CHAPTER III
EXPERIMENTAL

3.1 Materials

Chitosan with a degree of deacetylation of 75.8% was a gift from Prof. Suwalee Chandrkrachang, the Asian Institute of Technology, Thailand. Ammonium sulfate, dipotassium hydrogen orthophosphate, pyridine, sodium acetate, hydrochloric acid, *N,N*-dimethylformamide, and sodium hydroxide were the products of UNIVAR, Australia. Polyethylene glycol (MW 6 000), potassium dihydrogen phosphate, sodium carbonate anhydrous, lauric acid, phthalic anhydride, toluene-4-sulfonyl chloride and N-acetyl-D-glucosamine were purchased from Fluka Chemical, Switzerland. Methanol and glacial acetic acid were from J.T. Baker, U.S.A. Magnesium sulfate was from CARLO ERBA, France. Ethanol and potassium ferricyanide were purchased from BDH Laboratory Supplies, England.

3.2 Instruments and Equipment

3.2.1 Fourier Transform Infrared Spectrophotometer (FT-IR)

Quantitative and Qualitative FT-IR spectra were obtained from VECTOR 3.0 BRUKER spectrometer with 64 scans at a resolution of 4 cm^{-1}. A frequency range of 4000-400 cm^{-1} was observed using a deuterated triglycinesulfate detector (DTGS).

3.2.2 Ultraviolet-Visible Spectrophotometer (UV-VIS)

A lambda-16 UV-VIS spectrophotometer from Perkin-Elmer was used for quantitative analysis. The unit activity of enzyme was determined by colorimetric assay at absorbance 420 nm (A_{420}). The stability of the enzyme was evaluated by turbidimetric assay at transmission 650 nm (T_{650}). Bousch & Lomb STECTRONIC 2000 was used to measure the enzyme activity by turbidimetric assay at T_{650} nm during enzyme production.

3.2.3 Elemental Analysis (EA)

The percent elements were obtained from YANAKO CHN CORDER MT-3, MT-5 with combustion temperature 950°C. The sample were run under He (flowing rate 200 mL/min) and oxygen (flowing rate 20 mL/min).

3.2.4 Thermal Gravimetric Analysis (TGA)

Thermogravimetric analyzer, Du Pont TGA 2950 was applied for studying the weight loss of chitosan derivatives. Samples (approximately 5-7 mg) were loaded in a platinum pan and heated under a N_2 flowing rate of 20 mL/min. The heating rate was 20°C/min from 30°C to 600°C.

3.2.5 X-ray Diffraction (XRD)

X-ray diffraction patterns were obtained from a RIGAKU RINT2000. CuKα (λ = 0.154 nm) was used as an X-ray source and operated at 40 kV, 30 mA with Ni filter. Sample (0.1-0.2 g) was ground with agate mortar and spread on a glass slide specimen holder to examine 2θ of 5-50°.

3.2.6 Nuclear Magnetic Resonance Spectrometer (NMR)

Solution state ^{1}H-NMR spectra were analyzed by JEOL: JNM-LA500 and Varian INITY plus 600.

3.2.7 Brookfield Viscometer

Brookfield viscometer RVDV-III, with a small sample adapter (SSA 21/13R) and a water bath, was used to measure the chitosan viscosity before and after treatment with the enzyme. The measurement was operated at 37°C by controlling the temperature in the water bath.

3.2.8 Capillary Viscometer

The Cannon-Ubbelohde viscometer Cole Palmer, U.S.A., capillary no. 50 B582, with thermostatic bath DT-2 Heto, Denmark was applied to measure the chitosan viscosity in 0.1M sodium acetate / 0.2M acetic acid at 30°C.

3.2.9 Lyophilizer

The chitinase enzyme solution was frozen under liquid nitrogen and lyophilized by Flexi-Dry FTS, STONE RIDGE, New York, U.S.A.

3.2.10 Laminar Flow

The procedures of isolation, transferring, to prepare enzyme was done in Laminar flow, International Scientific Supply Co., Ltd, to avoid the other microorganisms and impurity in the air.

3.2.11 Shaker

The Shaker, New Brunswick Scientific Co., Inc, was applied to control the shaking and temperature in chitosan degradation studies.

3.2.12 Autoclave

All of equipments for enzyme and culture media were sterilized by autoclave, Kokusan Ensinki Co., Ltd., before use.

