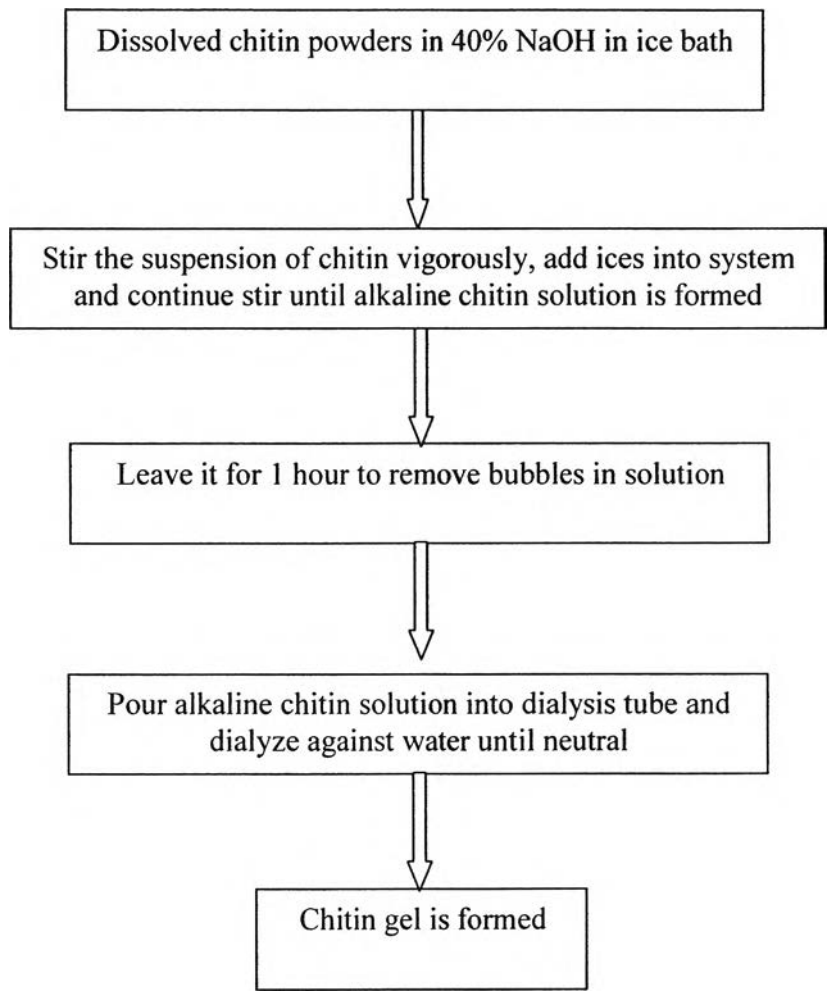
where

% DD = degree of deacetylation (%)

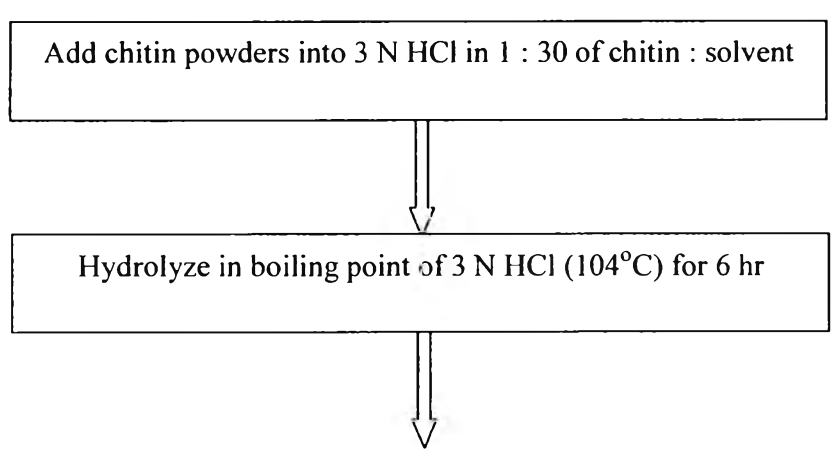
A_{1655} = absorbance at 1655 cm-1 (the C=O stretching)

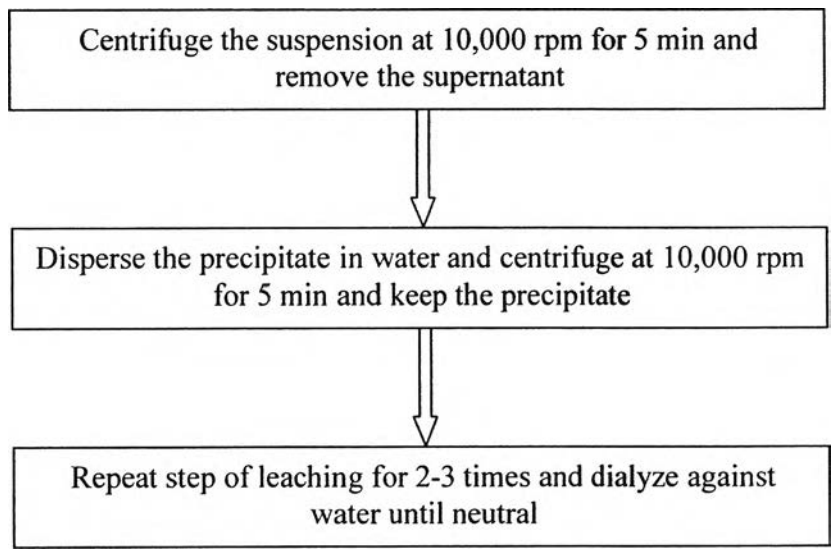
A_{3450} = absorbance at 3450 cm-1 (the O-H stretching)

