CHAPTER IV
MISCIBILITY AND BIODEGRADABILITY OF SILK FIBROIN/CARBOXYMETHYL CHITIN BLEND FILMS

4.1 Abstract

Blend films of silk fibroin and carboxymethyl chitin (CM-chitin) were prepared by solution casting technique using water as a cosolvent. The blend films were subjected to post treatment with an aqueous methanol solution to induce β-sheet formation of silk fibroin. The miscibility of the blend films with both before and after methanol treatments were investigated in terms of chemical interactions, morphologies, thermal properties, and crystal structures by using FTIR spectroscopy, SEM, DSC, and XRD, respectively. The results indicate that the blend between silk fibroin and CM-chitin was semi-miscible because only amorphous part of those polymers were compatible with each other. The enzymatic degradation experiment showed that the incorporation of CM-chitin enhanced biodegradability and swelling ability of silk fibroin.

Keyword: Silk fibroin; Carboxymethyl chitin; Biodegradability; Polymer blend; Miscibility

4.2 Introduction

In the past several decades, the binary blend of polymers has received a considerable attention from many researchers because it is a cost-effective way to prepare new materials with the desired physicochemical, thermal, mechanical properties and/or biological responses. The final properties of the blends are generally governed by the miscibility between the two polymers. For example, an immiscible blend usually shows two distinct glass transition temperatures while a miscible blend exhibits only one glass transition temperature. In the case of semi-miscible blend, a broad glass transition

temperature is observed.[1] For biodegradability, when immiscible blends are attained, the degradation behavior depends on the intrinsic biodegradability of each component, phase distribution and substrate accessibility to degrading enzymes. On the other hand, when the two components are miscible, biodegradability is strongly affected by mobility of the mixed amorphous phase.[2]

Silk fiber produced by the silkworm, *Bombyx mori*, has been used as a suture material for centuries. After proper removal of sericin, which is a glue-like protein coated on the silk fibroin fiber, silk fibroin is obtained. Silk fibroin has been proven to be effectively used in many clinical applications[3] and is one of the most attractive biopolymers as in both native fiber[4] and regenerated forms (i.e. films[5], electrospun fibers[6], wet-spun fibers[7], and sponges[8-11]). The conformation of the fibroin proteins can be classified into three forms; random coil, silk I, and silk II (β-sheet). Normally, the formation of β-sheet can be induced by treating with organic solvents such as aqueous alcohol solution[12], by physical stretching[13], or by heat treatment[14] resulting in water-insoluble materials. The simple techniques used to characterize crystal structures of silk fibroin were FTIR spectroscopy[15,16] and XRD[13,16]. However, Asakura *et al.*[16] suggested that FTIR spectroscopy could not distinguish conformation difference between random coil and silk I forms.

Nowadays, the potential utility of the fibroin protein as drug-delivery matrix and tissue-engineering scaffolds is being investigated because it exhibits a number of prerequisite requirements for biomedical applications, e.g. biocompatibility[17] and biodegradability[13,18,19], and long proven in clinical[3]. However, the slow biodegradability makes silk fibroin only suitable as tissue-engineering scaffolds for an organ that required a longer regeneration time such as ligament, bone and cartilage[3,9,11]. Thus, to extend the biomaterial utility of this protein, the slow biodegradability of the silk protein has to be solved.

During the decades, CM-chitin, which is a water-soluble chitin derivative, has been extensively investigated as drug-delivery carriers because of its low toxicity[20], non-cytotoxicity, and good swelling in water[21]. Ragnhild *et al.*[22] showed that its

biodegradability depended strongly on the degree of deacetylation (DD), the degree of substitution, and the substitution site. When compared to chitin and chitosan, CM-chitin was degraded with a higher rate *in vitro* by lysozyme regardless of the degree of substitution.

