CHAPTER III
EXPERIMENTAL

3.1 Materials

3.1.1 Shrimp Shells

The shells of *Penaeus merguiensis* shrimps were kindly provided by Surapon Foods Public Co., Ltd., Thailand.

3.1.2 Sodium Alginate

Sodium alginate was purchased from Carlo Erba Co., Ltd. The material was in the form of white powder.

3.1.3 Other Chemicals

Sodium hydroxide (NaOH) 50% w/w aqueous solution was kindly supplied by KPT Cooperation Co., Ltd., Thailand. Calcium chloride ($CaCl_2$), sodium acetate (CH_3COONa), sodium hydroxide anhydrous pellets (NaOH), sodium dihydrogen phosphate (NaH_2PO_4), potassium chloride (KCl), di-sodium hydrogen phosphate dodecahydrate (Na_2HPO_4), sodium borohydride ($NaBH_4$) and hydrochloric acid (HCl) 37% w/w were analytical grade purchased from Carlo Erba Co., Ltd. Glacial acetic acid 99.7% w/w was analytical grade purchased from Labscan Asia Co., Ltd., Thailand.

3.1.4 Model Drugs

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

3.2 Equipment

3.2.1 Capillary Viscometer

The viscosity-average molecular weights of chitosan and alginate were determined by using Cannon Ubbelohde-type viscometer number 50.

3.2.2 Fourier Transform Infrared (FTIR) Spectrophotometer

The FTIR spectra of chitosan, alginate and chitosan-coated alginate films were recorded with Bruker FTIR Spectrophotometer model Vector 3.0 with 32 scans at a resolution of 4 cm^{-1}. A wavenumber range of 4000-400 cm^{-1} was observed by using deuterated triglycerinesulfate detector (DTGS) with specific detectivity of $1x10^{9}$ $cm.Hz^{1/2}.w^{-1}$.

3.3.3 Lloyd Tensile Tester

The mechanical properties of the films were measured by using Lloyd Instrument LRX series of Lloyd tensile tester with the maximum load of 500 N.

3.2.4 UV/Visible Spectrophotometer

The amounts of drug released from calcium alginate films and chitosan-coated calcium alginate films at pH 2.0, 5.5, and 7.2 were determined by using a Perkin Elmer UV/Visible Spectrophotometer model Lambda10.

3.3 Methodology

3.3.1 Preparation of Chitin and Chitosan

3.3.1.1 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 performed 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.

3.3.1.2 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. After the deacetylation, the product was left to cool down at room temperature and the deacetylated product obtained was washed exhaustively with deionized water until the pH was neutral. The resulting chitosan flakes were dried in an oven at 60°C for 24 h. The process was repeated about four times to achieve chitosan with high degree of deacetylation.

3.3.2 Characterization of Chitosan

3.3.2.1 Degree of Deacetylation

The degree of deacetylation of chitosan was determined by an infrared spectroscopic measurement reported by Sannan (1978). Chitosan flakes were pulverized to powder that passed through a 200-mesh sieve and the powder was dried at 80°C overnight. About 1 mg of the sample powder and 60 mg of potassium bromide (KBr) were mixed, ground together and press 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 amideII band) were used to determine the degree of deacetylation. The degree of deacetylation was calculated from the 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).

3.3.2.2 Viscosity-Average Molecular Weight

The molecular weight of chitosan was determined by viscometric method. Chitosan solutions of different concentrations (0.00, 0.00625, 0.0125, 0.025, 0.05, and 0.1g/100 mL) in 0.2 M acetic acid/0.1M sodium acetate were prepared. An Ubbelohde viscometer was filled with 10 mL of sample, which was then mounted vertically in a water bath, regulated to 30 ± 0.1°C and left to equilibrate for 15–20 minutes. The sample was passed through the capillary once before the running times were measured. Each sample was measured at least 3 times. The running times of solvent and solutions were used to calculate the relative viscosity, specific viscosity, inherent viscosity, and reduced viscosity. The corresponding equations are:

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

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

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

$$\text{Inherent viscosity } (\eta_{inh}) = (\ln \eta_r)/c \quad (3.5)$$

$$\text{Intrinsic viscosity } [\eta] = (\eta_{red})_{c\rightarrow 0} \quad (3.6)$$

where:

t = flow time of chitin or chitosan solution (sec)

t_s = flow time of solvent (sec)

c = concentration of polymer solution in g/dl.

The values of reduced viscosity and inherent viscosity were plotted against the concentration. Then, the value of intrinsic viscosity was obtained from the intercept of the plot, multiplied by 100 to change the dimensions into mL/g, and followed by the calculation of the molecular weight based on the Mark–Houwink–Sakurada equation (shown as Equation 3.7):

$$[\eta] = KM^a \quad (3.7)$$

where [η] is intrinsic viscosity, M is viscosity-average molecular weight, K and a are constants determined based on the degree of deacetylation as mentioned in Equation 3.8 and 3.9.

$$K = 1.64 \times 10^{-30} \times DD^{14.0} \quad (3.8)$$

$$a = -1.02 \times 10^{-2} \times DD + 1.82 \quad (3.9)$$

where DD is degree of deacetylation.

