CHAPTER IV
RESULTS AND DISCUSIONS

4.1 Characterization of CM-chitin and CM-chitosan

FT-IR spectra of chitin, CM-chitin-Na salt, and CM-chitin films are shown in Figure 1. Obviously, the characteristic absorption peak of CM-chitin at 1733 cm^{-1} (–COOH) (Tokura *et al.*, 1983) was visible in the spectrum shown. Figure 2 illustrates FT-IR spectra of chitosan, CM-chitosan-Na salt, and CM-chitosan films. Apparently, the characteristic absorption peaks of CM-chitosan at 1734 cm^{-1} (-COOH) and 1621 and 1514 cm^{-1} ($-NH_3^+$) (Liu *et al.*, 2001) were visible in the resulting spectrum. Generally, the intensity of the absorption peak belonging to –COOH group increased with increasing degree of carboxymethylation.

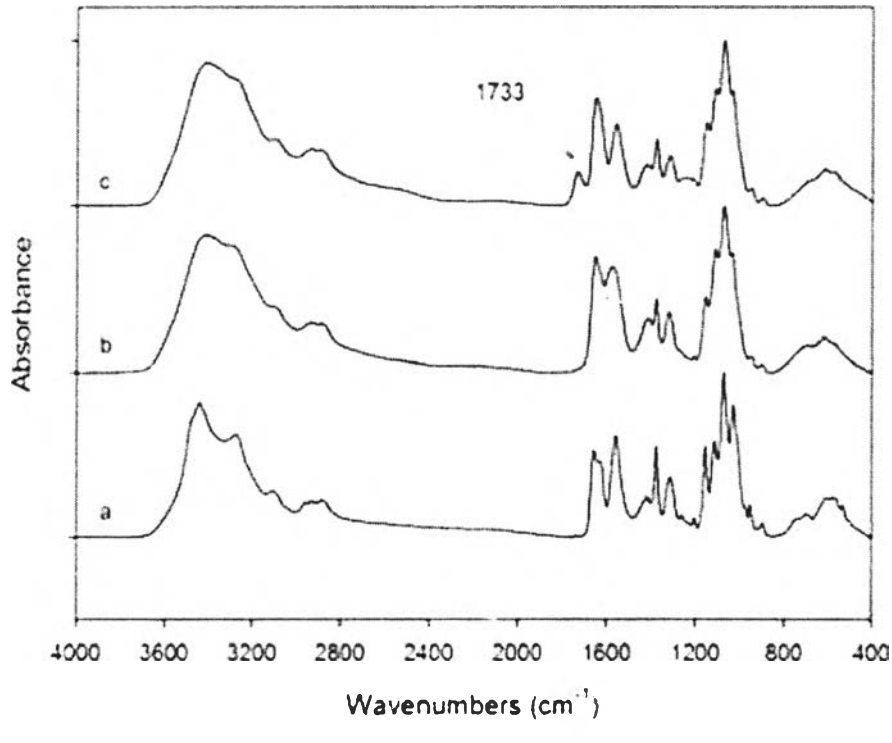


Figure 1 FT-IR spectra of (a) chitin, (b) CM-chitin-Na salt, and (c) CM-chitin films.

According to Figure 1, the intensity at 1733 cm^{-1} of CM-chitin was very low, indicating that the degree of substitution of carboxymethyl groups was very low. Elemental analysis (Nishimura *et al.*, 1986) result confirmed the low degree of substitution of the obtained CM-chitin at about 0.4 (i.e. Calcd. C 42.51 H 5.94 N 5.92; Found C 43.63 H 5.94 N 5.79). Contrarily to CM-chitin, the intensity at 1734 cm^{-1} of CM-chitosan was very high (see Figure 2), suggesting that the degree of substitution of carboxymethyl groups was very high. Indeed, the result from the elemental analysis indicated the degree of substitution of the obtained CM-chitosan to be about 1.0 (i.e. Calcd. C 38.50 H 5.23 N 5.57; Found C 38.35 H 5.52 N 5.56).

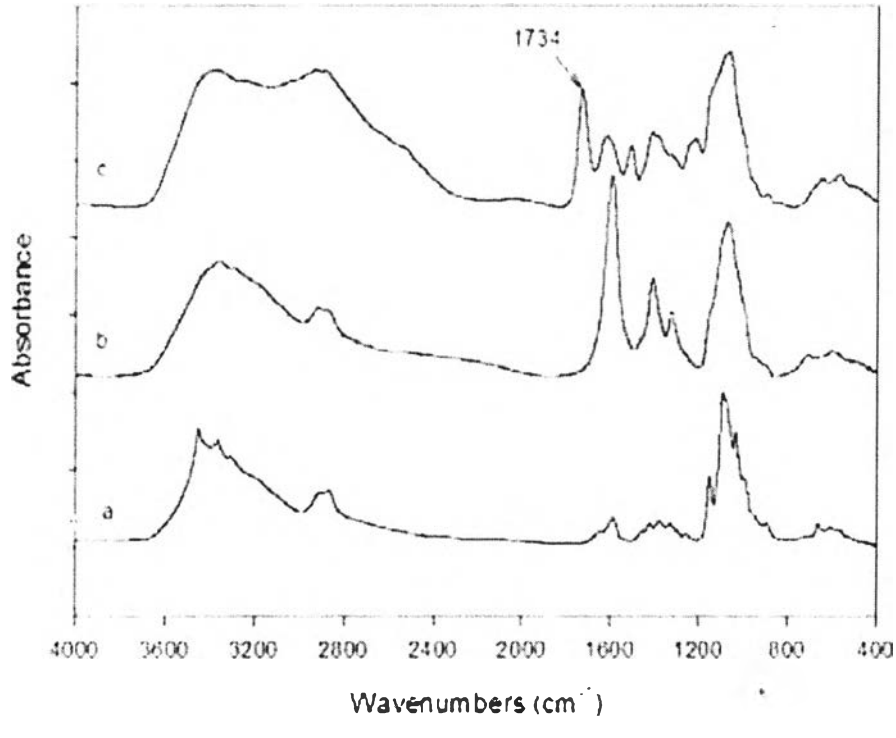


Figure 2 FT-IR spectra of (a) chitosan, (b) CM-chitosan-Na salt, and (c) CM-chitosan films.

Figure 3 shows DSC thermograms of chitin, chitosan, CM-chitin, and CM-chitosan films within the temperature range of 50 to 550°C at a heating rate of 10°C min^{-1}. Two different types of thermal transition were observed in these thermograms. It is postulated that the wide endothermic peak at

temperatures below 190°C was a result of the loss of moisture within the samples, while the wide exothermic peak (in exception of chitin) at the high temperature region was a result of the thermal decomposition of the samples. By careful consideration of the thermograms of chitosan and CM-chitosan films, the thermal decomposition of CM-chitosan occurred in two steps, which could be a direct result of the thermal decomposition of the highly-substituted regions and that of the pure chitosan segments (Kittur *et al.*, 2002).