3.3 Experimental Procedure

3.3.1 Preparation of Chitinase Enzyme

3.3.1.1 Preparation of Colloidal Chitin

Chitin (10 g) was added slowly into 400 mL of concentrated hydrochloric acid in ice-bath with vigorous stirring. After 2 h, the mixture was heated up to 37°C with moderate stirring for 30 min and then filtered through quartz wool. The filtrate was poured into 4 L of deionized cold water in ice-bath with stirring to obtain the colloidal chitin suspension. The suspension was kept overnight at 4°C. The supernatant was decanted and colloidal chitin was washed with deionized water until neutral. The colloidal chitin was resuspended in deionized water. The percent weight of colloidal chitin was determined after drying the suspension.

3.3.1.2 Preparation of Culture Media

Colloidal chitin liquid medium was used to grow bacteria for enzyme production. A litre of colloidal chitin medium (CM medium) containing colloidal chitin 0.2 %, yeast extract 0.5 g, $(NH_4)_2SO_4$ 1 g, $MgSO_4.7H_2O$ 0.3 g, KH_2PO_4 6 g, and K_2HPO_4 10 g was prepared. For solid medium, Bacto-Agar 20 g was added. Solid medium was used for isolating bacteria into a single colony. LB medium was used to generate innocumn. A litre of Luria–Burtani medium (LB Medium) contain Bacto–tryptone 10 g, Bacto–yeast extract 5 g, and NaCl 10 g, the pH of the LB medium was adjusted to 7.2–7.4 by 10 N NaOH. Before use, all of the media were sterilized by autoclave at 121°C for 20 min.

3.3.1.3 Production of Chitinase Enzyme

Staphylococcus species strain TU005 (E) was streaked to form single colonies on colloidal chitin medium agar medium. A single colony was inoculated to LB broth medium and grown overnight to produce an innocumn.

Chitinase enzyme was prepared as follows. The overnight bacteria cell culture was innoculated, 1:100 dilution, into CM medium. The culture was grown in water bath at 30°C for two days with shaking, 250 rpm. Every 3 h, the medium was sampled out to assay for the chitinase activity using turbidity assay. After the highest activity had reached, the culture medium containing crude enzyme was collected by centrifugation, at 7 000 rpm 20 min. The crude enzyme was put in a dialysis tube and placed in the box containing polyethylene glycol powder to reduce the volume of the solution. After the solution was reduced to 1/3 of the starting volume, the concentrated crude enzyme was dialyzed with phosphate buffer (10 mM) pH 7.0 and lyophilized to obtain powder chitinase.

3.3.1.4 Determination of Enzyme Activity

Turbidimetric Assay

Enzyme solution (1 mL) was added to a mixture of 0.5 µg/mL colloidal chitin (0.5 mL) and phosphate buffer pH 7.0 (0.5 mL). After adding enzyme, the transmittance (%T) at 650 nm was measuring every 5 min for 1 h at T_{650} nm. The enzyme activity was calculated from the slope of the graph between $\Delta\%T_{650}$ value ($\Delta\%T_{650} = T_t - T_0$) and the reaction time in unit $\Delta\%T_{650}$/min.

Calculation

$$\text{Enzyme Activity } (\Delta\%T_{650}/\text{min.mL or mg}) = \frac{\dfrac{T_{650_t} - T_{650_0}}{\text{measuring time (min)}}}{\text{amount enzyme used (mL or mg)}}$$

$$\text{Total activity} = \text{activity value } (\Delta\%T_{650}/\text{min .mL}) \times \text{total volume (mL)}$$

$(\Delta\%T_{650}/\text{min})$

$$\%\text{yield of enzyme purified} = \frac{\text{total activity of purified enzyme} \times 100\%}{\text{total activity of crude enzyme}}$$

Colorimetric Assay

The amount of enzyme (concentration 10 mg/mL) varied from 10 µL to 50 µL was added in the tubes containing excess colloidal chitin. The volume was made to be 1.5 mL with phosphate buffer pH 7.0 followed by shaking at 37°C for 30 min. The color reagent was prepared from 0.5g potassium ferriccyanide in 0.5 M sodium carbonate 1 L. The color reagent (2 mL) was added to the mixture and heat for 15 minutes in boiling water. After 15 min, the samples were centrifuge to remove the substrate and the color of mixture was determined by UV-VIS absorbance at 420 nm (A_1). The blank value (A_0) was measured by using phosphate buffer pH 7.0 replacing the enzyme solution in the reaction. The experiments were done at least 3 times and the values from the measurements were used in the calculation of ΔA, ($\Delta A = A_0 - A_1$).