In the present study, the blending of silk fibroin with CM-chitin was investigated in the aspects of miscibility and biodegradability. The miscibility between the two polymers with both before and after methanol treatment was studied in terms of chemical interactions, morphological, thermal, and structural properties. Furthermore, the biodegradability of crosslinked films at different blending compositions *in vitro* was also investigated using protease to determine the effect of CM-chitin content on the enzymatic degradation of silk fibroin in the blend films.

4.3 Experimental

4.3.1 Materials

Raw silk fibers, *Bombyx Mori*, were kindly supplied from Queen Sirikit Sericulture Center, Saraburi Province (Thailand). Protease (EC 3.4.24.31, 4 Umg^{-1}) from *Streptomyces griseus* was purchased from Sigma. Chitin was prepared from shells of *Penaeus merguiensis* shrimps (Surapon Foods Public Co., Ltd., Thailand). After decalcification and deproteinization, the degree of deacetylation of the obtained chitin was determined by Fourier-transformed infrared spectrometry (FTIR) following the method of Baxter *et al.*[23] and was found to be 25%. All of the materials were of analytical grade and were used as received.

4.3.2 Preparation of Regenerated Silk Fibroin Solution

The raw silk fibers of *Bombyx mori* were boiled for 15 min in an aqueous solution of 0.05% Na_2CO_3. The boiling process was repeated two times to remove sericin. The fibers were then rinsed thoroughly with hot water and dried at 40°C overnight. After drying, the degummed silk was dissolved in a solvent mixture of

$CaCl_2$:Ethanol:H_2O (molar ratio = 1:2:8) at 78°C. The protein solution was filtered with filter cloth. The filtrated solution was subsequently dialyzed in distilled water for 4 days by changing the media everyday, followed by centrifugation at 10,000 rpm for 10 min. The as-prepared aqueous silk fibroin concentration was 6.32 wt%. The fibroin solution was then diluted to 1 wt% with distilled water.

4.3.3 Preparation of CM-chitin

CM-chitin was prepared by a previously described method.[21] Briefly, the suspension of chitin powder was prepared in 42 wt% NaOH solution and left in a desiccator for 30 min under reduced pressure before adding crushed ice. The mixture was mechanically stirred for 30 min in order to obtain a viscous alkaline chitin solution. A monochloroacetic acid solution (28 wt% in 14 wt% NaOH solution) was then added dropwise into the alkaline chitin solution under stirring over a period of 30 min. After keeping at room temperature overnight, the mixture was neutralized with glacial acetic acid and subsequently dialyzed in running water for 2 days, followed by dialyzing in distilled water for 1 day. The dialysate was centrifuged at 5000 rpm for 20 min to remove insoluble substances and the supernatant was added dropwise into acetone. After being settled overnight, the precipitate was collected and further washed with acetone. The product (i.e. CM-chitin-Na salt) was resuspended in ethanol, collected, and dried at room temperature.

4.3.4 Preparation of Silk Fibroin/CM-chitin Blend Films

Silk fibroin blend films were prepared by adding 1 wt% CM-chitin solutions into the 1 wt% regenerated silk fibroin solutions at the following silk fibroin/CM-chitin blend ratios; 10/0, 8/2, 6/4, 5/5, 4/6, 2/8, and 0/10. The solutions were slowly stirred for 12 h at room temperature and then cast on polystyrene Petri dish at 40°C. For the crosslinked silk fibroin/CM-chitin blend films, glutaraldehyde used as a crosslinking agent was added into the blend solution to achieve the concentration of 0.0075%.

4.3.5 Methanol Treatment

Methanol treatment was carried out using a 90% (v/v) aqueous methanol solution. The silk fibroin, CM-chitin and the blend films were treated with this aqueous methanol for 10 min. Subsequently, the methanol-treated films were left to dry at room temperature under vacuum condition for at least 3 days and another 1 day in a desiccator prior to further characterization.

