CHAPTER III
EXPERIMENTAL

3.1 Materials

Shrimp shell was kindly supplied by Surapol Food Co., Ltd. Sodium hydroxide solution 50% (w/w) was kindly supplied by KPT Coorporation, Thailand. Hexanoyl chloride and sodium borohydride ($NaBH_4$) were purchased from Fluka. Lithium chloride (LiCl), dimethylacetamide, acetic acid (CH_3COOH), sodium acetate (CH_3COONa), pyridine, chloroform, dichloromethane, tetrahydrofuran, cyclohexane, and methanol were purchased from Labscan Co., Ltd (Thailand). Pyridine and chloroform were distilled and dried over molecular sieve prior to use. The other chemicals were analytical grade and were used without further purification. PLA was supplied as courtesy from Daiseru Chemicals, Japan. The viscosity-average molecular weight $\bar{M}_v$ of PLA was determined, based on viscosity measurements at 25°C in chloroform following the Mark-Houwink equation [17]: $[\eta] = 7.4 \times 10^{-5} \cdot \bar{M}_v^{0.87}$, to be ca. 70,000 g·mol^{-1}.

3.2 Experimental

3.2.1 Sample Preparation

3.2.1.1 Chitin Preparation

Chitin was prepared by acid and alkali treatment. Briefly, shrimp shells were cleaned and dried before grinding into smaller pieces. Deminerization was performed by immersing shrimp shells in 1 N HCl solution for 2 day with occasional stirring. The demineralized product was neutralized by washing with deionizing water and protein removal was performed in 4% (w/w) of NaOH solution by boiling at 80-90°C for 4 h. The deproteinized portion was washed with deionized water until neutral. Chitin obtained was dried at 60°C for 24 h.

3.2.1.2 Chitosan Preparation

Chitosan was obtained from deacetylation of α-chitin by using α-chitin flasks in 50% (w/w) NaOH solution. $NaBH_4$ 0.5% (w/w) was added based on the weight of chitin to prevent depolymerization. The mixture was heated in an autoclave at 110°C for 1 h. The deacetylated product was washed thoroughly with deionized water until neutral. The resulting chitosan flakes were dried in an oven at 60°C for 24 h. Chitosan powder was sieved using Restch Seived Machine type Vibro and the portion with the size of 70-75 μm was collected.

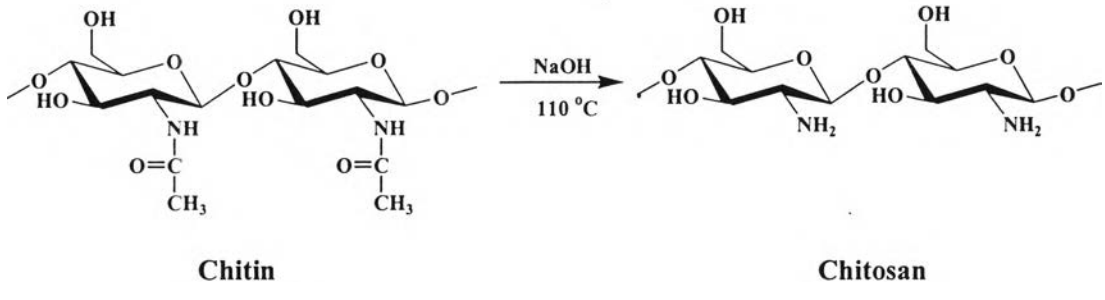

Figure 3.1 Deacetylation reaction of chitin.

3.2.1.3 H-chitosan Preparation

Chitosan (3.20 g, 19.13 mmol) was soaked in pyridine for one week and filtered off before further soaking in a mixture of pyridine (90 ml) and chloroform (45 ml) for one day. The mixture was cooled to -10°C in an ice-salt bath, and hexanoyl chloride (21.18 g, 160.67 mmol) dissolved in chloroform (15 ml) was added dropwise in 2 h. The mixture was then stirred for 2 h at room temperature and further refluxed for 6 h. at 98°C. A heterogeneous aggregation of the product was observed in the mixture. The resultant mixture was poured into methanol (300 ml), and the precipitated product was filtered off. The product was dissolved again in chloroform, then precipitated by pouring into methanol, filtered off, extracted in a Soxhlet extractor with methanol for 8 h, and dried in vacuum oven at 40°C for 24 h. The sticky yellowish product was obtained.

