CHAPTER III
EXPERIMENTAL

3.1 Materials

Chitin and Chitosan, with a degree of deacetylation of 22% and 85% respectively, were prepared from shrimp shells kindly supplied by the Surapol Food Public Co.,Ltd. Glacial acetic acid was purchased from J.T. Baker. Phthalic anhydride was obtained from Fluka Chemmika, Switzerland. N,N-dimethylformamide and methanol were supplied by Labscan Co.,Ltd. Hydrazine monohydrate was purchased from Nacalai Tesque, Japan. Hexanoyl chloride, dueterium chloroform and DMSO were obtained from Aldrich Co.,Ltd. Pyridine and Chloroform were distilled and dried over molecular sieve. The other chemicals were analytical grade used without further purification.

3.2 Experimental

A. Preparation of Chitosan and Hexanoyl Chitosan

3.2.1 Preparation of Chitin

Chitin was prepared using method adopted by Shimahara *et al.* (1988). The shrimp shells was cleaned and dried before ground into small pieces. Demineralization was performed by immersing shrimp shells in 1 N HCl solution for 2 days with occasional stirring. The demineralized product was neutralized by washing with deionized water and protein removal was performed in 4% w/w of NaOH solution by further 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.

3.2.2 Preparation of Chitosan

Chitosan was obtained from deacetylation of chitin by heating the chitin flakes in NaOH solution 50% (w/w). $NaBH_4$ 0.5% (w/w) was added based on the weight of chitin to prevent depolymerization and the ratio of chitin to NaOH solution was 1g/10ml. The deacetylation was performed in an autoclave at 110°C for 1 h. The deacetylated product was washed thoroughly with deionized water until neutral by checking the pH. The resulting chitosan flakes was dried in an oven at 60°C for 24 h. Chitosan powder was sieved using Restch Sieving Machine type Vibro and the portion with the size of <75μm was collected.

3.2.3 Preparation of Hexanoyl Chitosan (Method I)

(Varying degree of hexanoylation without protecting amino groups)

Chitosan powder (3.2 g, 19.13 mmol) was soaked in pyridine for one week and fitered off before futher soaking in the mixture of pyridine and chloroform (90 : 40 ml) for one day. The mixture was cooled to -10 ~ -5 °C in an ice-salt bath and hexanoyl chloride (21.18 ml, 160.67 mmol) dissolved in chloroform (15 ml) was added dropwisely for 2 h. The mixture was further stirred for 2 h at room temperature and then refluxed for 6 h at 98°C. The resulting mixture was concentrated using a rotary evaporator and then poured into methanol (300 ml). The precipitate was filtered off, dissolved in chloroform and then reprecipitated into methanol. The precipitate was filtered off, extracted in a Soxhlet extractor with methanol for 24 h and dried in vacuo at 40°C for 4 h. The sticky yellowish product was obtained.

The dried hexanoyl chitosan, fresh pyridine and chloroform were placed in a flask in the amounts described above. This procedure was repeated three times to obtain hexanoyl chitosans with three different degree of sustitution (H-1, H-2 and H-3 chitosan) with the third resulting derivative was completely substituted with hexanoyl groups.

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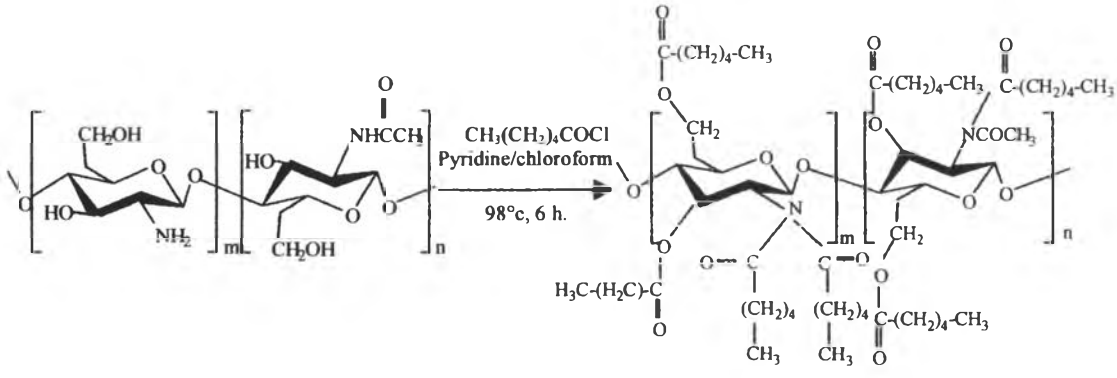


Chitosan Hexanoyl chitosan

Scheme 3.1 Synthesis procedure of hexanoyl chitosan.

