



CHAPTER III EXPERIMENTAL

3.1 Materials

3.1.1 Materials and Chemicals

Shells of *Metapenaeus dobsoni* shrimp were supported from Surapon Food Co. Ltd. Analytical grade hydrochloric acid 37 %w/w and analytical grade anhydrous sodium hydroxide pellets were purchased from Labscan. Analytical grade glacial acetic acid (J.T.Baker), commercial grade acetone, analytical grade monochloroacetic acid (Fluka), analytical grade ethanol (Labscan), glyoxal 40% in H₂O (Sigma-Aldrich), molecular biology grade Tris-HCl (Scharlau Chemie S.A.), Lysozyme from chicken egg white (Fluka), Methylene blue C.I. 52015 (UNILAB) and Methyl orange C.I. 13025 (LABCHEM) were used. The chemical structures of the dyes are illustrated in Figure 3.1.

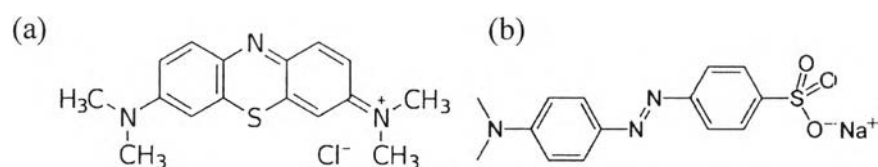


Figure 3.1 The chemical structures of (a) methylene Blue (cationic dye) and (b) Methyl Orange (anionic dye).

3.2 Methodology

3.2.1 Preparation of Chitin

Shrimp shells were first cleaned and dried under sunlight and then immersed in 1N HCl solution for 2 days with stirring to remove calcium carbonate. Hence, the samples were washed with distilled water until pH became neutral. The decalcified shells were later boiled in NaOH solution at 80 °C for 4 hours under

continuously stirring. Deproteinized shells were washed until pH became neutral again and dried in convection oven at 60 °C.

3.2.2 Preparation of CM-Chitin

Twenty gram of chitin powder was suspended in 400 g of 42%w/w NaOH solution. The suspension was placed in vacuum oven for 30 min to reduce pressure in the sample. Then, 640 gram of crushed ice was added to the suspension under vigorous stirring at 0-5 °C for 30 min. A pre-cooled acid solution, containing 108 g of monochloroacetic acid in 280 g of 14%w/w NaOH solution was slowly dropped into the mixture under stirring. The reaction temperature was kept at 0-5 °C for 30 min. The solution was settled at room temperature overnight and was then neutralized by glacial acetic acid. Afterwards, the sample was transferred to dialysis bags and placed in running water for 2 days, followed by distilled water for 1 day. The dialysate was centrifuged at 10,000 rpm for 10 min to remove insoluble material. The CM-chitin solid was obtained from the supernatant by adding it drop-wise into cool acetone and then centrifugation to separate the precipitate. Then, the product was washed with ethanol and dried at 50 °C.

3.2.3 Preparation of Chitin Whisker Suspension

Chitin whisker suspension was prepared by acid hydrolysis method (Nair and Dufrense, 2003; Wattanapanit, 2008). Chitin flakes were hydrolyzed with 3N HCl, which the ratio of chitin: HCl was 1g: 30mL, under continuously stirring at 104°C for 6 hours. The suspension was later diluted with distilled water, followed by centrifugation at 10,000 rpm for 10 minutes and repeated for two times. Afterward, the suspension was dialyzed in distilled water until neutral. The suspension was stored in refrigerator before used.

3.2.4 Preparation of CM-Chitin/Chitin Whiskers Bionanocomposite Films

CM-chitin solution was prepared at concentration of 2 %wt. Glyoxal solution, the crosslinking agent, was diluted from 40 %w/w to 1 g/L and then mixed with the CM-chitin solution at various pH to find the suitable condition for crosslinking. The mixture was transferred to the polystyrene petri dish (diameter: 25 mm) and dried at 40 °C to obtain CM-chitin films. Moreover, the glyoxal concentration in the solution was varied from 0.25-2.0 mM based on total volume.