3.3.3 Preparation of Chitin Gel



3.3.4 Preparation of Chitin Whisker

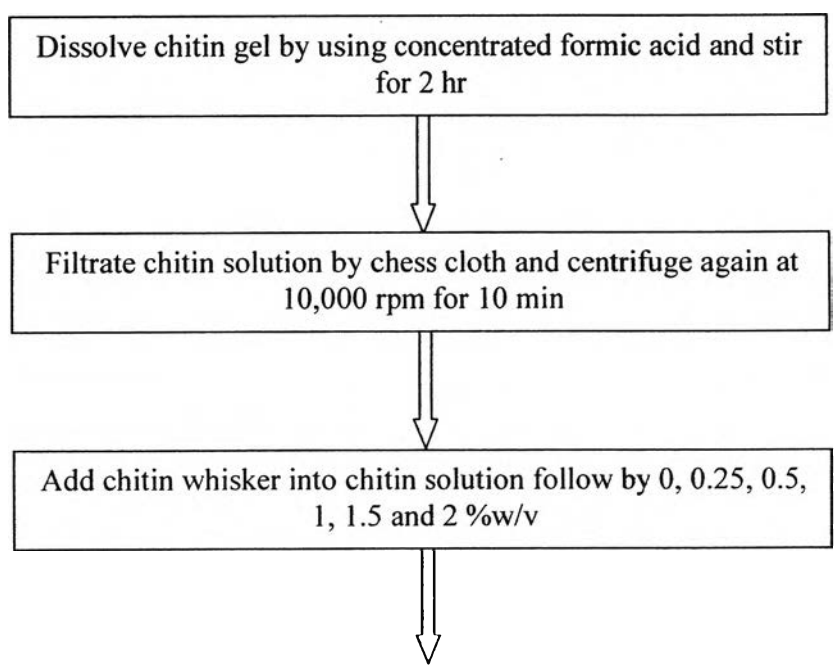


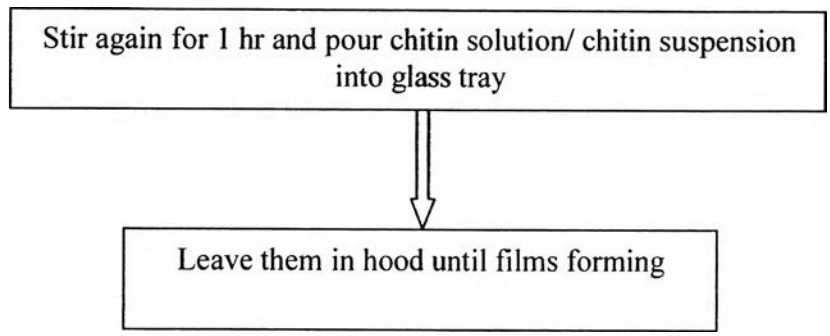


3.3.5 Characterization of α-Chitin Whisker

α-Chitin whisker was observed by using Transmission Electron Microscope on a JEOL JEM 1230 with an accelerating voltage of 100 kv. The diluted suspension was dropped onto a holey copper grid covered with a carbon film and then dried in vacuum oven. The sample was conducted on TEM without staining.

3.3.6 Preparation Chitin Film and Chitin Nanocomposite Films





3.3.7 Characterization of β-chitin Film and Chitin Nanocomposite Films

3.3.7.1 Tensile Properties of Chitin Nanocomposite Films

The pieces of films were cut into exact dimension (50x150 mm^2) following ASTM D882. The thickness of film was also determined by using thickness gauge meter. A Lloyd model LRX universal testing machine with a 500 N load cell at room temperature and cross-head speed was set at 50 mm min^{-1} was evaluated. The gauge length was 50 mm.

3.3.7.2 Thermal Stability

Thermogravimatric analysis (TGA) was carried out using A TG-DTA Perkin-Elmer Pyris Diamond instrument. Chitin film and chitin nanocomposite films were analyzed under nitrogen atmosphere from 50- 600°C at the heating rate of 10°C per min under flow rate of 20 mL/min of N_2. All of films were cut into small pieces and were put into Platinum pan about 5-10 mg.

3.3.7.3 Degree of Deacetylation of Films and the Interaction between α-Chitin Whisker and β-Chitin Matrix

The FTIR spectra of chitin powder, chitin film and chitin film nanocomposite were recorded with a Thermo Nicolet Nexus 671 FT-IR spectrophotometer, with 32 scans at a resolution of 4 cm^{-1}. The powder was mixed with KBr powder and formed into pellet, and then the chitin/KBr pellet was attached to the sample frames. The chitin film and nanocomposite films were produced in the thin films.