3.2.4 Preparation of 3,6-Di-O-Hexanoyl Chitosan (Method II)

(Protecting of amino groups by phthaloylation)

This method consisted of three modification steps as follows

3.2.4.1 *Synthesis of N-Phthalimido Chitosan*

Chitosan powder (5 g, 29.9 mmol) was stirred in N,N-dimethylformamide (120 ml) under vacuum condition and heated to 100°C. Phthalic anhydride (13.8 g, 93.1 mmol) was added into the solution. The mixture was further heated with stirring. After 5-7 h, the mixture became a clear and viscous solution. Then the temperature was adjusted to 60°C. The reaction mixture was left overnight under nitrogen atmosphere. Precipitate was obtained by pouring the solution into an ice-water slurry and was collected by filtration. It was then washed completely by Soxhlet extraction with ethanol and dried in vacuo to obtain the white product of N-phthalimido chitosan.

3.2.4.2 *Synthesis of 3,6-Di-O-Hexanoyl-N-Phthalimido Chitosan*

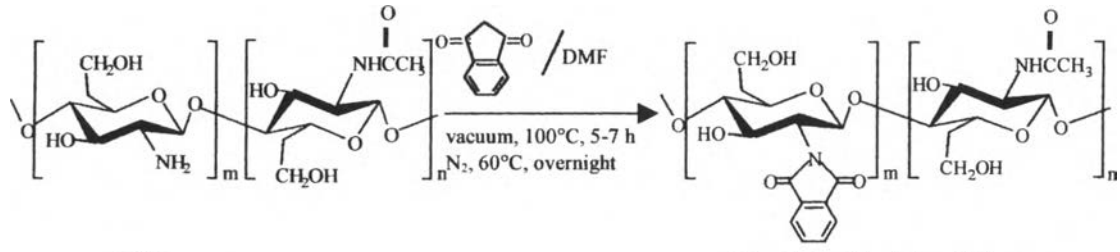
N-phthalimido chitosan (3.2 g, 11.45 mmol) was soaked in pyridine (54 ml) for one day. Chloroform (24 ml) was then added into the solution and stirred overnight. The mixture was cooled to -10 ~ -5 °C in an ice-salt bath and hexanoyl chloride (9.1 ml, 68.69 mmol) dissolved in chloroform (15 ml) was added dropwisely in 2 h. Then, the reaction was carried out in the same method used for preparation of hexanoyl chitosan as described in method I. The dried hexanoyl chitosan, fresh pyridine and chloroform were again placed in a flask in the amounts described above. This procedure was repeated twice until all or most -OH groups of chitosan were completely substituted.

3.2.4.3 *Synthesis of 3,6-Di-O-Hexanoyl Chitosan (H-P Chitosan) (Removal of Phthalimido Groups)*

The 3,6-di-O-hexanoyl chitosan was prepared by removing of phthalimido protecting group in 3,6-di-O-hexanoyl-N-phthalimido chitosan. A mixture of 3,6-di-O-hexanoyl-N-phthalimido chitosan (1 g, 2.04 mmol), hydrazine monohydrate (7.2 ml, 148.4 mmol) and DMF (56 ml) was heated with stirring for 5 min at 100°C under nitrogen atmosphere. After cooling, the mixture was precipitated in acetonitrile. The yellowish precipitate was collected by centrifugation, washed with ethanol and dried in vacuo.

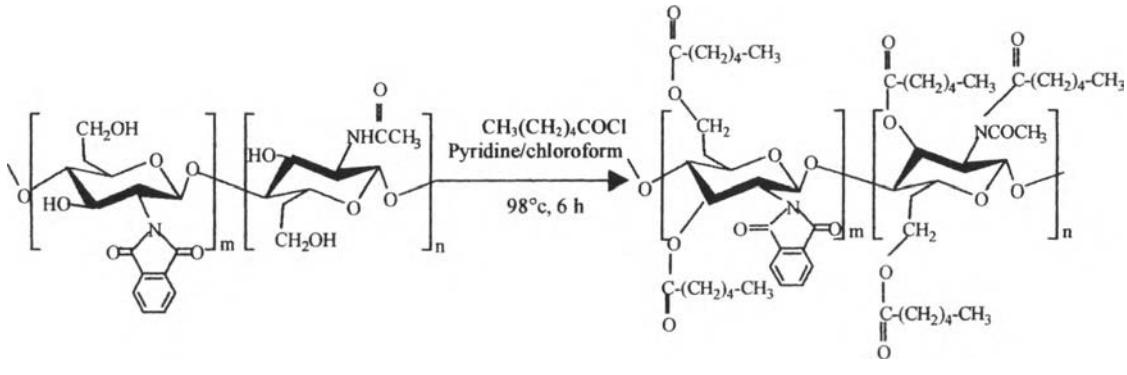
3.2.5 Preparation of Chitosan Film

Chitosan flake was dried at 110^0C for 1 h before use. Chitosan solution (1% w/w) was prepared by dissolution of chitosan in 1% (w/w) acetic acid solution. The chitosan solution was allowed to stand overnight at 4°C in a refrigerator to remove any trapped air bubbles before casted onto the clean dry petridishes in a dust free atmosphere at room temperature. The films were allowed to dry under the light for 72 h. The films obtained were soaked