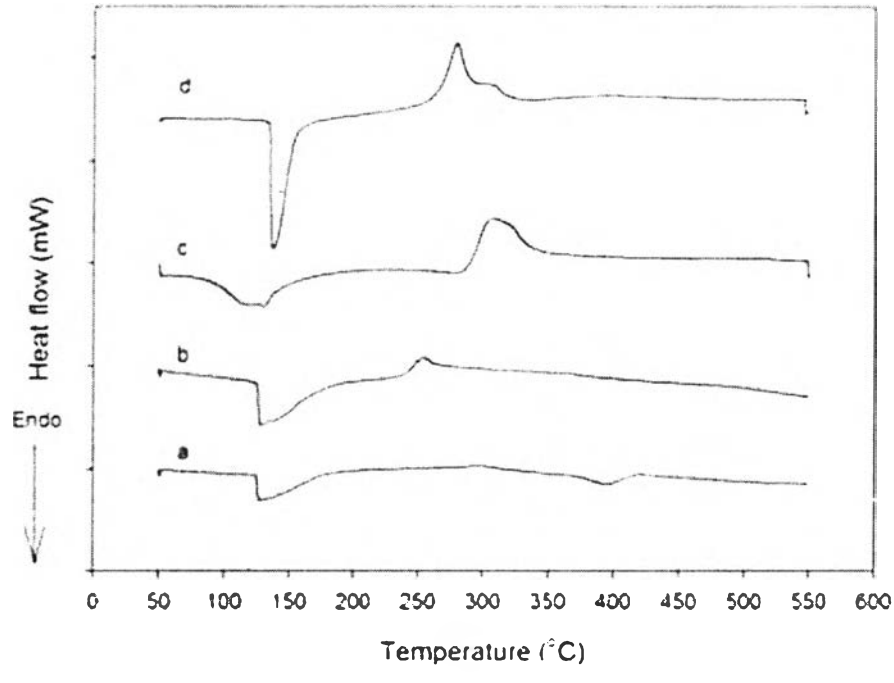
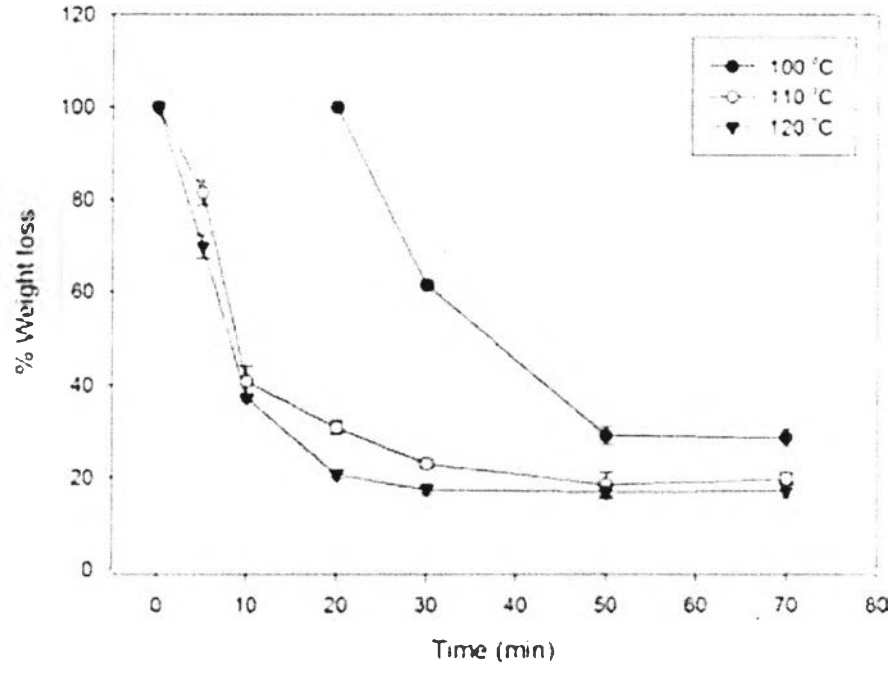


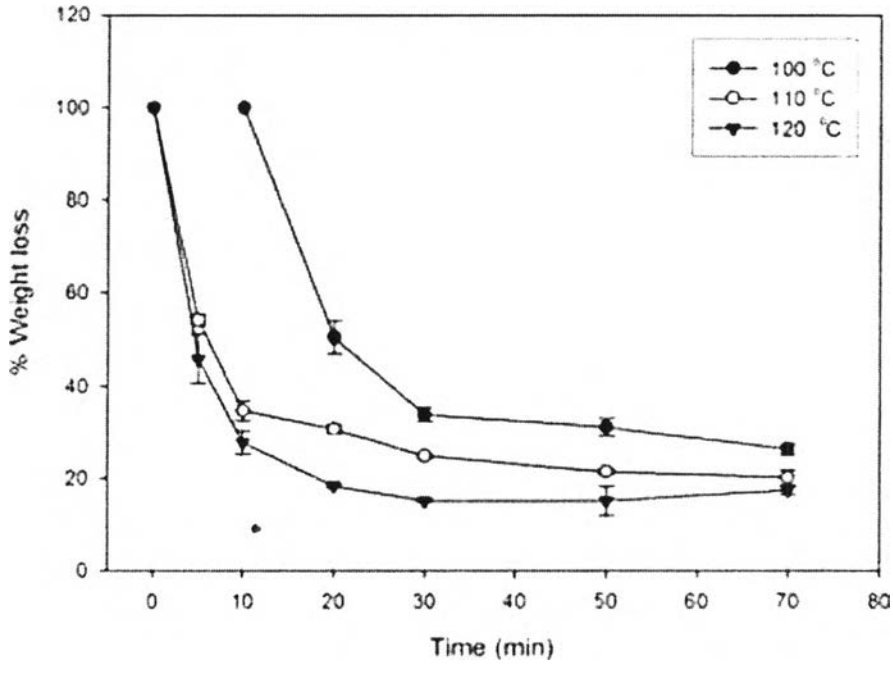
Figure 3 DSC thermograms of (a) chitin, (b) CM-chitin, (c) chitosan, and (d) CM-chitosan films.

4.2 Crosslinking of CM-chitin and CM-chitosan films

Upon crosslinking of CM-chitin and CM-chitosan films after microwave treatment, the treated films must not be dissolved in their good solvent. Figures 4 and 5 show the effects of temperature and time of microwave treatment on weight loss and swelling behavior of the treated CM-chitin and CM-chitosan films after submersion in distilled water at room temperature for 48 hours.

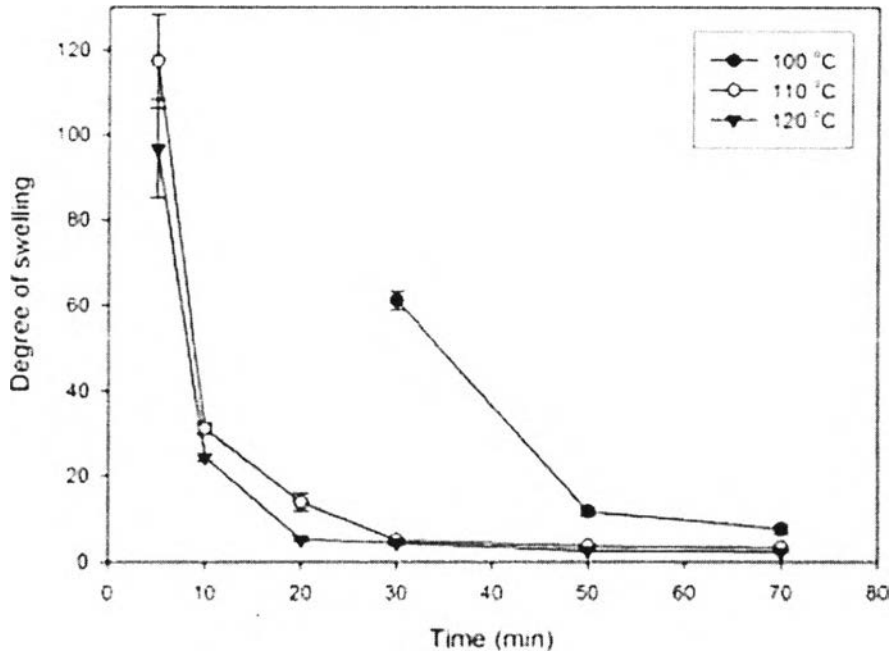


(A)

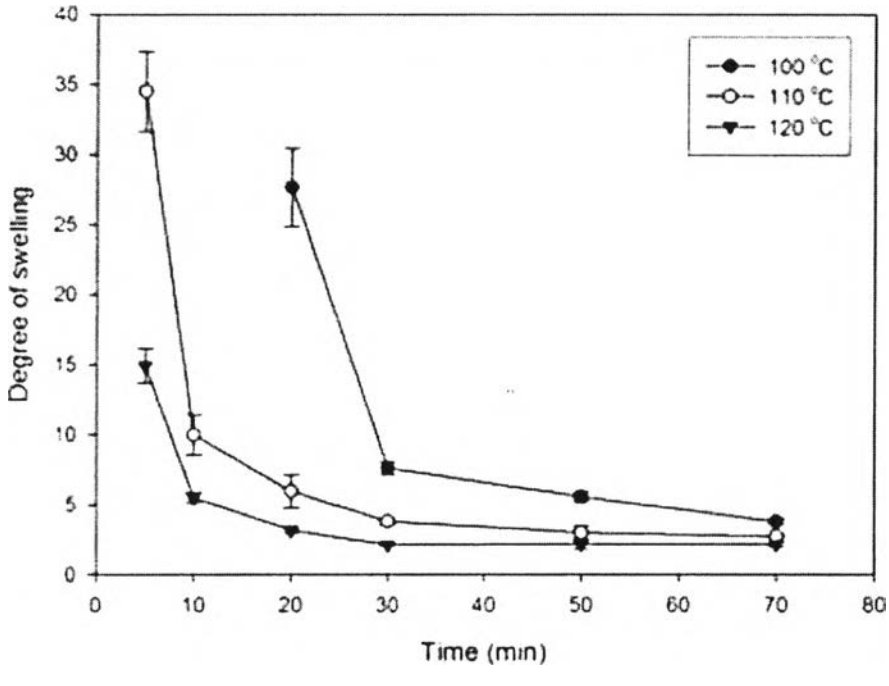


(B)

Figure 4 Percentage of weight loss of (A) microwave-treated CM-chitin and (B) microwave-treated CM-chitosan films as a function of treatment temperature and time. The measurements were carried out after submersion in distilled water for 48 hours at room temperature.



