CHAPTER IV
RESULTS AND DISCUSSION

4.1 Preparation of Chitosan

Shrimp shells generally compose of three major components, which are chitin, calcium carbonate, and protein.

In this research, chitin was prepared from shells of *Penaeus merguiensis* shrimp by decalcification with hydrochloric acid solution and deproteinization with aqueous sodium hydroxide solution in order to remove calcium carbonate and protein, respectively. Chitin obtained was further deacetylation in 50% w/w of sodium hydroxide solution. Then chitosan with mainly reactive amino groups would be obtained.

Table 4.1 Yields of chitin produced from shrimp shell

Chitin Batch no.	Weight of shrimp shells (g)	Weight of chitin (g)	% Yield
1	360	207.81	57.725
2	360	206.43	57.342

Table 4.2 Conversion of chitosan from chitin

Chitosan Batch no.	Weight of chitin (g)	Weight of chitosan obtained (g)	% Yield
1	75	56.40	75.20
2	75	58.68	78.24

4.2 Characterization of Chitosan

4.2.1 Structural Characterization

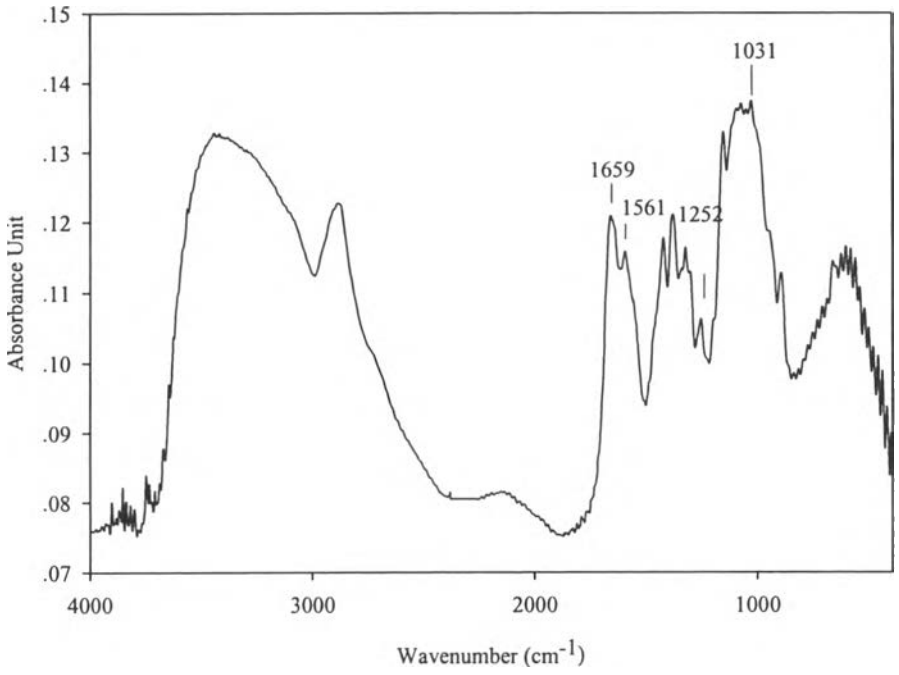

Figure 4.1 FTIR spectrum of chitosan.

Chitosan obtained was characterized by using FTIR. The FTIR spectrum of chitosan was shown in Figure 4.1 and the absorption frequencies of characteristic bands of chitosan were summarized in Table 4.3.

Table 4.3 FTIR characteristic absorption bands of chitosan

Frequencies (cm^{-1})	Assignment and Remarks
1659	C=O stretching
1561	NH deformation
1252	CN stretching
1080 and 1031	C-O stretching vibration

4.2.2 Degree of Deacetylation

The degree of deacetylation of chitosan was determined, based on an infrared spectroscopic measurement. From the step of preparation of chitosan, the deacetylation was performed repeatedly to achieve chitosan with higher degree of deacetylation. The degree of deacetylation of chitosan in each treatment was shown in Table 4.4.

Table 4.4 Degree of deacetylation of chitosan

No. of Treatment	% Degree of Deacetylation
1	83.580
2	92.456
3	93.163
4	93.546

4.2.3 Viscosity-Average Molecular Weight

The molecular weight of chitosan was determined by viscometric method. The molecular weight of chitosan was derived from its intrinsic viscosity. The plot of reduced viscosity ($\frac{\eta_{sp}}{C}$) and inherent viscosity (ln ($\frac{\eta_{rel}}{C}$) versus concentration of chitosan solution was shown in Figure 4.2.

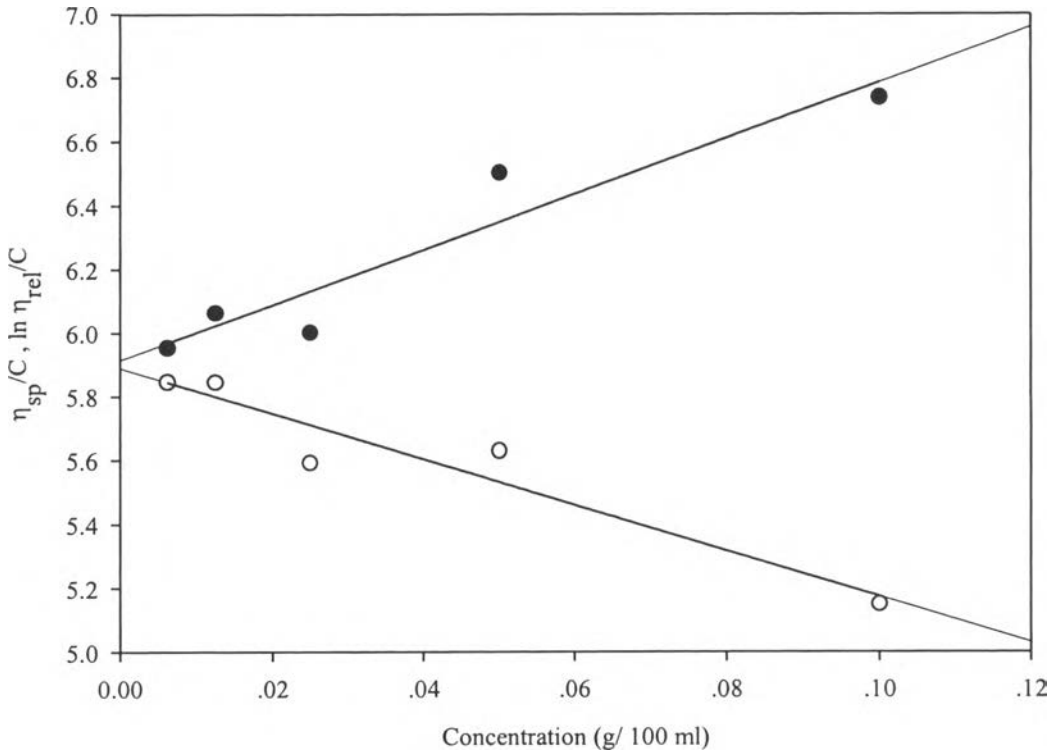

Figure 4.2 Plot of reduced viscosity and inherent viscosity versus concentration of chitosan solution.

From the calculation, the viscosity-average molecular weight of chitosan in each treatment were shown in Table 4.5.

Table 4.5 The viscosity-average molecular weight of chitosan

No. of Treatment	Viscosity-average molecular weight
1	822,697
2	619,108
3	549,136
4	538,098

4.3 Characterization of Alginate

4.3.1 Structural Characterization

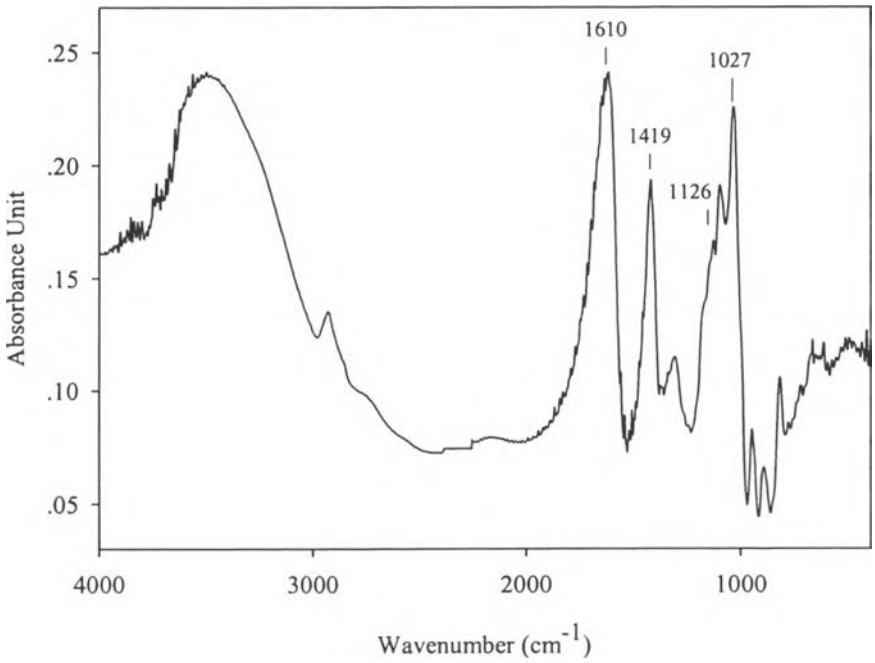

Figure 4.3 FTIR spectrum of alginate.