The dry acylated derivatives of chitosan, fresh pyridine, and chloroform were placed in a flask in the amount described above. This procedure

was repeated several times in order to vary the degree of substitution of resultant hexanoyl chitosan (Zong *et al.*, 2000).

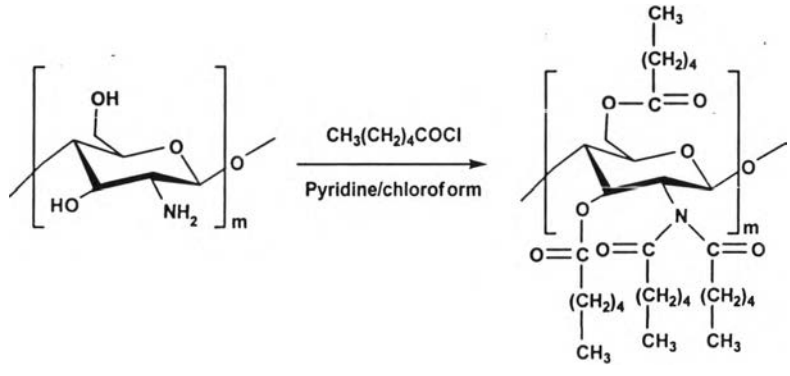

Figure 3.2 A synthesis route for fully substituted H-chitosan.

3.2.2 Characterization Techniques of Chitin and Chitosan

3.2.2.1 Degree of Deacetylation of Chitin and Chitosan

The degree of deacetylation of chitin and chitosan were determined based on infrared spectroscopic measurement. Absorbance of peaks at wave number of 3450 cm^{-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 based line method. The degree of deacetylation was calculated from the following equation (Sabnis and Block, 1997):

$$DD = 98.03 - [34.68 \times (A_{1550}/A_{2878})] \text{ for chitin}$$

$$DD = 97.67 - [26.486 \times (A_{1655}/A_{3450})] \text{ for chitosan}$$

where DD = degree of deacetylation (%)

A_{1550} = absorbance at 1550 cm^{-1} (the amide II band)

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

A_{2878} = absorbance at 2878 cm^{-1} (the C-H stretching) and

A_{3450} = absorbance at 3550 cm^{-1} (the hydroxyl band).

3.2.2.2 Viscosity-Average Molecular Weight of Chitin and Chitosan

The different concentrations (0.01, 0.02, 0.03, 0.04 and 0.05 g/100ml) of chitin and chitosan solution dissolved in suitable solvents were prepared. Solvents of chitin and chitosan are 5% LiCl in dimethylacetamide and 0.2 M

CH_3COOH/0.1 M CH_3COONa, respectively. 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 polymer solutions were used to calculate the relative viscosity, specific viscosity, and reduced viscosity.

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

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

Reduced viscosity (η_{red}) = η_{sp}/C

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

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

The plot of reduced viscosity (η_{sp}/C) and [$\ln(\eta_{rel})/C$] versus concentration of chitin and its derivatives showed that the extrapolated value of each line reached the same position and this value was referred to intrinsic viscosity of chitin and its derivatives.

The viscosity-average molecular weight of chitin and chitosan was determined based on Mark-Houwink equation. The "K" and "a" values were depended on the type of solvent and measured temperature (Lee, 1974; Kaneko, 1982).

$[\eta] = 8.93 \times 10^{-4} M^{0.71}$ in 5% LiCl/dimethylacetamide at 30°C

$[\eta] = 6.95 \times 10^{-5} M^{0.88}$ in 0.2 M CH_3COOH/0.1 M CH_3COONa at 30°C

where [η] is intrinsic viscosity and "M" is viscosity-average molecular weight.

3.2.3 Characterization Techniques of H-Chitosan Preparation

FTIR spectroscopic analysis was conducted using Bruker Instrument (EQUINOX55) with a resolution of 4 cm^{-1}. The solid samples were prepared by mixing 1% of sample with dried KBr, while the liquid samples were analyzed using Zn-Se window cell and the film samples were prepared with the thickness of 10-20 μm and attached to the sample holder.

