CHAPTER III
EXPERIMENTAL

3.1 Materials

Shrimp shell was kindly provided by Surapon Food Public Co., Ltd. Silk fiber (*Bombyx mori*) was degummed by treatment with 0.5% Na_2CO_3 at 100°C for 30 min, followed by washing with boiling distilled water. The degummed silk was dried at 60°C for 24 h in an oven. Afterwards, the silk fibroin was dissolved in a triad solvent $CaCl_2$: EtOH: H_2O with mole ratio of 1:2:8 at 100°C for 15 min. The silk solution was then dialyzed against distilled water for 7 days. The solution was next filtered through the sintered glass filter, and subsequently diluted to achieve a concentration of 1% w/w.

Sodium hydroxide 50% w/w solution was kindly supplied by KPT Cooperation (Thailand). Glacial acetic acid 99.9% w/w purchased from J.T. Baker was analytical grade. Glutaraldehyde 50% w/w was purchased from Fluka.

Theophylline was purchased from Shanghai Wandai Pharmaceuticals, China. Diclofenac sodium was purchased from Tangyin Yongqi Chemical Industry Co., Ltd., China. Salicylic acid was purchased from Ajax Chemicals, Australia. Amoxicillin trihydrate was purchased from Antibiotics Co., Ltd., Spain.

3.2 Equipment

3.2.1 Restch Seiving Machine

The chitosan powder with the size of 38 to 75 μm was sieved and collected separately by using Retsch Seiving Machine type Vibro.

3.2.2 Capillary Viscometer

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

3.2.3 FTIR Spectrophometer

The FTIR spectrum of chitosan was recorded with Vector 3.0 Bruker FTIR Spectrophotometer with 16 scans at a resolution of $4cm^{-1}$. A frequency of 4000-400 cm-1 was observed by using deuterated triglycerinesulfate detector (DTGS) with specific detectivity of $1x10^{9}$ $cm.Hz^{1/2}.w^{-1}$.

3.2.4 UV/Visible Spectrophotometer

The amount of drug release from chitosan films and blend films at pH 2.0, 5.5, and 7.2 was determined by using UV/Visible Spectrophotometer Lambda10, Perkin Elmer.

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 HCl solution for 2 days with occasional stirring. The decalcified product was washed with distilled water until neutral. Deproteinization was followed by boiling in 4% w/w of NaOH solution at 80-90°C for 4 h. After NaOH solution was decanted, the chips were washed with deionized water until neutral. The product obtained was dried at 60°C in a convective oven for 24 h.

3.3.2 Preparation of Chitosan

Chitin was deacetylated by heating in 50% w/w NaOH solution containing 0.5% w/w sodium borohydride ($NaBH_4$) to prevent depolymerization. The ratio of chitin to NaOH solution was 1 g of chitin in 10 ml of NaOH solution. The deacetylation was performed in an autoclave at 110°C for 1 h. The deacetylated product obtained was washed exhaustedly with deionized water until neutral. The resulting chitosan flakes was dried in an oven at 60°C for 24 h.

3.3.3 Degree of Deacetylation of Chitosan

The degree of deacetylation of chitosan was determined, based on an IR spectroscopic method reported by Sannan (1978). About 3 mg of chitosan powder, passed through a 200-mesh sieve, was mechanically mixed with 400 mg of potassium bromide 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 (the amideII band) were used to quantitate the degree of deacetylation. The degree of deacetylation was calculated from the equation 3.1.

$$D = 98.03\text{-}34.68(A_{1550}/A_{2878}) \quad (3.1)$$

where D = degree of deacetylation (%)

A_{1550} = absorbance at 1550 cm^{-1}

A_{2878} = absorbance at 2878 cm^{-1}.

3.3.4 Viscosity-Average Molecular Weight of Chitosan

Chitosan solutions of different concentrations (0.00, 0.00625, 0.0125, 0.025, 0.05, and 0.1g/100mL) in 0.2 M acetic acid: 0.1M NaCl: 4.0 M urea were prepared. An Ubbelohde viscometer was filled with 10 mL of sample, which maintained the temperature at 25°C. The sample was passed through the capillary once before the running times were measured. Each sample was measured 3 times. The running times of solvent and solutions were used to calculate the relative viscosity, specific viscosity, and reduced viscosity. The reduced viscosity was plotted against the concentration and the intrinsic viscosity determined from the intercept. The corresponding equations are:

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

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

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

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

where t is the flow time in seconds of chitosan solution, t_s is the flow time in seconds of solvent and C is the concentration of chitosan solution in g/100 mL.

The viscosity average molecular weight of chitosan was determined based on the Mark-Houwink equation (Lee *et al.*, 1974)

$$[\eta] = 8.93x\ 10^{-4}\ M^{0.71} \quad (3.6)$$

where $[\eta]$ is the intrinsic viscosity and M is viscosity average molecular weight.

3.3.5 Preparation of Chitosan Solution

Chitosan flake was dried at 110°C for 1 h before use. Chitosan solution was prepared by dissolution of chitosan in 1% w/w acetic acid. The chitosan solution was allowed to stand overnight at room temperature to get rid of air bubbles before preparation of films.

3.3.6 Preparation of Crosslinked Dug-Loaded Blend Films

Solutions containing chitosan and silk fibroin were prepared by mixing various ratios of 1% w/w of silk fibroin solution and 1% w/w of chitosan solution. Glutaraldehyde, used as crosslinking agent, was added to the blend solutions at the amount of 0.01 mole/glucosamine unit of chitosan. 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. The blend solution containing a model drug was stirred slowly for 12 h and 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 ambient temperature for 72 h and then stored over silica in a desiccator before use.

3.3.7 Drug Release Studies

To study the release characteristics of the model drugs from the films, drug-loaded blend films were immersed in buffer solutions at pH 2.0, pH 5.5 and pH 7.2. At time intervals, 1-mL aliquots were withdrawn and assayed for the amount of drug released. Theophylline, diclofenac sodium, amoxicillin trihydrate and salicylic acid released in the solutions were determined by a UV-Visible spectrophotometer

(Perkin Elmer, Lambda 10) at 272, 275, 272, 299 nm, respectively, using calibration curves for each drug. The experiments were performed in triplicate. The percentages of released drugs were from average value of repeated three times.