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CHAPTERS III
EXPERIMENTAL

3.1 Materials

Chitin was prepared from shrimp shell (Penaeus merguiensis) that was kindly supplied from Surapol Food Publics Co., Ltd. by the method of Shimahara and Takigushi (1988). The degree of deacetylation of chitin was determined by infrared spectroscopic measurement according to the method of Sannan et al. (1978). Chitin was ground to powder with the size of 71-75 μm before use. Chitosan was purchased from Seafresh Chitosan (Lab) Company Limited. The molecular weight and %degree of deacetylation are 500,000 and 90% respectively. Poly(vinyl pyrrolidone) K 30 and Poly(vinyl alcohol) was purchased from Fluka. Glutaraldehyde 50% w/w was purchased from Fluka. Monochloroacetic acid, glacial acetic acid 99.9% w/w and sodium hydroxide were analytical grade. Sodium hydroxide 50% w/w was kindly supplied by KPT Cooperation (Thailand).

Theophylline was purchased from Shanghai Wandai Pharmaceuticals, China. Salicylic acid was purchased from Ajax Chemicals, Australia.

3.2 Equipment

3.2.1 Restch Sieving Machine

The chitin powder was sieved by using Restch Sieving Machine type Vibro and chitin with size of 71-75 microns was collected for using in the experiment.

3.2.2 Capillary Viscometer

The viscosity-average molecular weight of CM-chitin was determined by using Cannon Ubbelohde-type viscometer number 50.

The viscosity-average molecular weight of chitin was determined by using Cannon Ubbelohde-type viscometer number 75.

3.2.3 Elemental Analysis

The degree of substitution of CM-chitin was estimated by elemental analysis using CHNS/O Analyzer (PERKIN ELMER PE 2400Series II) 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).

3.2.4 FTIR Spectrophotometer

The FTIR spectrum of PVP/CM-chitin blend films were recorded with a Bruker FTIR Spectrophotometer, model Vector 3.0, with 32 scans at a resolution of 4 cm^{-1}. The samples with the thickness f 10 μm were attached to the sample frames. A frequency of 4000-400 cm^{-1} was observed by using deuterated triglycinesulfate detector (DTGS) with specific detectivity of $1x10^{9}$ cm. $Hz^{1/2}W^{-1}$.

3.2.5 Nuclear Magnetic Resonance Spectrometry

^{1}H-NMR and ^{13}C-NMR spectra were recorded by using FT-NMR 500 MHz. Spectrometer (JEOL, JNM-A500). CM-chitin was dissolved in D_2O and used tetramethylsilane (TMS) as a reference for chemical shift measurement.

3.2.6 UV/Visible Spectrophotometer

The amount of drug release from CM-chitin films and blend films at pH 5.5 was determined by using UV/Visible Spectrophotometer model Shimazsu.

3.3 Methodology

3.3.1 Preparation of Chitin

Chitin was prepared from shrimp shell by decalcification and deproteinization to remove calcium carbonate and protein, respectively. The shrimp shells were cleaned and dried under sunlight before grinding into small pieces. Shrimp shell chips were treated by immersion in 1 N HCI solution for 2 days with occasional stirring. The decalcified product was washed with distilled water until neutral. Deproteinization was followed by boiling the decalcified product in 4% w/w

of NaOH solution at 80-90°C for 4 h. After NaOH solution was decanted, the product was washed with deionized water until neutral and dried at 60°C in a convective oven for 24 h.

3.3.2 Viscosity-Average Molecular Weight of Chitin

The different concentrations (0.01, 0.02, 0.03m 0.04 and 0.05 g/100 ml) of Chitin solutions dissolved in 5% LiCI/N,N-dimethylacetamide were prepared. All of samples were passed through filter papers before use. The Ubbelohde viscometer was filled with 10 ml of sample solution and then equilibrated in water bath, which was maintained the temperature at 30°C. The sample was passed through the capillary once before the running time was measured. Each sample was measured 5 times. The running times of solvent and solutions were used to calculate the relative viscosity, specific viscosity, and reduced viscosity, by the following equations:

$$\text{Relative viscosity } (\eta_{rel}) = (t/t_s) \quad (3.1)$$

$$\text{Specific viscosity } (\eta_{sp}) = t/_{ts})-1 \quad (3.2)$$

$$\text{Reduced viscosity } (\eta_{red}) = \eta_{sp}/C \quad (3.3)$$

$$\text{Intrinsic viscosity } [\eta] = (\eta_{sp})c \rightarrow 0 \quad (3.4)$$

Where t is the running time of CM-chitin solution, ts is the running time of solvent and C is the concentration in g/100 ml.

