CHAPTER IV
RESULTS AND DISCUSSION

4.1 Purification of Biopolymerand Characterization

The aim for purification of biopolymer is to increase degree of deacetylation (% DD) by removing acetyl group from the biopoylymer structure and to increase the amine functional group that can react with CO_2. A degree of deacetylation of purified biopolymer determined by FT-IR and titration method are shown in Figure 4.1 and Figure 4.2, respectively. Figure 4.1 compares the infrared spectra of original biopolymer with the purified biopolymer. Figure 4.2 shows titration curve between pH and volume of NaOH used.

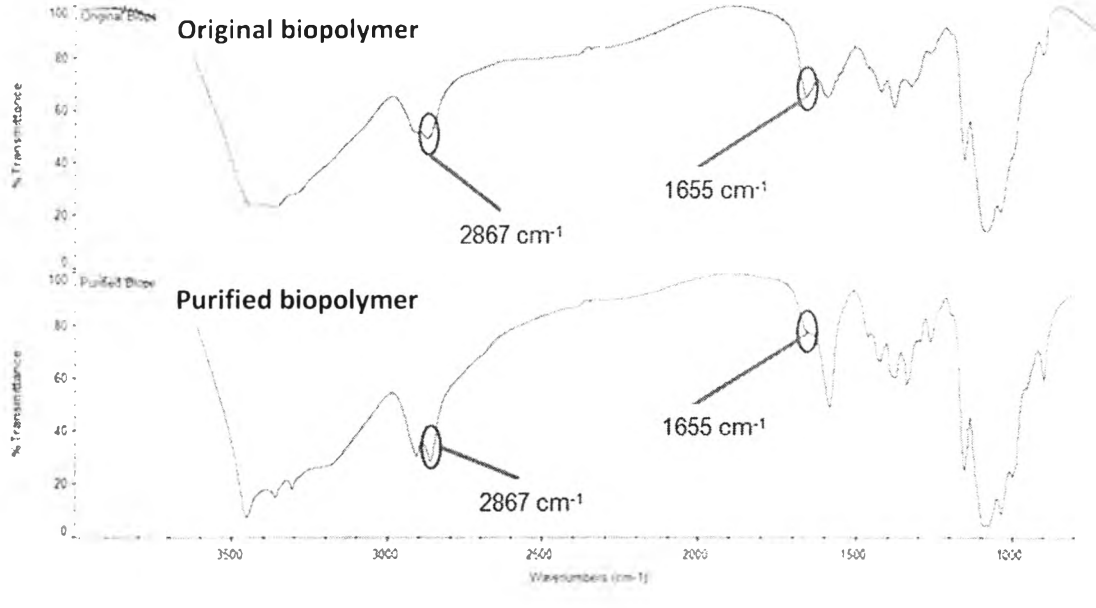

Figure 4.1 Comparison of infrared spectra of original biopolymer and purified biopolymer.

The purified biopolymer (PB) gave spectrum quite similar to the original biopolymer. However, the decrease of characteristic peak intensity at 1655 cm^{-1} corresponding to amide band I, which was used to determine the residual N-acetylglucosamine (CONH), was observed for highly purified biopolymer. Second

was the relation of the absorbance ratio of the amide band I at 1655 cm^{-1} to the CH stretching band at 2867 cm^{-1} (Miya *et al.*, 1980). The result is 96.80% with 0.358% standard deviation. Calculation was provided in greater detail in Appendix A.