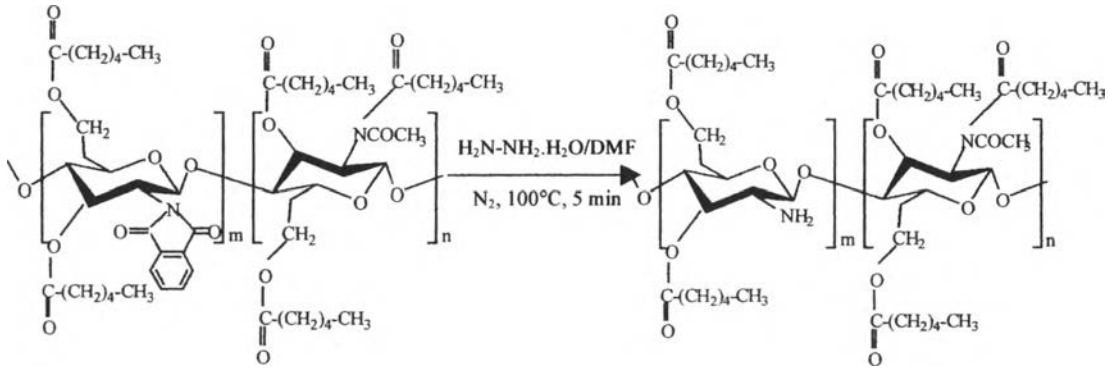
Chitosan N-phthalimido chitosan

Scheme 3.2 Synthesis procedure of N-phthalimido chitosan.



N-phthalimido chitosan 3,6-di-O-hexanoyl-N-phthalimido chitosan

Scheme 3.3 Synthesis procedure of 3,6-di-O-hexanoyl-N-phthalimido chitosan.



3,6-di-O-hexanoyl-N-phthalimido chitosan 3,6-di-O-hexanoyl chitosan

Scheme 3.4 Synthesis procedure of 3,6-di-O-hexanoyl chitosan.

in 0.5% (w/w) of NaOH in methanol(24 h) to neutralize any acid left and then washed thoroughly with methanol.

3.2.6 Preparation of Hexanoylated Chitosan Film

Hexanoylated chitosan (1% w/w) was dissolved in toluene with stirring. The solution was casted onto petridishes and dried at room temperature.

B. Characterization of Chitosan and Its Derivatives

3.2.7 Degree of Deacetylation of Chitosan

The method used to determine the degree of deacetylation of chitosan was based on infrared spectroscopic measurement. One mg of chitosan powder which was passed through 75 μm seive, was mixed and ground with 40 mg of potassium bromide powder and pressed to give a KBr disk. FT-IR spectra of chitosan was recorded in a range between 4000 to 400 cm^{-1}. Absorbances of peaks at wave number of 2878 cm^{-1} (the C-H stretching) and 1550 cm^{-1} (the amide II band) were evaluated by the baseline method (Sannan, 1978). The degree of deacetylation was calculated from equation (3.1):

$$DD = 98.03-34.68\ (A_{1550}/A_{2878}) \qquad (3.1)$$

Where DD = degree of deacetylation (%)

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

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

3.2.8 Viscosity-Average Molegular Weight of Chitosan

Viscosity-average molecular weight of chitin and chitosan were determined by Cannon Ubbelohde type number 150 and 75 of capillary viscometer respectively. The solutions with different concentration of chitosan (0.00, 0.01, 0.02, 0.03, 0.04, 0.05 and 0.1g/100ml) in 0.2 M acetic acid, 0.1 M NaCl and 4.0 M urea were prepared. The solution was placed in an Ubbelohde viscometer and equilibrated in water bath, which maintained the temperature at 25°C. The running time of both solvent and solutions were determined and repeated at least 3 constant values (±0.2 sec.) were obtained for each samples. These flow times were used to calculate the relative viscosity, specific viscosity and reduced viscosity. The values of reduced viscosity were plotted against the concentration and the value of intrinsic viscosity was obtained from the intercept of this plot.

$$\text{Relative viscosity } (\eta_{rel}) = t/t_s \quad (3.2)$$

$$\text{Specific viscosity } (\eta_{sp}) = (t/t_s) - 1 \quad (3.3)$$

$$\text{Reduced viscosity } (\eta_{red}) = \eta_{sp}/c \quad (3.4)$$

$$\text{Intrinsic viscosity } [\eta] = (\eta_{red})_{c \to 0} \quad (3.5)$$

Where t is the running time of chitosan solution, t_s is the running time of solvent and c is the concentration of polymer solution in g/100ml.