(A)



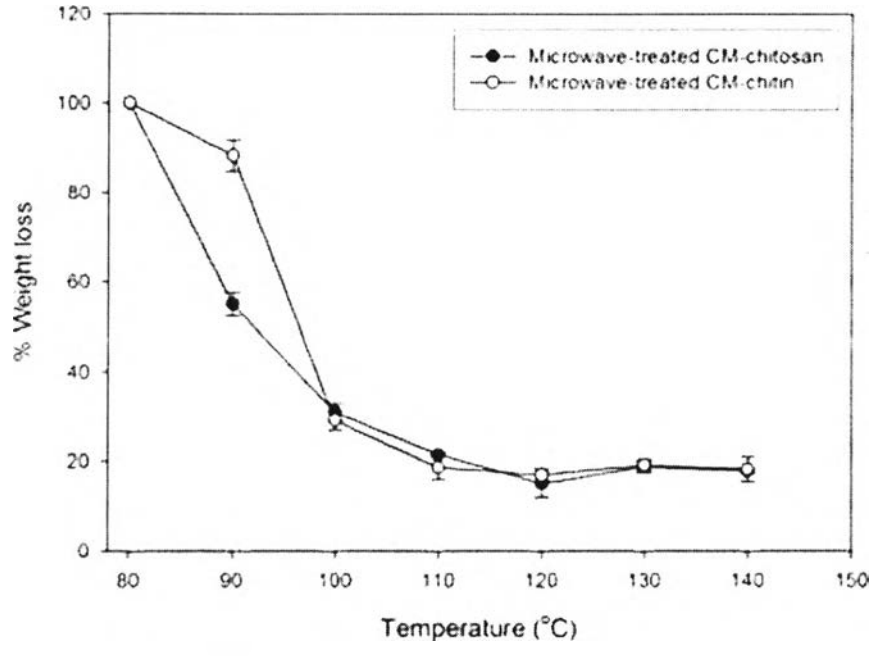
(B)

Figure 5 Degree of swelling of (A) microwave-treated CM-chitin and (B) microwave-treated CM-chitosan films as a function of treatment temperature and time. The measurements were carried out after submersion in distilled water for 48 hours at room temperature.

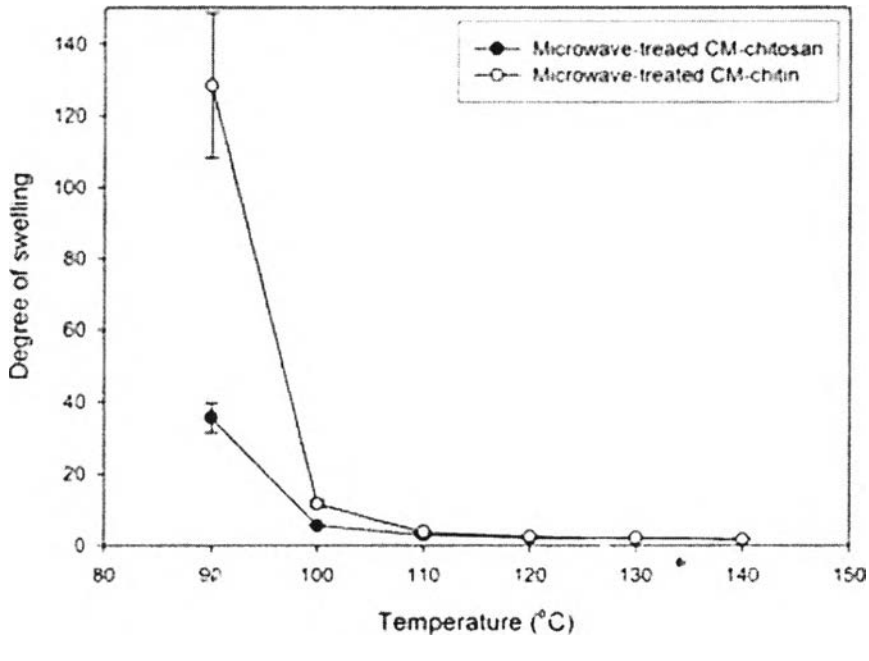
It should be noted that, without the microwave treatment, neat CM-chitin and CM-chitosan films completely dissolved in distilled water within 5 and 1 min, respectively. According to Figure 4, it is obvious that, for a fixed treatment temperature, the percentage of weight loss of both types of microwave-treated films decreased appreciably with initial increase in the treatment time and finally reached a plateau value at longer treatment times. With increasing treatment temperature, the percentage of weight loss was found to decrease. According to Figure 5, it is apparent that, for a fixed treatment temperature, the degree of swelling of both types of microwave-treated films decreased appreciably with initial increase in the treatment time and finally reached a plateau value at longer treatment times. In analogy to the percentage of weight loss results, the degree of swelling was found to decrease with increasing treatment temperature.

Both the percentage of weight loss and the degree of swelling results suggested that the crosslinking density of the microwave-treated films increased with increasing treatment temperature and time. Interestingly, for a given treatment condition, the percentage of weight loss and the degree of swelling of microwave-treated CM-chitosan was lower than that of microwave-treated CM-chitin, suggesting that the crosslinking density of microwave-treated CM-chitosan films was greater than that of the CM-chitin counterparts.

Figure 6 shows the effect of treatment temperature on the percentage of weight loss and the degree of swelling of microwave-treated CM-chitin and microwave-treated CM-chitosan films. It should be noted that these films were treated for a fixed treatment time interval of 50 min. Both the percentage of weight loss and the degree of swelling of the microwave-treated films decreased monotonically with increasing treatment temperature from 90 to about 110°C, after which temperature both the percentage of weight loss and the degree of swelling became independent of the treatment temperature used. The results obtained confirmed that the crosslinking density increased with increasing treatment temperature up to about 110°C, after which temperature the crosslinking density was pretty much constant.



(A)



(B)

Figure 6 (A) Percentage of weight loss and (B) degree of swelling of (o) microwave-treated CM-chitin and (•) microwave-treated CM-chitosan films as a function of treatment temperature. The films were microwave-treated for a fixed treatment time of 50 min. The measurements were carried out after submersion in distilled water for 48 hours at room temperature.

It should be noted that the treatment temperature of 80°C resulted in the films that completely dissolved in distilled water, however the time required for the dissolution for each type of microwave-treated films was still much longer than that for untreated films.

4.3 Characterization of Microwave-Treated CM-chitin and CM-chitosan Films

The profiles of the percentage of weight loss and the degree of swelling of both microwave-treated CM-chitin and microwave-treated CM-chitosan films were essentially similar. However, as previously mentioned, microwave-treated CM-chitin films exhibited slightly more weight loss and swelling than microwave-treated CM-chitosan films, which could be a result of the difference in the degree of carboxymethylation, molecular weight, and/or the activity of the functional groups involving in the formation of crosslinking points.

Figure 7 shows FT-IR spectra of microwave-treated CM-chitin films prepared under different treatment time intervals at a fixed treatment temperature of 110°C. Obviously, the spectrum of the as-prepared crosslinked CM-chitin films is essentially similar to one another and to that of the untreated CM-chitin films (Muramatsu *et al.*, 2003). However, when the spectra shown in Figure 7A were zoomed in to cover range of 1300-890 cm^{-1} (see Figure 7B), the absorption peak at 1112 cm^{-1} was observed to decrease gradually with increasing the treatment time. The results suggested that the formation of crosslinking points in microwave-treated CM-chitin films involved mainly the –OH groups at the C-3 position, because the peak at 1112 cm-1 belongs to a secondary alcohol (Nishi *et al.*, 1979).