Hence, CM-chitin/CTW composite films were prepared by mixing CM-chitin solution with chitin whisker (CTW) suspension by varying weight ratio of CM-chitin:CTW as following: 90:10, 80:20, 70:30, 60:40, 50:50 and 40:60 under stirring until homogeneous solution was obtained. Afterwards, the composite solution was crosslinked with glyoxal by the same method as CM-chitin film.

3.2.5 Preparation of Dyes Stocks

The dyes used in this study were Methylene blue, a cationic dye and Methyl Orange, an anionic dye. The dye stock solutions were prepared by dissolving dyes in the solvents to the concentration of 500 ppm. The experimental solvents were the mixture of distilled water and ethanol having volume ratio as following: 100:0, 80:20, 50:50 and 20:80. The known concentrations of the dyes were dilute from the dyes stocks to make the calibration curve. The absorbance of each solution was measured at the maximum wavelength of each solvent. Consequently, the dye stock was diluted to 25 ppm as an initial concentration.

3.3 Characterization

3.3.1 Fourier Transform Infrared Spectroscopy (FTIR)

Chemical structures of chitin, CM-chitin and crosslinked CM-chitin were confirmed by FTIR. The characterization was performed with Thermo Nicolet Nexus 670 spectrophotometer using total 64 scans at a resolution of 4 cm^{-1} for each sample. A frequency range of $4000\text{-}400\text{ cm}^{-1}$ was observed. Degree of deacetylation of chitin was determined following the method of Sannan *et al.* (1977) which estimated from the absorbance ratio of amide II band at 1550 cm^{-1} and C-H band at 2878 cm^{-1} as following equation:

$$DD = 101 - 35.71(\text{Abs}_{1550}/\text{Abs}_{2878}) \quad (1)$$

3.3.2 Elemental Analysis (EA)

The degree of substitution of the synthesized CM-chitin was estimated from the relationship between weight ratio of carbon to nitrogen and DD (Huang *et al.*, 2010) as following equation:

$$DS = DD + 0.5833 \times C/N - 4 \quad (2)$$

The percentage of carbon and nitrogen contained in CM-chitin were characterized by elemental analysis using a Perkin Elmer PE2400 Series II CHNS/O analyzer at combustion and reduction temperatures of 950 and 500 °C, respectively. Each sample was analyzed in air (60 psi), using oxygen as a combustion gas (15 psi) and helium as a carrier gas (20 psi).

3.3.3 Viscosity-Average Molecular Weight Measurement

The viscosity of CM-chitin solution was measured by an Ubbelohde type viscometer at 25 °C followed the method of Tokura *et al.* (1983). An aqueous solution of CM-chitin was dialysed against 0.1M NaCl solution at room temperature. The concentration of CM-chitin was diluted to be 0.05, 0.1, 0.15, 0.2 and 0.3 %w/v. The reduced viscosity was plotted against the concentration and the intrinsic viscosity was obtained from the intercept.

$$\text{Relative viscosity } (\eta_{rel}) = t/t_s \quad (3)$$

$$\text{Specific viscosity } (\eta_{sp}) = (t/t_s) - 1 \quad (4)$$

$$\text{Reduced viscosity } (\eta_{red}) = (\eta_{sp})/C \quad (5)$$

$$\text{Intrinsic viscosity } [\eta] = (\eta_{red})_c \quad (6)$$

where t is the running time of CM-chitin, t_s is the running time of the solvent and C is the concentration in %w/v.

The viscosity-average molecular weight was determined based on Mark-Houwink equation following:

$$[\eta] = KM^\alpha \quad (7)$$

where $[\eta]$ is the intrinsic viscosity and M is the viscosity-average molecular weight. According to the viscosity equation proposed by Kaneko *et al.* (1982), K is 7.92×10^{-5} and $\alpha = 1.00$.

3.3.4 Scanning Electron Microscopy (SEM)

For the morphological study, samples were placed onto stubs, coated with gold using a JEOL JFC-1100 sputtering device, and observed for their microscopic morphology using JEOL JSM-5200 scanning electron microscopy (SEM). In addition, the cross-sectional micrographs of the composite films were investigated after breaking films in liquid nitrogen.

3.3.5 Transmission Electron Microscopy (TEM)

The transmission electron microscope observations were observed by JEOL model JEM 2100 operating voltage of 200 kV. The samples were prepared by air-drying the particles onto a carbon-coated copper grid and air-dried.