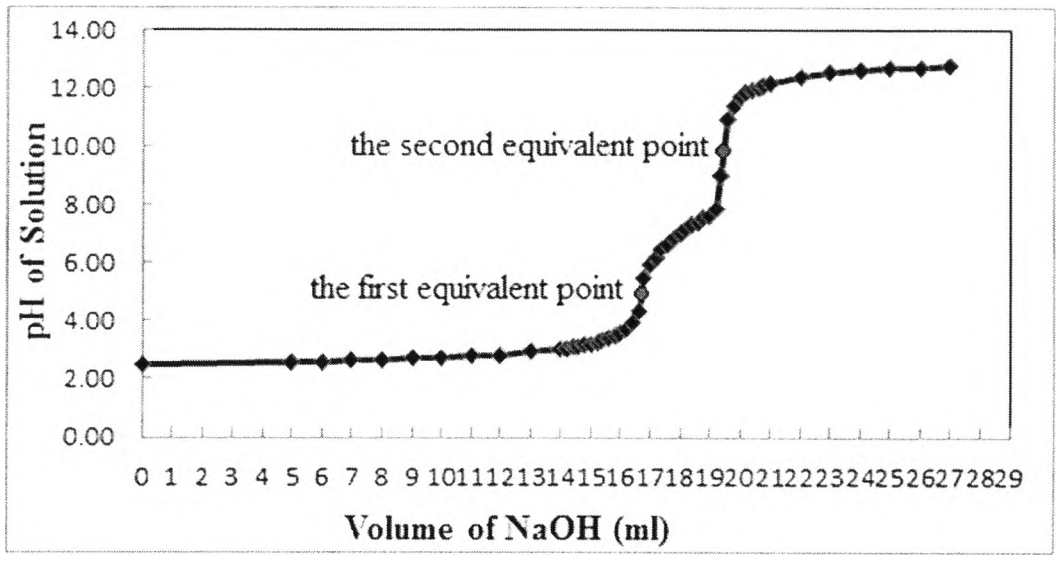

Figure 4.2 Titration curve for determining degree of deacetylation of purified biopolymer.

To confirm degree of deacetylation of purified biopolymer, titration was used. There are two equivalent points from titration curve as shown in Figure 4.2. Those are related to excess of HCl and protonated amino groups. The first equivalent point is due to neutralization of the excess HCl with NaOH solution and the second equivalent point is due to displacement of HCl bound to the primary amino groups of biopolymer. The correct position of two equivalent points can be determined by linear extrapolation of the adjacent portions of the titration curve. The degree of deacetylation calculated from the two equivalent points using Equation 3.1 was 96.05% with 0.066% standard deviation. Calculation was provided in greater detail in Appendix B.

The result from FT-IR method is similar likely with result from titration method. It can confirm degree of deacetylation of purified biopolymer was

96.43% with 0.53% standard deviation. The calculation was carried out according to the method proposed by Avadi *et al.*(2004).

4.2 Synthesis of Modified Biopolymer–Arginine and Characterization

The purpose of this study was to find the best way to bond the arginine onto biopolymer with highest degree of substitution. The effects for this study were effect of coupling agents, effect of reaction time, and effect of arginine. The reaction of biopolymer and arginine to form biopolymer-arginine showed in Figure 4.3.

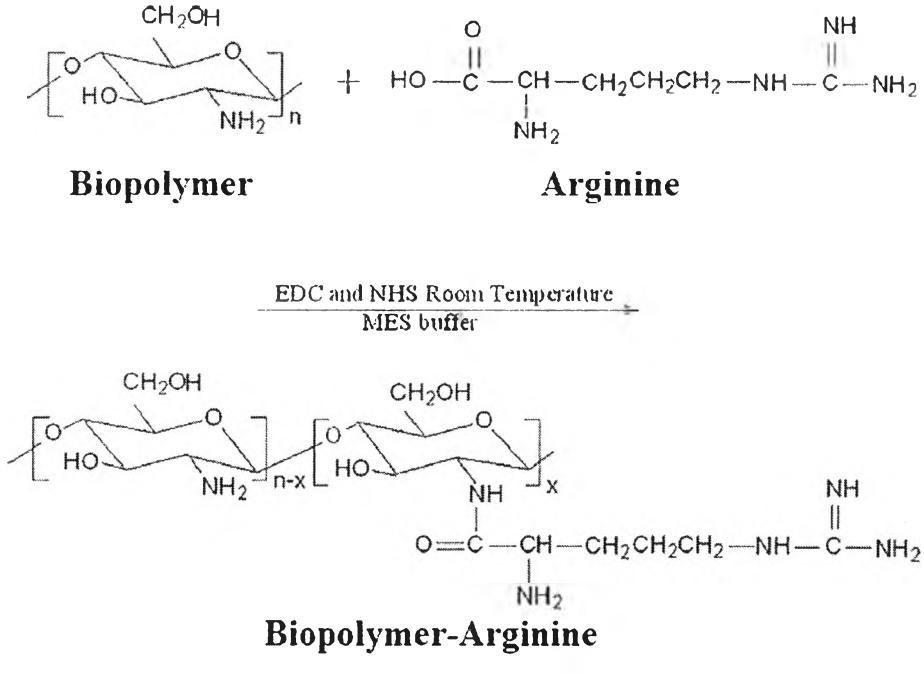

Figure 4.3 Reaction between biopolymer and arginine to form biopolymer-arginine using EDC.HCl and sulfo-NHS coupling agents in MES buffer solution.