4.3.6 *In Vitro* Biodegradation and Equilibrium Water Content

The crosslinked methanol-treated silk fibroin, CM-chitin and the silk fibroin/CM-chitin blend films at 9:1, 7:3, 4:6 blend ratios (circle-shaped films with diameter of 14 mm and thickness of ca. 30 μm) were incubated at 37°C for 2 and 6 days in a 1 $mg \cdot mL^{-1}$ protease in phosphate buffer saline (PBS) at pH 7.4. Samples without the enzyme served as a negative control. After reaching the desired time, the films were washed with distilled water for 3 times, then left to dry at room temperature under vacuum for at least 3 days and another 1 day in a desiccator before weighing.

Equilibrium water content (H) was determined by the following equation [24];

$$H = \frac{W_s - W_d}{W_s} \quad (1)$$

where W_s is the weight of the swollen hydrogel after submerging in PBS at pH 7.4 for 48 h and W_d is the weight of the dry hydrogel after extensively washed with distilled water to remove buffer salts. To measure W_s, the swollen hydrogels were removed and then gently blotted with lint-free wipe prior to weigh.

4.3.7 Characterization of Silk Fibroin, CM-chitin and Their Blend Films

A Mettler-Toledo Differential Scanning Calorimetry (DSC, moedel 822e/400) was used to evaluate the thermal properties of silk fibroin, CM-chitin, the

blend films, and the corresponding methanol-treated films (about 3 mg) under N_2 atmosphere at a heating rate of 10 $K \cdot min^{-1}$ from 50 to 450°C.

Morphology of the fractured cross-sections and surfaces of silk fibroin, CM-chitin, the blend films, and the corresponding methanol-treated films were observed by a scanning electron microscope (SEM, JSM-5410LV, Jeol, Japan) at a voltage of 15 kV. The fractured cross-section of the films was carried out by cracking them after immersion in liquid nitrogen. The further investigation of the miscibility between silk fibroin and CM-chitin was done by observation of films morphology after solvent extraction of water-soluble component from the blend films. Surfaces of the extracted films were observed by SEM after submerging the methanol-treated silk fibroin and the blend films in distilled water at room temperature for 48 h to extract the water-soluble fraction. All of samples were coated with gold on the ion sputter (SCD040, Lichtenstein) at 50 mTorr and 15 mA for 3 min.

FTIR spectroscopy was used to verify the chemical structure and conformation of silk fibroin, CM-chitin, the blend films and the corresponding methanol-treated films. The measurements were carried out on a Thermo Nicolet Nexus 671 FT-IR (32 scans at a resolution of 4 cm^{-1}). A Rigaku Rint2000 wide-angle X-ray diffractometer (XRD) was used to investigate the crystal structure of the as-cast silk fibroin, CM-chitin, the blend films and the corresponding methanol-treated films. The X-ray source was Ni-filtered Cu-Ka radiation (40 kV, 30 mA).

4.4 Results and Discussion

4.4.1 Miscibility Between Silk Fibroin and CM-chitin

4.4.1.1 Chemical Interactions

Figure 4.1 shows FTIR spectra of the as-cast silk fibroin, CM-chitin, and blend films at various blend ratios (see Figure 4.1A), and the corresponding methanol-treated films (see Figure 4.1B). The as-cast silk fibroin film exhibited the

characteristic absorption peaks which were assigned to the random coil conformation at 1652 (amide I), 1539 (amide II) and 1240 cm^{-1} (amide III).[15] After this film was treated with methanol, the amide I and amide II were shifted to 1627, 1527 cm^{-1}, respectively, and a new absorption shoulder, which are the characteristic absorptions of silk II or β-sheet form of silk fibroin, was observed at 1697 and 1268 cm^{-1}.[16]

The characteristic absorption peaks of CM-chitin were presented at 1649, 1593, 1320, 1111 and 1070 cm^{-1}, assigned to amide I, amide II, amide III, secondary alcohol, and primary alcohol, respectively (see Figure 4.1A).[25] After CM-chitin film was treated with methanol, its FTIR spectrum was almost identical to that of the non-treated CM-chitin films, except a peak at 1649 cm^{-1} was shifted to 1655 cm^{-1} (see Figure 4.1B).