Alginate was characterized by using FTIR. The FTIR spectrum of alginate was shown in Figure 4.3 and the absorption frequencies of characteristic bands of alginate were summarized in Table 4.6.

Table 4.6 FTIR characteristic absorption bands of alginate

Frequencies (cm^{-1})	**Assignment and Remarks**
1610	symmetrical C=O stretching
1419	asymmetrical C=O stretching
1126	C-O stretching
1027	C-O-C stretching

4.3.2 Viscosity-Average Molecular Weight

The molecular weight of alginate was determined by viscometric method. The molecular weight of alginate was derived from its intrinsic viscosity. The plot of reduced viscosity ($\frac{\eta_{sp}}{C}$) and inherent viscosity (ln ($\frac{\eta_{rel}}{C}$) versus concentration of alginate solution was shown in Figure 4.4.

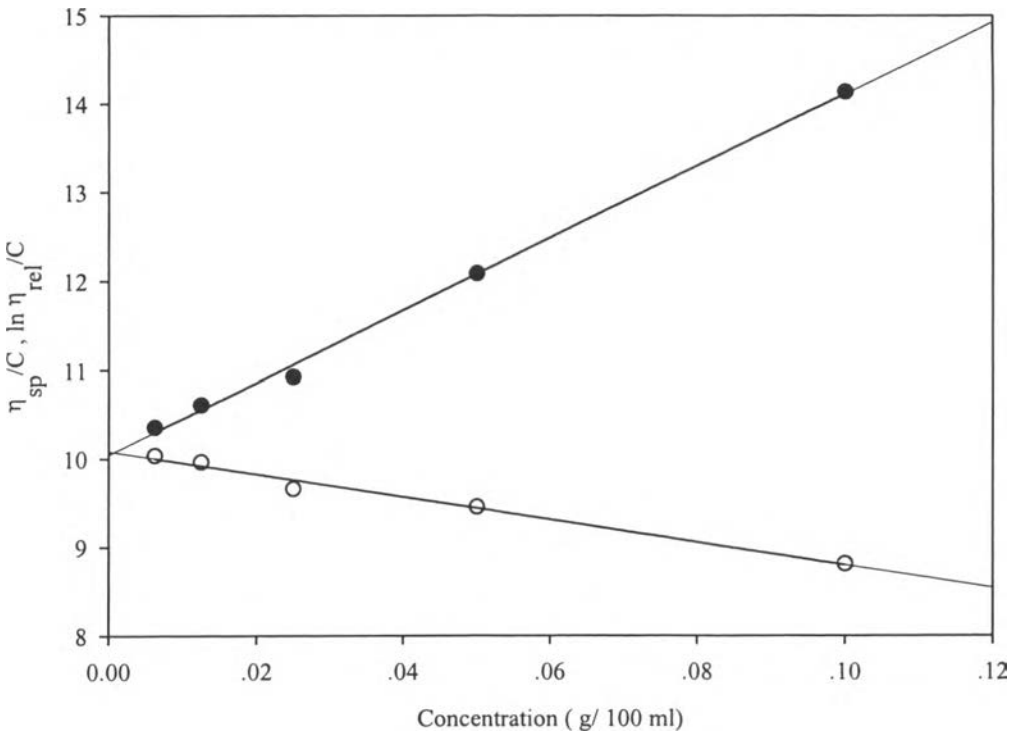

Figure 4.4 Plot of reduced viscosity and inherent viscosity versus concentration of alginate solution.

From Mark-Houwink equation, when a is 1.13 and K is 6.9×10^{-6}, the viscosity-average molecular weight of alginate obtained from the calculation was 284,981.

4.4 Characterization and Testing of Films

4.4.1 FTIR Spectra Characterization

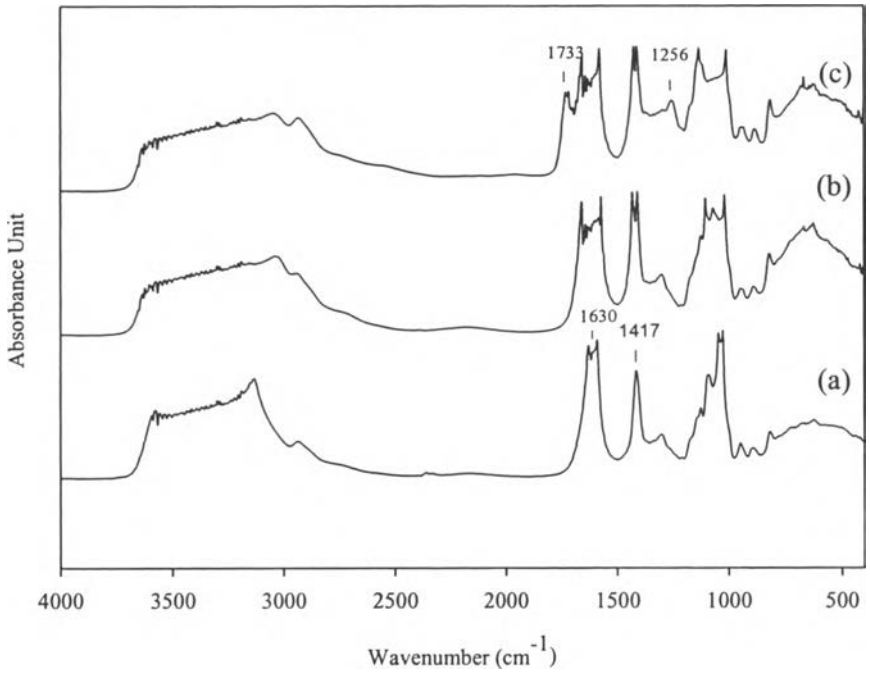

Figure 4.5 FTIR spectra of (a) pure alginate film, (b) calcium alginate film, and (c) chitosan-coated calcium alginate film.

The FTIR spectra of alginate, calcium alginate, and chitosan-coated calcium alginate films are shown in Figure 4.5. The characteristic peaks of alginate (Figure 4.5 (a)) at around 1630 and 1417 cm^{-1} were attributed to the asymmetrical and symmetrical stretching of COO^- groups, respectively (Zhang *et al.*, 2000). In the case of cross-linking the alginate films with calcium ions (Figure 4.5 (b)), there is no shift in characteristic peaks of alginate. The absorption bands of chitosan-coated calcium alginate film were shown in Figure 4.5 (c). The coated films had an absorption band at 1256 and 1733 cm^{-1}, referred to the C-N stretching of chitosan and the electrostatic interaction between $-COO^-$ groups of alginate and $-NH_2$ groups of chitosan (Zhang *et al.*, 2001). The FTIR results indicated that chitosan was coated on calcium alginate films.