4.2.1 Effect of Coupling Agents to Biopolymer

A role of coupling agents is to facilitating a change of carboxylic of arginine to amides as a similar reaction described by Hermanson, (2008) and shown in Figure 4.4. Two coupling agents were used, i.e. EDC.HCl and sulfo-NHS. EDC firstly reacts with carboxylic acid of arginine to form an active ester intermediate,

which is not stable. Sulfo-NHS replaces EDC in the intermediate, which makes the new intermediate more soluble, stable, and available for a reaction with amine.

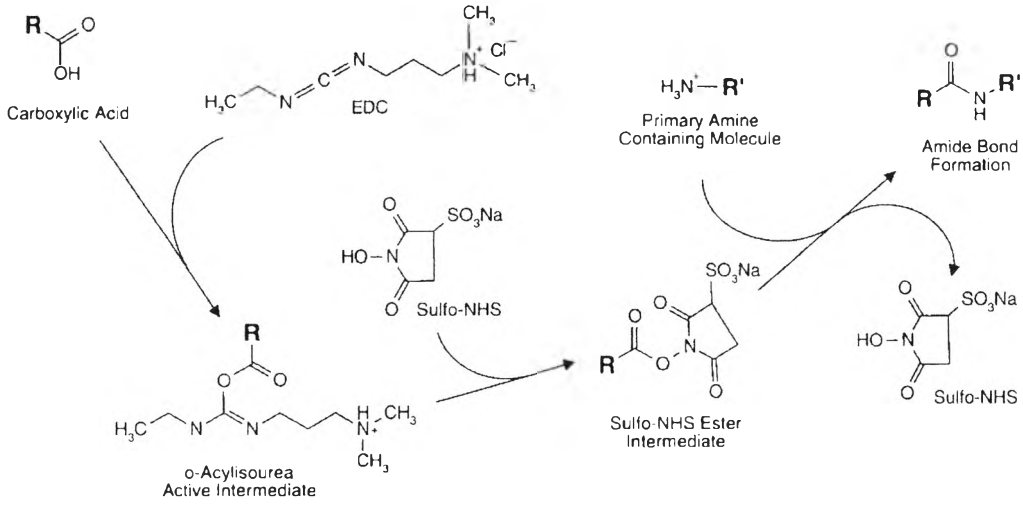

Figure 4.4 Reaction of EDC.HCl and sulfo-NHS to change carboxylic group to amide group (Hermanson, 2008).

The result of the reaction efficiency was compared between using and not using the coupling agents. With the coupling agents, a mole ratio of coupling agents of EDC.HCl to sulfo-NHS was 1:1. FTIR spectra are shown in Figure 4.5 for purified biopolymer and arginine and in Figure 4.6 for biopolymer- arginine with and without coupling agents in the reaction.

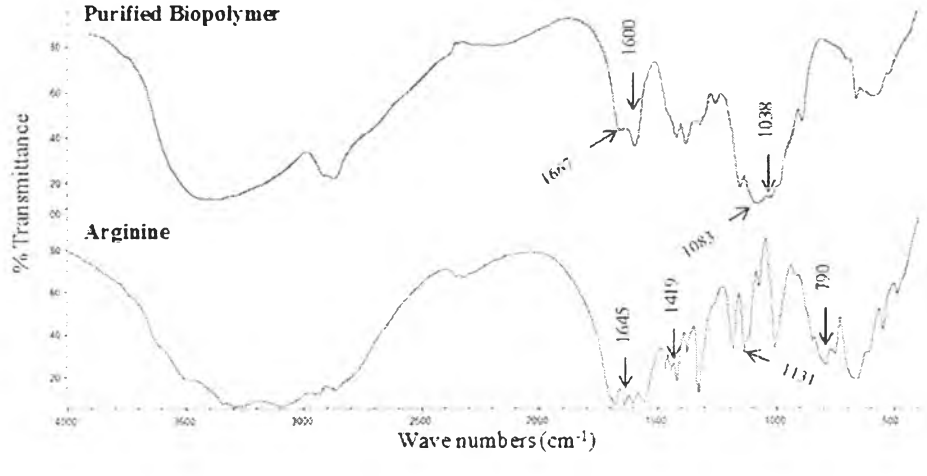

Figure 4.5 Infrared spectra of purified biopolymer and arginine.