By consideration of the blend films, FTIR spectra of the as-cast blend films at all ratios clearly indicated that the peak intensity at 1652, 1539, and 1240 cm^{-1}, which are the characteristic absorption peaks of random coil form of silk fibroin, were proportionally decreased with an increasing composition of CM-chitin (see Figure 4.1A). After methanol treatment, the characteristic absorption peaks of the silk II obviously appeared at all blend ratios (1627, 1527, and 1268 cm^{-1}, see Figure 4.1B). These demonstrated that CM-chitin chains did not disrupt the transformation of silk fibroin from random coil conformation to the silk II form during methanol treatment. It should be noted that although random coil conformation of the as-cast silk fibroin and blend films was mentioned throughout of the discussion of the FTIR result, Asakura *et al.*[16] suggested that this technique could not distinguish between the random coil conformation and silk I form in the films. It was found in the present study that the random coil conformation and silk I form were co-existent in the as-cast silk fibroin and blend films as revealed from XRD data (see section 4.4.1.4).

It is also important to be noted that some of FTIR peaks of silk fibroin and CM-chitin both before and after methanol treatment gradually shifted when the blend composition was varied. It is well accepted that spectral shifts of small magnitude (<10 cm^{-1}) provide a useful criterion for miscibility and also give the

information about the nature of the specific interaction in a variety of blends[26]. Table 4.1 summarizes spectral shifts of amide I, amide II, and amide III of the as-cast blend films and the corresponding methanol-treated films. It was observed that the spectral shift of the amide II was the strongest. In addition, the spectral shift of the amide II of the as-cast films decreased with an increasing of CM-chitin composition. Moreover, after methanol treatment, the stronger in spectral shift were observed.

From observation of the spectral shift of the amide II, the interaction between silk fibroin and CM-chitin thus mainly involved in the amide II of silk fibroin and/or CM-chitin. Furthermore, silk fibroin/CM-chitin blend system might be supposed to exhibit the miscible blending; however, the morphology of the blend films should be taken into account.

4.4.1.2 Cross-sectional and Surface Morphology

SEM micrographs of the fractured cross-sections and surfaces of the methanol-treated silk fibroin, CM-chitin, and the blend films at various blend ratios are shown in Figure 4.2 and 4.3, respectively. The fractured cross-section of pure silk fibroin and CM-chitin (see Figure 4.2A and 4.2G) exhibited the smooth surface while that of the silk fibroin/CM-chitin blend films exhibited quite rough surfaces (see Figure 4.2B, 4.2C, 4.2D, 4.2E, and 4.2F); however, the phase separation in the silk fibroin/CM-chitin blend films could not be clearly observed.

According to Figure 4.3, it was found that the SEM micrographs of the surfaces of the methanol-treated blend films at all ratios (see Figure 4.3B, 4.3C, 4.3D, 4.3E, and 4.3F) showed clear surface topography with the globular structure. The size of these globules that formed throughout on the surfaces of the blend films was smaller as the CM-chitin composition increased. In contrast, the methanol-treated CM-chitin film displayed a very smooth surface (see Figure 4.3G). Furthermore, the globular structure was not observed in the methanol-treated silk fibroin films (see Figure 4.3A).

To further investigate the miscibility between silk fibroin and CM-chitin by the observation of film morphology, the soluble fraction of the methanol-treated silk fibroin and blend films were extracted by submerging the films in distilled

water at room temperature for 48 h. Figure 4.4 is SEM micrographs illustrating the surface morphology of the as-cast silk fibroin and silk fibroin/CM-chitin blend films at 8/2 and 6/4 blend ratios (see Figure 4.4A, 4.4D and 4.4G), the corresponding methanol-treated films (see Figure 4.4B, 4.4E and 4.4H), and the methanol-treated silk fibroin and silk fibroin/CM-chitin blend films at 8/2 and 6/4 blend ratios after extracting the soluble fraction (see Figure 4.4C, 4.4F and 4.4I). Interestingly, after extracting the soluble fraction, the unique globular structure appearing on the surfaces of silk fibroin/CM-chitin blend films at both 8/2 and 6/4 blend ratios were still observed with more clearer in spherical shape, and larger gaps occurred between each globular unit.