The standard curve of N-acetylglucosamine was performed using concentrations of 0.09, 0.18, 0.27, 0.36, and 0.45 µmole of GlcNAc.

The enzyme activity was obtained by calculating the amount of GlcNAc produced in the reaction using the N-acetylglucosamine standard curve. The enzyme activity was calculated in the unit of µmole of reducing sugar produced/min.mg sample (U/mg sample).

Calculation

$$\text{Activity} = \frac{\text{Released of GlcNAc by enzyme}}{\text{amount of enzyme used (mg)} \times \text{degradation time (min)}}$$

$(\Delta \%T_{650} / \text{min.mg})$

3.3.2 Study on the Factors Effecting Enzyme Hydrolysis on Chitosan Chain

3.3.2.1 Effect of Hydrolysis time

Enzyme stability was measured by incubating chitinase solution (concentration 0.6 U/mL) for different length of time at 37°C. Every half an hour, chitinase solution was aliquot and assayed for enzymatic activity using turbidity assay. The enzyme stability was also considered by using acetate buffer pH 4.5 compared with phosphate buffer pH 7.0.

3.3.2.2 Effect of Substrate Concentration

The decrease of viscosity was measured by Brookfield viscometer to evaluate the chitosan concentration effect. Chitosan concentration was varied from 2% to 4% while the amount of enzyme was maintained the same (18mU/mL).

3.3.2.3 Effect of Enzyme Concentration

The amount of enzyme was varied from 4.5 mU/mL to 30 mU/mL while the 4% chitosan concentration was used. Enzyme hydrolysis was also measured by the reduction of reaction viscosity.

After the optimum condition was obtained. The product was reprecipitated by NaOH (1 M) to collect the oligochitosan.

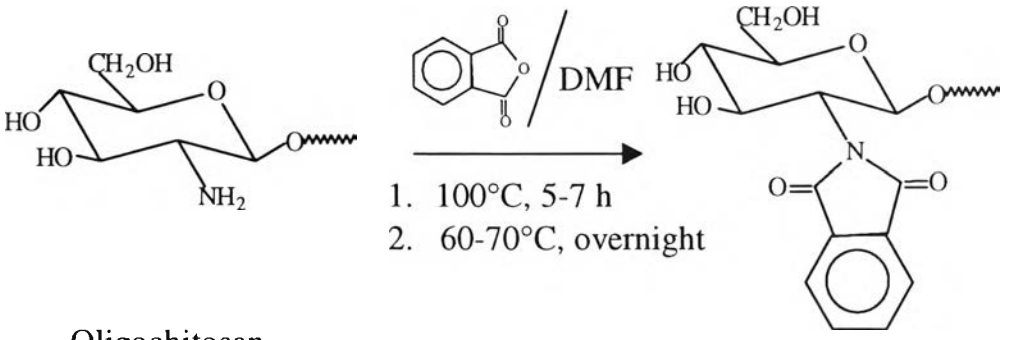
3.3.3 Preparation of Oligochitosan Derivatives

3.3.3.1 Preparation of N-Phthaloyloligochitosan

N-Phthaloyloligochitosan (Scheme 3.1) was prepared by using excess phthalic anhydride to react with chitosan in *N,N*-dimethylformamide (DMF) as referred to N-phthaloylchitosan (Nishimura *et al.*, 1991). One gram of oligochitosan was dispersed in 20 mL of *N,N*-dimethylformamide under vacuum condition and heated to 100°C. Three-fold excess phthalic anhydride were added and reaction was proceed at 100°C. After 7 h, the nitrogen atmosphere was changed to vacuum and stirred overnight. The

obtained product was reprecipitated in cold water and washed thoroughly with ethanol. After drying *in vacuo*, the product was characterized by FT-IR, EA, XRD, TGA, UV, and NMR.