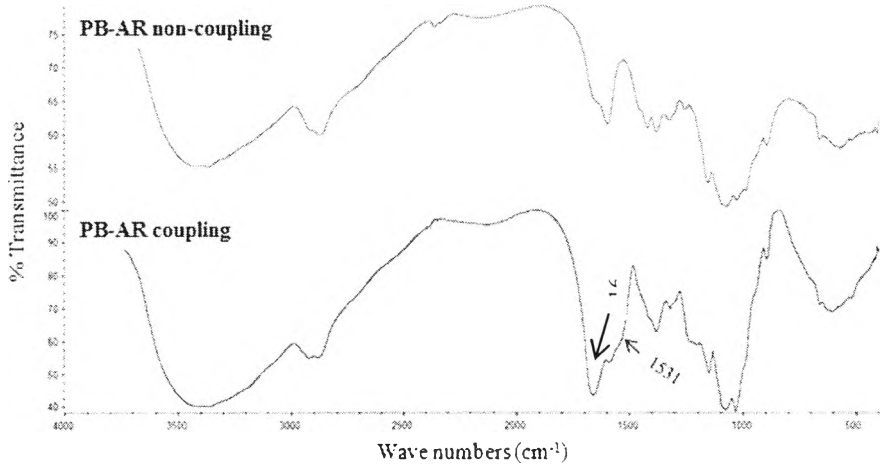

Figure 4.6 Infrared spectra of biopolymer-arginine with coupling agents and biopolymer-arginine with no coupling agents. A ratio of biopolymer/arginine/ coupling agents was 1:1:1.

FT-IR spectrum of biopolymer exhibits the characteristic bands of C=O stretch of the amide I at 1667 cm^{-1}, NH_2 bands of biopolymer at 1600 cm^{-1}, and C-O stretching vibrations of the pyranose ring at 1083-1038 cm^{-1}. For arginine, the absorption band at 1645 cm^{-1} is assigned to the guanido group, and the band at 1419 cm^{-1} is attributed to COO^- symmetric bending. The C-C-N asymmetric bending and COO^- scissoring modes are found at 1131 and 790 cm^{-1}, respectively. If biopolymer bond with arginine, it can be seen that there appear characteristic bands of arginine around 1630 cm^{-1} (guanido group). The new band around 1530 cm^{-1} is most likely due to an amide bond linking between biopolymer and arginine. In the presence of the coupling agents, the amide bond linkage and guanido group were observed, but the peak did not appear in the absence of coupling agents.

When the mole ratios of biopolymer/coupling agents was varied, 1:1, 1:2 and 1:3 while keeping mole ratio of biopolymer to arginine constant at 1:1, the infrared spectra are shown in Figure 4.7. The amide bond linkage was observed, however, the biopolymer to coupling agents ratio of 1:3 showed the lowest intensity as compared to the ratios 1:1, and 1:2.

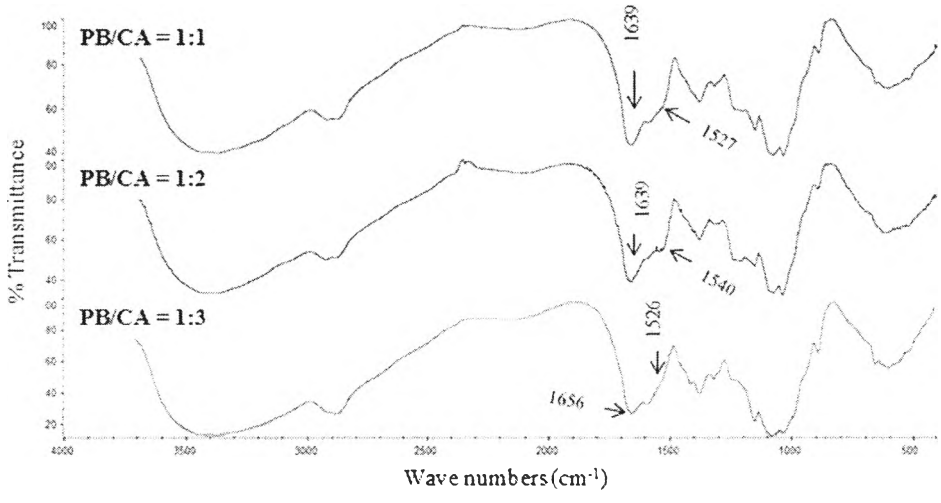

Figure 4.7 Infrared spectra of purified biopolymer-coupling agents used at ratio 1:1, 1:2 and 1:3, while biopolymer/arginine was kept constant at 1:1 at reaction time 48 h.