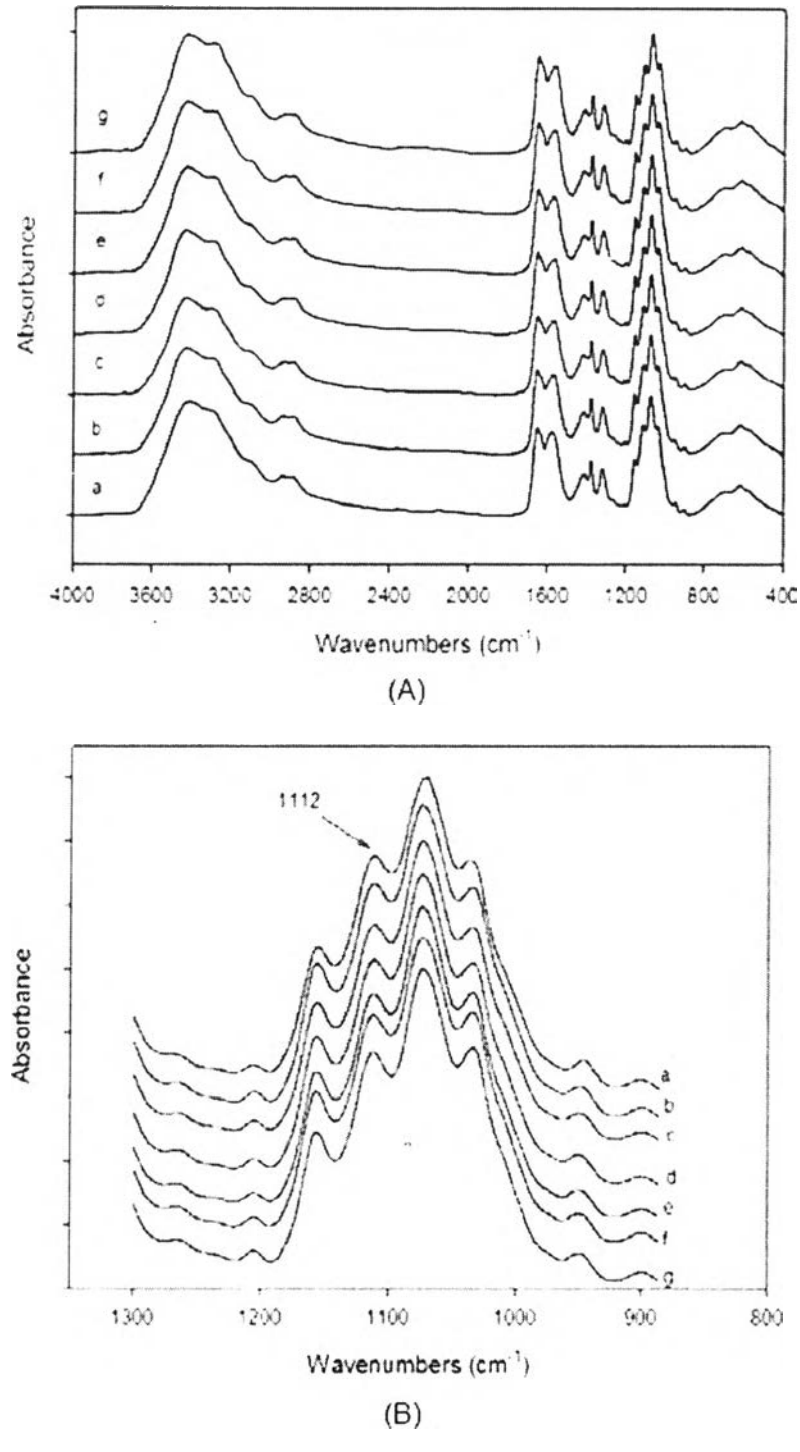


Figure 7 FT-IR spectra over the wavenumber range of (A) 4000 to 400 cm^{-1} and (B) 1300 to 890 cm^{-1} of microwave-treated CM-chitin films prepared at the treatment temperature of 110°C for various treatment time intervals of (a) 0, (b) 5, (c) 10, (d) 20, (e) 30, (f) 50, and (g) 70 min.

Simiiarly, Figure 8 illustrates FT-IR spectra of microwave-treated CM-chitosan films prepared under different treatment time intervals at a fixed treatment temperature of 110°C. Apparently, the spectrum of the as-prepared crosslinked CM-chitosan films is essentially similar to one another and to that of the untreated CM-chitosan films, excepted for that there was a new absorption peak appearing at 1661 cm^{-1} (C=O of amide I) after the microwave treatment. Interestingly, the intensity of this new peak increased with increasing the treatment time. In a separate experiment, chitosan films were treated in the microwave in the same conditions that produced crosslinked CM-chitosan films, but the resulting chitosan films still appeared to be soluble in aqueous acetic acid. This result along with the observation of the absorption peak at 1661 cm^{-1} suggested that the functional groups involved the crosslinking reaction of the microwave-treated CM-chitosan were the amino and the carboxylate groups (Janvikul *et al.*, 2001).

As previously discussed, crosslinking of CM-chitin and CM-chitosan films should involve carboxylate and hydroxyl as well as amino groups. Generally, it is known that an amino group exhibited higher nucleophilicity than a hydroxyl group. Hence, the crosslinking density of microwave-treated CM-chitosan films should be higher than that of the CM-chitin counterpart (see Section 3.2 on the discussion regarding the percentage of weight loss and the degree of swelling results). Notwithstanding, the effectiveness in the crosslinking reaction should depend on the degree of substitution, the distribution of the carboxymethyl groups, the molecular weight, the degree of deacetylation, and the crystallinity (if any).