4.4.2 Ninhydrin Staining

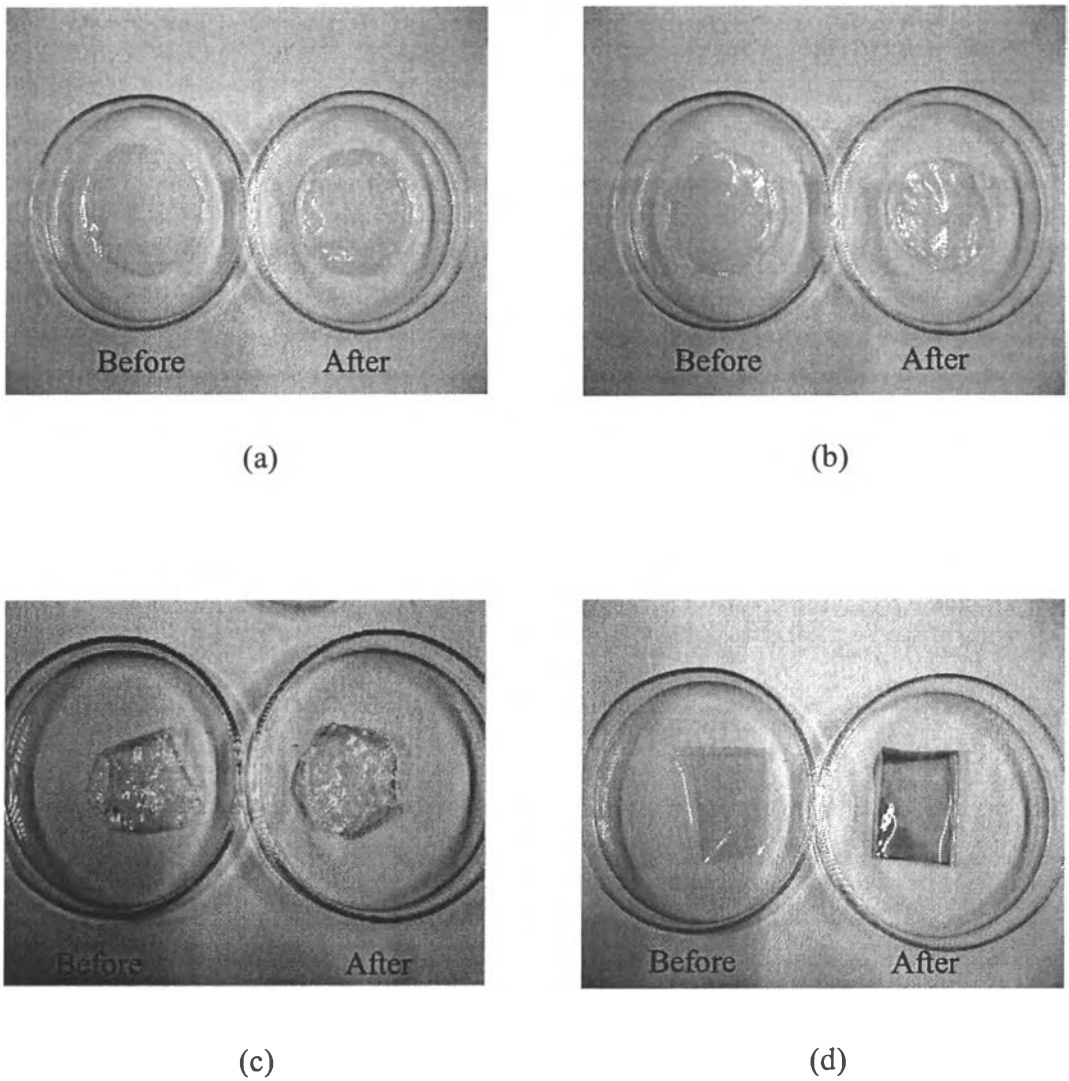

Figure 4.6 Ninhydrin test for (a) pure alginate film, (b) calcium alginate film, (c) chitosan-coated calcium alginate film and (d) chitosan film.

Figure 4.6 showed photographs of ninhydrin test on the films. Chitosan-coated calcium alginate films were tested to confirm the coating of chitosan on alginate film by using ninhydrin solution. Ninhydrin is a reagent for detecting amino groups. Chitosan film was used as a control. The result of ninhydrin test of chitosan films was shown in Figure 4.6 (d). The photographs showed that after spraying with ninhydrin, the color of chitosan films changed to purple color. The results of ninhydrin test of alginate film and calcium alginate film were shown in

Figure 4.6 (a) and (b), respectively. The photographs showed that after spraying with ninhydrin, no change in color of alginate and calcium alginate films was observed. The ninhydrin staining result for chitosan-coated calcium alginate films was shown in Figure 4.6 (c). The photographs showed that the color of the chitosan-coated calcium alginate films changed to light purple color after spraying with ninhydrin. The results confirmed that chitosan was coated on calcium alginate films.

4.4.3 Mechanical Properties

4.4.3.1 Tensile Strength

Tensile strengths of alginate, chitosan, calcium alginate and chitosan-coated calcium alginate films in dry state are shown in Figure 4.7. The results showed that alginate and chitosan films had a tensile strength of 74.40 and 70.18 MPa, respectively. When alginate films were cross-linked with calcium ions, calcium alginate films had higher tensile strength than alginate films. Chitosan-coated calcium alginate films had higher tensile strength than calcium alginate films. The results indicated that ionic cross-linking and chitosan coating played important roles in the improvement in tensile strength of the films. It is known that alginate can be cross-linked with Ca^{2+} through an ionic cross-linking to obtain alginate-calcium complex (Zhang *et* al., 2000). The calcium ions bind the adjacent alginate chains resulting in a restriction in mobility due to ionic cross-linking, leading to an increase in tensile strength. In the case of chitosan-coated calcium alginate films, the chitosan coating on calcium alginate films led to the electrostatic interaction between carboxyl groups of alginate and amino groups of chitosan, resulting in increasing of tensile strength of the films.

Tensile strengths of calcium alginate and chitosan-coated calcium alginate films in wet state are shown in Figure 4.8. The results of alginate and chitosan films are not shown in this Figure because both alginate and chitosan films dissolved in water. Tensile strength of chitosan-coated calcium alginate films was lower than that of calcium alginate films. Tensile strengths of calcium alginate and chitosan-coated calcium alginate films in the wet state decreased remarkably in comparison with those in dry state. Because alginate and chitosan are hydrophilic

polymers, the interaction between polymer chains within the films was diminished due to the interruption of surrounding water molecules, resulting in lower tensile strength.

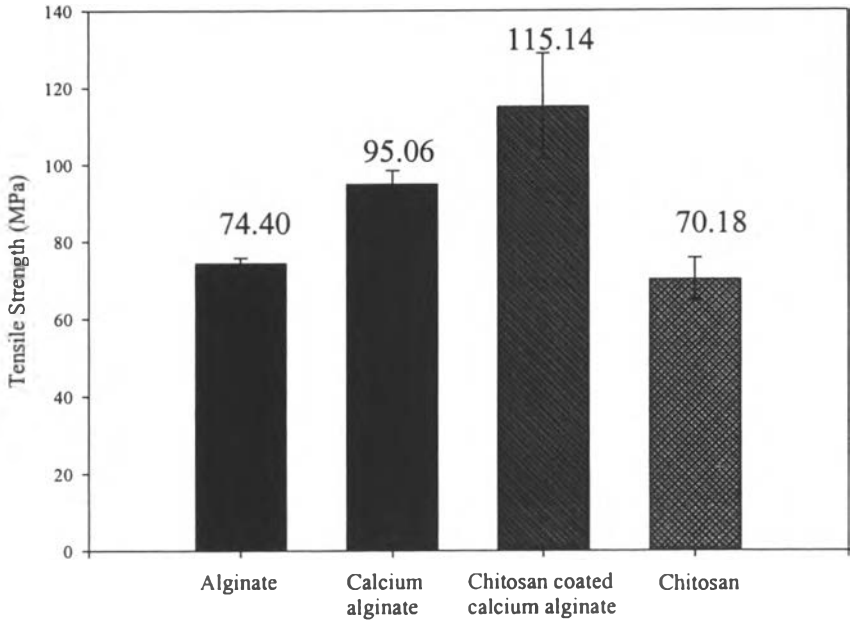

Figure 4.7 Tensile strength of the films in dry state.

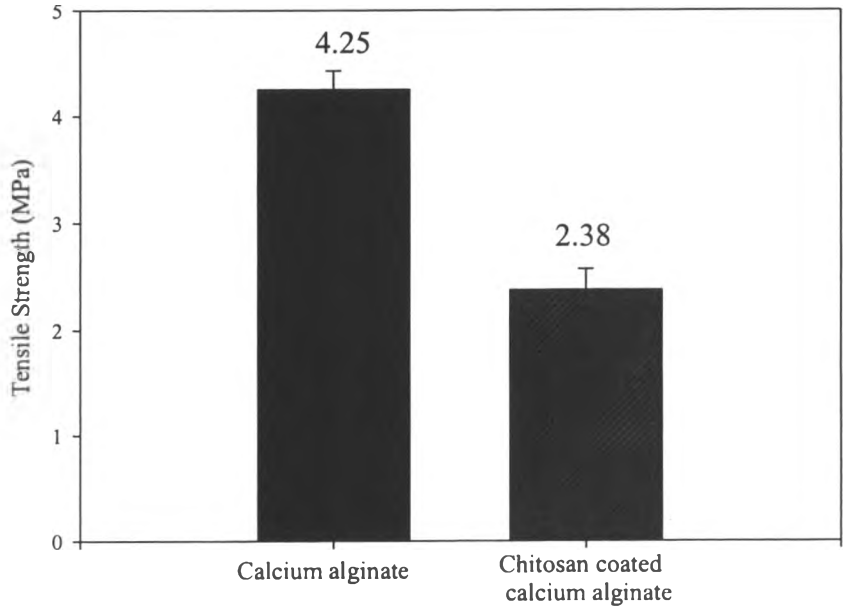

Figure 4.8 Tensile strength of the films in the wet state.

4.4.3.2 Young's Modulus

Young's modulus of alginate, chitosan, calcium alginate and chitosan coated calcium alginate films in dry state are shown in Figure 4.9. The Young's modulus of alginate and chitosan films were 1828.5 and 1219.4 MPa, respectively. Alginate films cross-linked with calcium ions became harder and had higher Young's modulus than alginate films. The chitosan-coated calcium alginate films were more brittle and had higher Young's modulus than calcium alginate films.