It was seemed that water-soluble CM-chitin presented at around these globular structures (see Figure 4.4B, 4.4E and 4.4H) and were washed out during submerging in water (see Figure 4.4C, 4.4F and 4.4I). However, after submerging in water for 48 h, the weight losses of the silk fibroin/CM-chitin blend ratios at 10/0, 8/2, and 6/4, were 2.42±1.30, 11.19±1.87, and 33.94±0.53, respectively. This result suggested that there was some CM-chitin fraction remaining in the extracted films because the weight loss of the films was lower than the amount of CM-chitin in the blend films. It was found that the methanol-treated CM-chitin film was completely soluble in water within 10 minutes.

Despite the fact that the globular structure was observed on the surfaces of all blend films, the cross-sectional SEM micrographs of the blend films did not clearly exhibit the phase separation. Furthermore, there was some CM-chitin fraction remaining in the blend film even the films were submerged in water for 48 h. These phenomena together with the FTIR analysis which indicated an intermolecular interaction between silk fibroin and CM-chitin occurred throughout the blend range, suggested that the miscibility of silk fibroin and CM-chitin occurred in some intermediate between the miscible blend and phase separation, i.e. so called semi-miscible over the blend range.

4.4.1.3 Thermal Behavior

It is well accepted that the glass transition temperature of a polymer blend could give the information about miscibility; an immiscible blend shows two distinct glass transitions; an miscible blend has one glass transition; or a semi-miscible blend usually possesses one very broad glass transitions.[1] However, the glass transitions of silk fibroin and CM-chitin both before and after methanol treatment were not observed within the temperature range of 50 to 450°C as shown in the DSC thermograms in Figure 4.5A and 4.5B.

Figure 4.5 shows DSC thermograms of silk fibroin, CM-chitin and silk fibroin/CM-chitin blend films at various blend ratios both before and after methanol treatment. Two mainly different types of thermal transitions were observed. It was 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 (except for CM-chitin) at the higher temperature region was a result of the thermal decomposition of the samples. Interestingly, the thermal decomposition temperature gradually decreased with an increasing of the CM-chitin composition in the blend films before and after methanol treatment. These results suggested that a strong interaction as indicated by FTIR analysis in section 4.4.1.1 between silk fibroin and CM-chitin might cause the shifting of thermal decomposition temperature between those of the pure polymer components.

In addition, DSC thermogram of the as-cast silk fibroin films before methanol treatment exhibited two small endo- and exo- thermic peaks at 68 and 107°C, respectively. Both of them disappeared after methanol treatment (see Figure 4.5) so this might be explained that these two peaks were the thermal characteristic peaks of random coil conformation and/or silk I form of the as-cast silk fibroin films. When the CM-chitin was blended with the silk fibroin, DSC thermograms of the as-cast blend films still exhibited those two characteristic peaks and decreased in intensity with an increasing in the CM-chitin composition. In addition, those two peaks were not observed after methanol treatment regardless of any blend compositions. These data indicated that the presence of CM-chitin in the blend films did not disrupt the crystallization of silk fibroin.

4.4.1.4 Crystal Structures

After silk fibroin, CM-chitin, and silk fibroin/CM-chitin blend films were examined in terms of chemical structure, morphology, and thermal characteristic, the crystal structures of the polymers should be revealed in order to observe a conformation of polymers and the conformation transition after methanol treatment. Figure 4.6 illustrates XRD patterns of silk fibroin, CM-chitin, and silk fibroin/CM-chitin blend films at various blend ratios both before (see Figure 4.6A) and after methanol treatment (see Figure 4.6B).

