CHAPTER III
EXPERIMENTAL

3.1 Materials and Equipment

Biopolymer flake (Mw 200 kDa) was purchased from Bio21 Co.,Ltd (Thailand). Purified biopolymer was obtained by highly deacetylated biopolymer using a literature method (Miya *et al.*, 1980). 2-(N-morpholino) ethanesulfonic acid sodium salt (MES) (ULTROL grade) was purchased from Calbiochem (Bangkok, Thailand). L-Arginine was purchased from Sigma-Aldrich. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimidehydrochloride (EDC.HCl) (purity 99%) and N-hydroxysulfosuccinimide sodium salt (sulfo-NHS) (purity 98%) were purchased from Suzhou Highfine Biotech Co.,Ltd. (China). Sodium hydroxide (NaOH) (lab grade) was purchased from Merck (Bangkok, Thailand). Sodium borohydride ($NaBH_4$) and Hydroxylamine (NH_2OH) were purchased from Labchem (Bangkok, Thailand). Hydrochloric acid (HCl, AR grade) was purchased from RCI Labscan (Bangkok, Thailand).

3.2 Experimental Procedures

3.2.1 Purification of Biopolymer

Deacetylation was used to obtain higher purity of biopolymer by removing acetyl functional group ($COCH_3$). 100 g of biopolymer and 0.5 g of $NaBH_4$ were immersed in 2 L of 50% w/w NaOH solution for 1 h at 120 °C in an auto-clave. After deacetylation process, the product was filtered and washed with water until the washed water was neutralized. The purified biopolymer was dried at 80 °C overnight in a vacuum oven and kept in desiccators. Deacetylation reaction is shown in Figure 3.1.

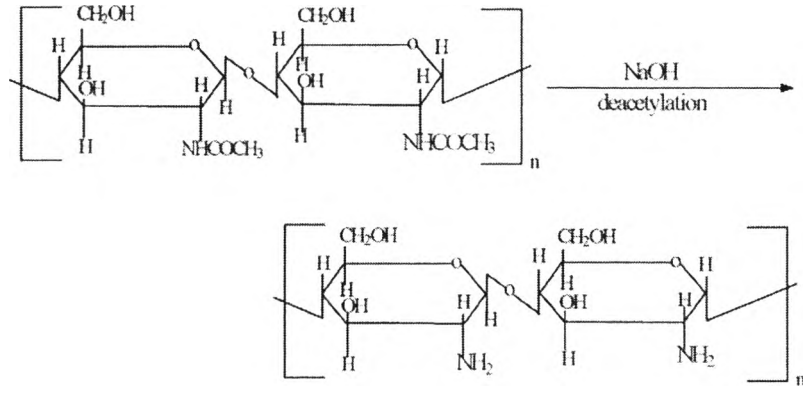

Figure 3.1 Deacetylation reaction of biopolymer (Kumar, 2000).

3.2.2 Determination of Degree of Deacetylation of Biopolymer

3.2.2.1 Fourier Transform Infrared Spectroscopy (FT-IR)

Biopolymer films were prepared by solvent casting method. Purified biopolymer powder of 0.2103 g was dissolved in 25 mL 1% (v/v) acetic acid, stirred until mixed well. The casting films were made by pouring 1 mL of biopolymer solution into a teflon coated glass mould and subsequently dried in an oven at 80 °C for 24 h. The film was subsequently neutralized with 0.5 M NaOH solution and washed thoroughly with deionized water until the washed solution was neutral. Finally, it was dried in an oven at 80 °C. The product called purified biopolymer (PB).

Fourier transform infrared spectroscopy 400 to 4000 cm^{-1} range was used to analyze functional groups in the purified biopolymer. Interaction of hydroxyl of alkali and amide group was also determined to give degree of deacetylation.

3.2.2.2 Titration

According to an acid–base titration of Avadi's method, 0.05 g of purified biopolymer was dissolved in 20.0 mL of 0.10 N HCl in an erlenmeyer flask. The titration was carried out by initially adding 1 mL of standardized solution of 0.10 N NaOH solution into the mixture solution and then pH was recorded. The titration was continued until the pH of the solution was constant. The titration curve contains two equivalent points which are related to excess of HCl and protonated

amino groups are plotted between pH of solution and the consumed volume of NaOH. Degree of deacetylation (DD) was calculated by using equation (3.1).

$$DD = \frac{16.1(Y-X)f}{w} \qquad \textbf{(Equation 3.1)}$$

Where X and Y are the consumed NaOH volume of each equivalent point.*f* is molarity of the NaOH solution and w is the initial purified biopolymer weight (Avadi *et al.*, 2004).