The viscosity-average molecular weight of CM-chitin was determined based on Marke-Houwink equation. The K and a values were 8.93×10^{-4} and 0.71, respectively, according to Kaneko *et al.*, (1982).

$$[\eta] = 8.93 \times 10^{-4} M^{0.71} \quad (3.5)$$

where $[\eta]$ is the intrinsic viscosity

M is viscosity-average molecular weight.

3.3.3 Degree of Deacetylation of Chitin

The method used to determine the degree of deacetylation of chitin is based on quantitative infrared spectroscopic technique (Sannan et al., 1978). About 3 mg of chitin powder, passed through a 200-mesh sieve, was mechanically mixed with 400 mg of potassium bromide powder to prepare a KBr disk. An infrared spectrum was recorded in a range from 4000 to 400 cm^{-1}. The absorbances at 2878 cm^{-1} (the C-H band) and 1550 cm^{-1} (the amid II band) were evaluated by the baseline method. The degree of deacetylation (DD) was calculated from the following equation:

$$DD\ (\%) = 98.03-34.68(A_{1550}/A_{2878}) \quad (3.6)$$

Where DD = Degree of deacetylation (%)

A_{1550} = The absorbances at 1550 cm^{-1} (the amid II band)

A_{2878} = The absorbances at 2878 cm^{-1} (the C-H band)

3.3.4 Preparation of CM-chitin

CM-chitin was prepared by the method of Hirano (1988). Alkaline chitin was prepared by suspending chitin power (4 g) in 42% NaOH solution (80 ml). After the suspention was allowed in desiccator for 30 min under reduced pressure, crush ice (160 g) was added and the mixture was mechanically stirred for 30 min in an ice bath to dissolve chitin. A viscous alkaline chitin solution was obtained. For successful synthesis of CM-chitin, the concentration of NaOH solution should not be less than 14%. Monochloroacetic acid solution was prepared by dissolving in 14% NaOH solution in an ice bath and was added dropwise into the alkaline chitin solution with stirring over 30 min. After standing overnight at room temperature, the mixture was neutralized with acetic acid under cooling in an ice bath and dialyzed against running water for 2 days, followed by dialysis against distilled water for 1 day. The dialysate was centrifuged at 5000 rpm for 20 min, in order to remove insoluble material, and the supernatant was added to 3 volumes of acetone. After

standing overnight, the precipitate was collected by centrifugation and washed with acetone. The product was resuspended in ethanol and collected by filtration. After drying at room temperature, CM-chitin sodium salt was obtained.

3.3.5 Degree of Substitution of CM-chitin

The degree of substitution was estimated by elemental analysis with combusion 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 with He as a carrier gas (flow rate of 200 ml/min).

3.3.6 Viscosity-Average Molecular Weight of CM-chitin

The different concentrations (0.00625, 0.0125, 0.025, 0.05 and 0.05 g/100 ml) of CM-chitin solutions dissolved in 0.1 M NaCl were prepared. All of samples were passed through filter papers before use. The Ubbelohde viscometer was filled with 10 ml of sample solution and then equilibrated in water bath, which was maintained the temperature at 30°C. The sample was passed through the capillary once before the running time was measured. Each sample was measured 5 times. The running times of solvent and solutions were used to calculate the relative viscosity, specific viscosity, and reduced viscosity, by the following equations:

$$\text{Relative viscosity } (\eta_{rel}) = (t/t_s) \quad (3.1)$$

$$\text{Specific viscosity } (\eta_{sp}) = t/_{ts})-1 \quad (3.2)$$

$$\text{Reduced viscosity } (\eta_{red}) = \eta_{sp}/C \quad (3.3)$$

$$\text{Intrinsic viscosity } [\eta] = (\eta_{sp})c \rightarrow 0 \quad (3.4)$$

Where t is the running time of CM-chitin solution, ts is the running time of solvent and C is the concentration in g/100 ml.