Normally, the transition of silk fibroin from random coil and/or silk I to β-sheet conformation is induced after methanol treatment. From the XRD pattern of the as-cast silk fibroin film, it showed diffraction peaks at 12.1° (moderate), 21.4° (moderate) and 27.7° (weak), corresponding to 7.3, 4.1, and 3.2 angstronm, respectively. These *d*-spacing values are the characteristic *d*-spacing of silk I[13,16] so it was indicated that silk I structure was presented in the absence of silk II. Thus silk fibroin in this film is mostly in an amorphous with some silk I form. After methanol treatment, only the *d*-spacing value of 4.1 angstronm disappeared while new diffraction patterns at 4.3 (moderate) and 3.7 angstronm (weak), which are the characteristic *d*-spacing of silk II[13,16], were observed. This indicated that some of silk I structure still remained while the rest had been transformed to silk II[16].

Considering the neat CM-chitin, the XRD patterns of the as-cast CM-chitin films exhibited the diffraction peaks at 9.2° (weak), 11.7° (moderate), 19.1° (strong) and 27.7° (weak), corresponding to 9.6, 7.5, 4.6, and 3.2 angstronm, respectively. After methanol treatment, the methanol-treated CM-chitin films showed the similar diffraction patterns as that before methanol treatment; however, the *d*-spacing at 4.6 angstronm was reduced from strong to moderate intensity.

By consideration of the blend films, the as-cast blend films yielded the superimposed XRD patterns of both silk fibroin and CM-chitin, suggesting that the crystal structures of the two polymers were not interfered with each other. In other word, the crystalline regions of the two polymers did not interact with each other,

thus the crystalline parts of these two polymers were not compatible to each other and the intermolecular interaction between the two polymers as indicated by FTIR analysis occur only in their amorphous regions.

Moreover, the presence of the diffraction pattern at 4.3 angstronm (characteristic *d*-spacing of silk II) after methanol treatment of the films at all blend ratios (except for silk fibroin/CM-chitin at 2/8 blend ratio) indicated that the presence of CM-chitin chains did not disrupt the crystallization of silk fibroin in the blends, which was in good agreement with FTIR and DSC analysis. To explain this behavior, the physicochemical properties of silk fibroin should be considered.

Silk fibroin contains at least two major fibroin proteins, light and heavy chains, 25 and 350 kDa, respectively. Considering the peptide sequence of the heavy chain, this chain can be divided into two blocks, i.e. (six) smaller internal hydrophilic blocks and (seven) internal hydrophobic blocks. In general, these two blocks alternatively connected to each other with larger hydrophilic blocks at the chain ends, resulting in the possibility to form micellar structures which exhibited typical size in the range of 100-200 nm in diameter.[27] When blending silk fibroin with CM-chitin solution, the larger hydrophilic terminal blocks present on the outer edges of the fibroin micelles was probably in the position that could interact with the CM-chitin chains via the hydrogen bonding. As a result, the mobility of CM-chitin chains was restricted at the interface. During casting to form a film, CM-chitin chains could not fold themselves to form ordered structure. Thus, it could be implied that an intermolecular interaction between the two polymers should occur between amorphous region of the disordered CM-chitin chains and hydrophilic block of silk fibroin.

In contrast, the interaction of CM-chitin chains with the hydrophobic blocks of silk fibroin could not easily occur due to the formation of micellar structure and the hydrophobic nature of the hydrophobic blocks of silk fibroin. It is known that the crystalline region is usually dominated by the hydrophobic blocks of the silk fibroin chains.[28] Therefore, when silk fibroin was blended with CM-chitin, the

presence of CM-chitin chains did not disrupt the crystallization of fibroin proteins after methanol treatment.

At this point, we proposed a model of the interaction between silk fibroin and CM-chitin as shown in Figure 4.7B. The proposed model showed the semi-miscibility between silk fibroin and CM-chitin because at the molecular level only amorphous parts of both polymer chains had the interaction to each other. In contrast, the crystalline phases of each polymer were not involved with each other.