The unreacted arginine remaining in the solution in which the mole ratios of biopolymer/coupling agents were varied, 1:1, 1:2 and 1:3 while keeping mole ratio of biopolymer to arginine constant to 1:1 at reaction time 48 h, were analyzed by HPLC. Arginine peak were detected with retention time 3.5 sec. The percentage of arginine was determined by comparing peak area with the arginine standards curve which was plots of peak area against the moles of standard arginine as shown in Figure 4.8. The HPLC chromatograms of 4% wt/v arginine standard and the unreacted arginine in the solution are shown in Figure 4.9, and 4.10, respectively. The degrees of substitution are summarized in Table 4.1. The ratio of biopolymer (PB) to arginine (AR) to coupling agents (CA) of 1:1:1 at reaction time 48 h give the highest degree of substitution (34.84%). The greater details of calculation showed in appendix D.

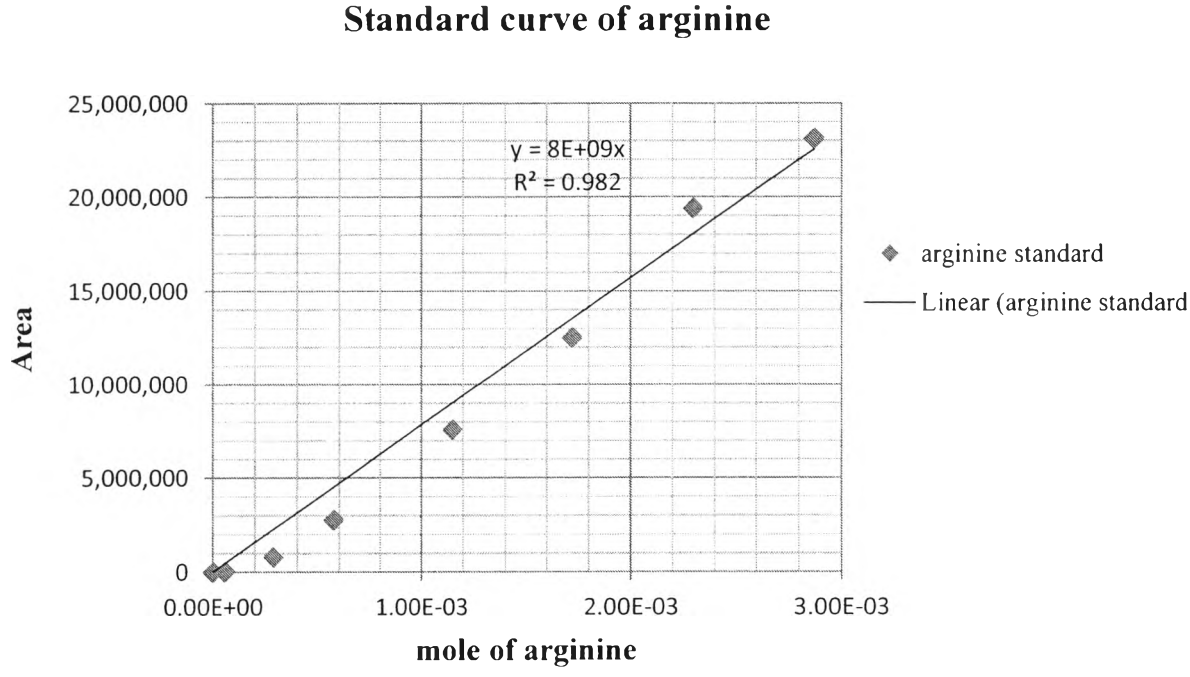

Figure 4.8 Standard calibration curve of arginine.