Figure 4.10 shows the elongation at break of calcium alginate and chitosan-coated calcium alginate films in wet state. Young's modulus of chitosan-coated calcium alginate films was lower than that of calcium alginate films. Young's modulus of the films in wet state decreased remarkably in comparison with those in dry state. It may be explained that the water molecules acted as a plasticizer resulting in lower Young's modulus.

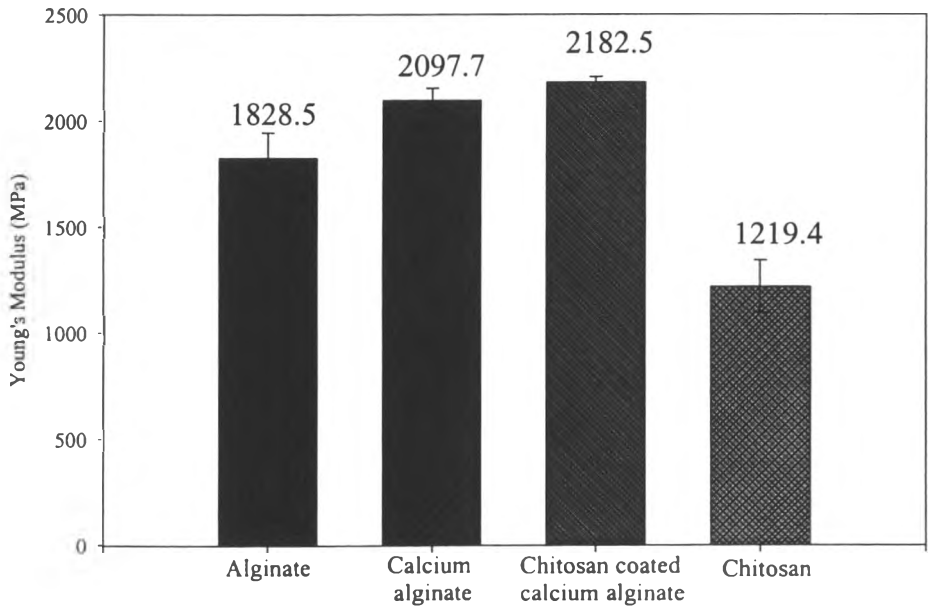

Figure 4.9 Young's modulus of the films in dry state.

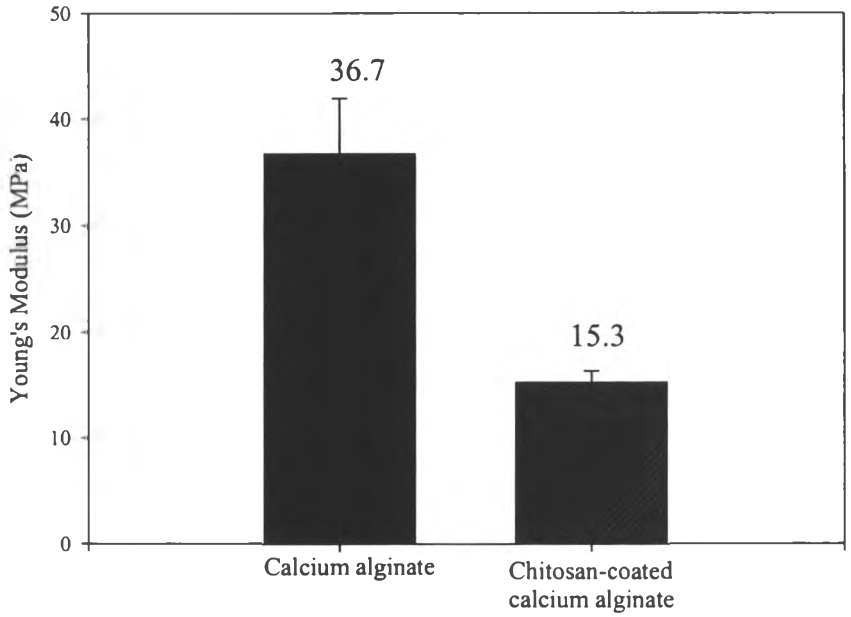

Figure 4.10 Young's modulus of the films in wet state.

4.4.3.3 Elongation at Break

Figure 4.11 shows the elongation at break of alginate, chitosan, calcium alginate and chitosan-coated calcium alginate films in dry state. The results showed that the elongation at break of alginate and chitosan films were 11.59 and 12.82%, respectively. However, alginate films cross-linked with calcium ions had lower elongation at break than alginate films. In addition chitosan-coated calcium alginate films had lower elongation at break than calcium alginate film. This may be explained that calcium ions bound the adjacent alginate chains, resulting in restriction in mobility of the polymer chains, leading to a decrease in elongation at break. When calcium alginate films were coated with chitosan, electrostatic interaction between carboxyl groups of alginate and amino groups of chitosan was formed, resulting in the decreasing of elongation at break.

The elongation at break of calcium alginate and chitosan-coated calcium alginate films in wet state are shown in Figure 4.12. The results of alginate and chitosan films are not shown because both alginate and chitosan films dissolved in water. The elongation at break of chitosan-coated calcium alginate film was higher than that of calcium alginate film. The elongation at break of the films in

wet state increased remarkably in comparison with those in dry state. It may be explained that the water molecules facilitate the chain mobility of the polymers resulting in higher elongation at break.

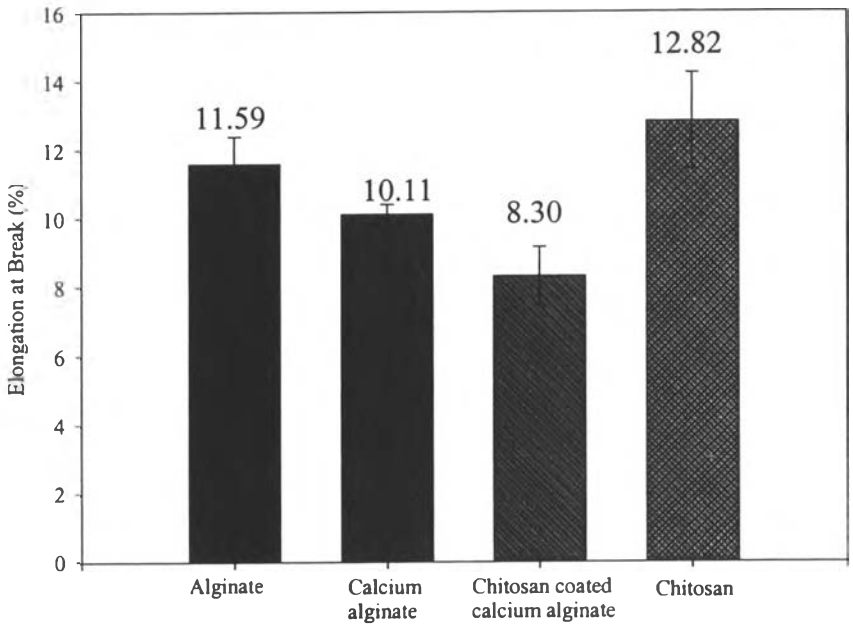

Figure 4.11 Elongation at break of the films in dry state.

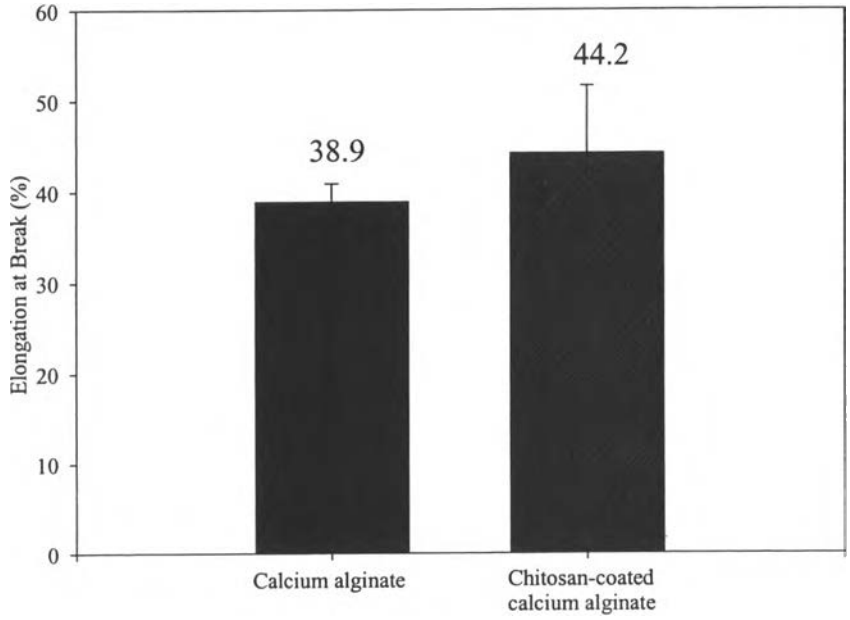

Figure 4.12 Elongation at break of the films in wet state.