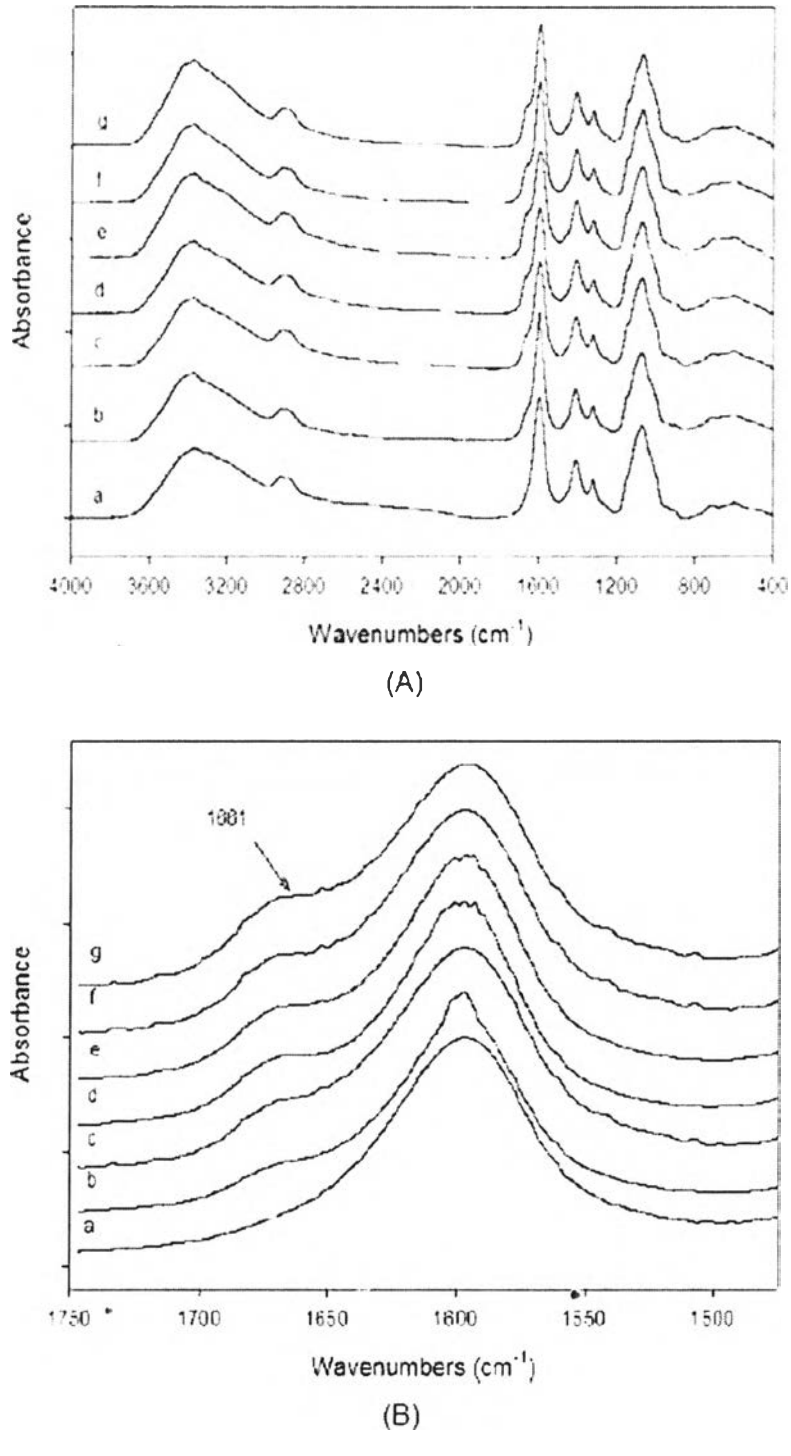


Figure 8 FT-IR spectra over the wavenumber range of (A) 4000 to 400 cm^{-1} and (B) 1740 to 1430 cm^{-1} of microwave-treated CM-chitosan films prepared at the treatment temperature of 110°C for various treatment time intervals of (a) 0, (b) 5, (c) 10, (d) 20, (e) 30, (f) 50, and (g) 70 min.

In order to observe the packing ability of CM-chitin and CM-chitosan molecules before and after the microwave treatment, WAXD technique was employed. Figure 9 shows diffractograms of CM-chitin and CM-chitosan films which were treated in microwave at 110°C at different treatment time intervals. Obviously from the results obtained, neat CM-chitin and CM-chitosan films were semicrystalline and amorphous, respectively. For microwave-treated CM-chitin films, initial increase in the treatment time interval from 0 to around less than 30 min, the resulting diffractograms were essentially unchanged, but, with further increase in the treatment time interval from 30 to 70 min, the crystallinity of the resulting microwave-treated CM-chitin films was found to increase (see Figure 9A). Both the percentage of weight loss and the degree of swelling results (see Figures 4 and 5) of CM-chitin films treated in microwave at 110°C suggested that the crosslinking density increased appreciably up to the treatment time interval of 30 min, after which time it was found to be constant. Coupled with the WAXD results, it is postulated that the crosslinking reaction occurred appreciably during the first 30 min, after which time the chain scission reaction resulted in an increase in the number of highly mobile molecular segments that could contribute to the observed increase in the crystallinity of the resulting films.

On the other hand, no strong indication about the ordered structure was observed in the diffractograms of microwave-treated CM-chitosan films, despite the observation of sharp peaks of very weak intensity at the diffraction angles of about 9.2, 21.2, and 28.4°, respectively (see Figure 9b). The results indicated that all of the microwave-treated CM-chitosan films were predominantly amorphous.

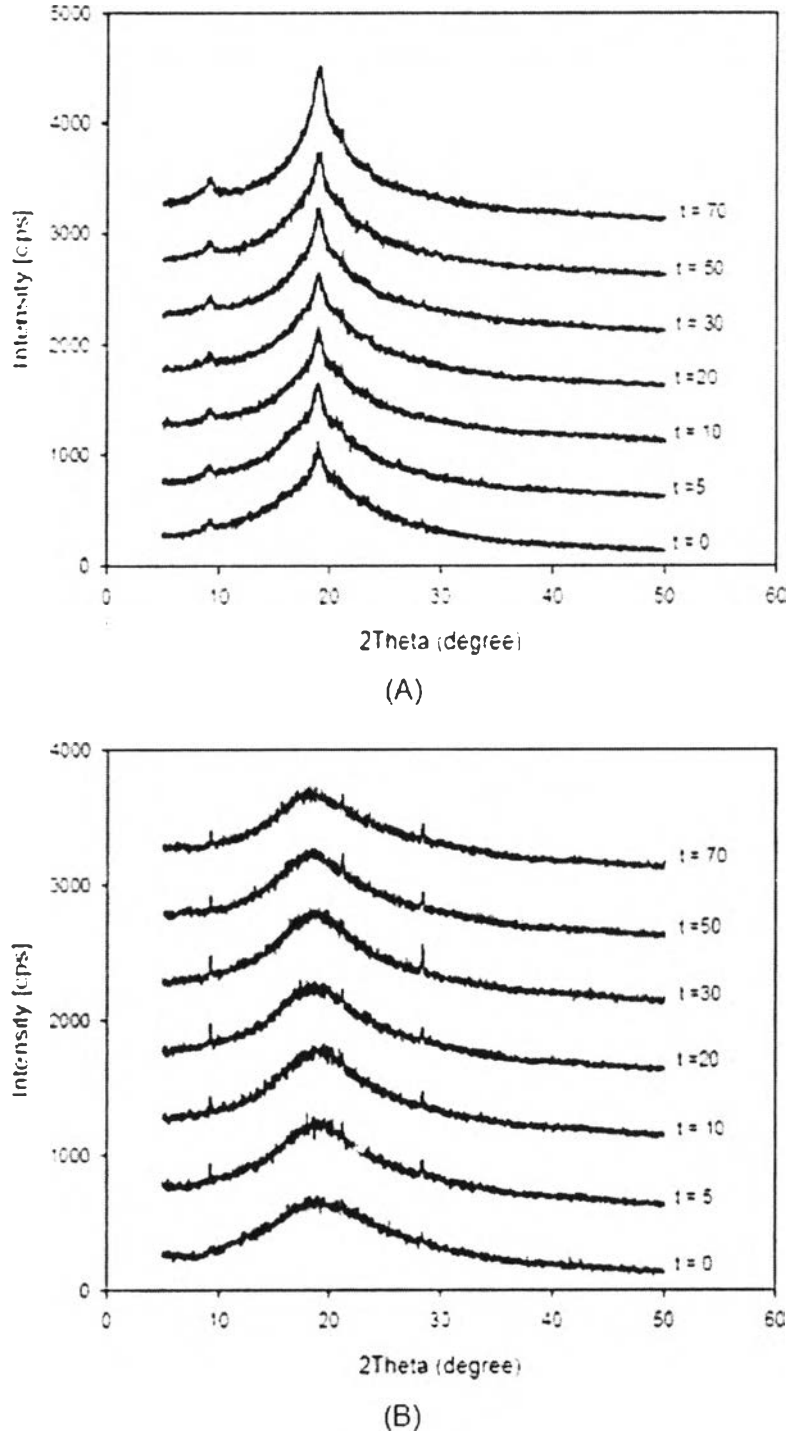


Figure 9 WAXD patterns of (A) microwave-treated CM-chitin and (B) microwave-treated CM-chitosan films prepared at the treatment temperature of 110°C for various treatment time intervals as indicated.

The discussion about the characteristics of the microwave-treated CM-chitin and microwave-treated CM-chitosan films cannot be completed without the visual characteristics of the films. Figure 10 illustrates SEM images of crosslinked CM-chitin and CM-chitosan films which were treated in microwave at 120°C for 30 min in comparison with the as-cast CM-chitin and CM-chitosan films as well as the crosslinked films which were submerged in distilled water for 48 hours at room temperature to remove soluble materials. It should be noted that the treatment conditions of 120°C and 30 min were chosen based mainly on the practicality in sterilization of the film products for future uses. According to the SEM images, the surface of the as-cast CM-chitin and CM-chitosan films was smooth. After microwave treatment, the surface of the crosslinked films was still smooth and the shrinkage of these films was not observed. After the crosslinked films were soaked in distilled water for 48 hours to remove the soluble components, the surface of the films was still practically smooth, despite the fact that slight shrinkage was observed in those films (viz. the films losed about 18% of their weight, see Figure 4).