3.4 Water Absorption Properties

The CM-chitin/CTW composite films were immersed in bottles containing 5 mL of distilled water for 24 hours and later taken out from the bottles. The excess water from each sample was absorbed by tissue papers. The dimension stability of samples in wet state was also evaluated in term of degree of swelling and weight loss. The degree of swelling was evaluated according to the following equation:

$$\text{Degree of swelling (\%)} = \frac{W_s - W_d}{W_d} \times 100 \quad (8)$$

where W_d is the weight of dried sample and W_s is the weight of the sample in the swollen state.

The samples were then dried in convection oven and measured the weight loss after immersion in water. The weight loss was determined according to the following equation:

$$\text{Weight loss (\%)} = \frac{W_d - W_i}{W_d} \times 100 \quad (9)$$

where W_i is the weight of dried sample after immersion in water for 24 hours.

3.5 Dye Adsorption and Desorption Characteristics

3.5.1 Dye Adsorption Characteristics

In order to investigate the adsorption and desorption characteristics of the composite films, methylene blue and methyl orange were used as the cationic and anionic model compounds. The composite films were placed in the glass bottles containing 40 ml of dye solutions in various solvents at room temperature. At various time, 100 μ L of dye solution was taken out and transferred to the well plate. The absorbance of the sample was measured by the UV-VIS Spectrophotometer (Tecan, Infinite M200). The λ_{max} for methylene blue in the mixture of distilled water and ethanol having volume ratio as following: 100:0, 80:20, 50:50 and 20:80 were 668, 664, 662 and 660 nm, respectively. Moreover, the λ_{max} for methylene blue in the mixture of distilled water and ethanol having volume ratio as following: 100:0, 80:20, 50:50 and 20:80 were 466, 468, 448 and 430 nm, respectively. The accumulative adsorption of the films was calculated from following equation (Jeon *et al.*, 2008):

$$\text{Accumulative adsorption} = \frac{C_0 - C_t}{C_0} \times 100 \quad (10)$$

where C_0 (ppm) and C_t (ppm) are the initial concentration of dye and the concentration of dye at time t , respectively. The amounts of dyes sorbed by the films were calculated from following equation (Gong *et al.*, 2005):

$$q_s = \frac{(C_0 - C_t)V}{W} \quad (11)$$

where q_s (mg/g) is the amount of dye sorbed by the film, V (l) is the initial volume of dye solution and W (g) is the weight of the film. Note that ppm is equal to mg/l.

In addition, the effect of blend compositions to the adsorption characteristic was also evaluated from the CM-chitin/CTW composite films at the weight ratio of 90:10, 80:20 and 70:30.

3.5.2 Dye Desorption Characteristics

The films containing the dye were placed in 40 ml of 0.01M 2-Amino-2-hydroxymethyl-1,3-propanediol hydrochloride or Tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) solution at pH 7.4 with shaking rate 100 rpm at 37°C, and compared condition with and without 0.001 %w/v lysozyme. The buffer solution was taken out at the various time and transferred to the well plate and the absorbance of the sample was later measured. In case of the presence of lysozyme, the mixture was centrifuged at 10000 rpm for 5 min before transferring to the well plate. The concentration of dye release was determined in compared with the standard curve. Furthermore, the effect of ionic strength to the desorption characteristics was estimated by varying the concentration of NaCl in buffer solution as 0.01, 0.1 and 0.5 M. The amounts of dyes desorbed from the films were calculated from the equation modified from Gong *et al.* (2005) as following:

$$q_d = \frac{(C_{tb})V_b}{W} \quad (12)$$

where q_d (mg/g) is the amount of dye desorbed from the film, C_{td} (ppm) are the concentration of dye in buffer solution time t , V_b (l) is the volume of buffer solution and W (g) is the weight of the film. Note that ppm is equal to mg/l.

Then, the percentage of dye desorption was determined from following equation:

$$\text{Dye desorption (\%)} = \frac{\text{total dye desorption capacity}}{\text{total dye adsorption capacity}} \times 100 = \frac{q_d}{q_s} \times 100 \quad (13)$$