4.4.4 Swelling Study

Swelling behavior of pure alginate and chitosan films could not be determined because the films were dissolved in water and buffer solutions.

4.4.4.1 Equilibrium Water Content (EWC)

The equilibrium water contents of calcium alginate and chitosan-coated calcium alginate films are shown in Figure 4.13. The films were immersed in distilled water for 24 h. The water absorption of the films increased remarkably within 15 min after immersing the films in distilled water. This led to the rapid increase in water contents in the films. After that water contents of the films gradually increased when the immersion time increased and became rather constant within 4 h. The results showed that chitosan-coated calcium alginate film had higher equilibrium water content than calcium alginate film. This may be due to the addition of the hydrophilicity of chitosan in the outer layer of films.

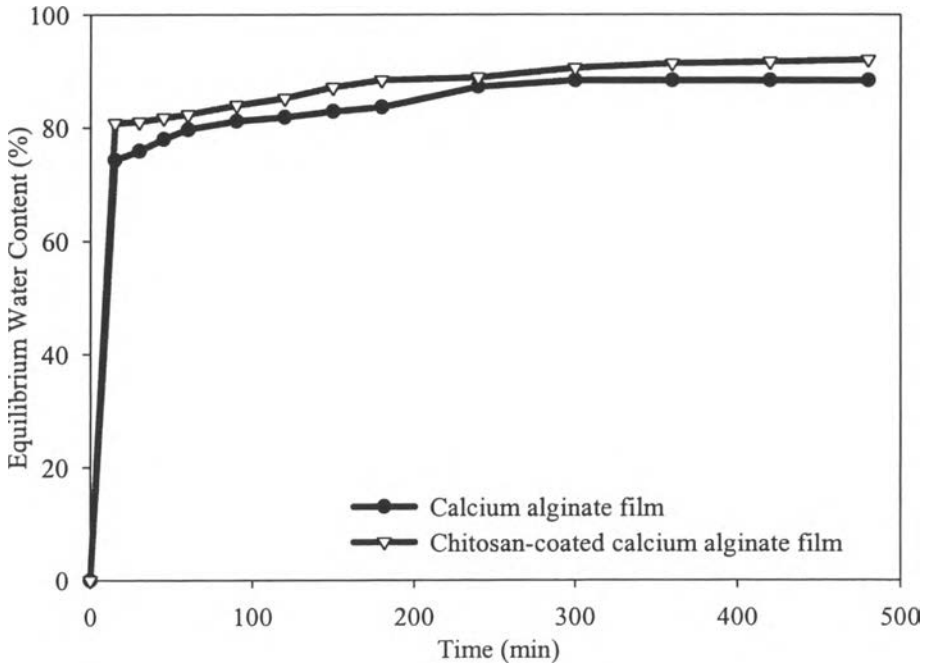

Figure 4.13 Equilibrium water content of calcium alginate and chitosan-coated calcium alginate films.

4.4.4.2 Effect of pH

In order to investigate pH sensitive performance, the effect of pH on the degree of swelling of calcium alginate and chitosan-coated calcium alginate films was determined and the results are shown in Figure 4.14. It was found that the degree of swelling of the films was dependent on pH values of the swelling medium. In addition, both of the films showed remarkable increasing in degree of swelling at pH higher than 4. The increase in degree of swelling at pH higher than 4 were attributed to the ionization of carboxyl groups (COOH) of alginate. Huguet *et al.* (1994) reported that alginate chains consists of mannuronic acid and guluronic acid units whose pKa are 3.38 and 3.65, respectively. Therefore, at pH higher than its pKa, the carboxyl groups of alginate were ionized to COO^- form. The chitosan-coated calcium alginate films showed higher degree of swelling than calcium alginate films. This may be due to the addition of the protonizable amino groups of chitosan at the outer layer of the films.

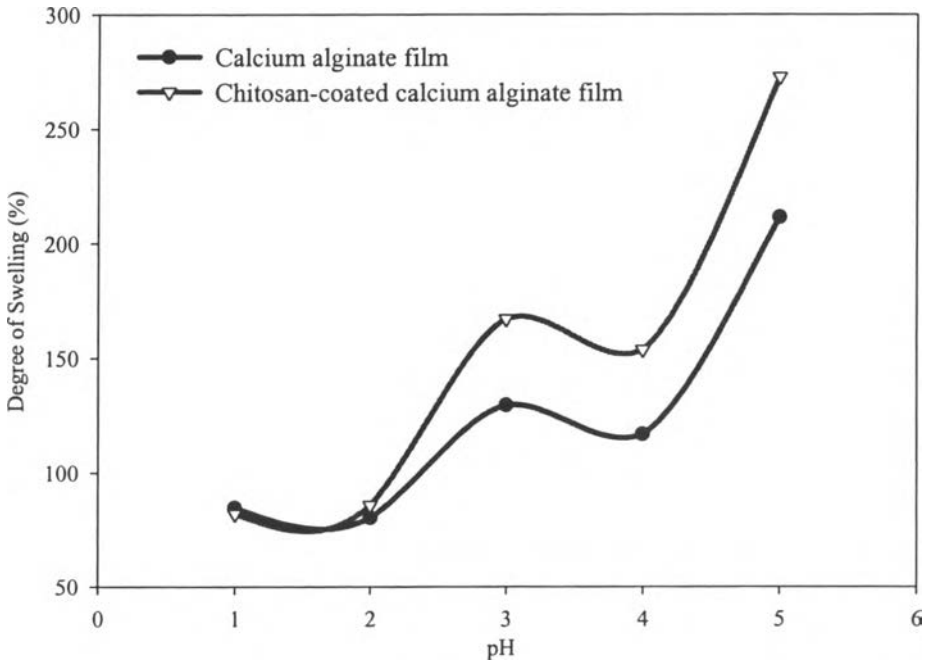

Figure 4.14 Swelling behavior of calcium alginate and chitosan-coated calcium alginate films as a function of pH.

Figure 4.15-4.17 shows the degree of swelling of calcium alginate and chitosan-coated calcium alginate films as a function of immersion time in buffer solutions pH 2, pH 5.5, and pH 7.2, respectively. It was found that the degrees of swelling of the films at pH 7.2 were higher than at pH 5.5 and pH 2, respectively. However, the degrees of swelling of calcium alginate and chitosan-coated calcium alginate films at pH 7.2 could be observed within 20 and 10 minutes, respectively, after that the dissolution of the films occurred. Chitosan-coated calcium alginate films showed higher degree of swelling than calcium alginate films. This may be due to the presence of chitosan at the outer layer of the films.

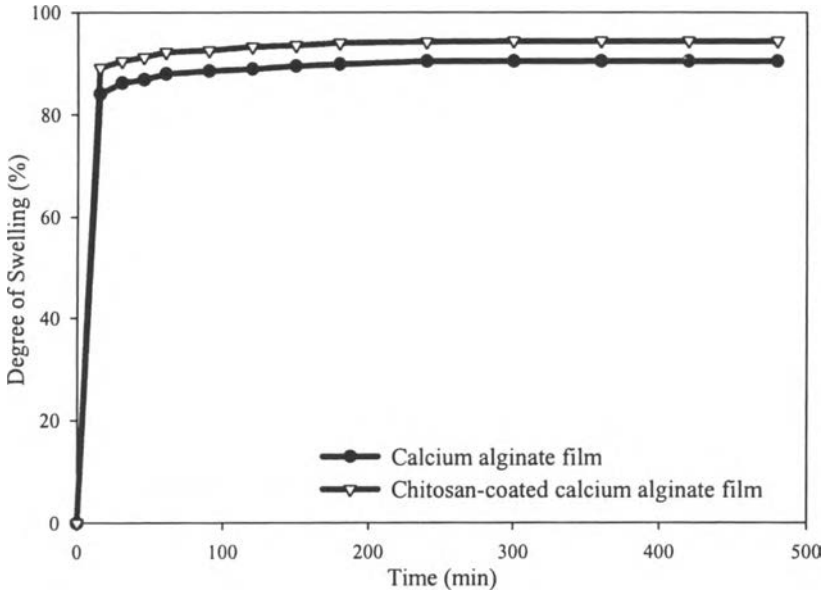

Figure 4.15 Swelling behavior of calcium alginate and chitosan-coated calcium alginate films at pH 2 as a function of immersion time.