The viscosity-average molecular weight of CM-chitin was determined based on Marke-Houwink equation. The K and a values were 7.92×10^{-5} and 1, respectively, according to Kaneko *et al.*, (1982).

$$[\eta] = 7.92 \times 10^{-5} M^{-1} \quad (3.7)$$

where $[\eta]$ is the intrinsic viscosity

M is viscosity-average molecular weight.

3.3.7 Swelling Behavior

The blend films were cut into disk form with diameter of 16 mm and 25-30 μm in thickness. The weights of the completely dried samples were measured, and the samples were immersed in water and buffer pH 5.5 at 37°C. The degrees of swelling of these samples were calculated from the following equation (Wang et al., 1997):

$$\text{Degree of swelling (5)} = \frac{Ws-Wd}{Wd} \times 100 \quad (3.8)$$

where Ws and Wd denote the weight of swollen and dried film, respectively.

3.3.8 Preparation of CM-chitin Solution

CM-chitin solution was prepared by dissolution of CM-chitin in distilled water to obtain the concentration of 1% by weight. The solution was stirred continuously overnight at room temperature.

3.3.9 Preparation of Crosslinked Durg-Loaded Blend Films

Solutions containing CM-chitin and PVP were prepared by mixing various ratios of 1% w/w of PVP solution and 1% w/w of CM-chitin solution. The model drugs (theophylline, diclofenac sodium, salicylic acid and amoxicillin trihydrate) were added to the blend solutions to reach a concentration of 0.1% w/w.

Glutaraldehyde, used as crosslinking agent, was added to the blend solutions at the amount of 0.01%. The blend solution containing a model drug was stirred slowly for 12 h and then left overnight to get rid of air bubbles before casting onto clean dry petri dishes in a dust-free atmosphere at room temperature. The films were allowed to dry at 40°C for 12 h and then stored over silica in a desiccator before use.

3.3.10 Skin Preparation

Permeation experiments which were performed with full-thickness pig skin were from a side of pigs. The whole pig skins were surgically removed and cleaned with sterile normal saline. The subcutaneous fat, tissue, blood vessel, and epidermal hair were carefully removed by blunt section. The skin was free of obvious holes or defects. The full thickness skin was cleaned with normal saline and finally with distilled water, blotted dry, wrapped with aluminium foil and stored frozen before use. To perform in-vitro skin permeation experiment, full thickness skin was thawed at room temperature and cut into pieces (peripheral of circumference cell cap area) and a unit of drug-loaded blend films was applied onto the stratum corneum surface of the skin and then mounted individually between the half cells.

3.3.11 *In vitro* Skin Permeation of Drug

The *in vitro* skin permeation of drug from prepared membrane was studied using a modified Franz diffusion cell. The full thickness pig skin was mounted onto the receptor compartment with the stratum corneum side facing upward into the donor compartment and the dermal side facing downward into the receptor compartment. A unit of drug-loaded blend film was placed over the skin and the whole assembly was then clamped together with the donor cap on the top. The receptor compartment was then filled with the acetate buffer solution pH 5.5 constantly stirred using a magnetic stirrer and maintained at 37°C by a circulating water bath. A portion (0.5 ml each) of buffer solutions were withdrawn from the receptor compartment at predetermined time intervals of $^1/_4$, $^1/_2$, $^3/_4$, 1, $1^1/_2$, 2, $2^1/_2$, 3, 4, 5, 6, 8,10, 12, 14, 16, 18, 20, 22 and 24 hours; the drug concentrations in these samples were determined by the UV/visible spectrophotometer. The analytical

wavelength of salicylic acid and theophylline were 296 nm and 272 nm, respectively, using calibration curve of each drug. The experiments were performed in triplicate. The percentages of released drugs were average values of repeated three experiments.