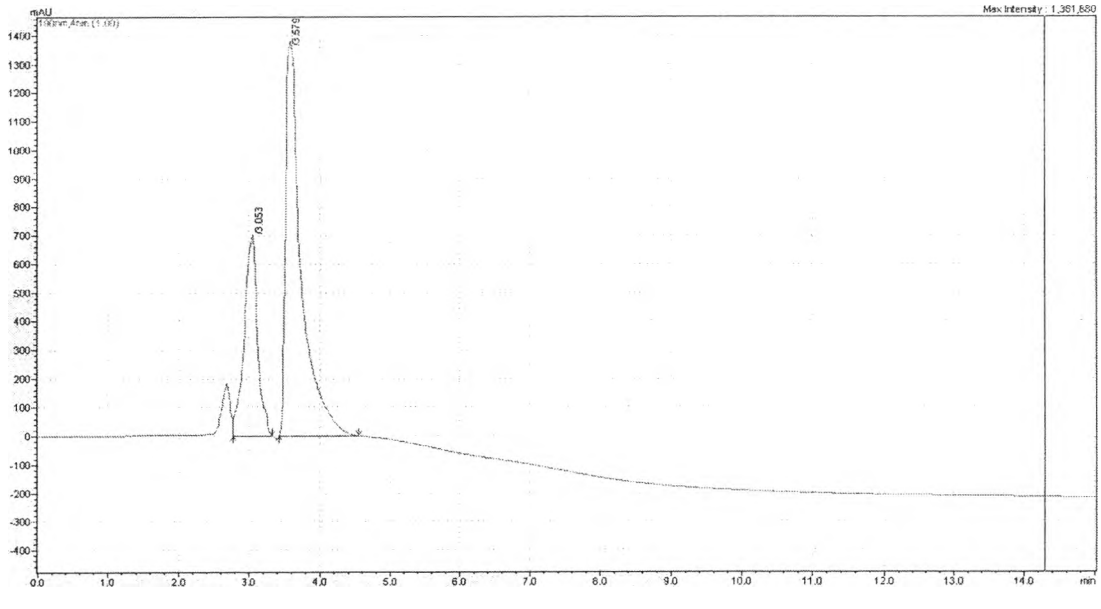

Figure 4.9 HPLC chromatogram of 4 (%wt/v) arginine standard.

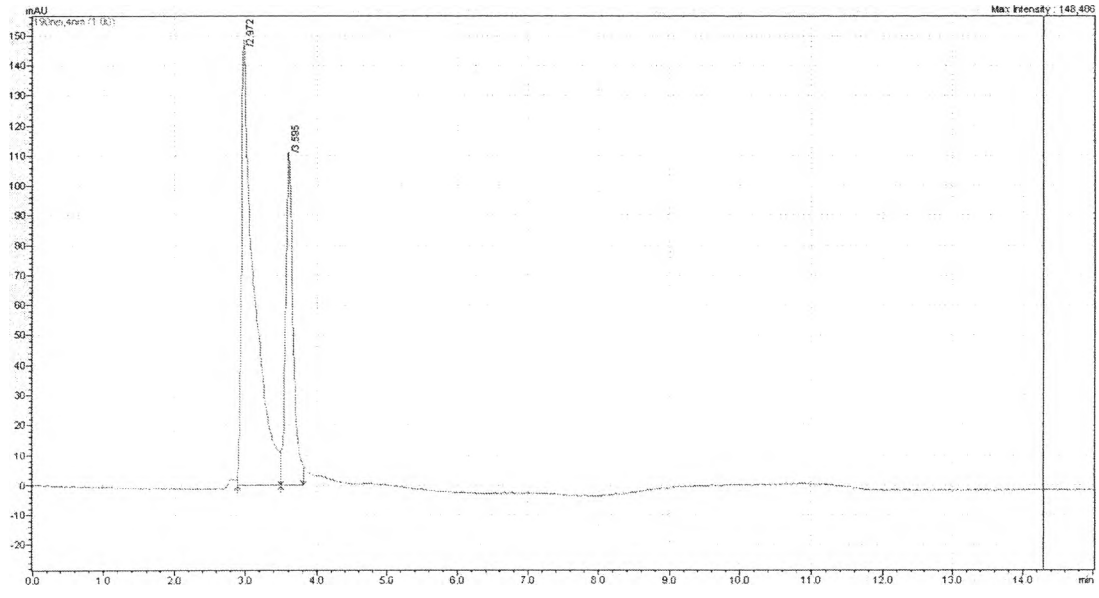

Figure 4.10 HPLC chromatogram of the product from the mole ratios of biopolymer/coupling agents was varied, 1:2 while keeping mole ratio of biopolymer to arginine constant at 1:1 at reaction time 48 h.

Table 4.1 Degree of substitution of arginine in biopolymer-arginine at various mole ratio of coupling agents to biopolymer and reaction time 48 h

Molar ratio			Reaction time (h)	%DS
PB	AR	CA		
1	1	1	48	34.84
1	1	2	48	7.04
1	1	3	48	5.21

4.2.2 Effect of Reaction Time

The purpose of this study is to investigate effect of reaction time to find the most suitable reaction time that gives the highest degree of substitution. For varying reaction time, the condition used at ratio biopolymer/arginine/coupling agents as 1:1:1, respectively. Three reaction times studied were; 24 h, 48 h and 72 h. The results from the infrared spectra are shown in Figure 4.11.