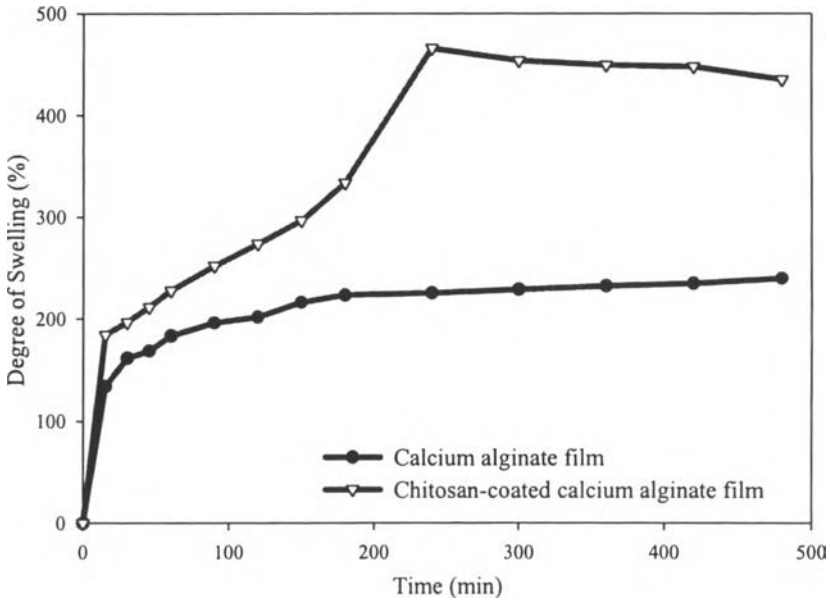

Figure 4.16 Swelling behavior of calcium alginate and chitosan-coated calcium alginate films at pH 5.5 as a function of immersion time.

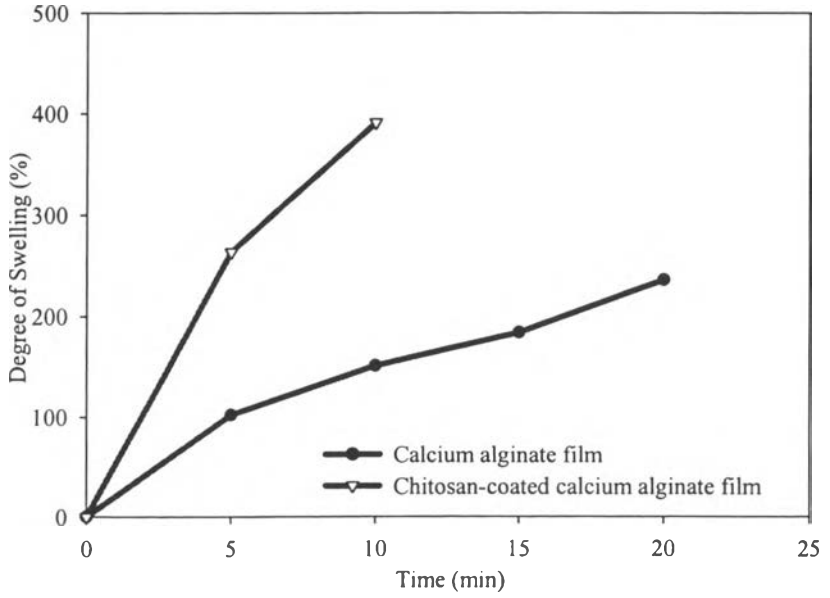

Figure 4.17 Swelling behavior of calcium alginate and chitosan-coated calcium alginate films at pH 7.2 as a function of immersion time.

4.4.5 Drug Release Studies

The drug release profiles of salicylic acid and theophylline from calcium alginate and chitosan-coated calcium alginate films as a function of immersion time in buffer solutions pH 2.0, pH 5.5 and pH 7.2 are illustrated in Figure 4.18-4.23. It was found that at pH 7.2, both calcium alginate and chitosan-coated calcium alginate films could not retain their shapes at this pH. The films dissolved in buffer solution pH 7.2 within 20 and 10 minutes for calcium alginate and chitosan-coated calcium alginate films, respectively. In the case of the release of salicylic acid from the films, there was a fast release occurred at the beginning of drug release profiles for both pH 2 and pH 5.5. The release of salicylic acid from calcium alginate and chitosan-coated calcium alginate films at pH 2 reached the equilibrium within 10 and 15 minutes, respectively. At pH 5.5, the salicylic acid released from calcium alginate and chitosan-coated calcium alginate films reached the equilibrium within 10 and 8 minutes, respectively. For the release of theophylline from the films, there was also a fast release occurring at the beginning for both pH 2 and pH 5.5. The release of theophylline from calcium alginate and chitosan-coated calcium alginate films at pH 2 reached the equilibrium within 10 and 6 minute, respectively. At pH 5.5, the release of theophylline from calcium alginate and chitosan-coated calcium alginate films reached the equilibrium within 10 and 15, respectively.

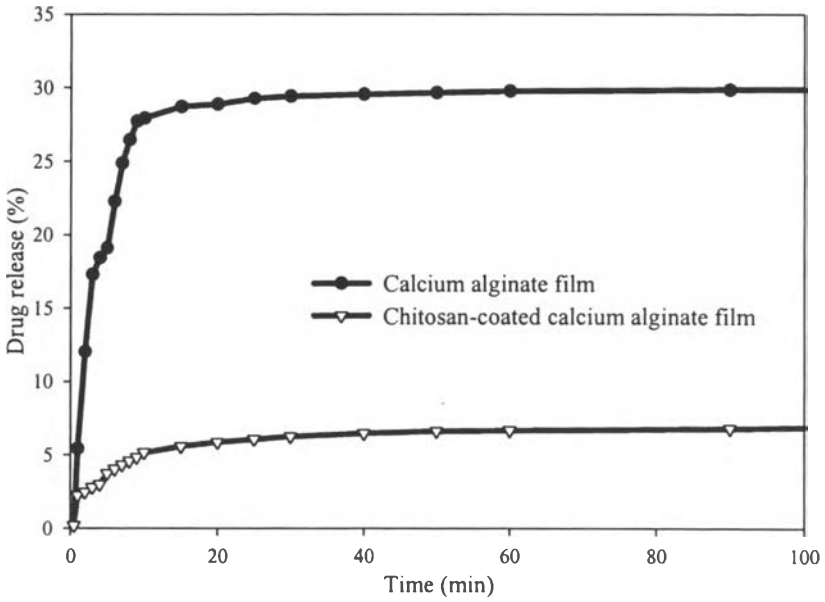

Figure 4.18 Salicylic acid release profile for calcium alginate and chitosan-coated calcium alginate films at pH 2.0.

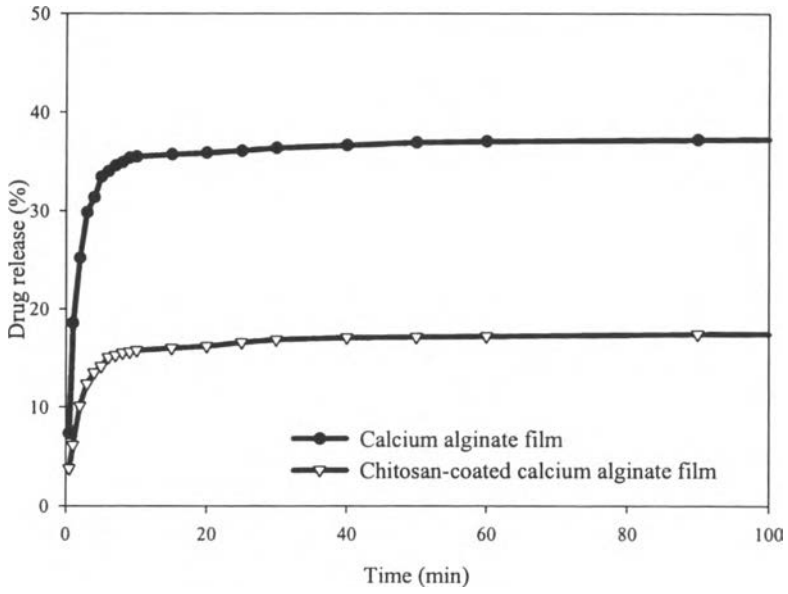

Figure 4.19 Salicylic acid release profile for calcium alginate and chitosan-coated calcium alginate films at pH 5.5.