Visual characteristics include the change in the color of the treated films as well. It is observed that the color of the treated CM-chitin and CM-chitosan films changed from transparency to pale yellow. The increase in the severity of the treatment conditions (i.e. increasing treatment temperature or time) resulted in the films exhibiting a darker color. Since the main chain of these polysaccharides consists of glucosamine units, the introduction of thermal energy and the presence of moisture could induce hydrolytic ring-openning reactions of these rings into aldehyde group at the chain ends. The aldehyde groups can react with the amino groups in the polymer backbone. The similar reactions between the aldehyde and the amino groups are reported as the main cause for the browning occurring in the roasting of meat or the baking of bread, i.e. the Maillard reaction (Hofmann, T., 2001). Coloration of chitosan after thermal treatment was also studied and reported by Lim *et al.*

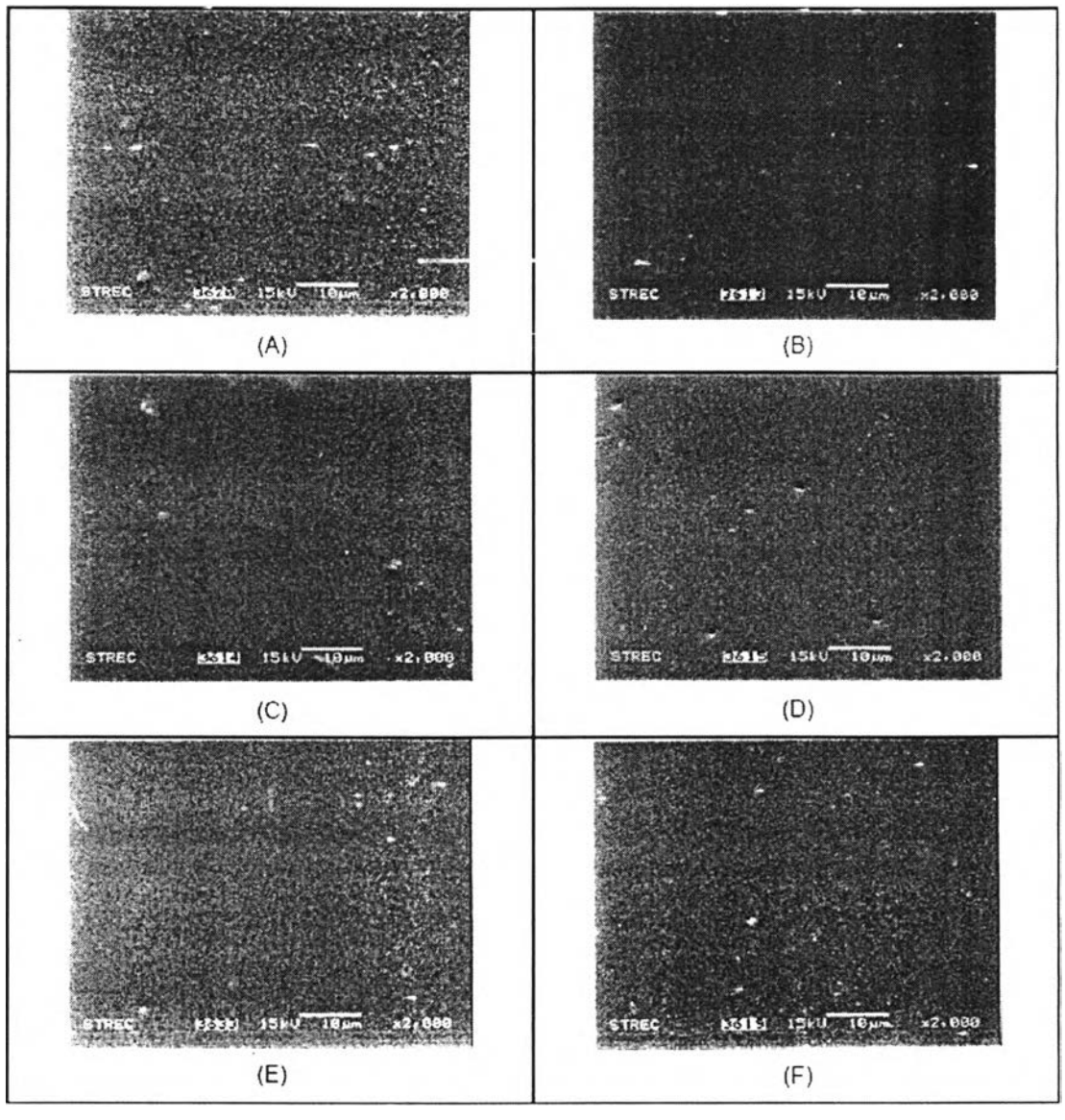


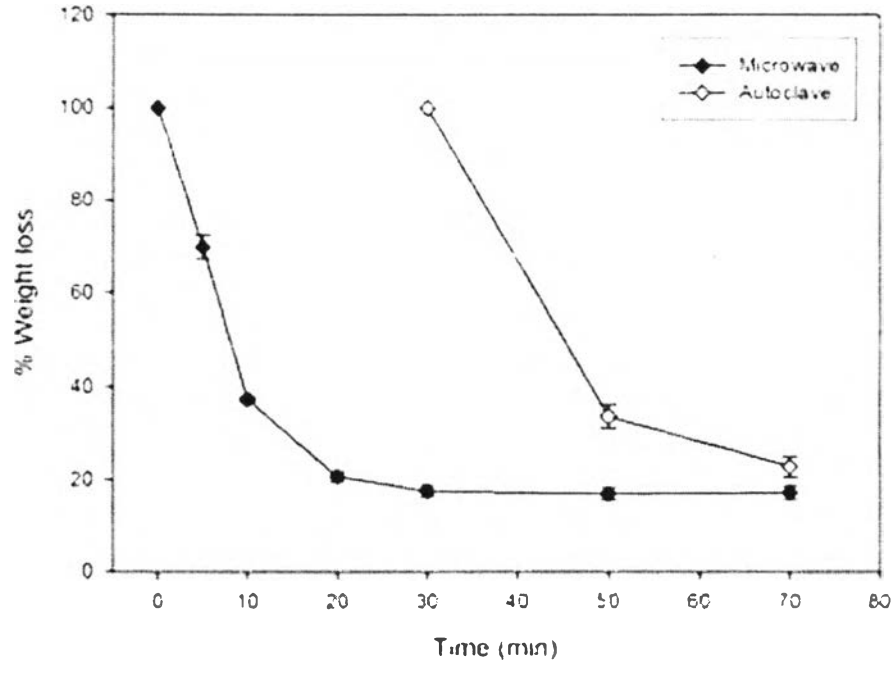
Figure 10 SEM images of (A) CM-chitin film, (B) CM-chitosan film, microwave-treated CM-chitin films (C) before and (D) after removal of soluble materials, and microwave-treated CM-chitosan films (E) before and (F) after removal of soluble materials.

4.4 Comparison Between Effectiveness of Microwave and Autoclave in Crosslinking CM-chitin and CM-chitosan Films

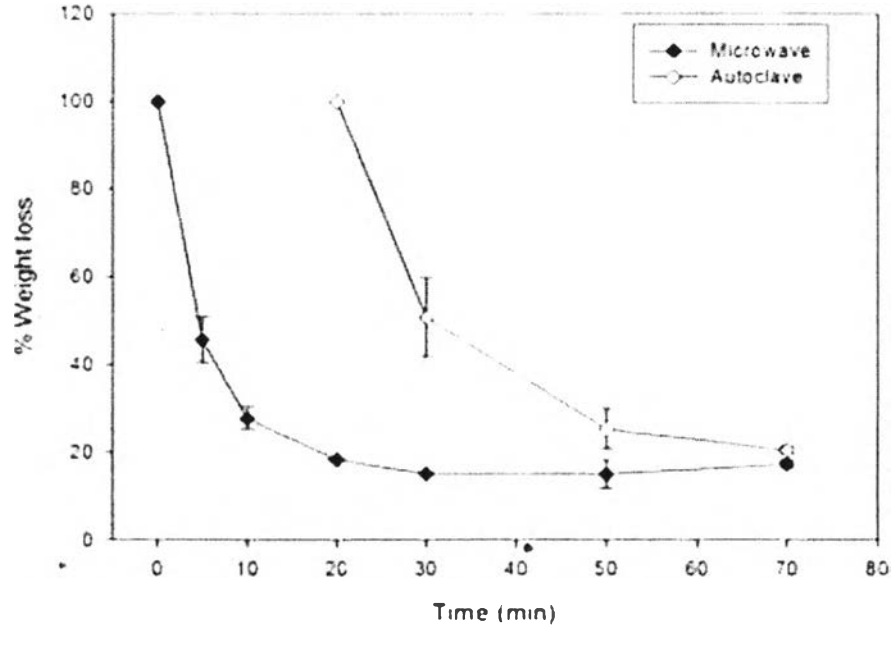
Before going further to the cell culture studies of the microwave-treated CM-chitin and microwave-treated CM-chitosan films, it is interesting to know the effectiveness of the microwave technique in crosslinking the

treated films in comparison with that of a conventional technique (e.g. autoclave treatment). Figure 11 shows the percentage of weight loss of CM-chitin and CM-chitosan films that were treated in microwave or autoclave at 120°C at different treatment time intervals. The weight loss of these films was measured after the films were submerged in distilled water for 48 hours. Evidently, the microwave-treated films showed much less percentage of weight loss than the autoclave-treated counterparts. For examples, in the autoclave treatment, the time required for the resulting crosslinked films to exhibit a percentage of weight loss of about 20% was about 70 min, while it was only about 20 min in the microwave treatment. Interestingly, like the crosslinked films obtained from the microwave treatment, the autoclave-treated CM-chitosan films exhibited much less percentage of weight loss than the CM-chitin counterparts.

