CHAPTER III
EXPERIMENTAL

3.1 Materials and Chemicals

Shells of *Metapenaeus dobsoni* shrimps were kindly provided by Surapon Foods Public Co., Ltd. (Thailand). 50% (w/w) NaOH solution (Chemical enterprise, commercial grade), glacial acetic acid (CH_3COOH, J.T.Baker, analytical grade), Anhydrous sodium hydroxide (NaOH) pellets, hydrochloric acid (HCl), methanol (CH_3OH), ethanol (CH_3CH_2OH), and propanol ($CH_3CH_2CH_2OH$, Labscan analytical science, analytical grade) were used in this

3.2 Methodology

3.2.1 Preparation of chitosan from shrimp shells

To prepare chitin, shrimp shells were first dried under sunlight for a few days. Then, 1 kg of dried shrimp shells was immersed in 10 L of a 1 N HCl solution with occasional stirring at room temperature for 3 days. The acidic solution was changed daily. The demineralized shrimp shells were subsequently neutralized by deionized water and dried at 60 °C for 48 hr. The demineralized shrimp shells were further deproteinized in a 4 % w/v NaOH solution at a ratio of NaOH solution to shrimp shells of 10:1 with continuous stirring at 80 °C for 4 hr. The obtained chitin was filtrated, neutralized by distilled water, and dried at 60 °C for 24 hr.

3.2.2 Preparation of Chitin hydrogel

There are three steps for preparation of chitin hydrogel. Firstly, to prepare calcium chloride-saturated methanol, 850 g calcium chloride dihydrate was added into 1000 mL of methanol (85%w/v) and refluxed at 60°C for 30 min, followed by standing over night at room temperature and subsequent filtration. Secondly, to prepare chitin solution, 20 g of chitin powder was dissolved in cacluim chloride-saturated methanol by refluxing at 60°C for several hours until the complete dissolution of chitin was accomplished. Finally, to prepare chitin hydrogel, a large amount of distilled water was added into the chitin solution with vigorous stirring to

induce the precipitation of chitin hydrogel. Then, the suspension was centrifuged at 12000 rpm for 30 min at 4°C to collect chitin hydrogel. Chitin hydrogel was dialyzed against distilled water for 1 week to remove salt and kept in a refrigerator before use.

3.2.3 Deacetylation of chitin by using solution plasma

The deacetylation reaction of chitin hydrogel was carried out by varying concentrations of NaOH in 90% methanol solutions to be 1%, 5%, 10%, and 12% w/v in order to obtain 2%w/v chitin hydrogel suspension. The chitin hydrogel suspension was added into the plasma reactor and the plasma treatment was operated at the fixed frequency, voltage and pulse width of 12.5 kHz, 2.4 kV and 2 s, respectively. During the plasma treatment, the temperature of chitin hydrogel suspension was at around 50–70°C. The plasma treatment time was one hour for one times of deacetylation reaction. In order to get high value of degree of deacetylation, the deacetylation reaction in association with plasma treatment was performed repeatedly with the change of alkali solutions. The repeated deacetylation reactions with plasma treatment were done up to five times. The deacetylation reaction of chitin hydrogel by using plasma treatment was studied in comparison with the corresponding deacetylation reaction by the conventional heat treatment.

3.3 Characterization

3.2.1 Fourier transformed infrared spectroscopy (FTIR)

A Thermo Nicolet Nexus 671 FTIR) spectrophotometer was used to characterize the chemical structure and degrees of deacetylation of chitin, chitin hydrogel and chitosan. The degree of deacetylation of chitin and chitosan was calculated by following the method of Sannan *et al.* (1977) which estimated from the absorbance of amide II band at 1550 cm^{-1} and C-H band at 2878 cm^{-1} and Baxter *et al.* (1999), which estimated from the absorbance of amide I band at 1655 cm^{-1} and O-H band at 3450 cm^{-1} respectively.

3.2.2 Nuclear magnetic resonance (NMR)

Chemical structures and degrees of deacetylation of chitin, chitin hydrogel and chitosan were determined by Nuclear magnetic resonance (NMR). The

degree of deacetylation of chitin and chitosan was calculated by following the method of Lavertu *et al.* (2003)

3.2.3 Gel permeation chromatography (GPC)

Gel permeation chromatography (GPC) was used to observe any changes in an average molecular weight of plasma treated chitin hydrogel sample at different reaction times and NaOH concentration. The test plasma treated chitin hydrogel sample was filtered through a nylon 66 membrane with the pore size of 0.45 μm (Millipore, USA) before injection into the GPC instrument (Waters, Water 600E) equipped with an refractive index (RI) detector using an ultrahydrogel linear column (molecular weight resolving range of 1.0×10^3 Da to 2.0×10^7 Da). The eluent used in the GPC analysis was an acetate buffer at pH 4.0 (a mixture of 0.5 M CH_3COOH and 0.5 M CH_3COONa). The sample injection volume was 20 μL while the flow rate of the mobile phase was set constant at 0.5mL min^{-1}. The GPC analysis was done at the chitosan concentration of 2 mg/mL and at the temperature of 30 °C. Pullulans with the molecular weight in the range of 2.17×10^4 Da to 8.05×10^5 Da were used as standard samples.

3.2.4 X-ray diffractometer (XRD)

The crystalline structure of chitin hydrogel and plasma treated chitin hydrogel was characterized by an X-ray diffractometer (Bruker AXS, D8 advance) operated with the use of Cu Kα as an X-ray source. The WAXD analysis was carried out in a continuous mode with a scan speed of 1° min^{-1} covering the scanning angle (2θ) from 5° to 50°.

3.2.5 Antibacterial activity

Antibacterial activity of the obtained chitosan from plasma solution treatment was investigated by using the colony forming count method according to the following procedure. A colony of Escherichia coli, a gram negative bacterium, or Staphylococcus aureus, a gram positive bacterium, was put into 20 ml. of culture medium containing 0.5%w/v peptone and 0.3%w/v beef extract dissolved in distilled water,. The culture broth was incubated in a shaking incubator at 37 °C and 110 rpm for 24 hr. Then the cell dilution was performed by transferring 0.1 ml. of culture broth to 9.9 ml. of a fresh medium. The dilution process was performed until an appropriate amount of cell concentration was obtained. Next, plasma treated chitin

hydrogel was put into a culture broth containg an appropriate amount of bacteria, followed by incubating in a shaking incubator at 37 °C and 110 rpm for 24 hr. After incubation, 0.1 ml. of culture broth was spread on a nutrient agar plate containing 0.3%w/v beef extract, 0.5%w/v peptone and 1.5%w/v agar dissolved in distilled water, followed by incubating at 37 °C for 24 hr. The colony-forming unit (CFU) was calculated and the average was taken of the three plates. The antibacterial activity against either *S. aureus* or *E. coli* of the obtained chitosan from plasma solution treatment was determined from the percent reduction in the number of viable bacterial cells