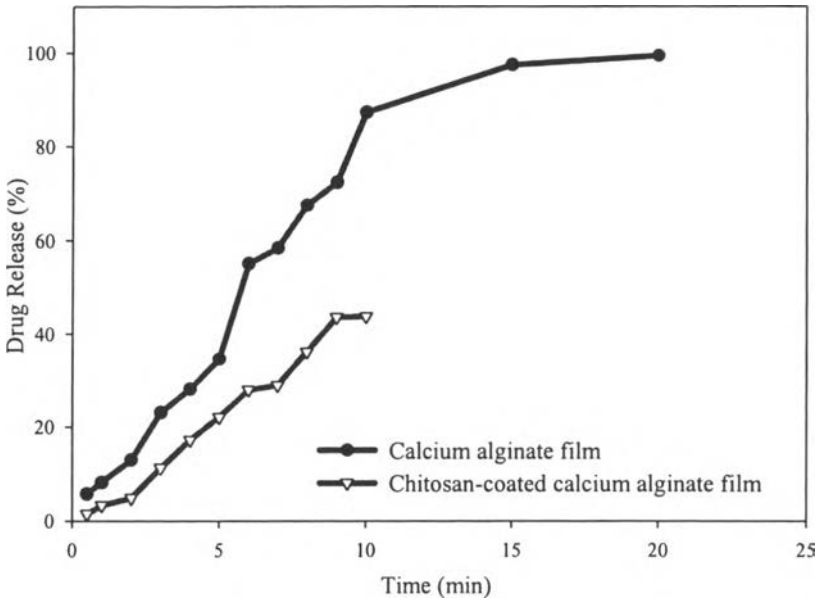

Figure 4.20 Salicylic acid release profile for calcium alginate and chitosan-coated calcium alginate films at pH 7.2.

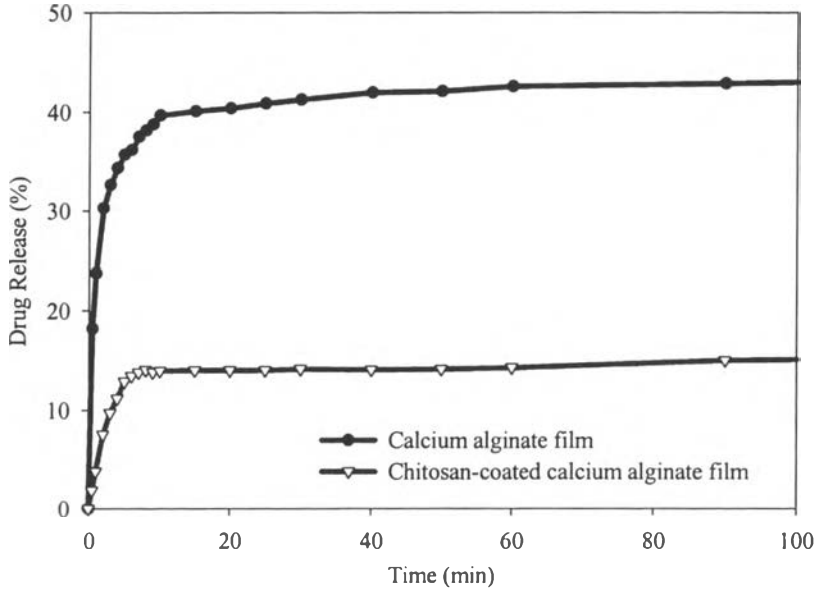

Figure 4.21 Theophylline release profile for calcium alginate and chitosan-coated calcium alginate films at pH 2.0.

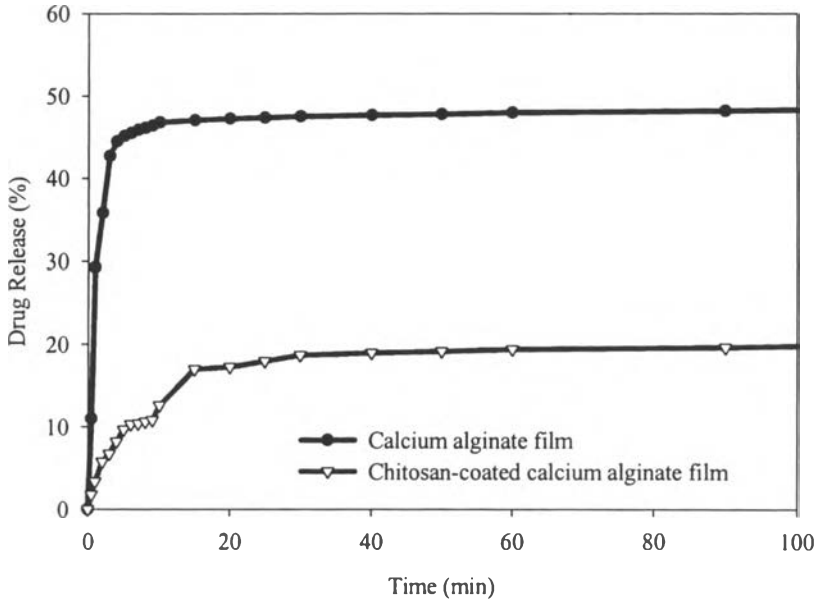

Figure 4.22 Theophylline release profile for calcium alginate and chitosan-coated calcium alginate films at pH 5.5.

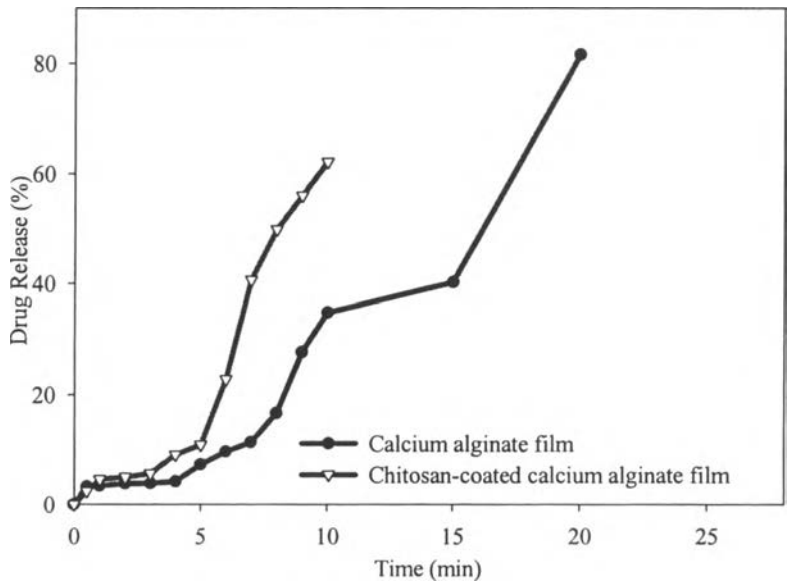

Figure 4.23 Theophylline release profile for calcium alginate and chitosan-coated calcium alginate films at pH 7.2.

The effect of pH on drug released from calcium alginate and chitosan-coated calcium alginate films are shown in Figure 4.24 and 4.25. It was found that the amount of drug release from both calcium alginate and chitosan-coated calcium alginate films at pH 5.5 were higher than at pH 2 for both model drugs. For calcium alginate film, the amount of salicylic acid released at pH 2 and pH 5.5 were 32.41 and 40.19 %, respectively. For chitosan-coated calcium alginate film, the amount of salicylic acid release at pH 2 and pH 5.5 were 8.50 and 19.24 %, respectively. In the case of theophylline released, the amount of theophylline release from calcium alginate film at pH 2 and pH 5.5 were 44.93 and 50.29 %, respectively. The amount of the release of theophylline from chitosan-coated calcium alginate film at pH 2 and pH 5.5 were 17.59 and 22.56 %, respectively. It is known that drug release from hydrogels is mainly controlled by swelling-controlled release mechanism. According to this, degrees of swelling of the films at pH 2.0 and pH 5.5 were determined and the results are shown in Table 4.7. It was found that drug release property corresponded with swelling behavior of the films. It appeared that the degree of swelling of the films at pH 5.5 was higher than at pH 2. It can be explained by the fact that at pH higher than pKa of alginate (pKa = 3.38 and 3.63), the carboxyl groups of alginate were ionized, resulting in the dissociation of the adjacent chains. On the other hand, the films exhibited lower degree of swelling when pH was lower than its pKa. This may be because the number of ionized carboxyl groups of alginate becoming lower. Table 4.7 also shows the weight loss of the films, it was found that besides the release of drug occurred due to swelling-controlled release mechanism, the drug release may be concerned with the erosion process.

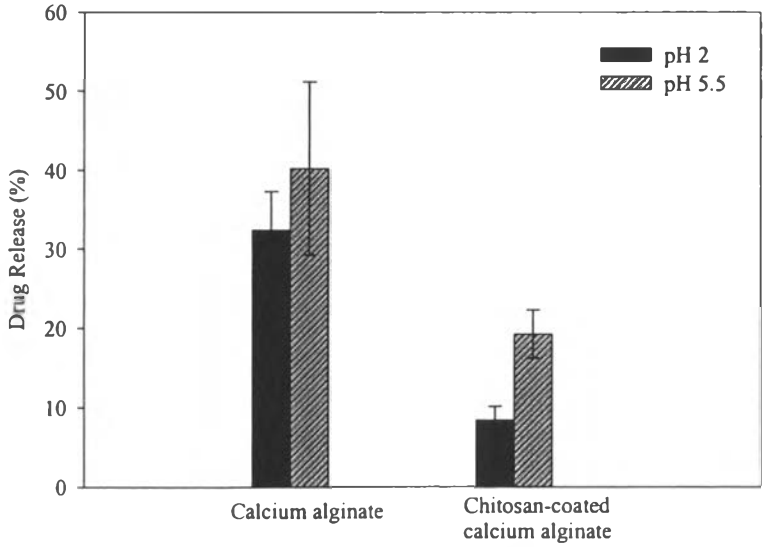

Figure 4.24 Effect of pH on salicylic acid released from calcium alginate and chitosan-coated calcium alginate films.