By considering the surface appearance based on the above-mentioned results and discussion, the morphology of the blend films was governed by the competitive interaction between the fibroin proteins themselves and the fibroin proteins with CM-chitin chains in the blend solution. Although the fibroin proteins were thermodynamically favorable to interact with themselves rather than with CM-chitin chains, the opportunity which CM-chitin chains could interact with silk fibroin became higher as the CM-chitin composition increased. Thus the presence of CM-chitin in the blend films caused local accumulation of fibroin proteins and the size of aggregated fibroin proteins was gradually decreased by increasing of CM-chitin composition. As a result, after casting and subsequently immersing the films in an aqueous methanol solution, the aggregated fibroin proteins were locally accumulated and contracted (due to β-sheet formation) in the blend films, which could be observed by the presence of irregularly globular structure throughout the film surface (see Figure 4.4F and 4.4I). These globules had irregular shape because some of CM-chitin chains were trapped in the aggregated fibroin proteins. In addition, size of this globular structure was decreased with an increasing of the CM-chitin composition (see Figure 4.3). It should be noted that after methanol treatment, pure silk fibroin film showed the random lines appearing throughout the film surface as a result of the contraction of silk fibroin after conformation transition but the globular structure could not be observed because fibroin proteins evenly distributed (see Figure 4.4B). Moreover, after submerging in water, the silk fibroin film displayed a very smooth surface (see Figure 4.4C).

4.4.2 Biodegradability In Vitro

To evaluate the *in vitro* biodegradability of a polymer blend system, it is difficult to find an appropriate choice of enzyme because each polymer component might be degraded with different types of enzymes. Among variety types of enzymes such as protease, α-chymotrysin, and collagenase, it has been reported that protease is the most effective one for digesting silk fibroin.[18] From our preliminary test (data not shown), it was found that protease from *Streptomyces griseus* could effectively degrade the methanol-treated silk fibroin as well as crosslinked CM-chitin films. Therefore, protease was used to investigate the biodegradability of the blend films in this study.

Figure 4.8A illustrates the quantitative changes in the weight of methanol-treated silk fibroin/CM-chitin films crosslinked with 0.0075% glutaraldehyde as a function of CM-chitin content. After the films were incubated with protease in PBS buffer solution at 37°C for 2 days (changing media daily), it was found that the weight of films decreased with an increasing of CM-chitin content.

Normally, a poor biodegradability of the silk fibroin is mainly due to its hydrophobic nature and high β-sheet content.[13] After blending with CM-chitin, as above mentioned, the miscibility of the two polymers occurred only in their amorphous parts and the β-sheet structure formation of silk fibroin induced by methanol treatment was not interfered by the presence of CM-chitin in the blend films; however, β-sheet content and hydrophobic nature of the blend films decreased with an increasing of CM-chitin content. Additionally, partial dissolution of CM-chitin fraction was also another factor of the decrease in the weight of the blend films during protease treatment as could be observed from the control in PBS solution without protease but it was only a small effect. Furthermore, a strong intermolecular interaction between silk fibroin and CM-chitin resulted in the good resistance of the blend films from dissolution in PBS solution (the maximum weight losses of the blend films was not higher than 8 wt% of their initial weights), while CM-chitin films crosslinked with glutaraldehyde at the same concentration (0.0075%) was dissolved in PBS solution ca. 25 wt% of their initial weights.

In order to explore the influence of the crosslinking on biodegradability, the degradation of the methanol-treated silk fibroin films with and without crosslinking was studied. Table 4.2 illustrates the weight of the methanol-treated silk fibroin films with and without cosslinking and the methanol-treated silk fibroin/CM-chitin blend films at 9/1 and 7/3 blend ratios incubated with protease in PBS buffer solution at 37°C for 6 days with changing of media daily. The data shown in Table 4.2 indicated that the *in vitro* biodegradability of the methanol-treated silk fibrin films with and without crosslinking were comparable. This was attributed to the use of a low concentration of crosslinking agent (0.0075%). It was found that when the CM-chitin content increased, the weight of films was decreased.