The difference in the crosslinking effectiveness of the microwave over the autoclave treatment technique is postulated to be the difference in the generation and the transport of the thermal energy within the materials. In autoclave, the generation of the thermal energy was by a furnace and the thermal energy was dissipated within the samples mainly by convective and conduction transport. In microwave, the generation of the thermal energy was by the rapid rotation of water molecules present within the samples. The rapid rotation of water molecules was induced by absorption of the microwave energy by water molecules. Based on the described mechanisms, it is apparent that microwave heating was more rapid and uniform than the autoclave heating. It has been found that utilization of microwave energy as the heating source for the esterification of cellulose and N-phthaloylation of chitosan resulted in a dramatic decrease in the reaction time when compared with the use of a conventional heating technique (Stage *et al.*, 2001; Liu *et al.*, 2004).



(A)



(B)

Figure 11 Percentage of weight loss of (A) CM-chitin and (B) CM-chitosan films after (♦) microwave or (◊) autoclave treatment at the treatment temperature of 120°C for various treatment time intervals. The measurements were carried out after submersion in distilled water for 48 hours at room temperature.

4.5 Cell Culture Studies

4.5.1 Cytotoxicity Tests

The direct evaluation of cytotoxicity of microwave-treated CM-chitin and microwave-treated CM-chitosan films was conducted using the normal human gingival fibroblast (NHGF) cells. Figure 12 illustrates the number of living NHGF cells (reported as the percentage of the controls) after the seeded cells were in direct contact with the film samples for 24 or 48 hours. Evidently, after either 24 or 48 hours in incubation, the number of living cells after the seeded cells was in direct contact with neat chitin, neat chitosan, and microwave-treated chitin films was still greater than or equal to 90% in comparison with that of the controls. However, the number of living cells when the seeded cells were exposed to microwave-treated CM-chitosan films remained only about 70 and 38% after either 24 or 48 hours in incubation. The results obtained suggest that only microwave-treated CM-chitosan films were detrimental to NHGF cells.

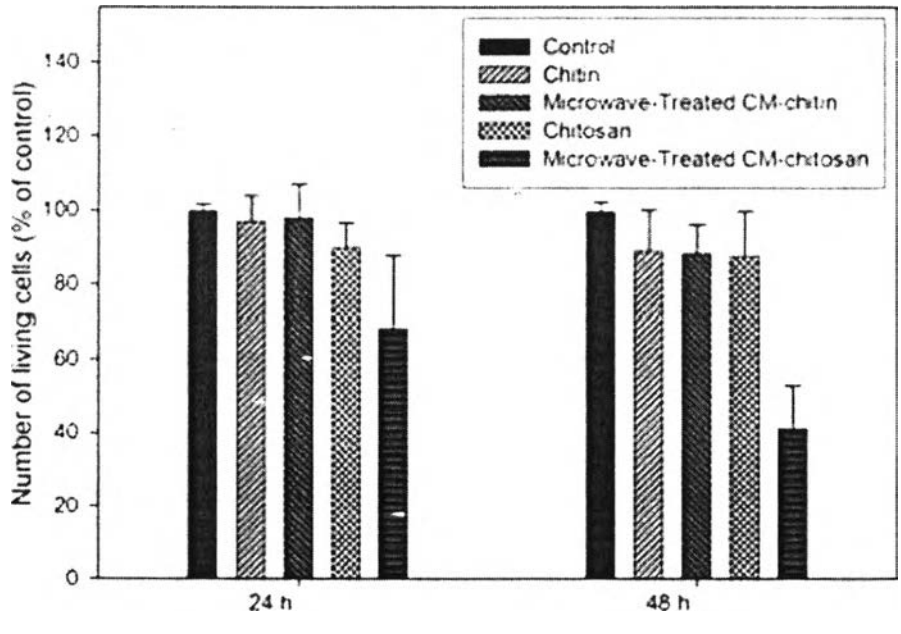
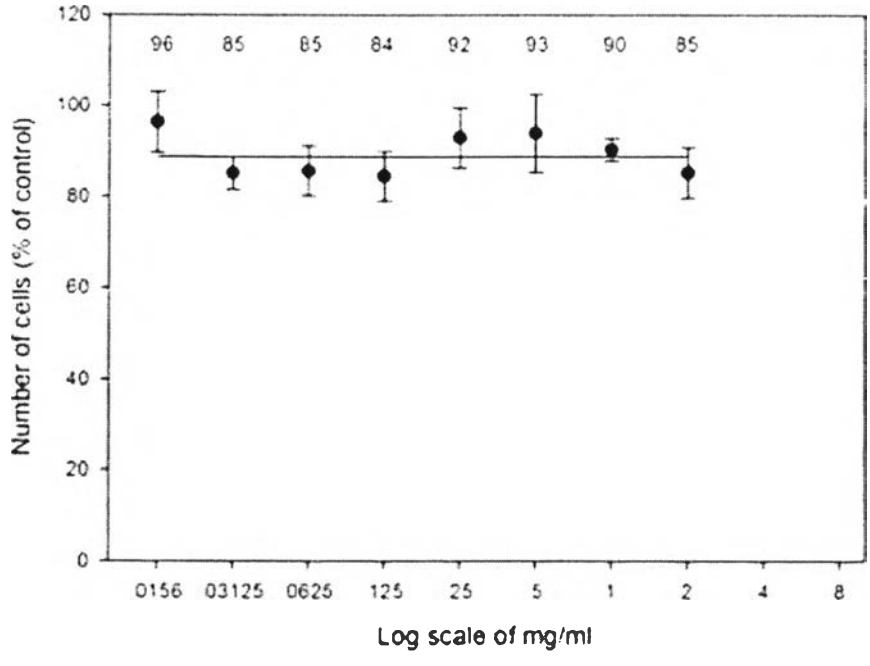
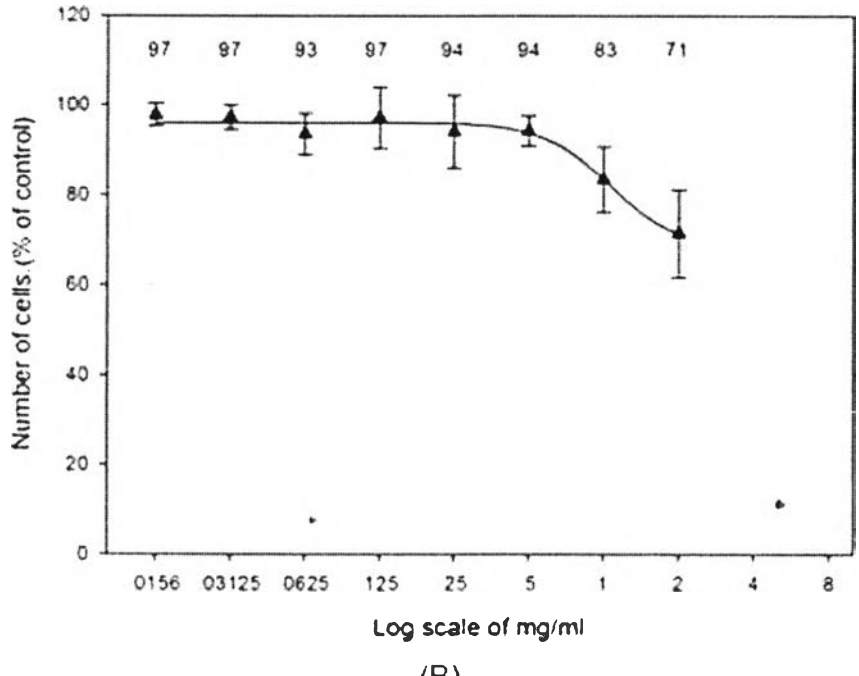


Figure 12 Number of living cells after chitin, microwave-treated CM-chitin, chitosan, and microwave-treated CM-chitosan films were deposited over NHGF confluence for a period of either 24 or 48 hours.