3.2.3 Modification of Purified Biopolymer with Arginine

3.2.3.1 Effect of Ratios of Coupling Agents to Biopolymer

Mole ratios of biopolymer to coupling agents were varied, 1:1, 1:2, and 1:3 while mole ratio of biopolymer/arginine kept constant at 1:1. Coupling agents were EDC.HCl and sulfo-NHS. When coupling agents ratio was one, it means used 1 mole of EDC.HCl and 1 mole of sulfo-NHS, and ratio was two which mean 2 mole of EDC.HCl and 2 mole of sulfo-NHS. For synthesis of modified biopolymer using coupling agents, 0.25 g of biopolymer was dissolved in 200 mL of MES buffer (25 mM) and the solution was adjusted for pH to 5.0 with an addition of 0.1 M HCl solution and left for activated arginine. For activated arginine, the carboxyl group of 0.271 g arginine was activated for 2 h in 300 mL of MES buffer with coupling agents (EDC.HCl and sulfo-NHS) at a fixed molar ratio of biopolymer/arginine/EDC.HCl/ sulfo-NHS of 1:1:1:1. The resultant mixture was prepared by adding the activated arginine solution into the biopolymer solution and the reaction was allowed to take place at ambient temperature with stirring for 48 h. The final product was opaque solution, which contained white precipitate). The reaction of final product was quenched by adding of 0.259 g hydroxylamine at a fixed molar ratio of biopolymer/hydroxylamine of 1:1, and the mixture pH was adjusted to 8.0 with 0.1 M NaOH solution. The collected products were taken to stirred cell for dialyzing. Before dialyzing process, a part of the permeate solution (clear solution) was taken for detected degree of substitution of arginine in biopolymer via unreacted arginine with HPLC. The collected products were dialyzed

using distilled water in a stirred cell with molecular weight cut off (MWCO) of 1000 to eliminate molecules lower than 1,000 dalton, i.e. unreacted arginine and salt, coupling agents, until the pH of permeate solution was neutral (pH7). After dialyzing, the retentate solution, which was still opaque containing unreacted biopolymer and biopolymer-arginine was freeze-dried (the solution was frozen and then the pressure of the system was reduced to allow the frozen water in the material to sublimate) (Xiao *et al.,* 2011). The final product was solid, named biopolymer-arginine which was taken to be analyzed by FT-IR and CHN elemental analyzer.

For the modified biopolymer without coupling agents, the procedure repeated the previous description, except no coupling agent (EDC.HCl and sulfo-NHS) was added. Summary of synthesis of modified biopolymer with/without coupling agents is shown in Table 3.1

Table 3.1 Effect of coupling agents on biopolymer (PB)/arginine (AR)/coupling agents (CA) at mole ratio of 1:1:0, 1:1:1, 1:1:2 and 1:1:3, respectively at reaction time 48 h

Mole ratio of PB/AR/CA	**Weight of chemical used (g)**				**Reaction time (h)**
	PB	**AR**	**EDC.HCl**	**Sulfo-NHS**	
1:1:0	0.2534	0.2712	-	-	48
1:1:1	0.2518	0.2743	0.3027	0.3391	48
1:1:2	0.2540	0.2775	0.5950	0.6744	48
1:1:3	0.2532	0.2719	0.8932	1.0102	48

In detail, calculations of chemical explain in Appendix C.

3.2.3.2 Effects of Reaction Time

The reaction times between biopolymer and arginine of 24, 48 and 72 h were varied while kept mole ratio of biopolymer/arginine/coupling agents was 1:1:1. The reaction condition was composed of 0.25 g of purified biopolymer being dissolved in 100 mL of MES buffer (25 mM) adjusted pH to 5.0 with addition of 0.1 M HCl solution and 0.271 g arginine activated for 2 h by sulfo-

NHS 0.337 g/EDC.HCl 0.298 g in MES buffer 200 mL (EDC.HCl:sulfo-NHS:arginine of 1:1:1). Summary of the effects of ratios of arginine to biopolymer is shown in Table 3.2. The resultant mixtures were allowed to react at ambient temperature with stirring for 24, 48 and 72 h. The reactions were quenched by adding hydroxylamine 0.259 g at fixed molar ratio of modified biopolymer/hydroxylamine of 1:1, and the pH of reaction systems was adjusted to 8.0 with addition of a 0.1 M NaOH solution. The collected products were dialyzed against distilled water until the pH of permeate was neutral (pH7) in a stirred cell (MWCO= 1000) and freeze-drying the retentate.