^{1}H-NMR spectrum was recorded by using FT-NMR 500 MHz. spectrometer (JEOL, JNM-A500). Hexanoyl chitosan was dissolved in $CDCl_3$ and used tetramethylsilane (TMS) as reference for chemical shift measurement.

Elemental analysis results were obtained from CHNS/O analyzer (Perkin Elmer PE2400 Series II: option CHN) with combustion temperature 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 He as a carrier gas (flow rate of 200 ml/min).

Thermal properties were analyzed by thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) mode. TGA thermograms were performed using Perkin Elmer: TGA7 analyzer while DSC thermograms were conducted on Mettlor DSC 822e/400 analyzer at a heating rate of 10°C/min under nitrogen atmosphere. Aluminum pans were used for DSC analysis with sample size of 5 – 10 mg, while platinum pan was used for TGA analysis with sample size of 10 – 20 mg.

Crystallinity of products was characterized using Rigaku X-ray diffractometer at scanning speed of 5 degree/sec using CuKα as a source and CuKβ as a filter. The working ranges were over the 2θ range of 2 to 40°.

3.2.4 Solution Properties of H-chitosan

3.2.4.1 Intrinsic viscosity measurement

H-chitosan solutions of various concentrations (0.1-0.5 g/dl) in different organic solvents were prepared. Each solution was passed through a filter (0.45 μm in pore size and 13 mm in diameter, PTFE filter) to remove insoluble materials. The capillary viscometer (Cannon-Fenske, No 50) was filled with 5 ml of polymer solution and thermally equilibrated in a water bath to maintain a fixed temperature at 30±0.1°C. The sample was passed through the capillary once before the running time was measured. For each sample, viscosity was measured 3 times.

3.2.4.2 Light scattering measurement

H-chitosan solutions of various concentrations (0.1-0.5 g/dl) in different organic solvents were prepared. Dynamic light scattering measurement was carried out at the temperature of 30°C, using a Malvern dynamic light scattering

spectrometer with a PCS7 stepper motor controller, a PCS8 temperature controller, and a photo multiplier.

3.2.4.3 Surface tension measurement

Surface tension of polymer solutions with difference solvents was measured by using a Kruss Drop Shape analyzer (Kruss Co., Germany) at 30°C.

3.2.5 H-chitosan/PLA Blend Films

3.2.5.1 Preparation of H-chitosan/PLA Blend Films

To prepare blend films of H-chitosan and PLA, solutions of H-chitosan and PLA were first separately prepared at the concentration of 1% w/w in selective solvents such as chloroform, dichloromethane, and tetrahydrofuran. Slight stirring was used to expedite the dissolution and to homogenize the solutions. Blends films of different compositions (i.e. the weight ratios between H-chitosan and PLA of 100/0, 80/20, 60/40, 50/50, 40/60, 20/80, and 0/100, respectively) were then prepared by casting a mixture of the solutions in a respective weight ratio on a Teflon dish. It should be noted that slight stirring was used to homogenize the mixture prior to pouring onto the dish. The casting was allowed to be dried at room temperature for one day and later dry at room temperature under vacuum for another two days.

3.2.5.2 Characterization Techniques of H-chitosan/PLA Blend Films

FT-IR spectroscopy was used to characterize chemical functional groups of as-synthesized H-chitosan and as-prepared blend films. FT-IR spectra were collected on a Bruker Instrument Equinox55 FT-IR spectrometer at a resolution of 4 cm^{-1}.

Thermal properties of the blend films were analyzed by differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA). DSC thermograms were recorded on a Mettler DSC 822e/400 analyzer at a heating rate of 10°C·min^{-1} under nitrogen atmosphere. Samples of around 5 to 10 mg were used for the DSC measurements. TGA patterns were measured on a Perkin Elmer Pyris Diamond TG-DTA analyzer at a heating rate of 10°C·min^{-1} under nitrogen atmosphere over the temperature range of 30 to 750°C. Samples of approximately 5 to 10 mg were used for the TGA measurements.