(A)



(B)

Figure 13 Number of living cells after cultured with extraction medium of varying concentration for a period of 24 hour for (A) microwave-treated CM-chitin and (B) microwave-treated CM-chitosan films.

The indirect evaluation of cytotoxicity of microwave-treated CM-chitin and microwave-treated CM-chitosan films was conducted using the mouse connective tissue, fibroblast-like L929 cells. Figure 13 shows the number of living L929 cells (reported as the percentage of the controls) after the seeded cells were cultured in the extraction medium solution at various concentrations for 24 hours. Apparently, the numbers of living cells after the seeded cells were cultured with the extraction medium from microwave-treated CM-chitin films in all of the concentrations investigated was more than 80%, with the average value being about 89%. On the other hand, the number of living cells after the seeded cells were cultured with the extraction medium from microwave-treated CM-chitosan films was found to be quite constant at about 95% up to the medium concentration of about 0.5 $mg \cdot ml^{-1}$, but, when the medium concentration increased to 1 and 2 $mg \cdot ml^{-1}$, the number of living cells decreased to about 80 and 70%, respectively.

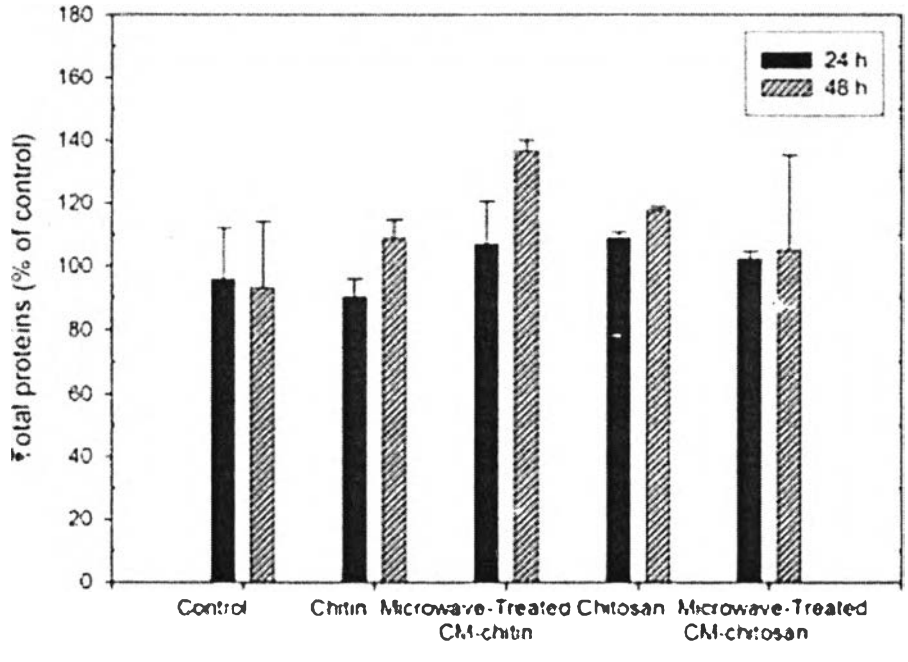


Figure 14 Total amount of protein synthesis of living NHGF cells after chitin, microwave-treated CM-chitin, chitosan, microwave-treated CM-chitosan films were deposited over NHGF confluence for a period of either 24 or 48 hours.

Based on both direct and indirect cytotoxicity evaluations, microwave-treated CM-chitin films were cytocompatible, while microwave-treated CM-chitosan films were not. Despite the indication of the cytoincompatibility of microwave-treated CM-chitosan films in comparison with other materials investigated, the results on the total protein synthesis of living cells when the seeded cells were in contact with this material after either 24 or 48 hours indicated that the activity of the living cells was normal, since the total amount of proteins produced appeared to be quite equivalent to that of the controls (see Figure 14).

Chen *et al.* (2002) demonstrated that CM-chitosan was not only cytocompatible, but also found to promote proliferation of normal skin fibroblast cells. After the microwave treatment, crosslinked CM-chitosan films appeared to be cytotoxic. During heat treatment, two possible reactions occurred: 1) crosslinking (the reaction between the carboxylate and the amino groups) and 2) Maillard reaction (the reaction between the aldehyde and the amino groups). At this point, it is logical to postulate that the chromophore products from the Maillard reaction were responsible for the observed cytotoxicity of microwave-treated CM-chitosan films. Even though, in the case of microwave-treated CM-chitin films, the Maillard reaction, which resulted in the coloration of the treated films, also occurred, but, due to the much lower amount of the amino groups along CM-chitin chains in comparison with that along CM-chitosan chains (i.e. about 25 versus 97%), the extent of the Maillard reaction may not be too great to exhibit an adverse effect on the cells. As a result, microwave-treated CM-chitin films were not found to be cytotoxic.

4.5.2 Cell Adhesion Tests

Figure 15 shows the number of living NHGF cells (reported as the percentage of the number of living cells for chitosan that was in culture for 1 hour) after the film samples were cultured with NHGF cells for either 1 or 2 hours, after which time the attachment of living cells of these film samples was assessed. Apparently, at a given incubation time, the number of living

cells attached on chitin films was greater, while that attached on microwave-treated films was lower, than that of chitosan films. With increasing incubation time, the number of cells attached on a given film was found to increase. Interestingly, despite the fact that microwave-treated CM-chitosan films exhibited cytotoxicity towards the NHGF cells in a greater extent than microwave-treated CM-chitin films (see Figure 12), the adhesion of cells on the surface of microwave-treated CM-chitosan films was better than on the surface of microwave-treated CM-chitin counterparts.

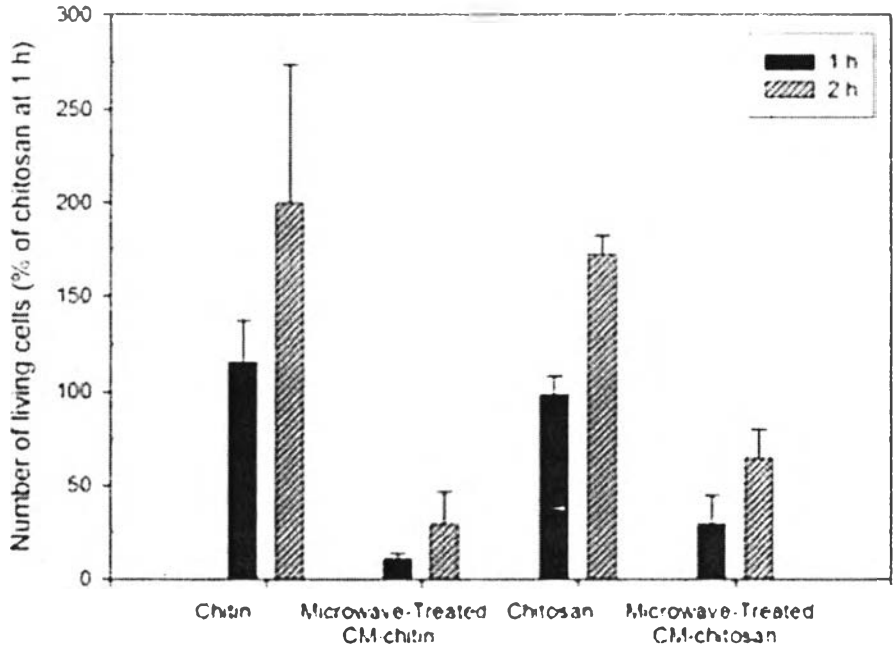


Figure 15 Adhesion of NHGF cells on chitin, microwave-treated CM-chitin, chitosan, and microwave-treated CM-chitosan films after cell culture for either 1 or 2 hours.

Moreover, the cells adhered on the surface of microwave-treated CM-chitosan films exhibited normal cell behavior, in that they spreaded out over the surface of the films after culture, while the cell adhered on the surface of microwave-treated CM-chitin films tended to flocculate rather than spread out over the surface of the films after culture. The better cell response towards the surface of microwave-treated CM-chitosan films over that of microwave-treated CM-

chitin ones should be due to the greater number of amino groups that are present on the microwave-treated CM-chitosan films than on the microwave-treated CM-chitin ones.