Table 3.2 Effect of reaction time on biopolymer (PB)/arginine (AR)/coupling agents (CA) of 1:1:1

Mol ratio of	**Weight of chemical used (g)**				**Reaction time**
PB/AR/CA	**PB**	**AR**	**EDC.HCl**	**Sulfo-NHS**	**(h)**
1:1:1	0.2535	0.2712	0.2980	0.3340	24
1:1:1	0.2518	0.2743	0.3027	0.3391	48
1:1:1	0.2541	0.2738	0.2960	0.3372	72

3.2.3.3 Effect of Ratios of Arginine to Biopolymer

The mole ratio of arginine was varied against biopolymer, 1:1, 2:1 and 3:1. At a fixed condition of the molar ratio of biopolymer/EDC.HCl/sulfo-NHS/arginine of 1:1:1 with reaction time 72h, the condition was composed of biopolymer (0.25g) being dissolved in 100 mL of MES buffer (25 mM) at pH 5.0, reacting with arginine, being activated by sulfo-NHS and EDC.HCl in 200 mL MES buffer. Summary of the effects of ratios of arginine to biopolymer is shown in Table 3.3. The resultant mixtures were allowed to react at ambient temperature with stirring for 72 h. The reactions were quenched by adding hydroxylamine 0.259 g at fixed molar ratio of modified biopolymer/hydroxylamine of 1:1. and the pH of reaction systems was adjusted to 8.0 with addition of a 0.1 M NaOH solution. The collected products were dialyzed against distilled water until the

pH of permeate was neutral (pH7) in a stirred cell (MWCO= 1000) and freeze-drying the retentate.

Table 3.3 Effect of arginine on biopolymer (PB)/arginine (AR)/coupling agents (CA) of 1:1:1, 1:2:1 and 1:3:1 with reaction time 72 h

Mol ratio of PB/AR/CA	Weight of chemical used (g)				Reaction time (h)
	PB	AR	EDC.HCl	Sulfo-NHS	
1:1:1	0.2541	0.2738	0.2960	0.3372	72
1:2:1	0.2519	0.5414	0.3210	0.3343	72
1:3:2	0.2512	0.8110	0.2982	0.3447	72

3.2.4 Characterization of Biopolymer-Arginine

Fourier Infrared Spectrometer

Infrared spectra of biopolymer and biopolymer-arginine samples were recorded using FT-IR (Nicolet/NEXUS 670 FTIR, Massachusetts, USA). 1.2 mg of powder sample was dried in an oven for 2 days and mixed with KBr 40 mg, ratio of 3:100 (w/w) in a mortar. The sample and KBr was thoroughly ground until mixed well. The mixture was then pelletized by hydraulic pressure. The IR spectrum was obtained with DTGS detector using 4 resolutions and 64 numbers of scan. A background spectrum was obtained with a pure KBr pellet.

CHN Elemental Analyzer

Degrees of substitution (DS) of biopolymer-arginine were determined by CHN elemental analyzer (LECO/Truspec CHNSO Model, Michigan, USA). 0.05 g of powder sample was dried in an oven for 2 days and weigh into tin foil and sealed. The tin foil sample was measured. A standard was obtained with EDTA standard.

High-Performance Liquid Chromatography

To confirm degrees of substitution (%DS) of biopolymer-arginine, the permeate solutions were determined by high-performance liquid chromatography (SHIMADZU LC-20AD, Kyoto, Japan). The unreacted arginine in the permeate

solution from the stirred cell was collected and analyzed. A made-up mobile phase was methanol/acetonitrile/water at the volume ratio of 45:45:10. HPLC was connected to photodiode array detector (PDA) which was set at λ= 190 nm for the detection., Column inertsil ODS-3 (5 um - particle size) with dimension of 250x4.6 mm was operated at 40 °C and the separation at the flow rate of 0.9 mL/min. The peak area of sample was measured and compared with the standard curve of arginine in which the peak areas of arginine was plotted against concentration. 0.1%, 0.5%, 1%, 2%, 3%, 4%, and 5% (%wt/v).

Thermo Gravimatric Analyzer

Thermal degradation of samples was monitored using a thermal analyzer (METTLER TOLEDO - TGA/DSC Mode, Ohio,USA).0.03 g of sample was heated in alumina pan from ambient temperature (25 °C) to 600 °C at a constant heating rate of 10 °C/min under a nitrogen atmosphere.