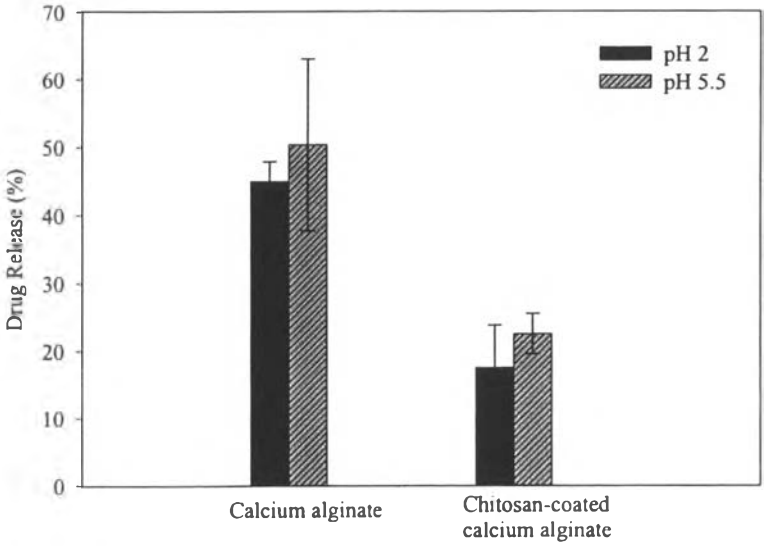

Figure 4.25 Effect of pH on theophylline released from calcium alginate and chitosan-coated calcium alginate films.

Table 4.7 Degree of swelling and percent weight loss of drug- loaded films

Model Drug	Film Type	Degree of Swelling (%)[a]		Weight Loss (%)[a]		
		PH 2	pH5.5	pH 2	pH5.5	PH 7.2
Salicylic acid	Calcium alginate	90.41	239.70	15.76	4.13	Dissolve
	Chitosan-coated calcium alginate	94.32	435.22	10.43	3.28	Dissolve
Theophylline	Calcium alginate	90.41	239.70	15.72	6.74	Dissolve
	Chitosan-coated calcium alginate	94.32	435.22	12.89	5.39	Dissolve

[a] After immersing in buffer solution for 8 h.

It was found that chitosan-coated calcium alginate films gave lower amount of drug release than calcium alginate films. This may be because the formation of the complex at the interface between the carboxyl groups of alginate and amino groups of chitosan retarded the drug release. Gonzalez *et al.* (2002) studied the release of sodium diclofenac from chitosan-coated calcium alginate particulate. It was found that the addition of chitosan decreased the amount of drug release.

Comparison of salicylic acid and theophylline released from calcium alginate and chitosan-coated calcium alginate films at pH 2 and pH 5.5 are shown in Figure 4.26 and 4.27, respectively. It was found that the releasing amounts of theophylline were higher than those of salicylic acid for both pH 2 and pH 5.5. A

factor that can affect the penetration of a drug from a carrier is the drug-polymer interaction. Salicylic acid and theophylline were anionic and neutral drugs, respectively. The drug-polymer interaction between salicylic acid and chitosan in chitosan-coated calcium alginate film might be occurred. It has been reported by Puttipipatkhachorn *et al.* (2001) that there was the drug-polymer interaction between salicylic acid and chitosan whereas there was no the drug-polymer interaction between theophylline and chitosan. Other factors that can affect the drug release are the solubility of drugs in the polymer solution and the molecular weight of drugs. Althrough the molecule of salicylic acid (MW = 138.12) (Figure 4.28) is smaller than theophylline (MW = 180.16) (Figure 4.29), it was found that the releasing amounts of salicylic acid were lower than those of theophylline. When the solubility of drug in the polymer solution was considered, it was found that theophylline could dissolve in the polymer solution better than salicylic acid. The solubilities in water of salicylic acid and theophylline are 2.17 mg/ml and 8.3 mg/ml, respectively (Florey (1975) and Brittain (1994). It might be said that the releases of drug from the films were affected by the combination of various factors.

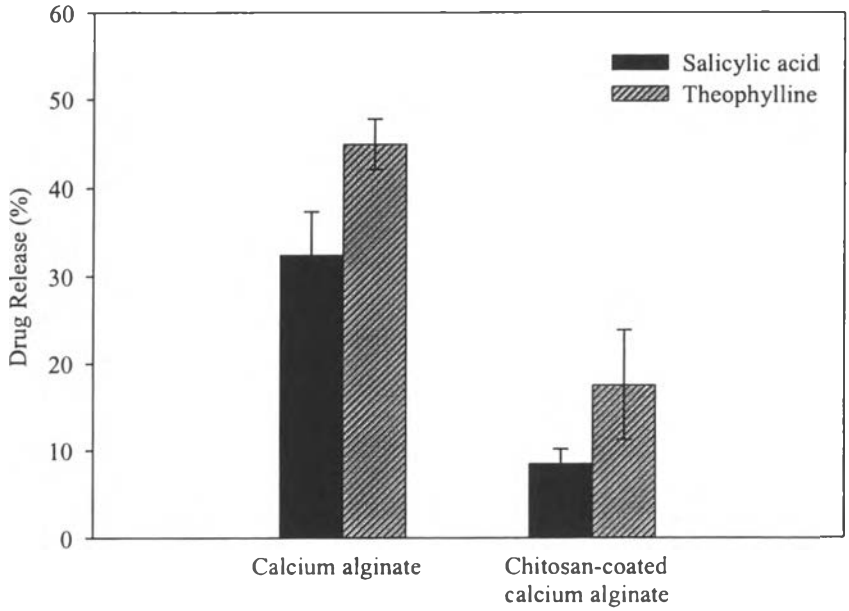

Figure 4.26 Comparison of the amounts of model drugs released from calcium alginate and chitosan-coated calcium alginate films at pH 2.0.

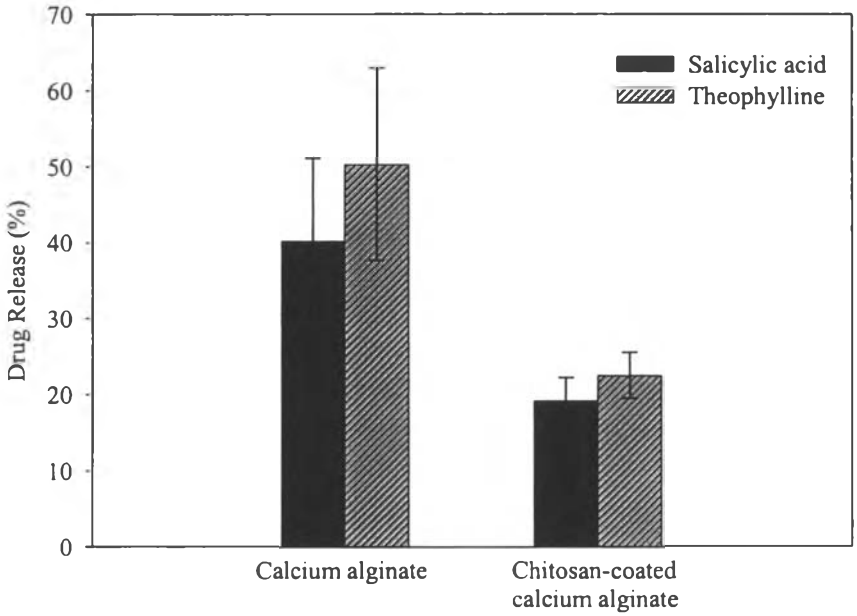

Figure 4.27 Comparison of the amounts of model drugs released from calcium alginate and chitosan-coated calcium alginate films at pH 5.5.

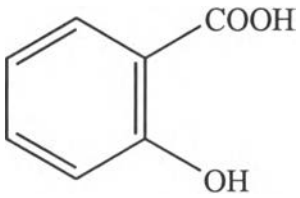

Figure 4.28 Chemical structure of salicylic acid.

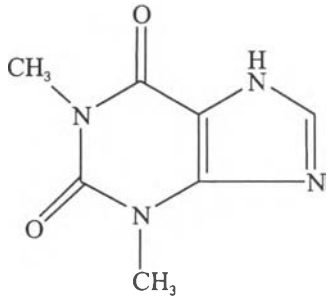

Figure 4.29 Chemical structure of anhydrous theophylline.