The viscosity-average molecular weight of chitosan was determined based on the Mark-Houwink equation:

$$[\eta] = KM^a \quad (3.6)$$

$$[\eta] = 8.93 \times 10^{-4} M^{0.71} \quad (3.7)$$

where [η] is the intrinsic viscosity, M is viscosity-average molecular weight and 8.93×10^{-4} and 0.71 are constants for chitosan in this solvent system(Lee, 1978).

Molecular weight of chitin can be found from the same method and equation used for chitin by dissolving chitin in 5%LiCl in DMAc (Lee, 1978).

3.2.9 Fourier Transform Infrared Spectroscopy (FT-IR)

Qualitative and quantitative FT-IR spectra were obtained from a VECTOR 3.0 BRUKER Spectrophotometer with a resolution of 4 cm^{-1}. A frequency range from 4000 to 400 cm^{-1} was observed using a deuterated triglycinesulfate detector (DTGS) with a specific detectivity, D^*, of 1×10^9 $cm.Hz^{1/2}w^{-1}$. The sample powder was ground with KBr, using 1 mg of sample for 40 mg of KBr, and pressed into a pellete form. Hexanoyl chitosan solution was prepared by dissolution in chloroform. The liquid sample was casted directly on the ZnSe plate and allowed the solvent to evaporate. The disk was attached to the frame and scanned with 36 scans.

3.2.10 Nuclear Magnetic Resonance Spectrometry (NMR)

^1H- and ^{13}C-NMR spectra were recorded using a Bruker-300 MHz spectrometer at Chemistry department, Faculty of Science, Chulalongkorn University. ^1H- and ^{13}C-NMR spectra were carried out at room temperature using deuterated DMSO-d6 and $CDCl_3$ as the solvent and reference for chemical shift measurements.

Solid state ^{13}C-NMR spectrum was recorded using a DPX-300 Advance 300 MHz Digital NMR Spectrometer of Bruker, Switzerland, by a courtesy of the National Metal and Materials Technology Center (MTEC), Ministry of Science and Technology, Thailand.

3.2.11 Thermogravimetric Analysis (TGA)

The thermal stability of chitosan and its derivatives were evaluated using a TGA 5.1 Dupont Instrument model 2950. The sample of 8-15 g was placed in the Pt pan. Thermogravimetric analysis of chitosan and its derivatives were carried out from 30°C to 700°C at the heating rate of 10°C/min under nitrogen atmosphere.

3.2.12 Crystallinity Determination

Wide-angle X-ray diffractograms of chitosan and its derivatives were recorded at room temperature using a RIGAKU RINT 2000. High intensity monochromatic Ni-Filtered CuKα radiation was generated at 40 kv and 30 mA. The sample powder was attached to the sample holders. The analysis was performed in the range of 5-50 degree with scan speed of 5 degree/min and 0.02 degree of scan step.

3.2.13 Degree of Substitution

The element analysis results were obtained from a CHNS/OAnalyzer (Perkin Elmer PE2400 Series II : option CHN) with combustion temperature at 950°C. The sample(1-2mg.) was filled in tin foil analysed under air with oxygen as a combustion gas (flowing rate of 20 ml/min.) and He as a carrier gas (flowing rate of 200 ml/min.). Degrees of substitution of chitosan derivatives were computed from the results obtained by elemental analysis.

3.2.14 Metal Adsorption

The metal adsorption of chitosan and its derivatives were determined using Atomic Adsorption Spectrometer (AAS) Varian Spectraa 300/400 System. The solution containing 10 ppm of Cu^{2+} was prepared by dissolution of $CuCl_2$ in a buffer solution (pH 7). The hexanoylated chitosan

films (dimension of 1.5×1.5 cm^2) were immersed in $CuCl_2$ solution with ratio of 0.31mg chitosan/1ml of Cu^{2+}solution with first 12 h shaking at ambient temperature. The solutions were collected at 0, 0.5, 1, 2, 3, 6, 12, 24, 48 and 72 h. The remaining concentration of metal ion in these solutions were determined using AAS. The metal adsorption of chitosan and its derivatives were calculated from equation (3.8).

$$\text{Metal adsorption (\%)} = [(C_i - C_t)/C_i] \times 100\% \qquad (3.8)$$

Where C_i is initial concentration of metal ion, C_t is concentration of metal ion at observed time.