3.3.3 Characterization of Sodium Alginate

Sodium alginate was characterized by its FTIR spectrum and molecular weight by using the same methods as those described for chitosan in section 3.3.2.1 and 3.3.2.2, respectively. For molecular weight determination, the solvent used for alginate was 0.1 M sodium chloride (NaCl) and the experiment was performed at 25 ± 0.1°C. The K and a constants are 6.9×10^{-6} and 1.13, respectively (Yan *et al.*, 2000).

3.3.3 Preparation of Chitosan-Coated Calcium Alginate Films

3.3.4.1 Preparation of Alginate Solution

Alginate solution was prepared by dissolution of alginate in distilled water. The above solutions were allowed to stand overnight at room temperature to get rid of air bubbles before preparation of films.

3.3.4.2 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 4% 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.

3.3.4.3 Preparation of Alginate Films

Alginate films were produced by a casting/solvent evaporation technique. Alginate solutions (2% w/v) were prepared by dissolving alginate in distilled water. The above solutions were left to stand until trapped air bubbles were removed, and poured on a polystyrene mould. The films were dried for 24 h in an oven at 40 °C.

3.3.4.4 Preparation of Model Drug-Loaded Alginate Films

To prepare drug-loaded alginate films, salicylic acid and theophylline (0.5% w/w) were dissolved or dispersed in alginate solution. Alginate solution containing the model drugs was casted onto clean dry polystyrene plate in a dust-free atmosphere. The films were allowed to dry at 40 °C for 24 h and then stored over silica in a desiccator before use.

3.3.4.5 Preparation of Calcium Alginate Films

Calcium cross-linked alginate films were prepared by soaking the alginate films in an aqueous solution of calcium chloride (3% w/v) for 5 min. The calcium alginate films were then washed with distilled water. The films were dried in the air at room temperature.

3.3.4.6 Preparation of Chitosan-Coated Calcium alginate Films

Chitosan-coated calcium alginate films were prepared by dipping calcium alginate films in chitosan solution (0.5 % w/v in 4% acetic acid) for 15 min and then the films were allowed to dry in air at ambient temperature. The films obtained were stored over silica in a desiccator before use.

3.3.5 Characterization and Testing of Films

3.3.5.1 Fourier Transform Infrared Spectra

The FTIR spectra of alginate, calcium alginate and chitosan-coated calcium alginate films were determined by using a Bruker FTIR spectrophotometer model Vector 3.0.

3.3.5.2 Ninhydrin Staining

Chitosan-coated calcium alginate films were detected for the chitosan coating on calcium alginate films by using ninhydrin staining technique. Ninhydrin solution (0.5% ninhydrin in butanol) was stained on a dry film and the film was heated in oven to 100°C for 10 minute. The purple color was developed for the presence of chitosan.

3.3.5.3 Mechanical Testing

Mechanical properties of dry and wet films were measured by a Lloyd Tensile Tester, at a gauge length of 50 mm and 20 mm/min of strain rate. The dimension of samples was 25 mm x 150 mm. For dry state, the films were dried at 60°C for 24 h before measurement. For wet state, the films were soaked in distilled water for 3 h to reach equilibrium before testing.

3.3.5.4 Swelling Behavior Determination

Swelling behavior of the films was studied in water and in different pH buffer solutions. The films with diameter of 16 mm were immersed in water and buffer solutions pH 2.0, pH 5.5 and pH 7.2. At predetermined time intervals, the swollen films were weighed after they were wiped with soft paper tissue. The degree of swelling for each sample was calculated by using the following equation:

$$\text{Degree of swelling (\%)} = \frac{W - W_0}{W_0}$$

where W and W_0 are the weights of the swollen films and the dry films, respectively.

3.3.5.5 Drug Loading Determination

To evaluate the amounts of model drugs inside the films, an indirect method was used. The model drug loss during cross-linking process was determined by measuring the UV absorption of drug in the cross-linking solutions. Aliquots from the filtered solutions remaining after removal of the films were assayed spectrophotometrically at 272 and 298 nm for theophylline and salicylic acid, respectively. The amounts of model drugs entrapped were calculated from the difference between the total amount of model drugs added and the model drugs found in the cross-linking solution.

3.3.5.6 Drug Release Studies

The releases of the model drugs from the films was evaluated by immersing the drug-loaded film in buffer solutions at pH 2.0, 5.5 and 7.2 (simulated pHs of gastric, skin, and intestinal juice, respectively), and incubated on a shaking water-bath at 37°C. At appropriate time intervals, the solutions were withdrawn and assayed for the amount of drug released. The contents of theophylline and salicylic acid were determined by measuring the absorption at 272 and 298 nm, respectively. The model drugs released into the medium from films were measured as a function of time. The experiments were performed in triplicate. The percentages of released drugs were average values of three repeated experiments.