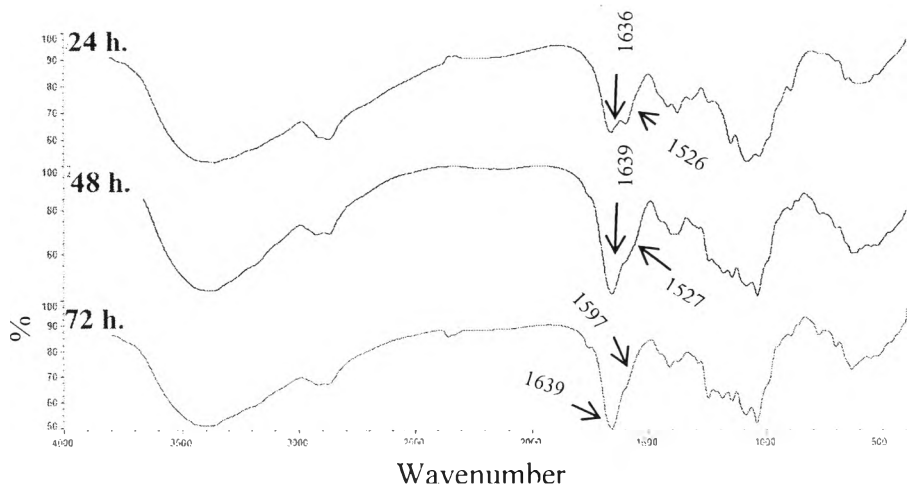

Figure 4.11 Infrared spectra of biopolymer-arginine at reaction time 24 h, 48 h and 72 h, while the ratio of biopolymer/arginine/coupling agents was constant at 1:1:1.

FT-IR spectra of biopolymer-arginine at reaction time 24 h, 48 h and 72 h are in Figure 4.11. The characteristic band of arginine around 1630 cm^{-1} and the amide linkage band around 1530 cm^{-1} were observed. The guanido group of arginine at around 1630 cm^{-1} gave higher intensity for longer reaction time.

The HPLC analysis gave the degrees of substitution as shown in Table 4.2. The ratio of biopolymer (PB) to arginine (AR) to coupling agents (CA) of 1:1:1 and at reaction time of 72 h, the highest degree of substitution of 76.41% was obtained followed by 48 and 24 hours.

Table 4.2 Degree of substitution of arginine in biopolymer-arginine at various reaction time while biopolymer/arginine/coupling agents was kept constant at 1:1:1

Molar ratio			Reaction time (h)	%DS
PB	AR	CA		
1	1	1	24	29.10
1	1	1	48	34.89
1	1	1	72	76.41

4.2.3 Effect of Arginine to Biopolymer

Arginine in biopolymer/arginine/coupling agents was varied as 1:1:1, 1:2:1, and 1:3:1. The experiment was studied at reaction time 72 h. The infrared spectra are shown in Figure 4.12.

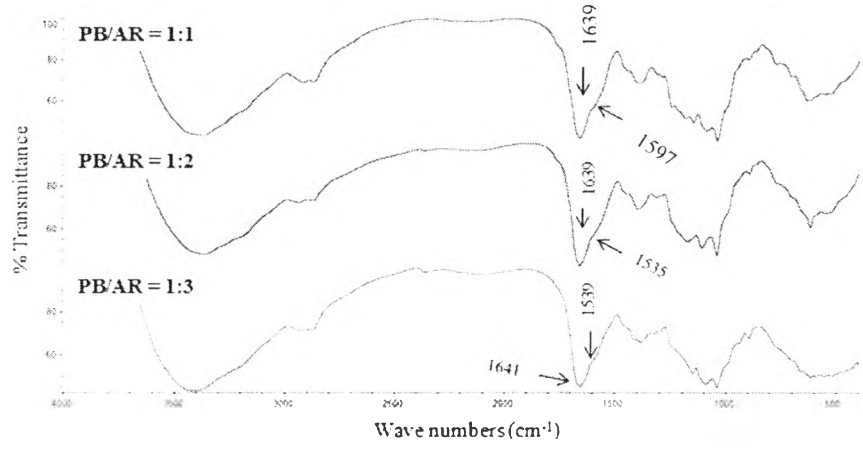

Figure 4.12 Infrared spectra of purified biopolymer-arginine used at ratio 1:1, 1:2 and 1:3, while biopolymer/coupling agents was kept constant at 1:1 at reaction time 72 h.

FT-IR spectra of purified biopolymer-arginine, there appear characteristic bands of arginine around 1630 cm^{-1} and the amide bond linking around 1530 cm^{-1}. The intensity at 1630 cm^{-1} of all ratios quite similar that can explained the degree of substitution of all ratios are not difference too much.

Degrees of substitution analyzed by HPLC method are shown in Table 4.3. The degree of substitution was independent with ratio of arginine because there was no different in degree of substitution among these three ratios. Although ratios of purified biopolymer (PB)/arginine (AR)/coupling agents (CA) used as 1:1:1 at reaction time 72 h give the highest degree of substitution (76.41%), it was very close to the value obtained from ratio 1:2, and 1:1: 1: 3 .