Scheme 3.1 Preparation of N-phthaloyloligochitosan



Oligochitosan

N-Phthaloyloligochitosan

3.3.3.2 Preparation of O-Tosyl-N-Phthaloyloligochitosan

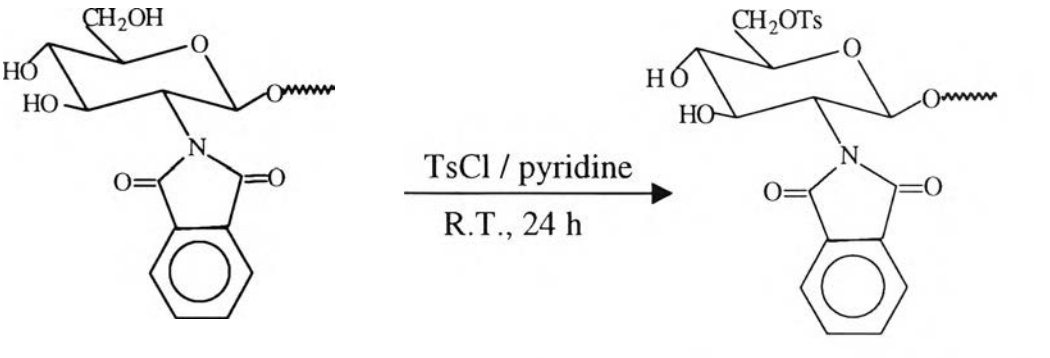
One gram of phthaloyloligochitosan was dissolved in pyridine 20 mL at room temperature. Ten-fold excess tosylsulfonylchloride were added, and the mixture was stirred at room temperature for 24 h (Scheme 3.2). The obtained product was reprecipitated in cold water and washed with ethanol. The product was characterized by FT-IR, EA, XRD, TGA and UV.

3.3.3.3 Preparation of O-Lauryl-N-Phthaloyloligochitosan

One gram of tosylphthaloyloligochitosan was dispersed in 40 mL pyridine under vacuum condition and heated to 80°C. Lauric acid (15 fold-excess) was added and reacted at 80°C. After 12 h, the nitrogen atmosphere was replaced to vacuum condition and stirred for 3 days (Scheme 3.3). After the reaction, the solution was reprecipitated in cold water and

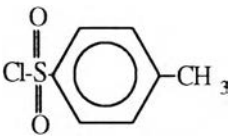
washed thoroughly by acetone and ethanol. The obtained product was dried *in vacuo* and characterized by FT-IR, NMR, EA, TGA and UV.

Scheme 3.2 Preparation of O-tosyl-N-phthaloyloligochitosan



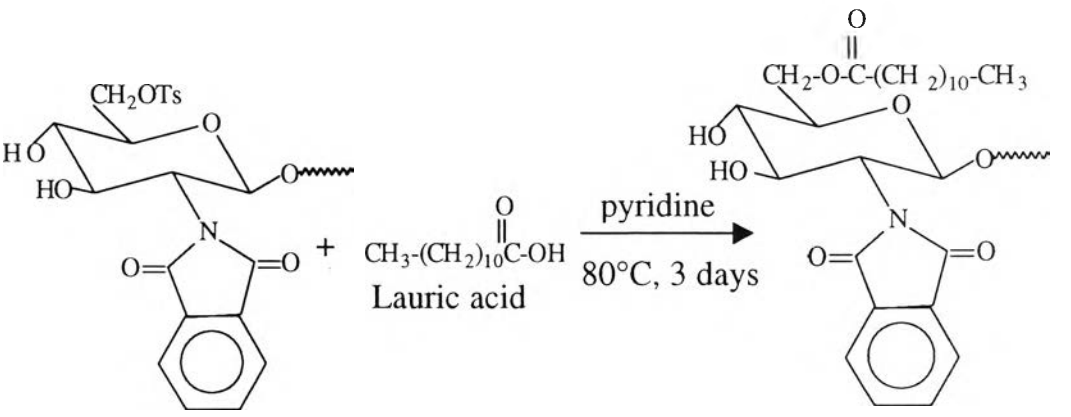
N-Phthaloyloligochitosan

O-Tosyl-N-Phthaloyloligochitosan



:- Toluene-4-sulfonyl chloride (TsCl)

Scheme 3.3 Preparation of O-lauryl-N-phthaloyloligochitosan



O-Tosyl-N-Phthaloyloligochitosan

O-Lauryl-N-Phthaloyloligochitosan