Wide-angle X-ray diffraction (WAXD) was used to observe the crystal structure of the blend films. WAXD patterns were obtained on a Rigaku Rint 2000 X-ray diffractometer. The X-ray source was CuKα. The measurements covered the scanning range of 5 to 40° at a scanning speed of 5 deg·sec^{-1}.

Phase morphology of the blend films was investigated by a JEOL 520-2AE scanning electron microscope (SEM). Prior to observation under SEM, the blend films were either etched with cyclohexane or concentrated acetic acid solution in order to remove H-chitosan or PLA, respectively, for two minutes at room temperature.

A Lloyd LRX universal testing machine was used to assess the mechanical properties of the blend films. A load cell of 500 N was used along with a 500-mm·min^{-1} cross-head speed and a 50-mm gauge length.

3.2.6 Electrospining of H-chitosan/PLA Blends

3.2.6.1 Preparation of H-chitosan/PLA Blend Fibers

To prepare as-spun H-chitosan/PLA blends fibers, stock solutions of H-chitosan and PLA were first prepared separately using chloroform, dichloromethane, or tetrahydrofuran as the solvent. The concentration of H-chitosan stock solution was 10% w/v, while the concentration of PLA stock solutions was either 20 or 24% w/v. Slight stirring was used to expedite the dissolution and to homogenize the solutions. It should be noted that the 20% w/v PLA solution was prepared in order to illustrate the effect of the solution concentration on the resulting as-spun fibers. The stock solutions of H-chitosan/PLA blends were conveniently prepared by mixing the stock solutions of the pure material in the following weight ratios (H-chitosan/PLA): 80/20, 60/40, 50/50, 40/60, and 20/80, respectively).

To electrospin each of the stock solutions prepared, about 3 ml of the solution was filled in a 5-ml syringe, with a blunt-end, stainless steel needle (inner diameter = 0.9 mm) being attached at the opening end. Both the syringe and the needle were tilted 45° from a vertical line. The needle was connected to the emitting electrode of a Gamma High Voltage Research ES30P-5W high-voltage supply capable of generating a DC voltage in the range of 0 to 30 kV. An aluminium sheet, used as the collective screen, was connected to the ground electrode of the

power supply and was placed perpendicular to the needle. The distance between the needle tip and the collective screen defines a collection distance. The applied electrostatic field strength was fixed at 16 kV/15 cm and the electrospinning process was carried out at room temperature (i.e. about 25°C) within a fixed collection time of 5 minutes.

3.2.6.2 Characterization Techniques of H-chitosan/PLA Blend Fibers

Shear viscosity of the H-chitosan/PLA blend solutions was measured using a Brookfield DV-III programmable viscometer at 25°C.

Morphological appearance of the as-spun products was examined by a JEOL JSM-5200 scanning electron microscope (SEM). The specimens for SEM observation were prepared by cutting an aluminium sheet covered with the as-spun webs and the cut section was carefully affixed on a copper stub. Each sample was coated with thin layer of gold using a JEOL JFC-1100E ion sputtering device prior to observation under SEM. For each spinning condition, at least 80 measurements for the fiber diameters were recorded. Statistical analysis of the data obtained was carried out, from which an arithmetic mean and a standard deviation were reported.

Thermal properties of the as-spun products were analyzed by thermo-gravimetric analysis (TGA) and differential scanning calorimetry (DSC), respectively. TGA patterns were measured on a Perkin-Elmer Diamond TG/DTA analyzer at a heating rate of 10°C·min^{-1} under nitrogen atmosphere over a scanning range of 50 to 500°C. Samples of about 5 to 10 mg were used. The melting characteristic and the crystalline structure of the as-spun products were analyzed by DSC and wide-angle X-ray diffraction (WAXD), respectively. DSC thermograms were recorded on a Mettler DSC 822e/400 anaiyzer at a heating rate of 10°C·min^{-1} under nitrogen atmosphere, using the standard 40 μl pans (containing about 3 to 4 mg of samples).

WAXD patterns were recorded on a Rigaku Rint2000 X-ray diffractometer. The X-ray source was Cu Kα. The scanning range and the scanning speed were 5 to 40° and 5 deg·s^{-1}, respectively.