Table 4.3 Degree of substitution of biopolymer-arginine at various mole ratio of arginine and reaction time 72 h

Molar ratio			Reaction time (h)	%DS
PB	AR	CA		
1	1	1	72	76.41
1	2	1	72	69.69
1	3	1	72	72.25

In conclusion, the highest degree of substitution was obtained from product ratio of biopolymer/arginine/coupling agents 1:1:1 with reaction time 72 h. This condition gave degree of substitution 76.41%.

4.2.4 Thermo Gravimetric Analysis (TGA)

Thermo gravimetric analysis of biopolymer-arginine obtained from the highest degree of substitution, i.e.at the ratio of biopolymer (PB) to arginine (AR) to coupling agents (CA) of 1:1:1 at reaction time 72 h is shown in Figure 4.13.

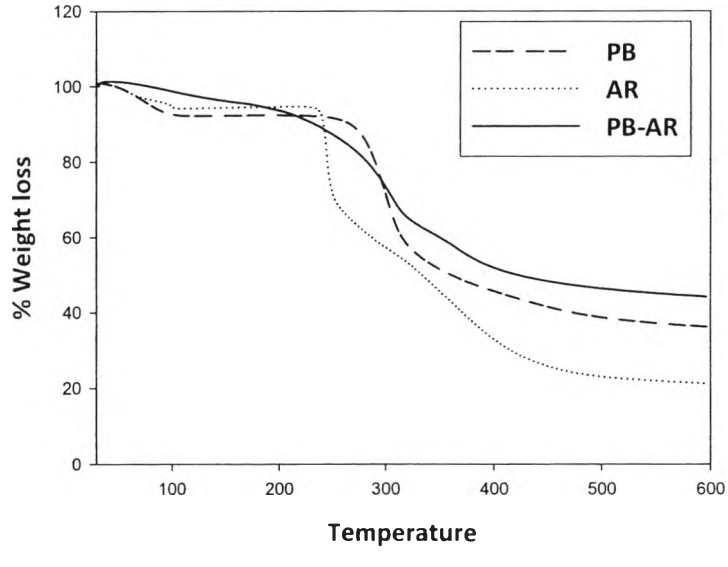

Figure 4.13 Thermograms of a) biopolymer, b) arginine and c) biopolymer-arginine with 76.29% degree of substitution, from 25°C-600°C at a constant heating rate of 10°C/min under a nitrogen atmosphere.

The thermogram of biopolymer (PB) indicated two weight loss stages. The first stage occurs between 50.83°C and 94.92°C (extrapolated temperature 73.03°C) was accounted for 7.81% loss in weight corresponding to the loss of bound water in the samples. The second one ranging from 278.46°C to 327.05°C (extrapolated temperature 298.96°C) was accounted for 53.5% loss in weight corresponding to depolymerisation of polymeric chains, decomposition of pyranose rings through dehydration and degradation of the samples (Bo Xiao *et al.*, 2011). For the thermogram of arginine (AR), it indicated three weight loss stages, the first stage which occurred between 37°C and 103°C (extrapolated temperature 56.25°C) was accounted for 5.63% loss in weight corresponding to the loss of loosely bound water in the samples. The second one ranging from 240.49°C to 249.14°C (extrapolated temperature 245.52°C) was accounted for 36.12% loss in weight corresponding to guanido group. The third one ranging from 331.86°C to 425.80°C (extrapolated temperature 359.50°C) was accounted for 35.74% loss in weight corresponding to degradation of main carbon chain (Mallik and Kar, 2005). For biopolymer-arginine (BP-AR) indicated two weight loss stages. The first stage ranging between 30.21°C and 150.34°C (extrapolated temperature 100.52°C) was accounted for 6.23% loss in weight corresponding to the evaporation of adsorbed and bound water. The second one started at 220°C and continues up to 400°C (extrapolated temperature 352.68°C) was accounted for about 39.8%, the degradation of biopolymer-arginine together with the breakage of the amide linkage of biopolymer-arginine (Bo Xiao *et al.*, 2011). Conclusion of decomposition temperature of biopolymer, arginine, and biopolymer-arginine showed in Table 4.4.

Table 4.4 Thermal decomposition temperature of biopolymer, arginine, and biopolymer-arginine

Sample	Decomposition temperature (°C)
Biopolymer	298.96
Arginine	245.52
Biopolymer-arginine	352.68