A frequency of 4000 to 400 cm^{-1} was observed by using deuterated triglycinesulfate detecter (DTGS) with a specific detectivity of 1x109 cm $Hz^{1/2}$ w^{-1}.

3.3.7.4 SEM Observation

Scanning electron microscope (SEM) observation was conducted on a JEOL JSM- 5200 with an acceleration voltage of 15 kv, magnification in the range of 150-3500x. The samples were coated with a thin layer of Au by an Ion Sputtering Device prior to SEM observation.

3.3.7.5 Water Adsorption Ability

The chitin and chitin nanocomposite films were cut into 10 mm diameter disk shapes. The known-weight samples were immersed in distilled water in an incubator at 37°C. For every hour for 7 hr, the samples were removed and placed on filter paper to remove the excess water, after which the sample was weighed. The water absorption of the samples at each time point was found by using Eq 2.

$$G = (W_t - W_o) / W_o \times 100 \quad (2)$$

where G is the percentage of swelling, W_t is the wet weight of films at each time point (g), W_o is the dry weight of films (g).

3.3.7.6 Gas Permeability

The flat samples were cut into 110 mm diameter disk shapes and were mounted in the diffusion chamber, with a transmission area of about 100 cm^2, of a GDP/E Brugger Munchen gas permeability tester. The procedure of the test was followed ASTM D1434 with oxygen gas. Tests were conducted at 25°C and all tests were performed in triplicate.

3.3.7.7 X-ray Diffraction

A RIGAKU RINT 2000, wide Angle x-ray diffraction mode (WAXD), was applied to detect the crystallinity of the chitin and the chitin nanocomposite films. The diffractometer was used to record the diffraction angel (2θ)

between 5 and 50°. Ni-filtered Cu Kα-radiation was used as an x-ray source and this system was operated at 40 kv and 30 mA.

3.3.7.8 In Vitro Biodegradability

The 10 mm diameter films were placed, at pH 7.4 in PBS with 1 mg/10 ml lysozyme, in an incubator at 37°C for 7 days. After the evaluation time, the films were removed from the lysozyme solution and then washed with distilled water three times, followed by drying in an oven at 60°C. The films were weighed after they were completely dried. The percentage of weight loss of the films was calculated according to equation Eq 3.

$$\text{Percentage of weigth loss} = (W_o - W_t) / W_o \times 100 \quad (3)$$

where W_o is the dry weight of films and W_t is the weight of the films after enzymatic hydrolysis at the evaluation time.

3.3.7.9 MTT Cytotoxicity Test

The films were sterilized using hot stream water at 102.5°C in an autoclave for 10 min. The film was checked for bacterial contamination by soaking it in a cell culture medium for 48 h. After that, the sample, at 1 piece per 1 ml of the medium, was further incubated at 37°C for 24 h. The cultured medium, with any leaching substance from the film was then diluted in the respective growth medium of the cells at a ratio of 1:2 giving 8 dilution ranges.

The MTT assay was carried out according to Plumb, J.A. *et al.* (1989) to confirm the viability of cells. L929 cells were seeded in a 96-well plate with 1000 cells/well and incubated for 24 h. The test samples were removed from the cell cultures and the cells were incubated for a further 24 h in fresh medium and then tested with MTT assay. 50 µl of MTT in PBS at 5 mg/ml was added to the medium in each well and the cells were incubated for 4 h. Medium and MTT were then aspirated from the wells, and formazan solubilized with 200 µl of DMSO and 25 µl of Sorensen's Glycine buffer, pH 10.5. The optical density was read with a plate reader at a

wavelength of 570 nm. Two controls were set up, one with fresh medium, and the second with medium incubated at 37°C for 24 h. A dose-response curve was derived from 8 concentrations in the test ranges using 4 wells per concentration. Within each experiment, 2-dose response curves were obtained. The results of toxic compounds are expressed as the concentration of sample required to kill 50% (IC_{50}) of the cells, compared to controls.

3.3.7.10 Cell Culture

The L929 cells (mouse connective tissue ECACC Cat. No. 85011425) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 2 mM L-glutamine. The L929 cells were incubated at 37°C in a fully humidified 5% CO_2 atmosphere. The cells were harvested by using 0.05% trypsin-EDTA and suspended in 50 µl of DMEM supplemented with 10% fetal bovine serum and 2 mM L-glutamine. The L929 cells were seeded in a plate containing the film with 1000 cells concentration per plate and were then incubated for 30 min. After that, fresh medium was added to the plate and further incubated at 37°C in a fully humidified 5% CO_2 atmosphere for 1 to 3 days. To reach the evaluation time, the L929 cultured film was fixed using a 2.5% glutaraldehyde solution for 2 h. Then it was rinsed with PBS and dehydrated the films with a series concentration of ethanol at 30, 50, 70, 90 and 100% for 2 min at each step. The dehydrated film was dried in a vacuum oven for 24 h. The dried film was cut and placed on the SEM stub and coated with a thin layer of Au prior to SEM observation.