The blend of silk fibroin with CM-chitin not only increased the biodegradability but also promoted the swelling ability of the blend films. Figure 4.8B illustrates equilibrium water content of the methanol-treated silk fibroin films as a function of the CM-chitin content. Apparently, the equilibrium water content of the blend films increased with an increasing of CM-chitin content. This might be explained that hydrophilicity of the blend films was enhanced due to the presence of the ionizable functional groups in CM-chitin and the β-sheet content of silk fibroin was decreased when the CM-chitin content in the blend increased. Therefore, the swelling of silk fibroin was improved by blending with CM-chitin.

In addition, to examine the degradation behavior of the methanol-treated silk fibroin and blend films, the surface morphology had to be revealed after incubation in protease solution. Figure 4.9 is the SEM micrographs illustrate the surface of the methanol-treated silk fibroin films with and without crosslinking (see Figure 4.9A and 4.9B) and that of the silk fibroin/CM-chitin at 9/1 and 7/3 blend ratios (see Figure 4.9C and 4.9D) after incubation for 6 days in protease solution. The similar fashion in surface morphology was observed between the methanol-treated silk fibroin films with and without crosslinking. This was due to a low concentration of glutaraldehyde used for the crosslinking.

Interestingly, according to Figure 4.9C, the globular structure appearing in the silk fibroin/CM-chitin film at 9/1 blend ratio was still observed with larger gaps between each globule. It was seemed that the globules were hydrolyzed first from the outer surface and gradually lost toward to the central core (surface erosion). However, the degradation of the films in this fashion could not be clearly observed for the silk fibroin/CM-chitin at 7/3 blend ratio (see Figure 4.9D). This might be attributed to the higher CM-chitin composition and smaller in size of globular structure.

4.5 Conclusion

The multiblock copolymeric structure of silk fibroin can be divided into two major regions; hydrophobic and hydrophilic parts. When blending with CM-chitin, only the hydrophilic part (amorphous part) of silk fibroin was compatible with amorphous part of CM-chitin chains while crystalline phases of those polymers were not compatible. Thus the blend of silk fibroin with CM-chitin exhibited the semi-miscible blend. After crosslinking, the presence of CM-chitin in the blend films could improve the biodegradability and swelling ability of silk fibroin. Although the presence of CM-chitin did not directly affected the β-sheet structure of silk fibroin after methanol treatment, the content and hydrophobic nature of the blend films were decreased as CM-chitin content increased.

4.6 Acknowledgements

The authors would like to acknowledge Professor Seiichi Tokura and Associate Professor Pitt Supaphol for their stimulating discussion. This work was supported in part by Chulalongkorn University (through a research grant from the Ratchadaphisek Somphot Endowment Fund), the Thailand Research Fund (through the Royal Golden Jubilee Ph.D Program i.e., RGJ grant), the Petroleum and Petrochemical Technology Consortium (through a governmental loan from the Asian Development Bank), and the

Petroleum and Petrochemical College, Chulalongkorn University. The authors wish to thank Surapon Foods Public Co., Ltd. (Thailand) and Queen Sirikit Sericulture Center for supplying shrimp shells and raw silk fibers, respectively.

4.7 Reference

[1] L.H. Sperling, *"Introduction to Physical Polymer Science"*, Wiley-interscience, New York 2001.

[2] L. Finelli, M. Scandola , P. Sadocco, *Macromol. Chem. Phys.* **1998**, 199, 695.

[3] G.H. Altman, F. Diaz, C. Jakuba, T. Calabro,R.L. Horan, J. Chen, H. Lu, J. Richmond, D.L. Kaplan, *Biomaterials* **2003**, 24, 401.

[4] G.H. Altman, R.L. Horan, H.H. Lu, J. Moreau, I. Martin, J.C. Richmond, D.L. Kaplan, *Biomaterials* **2002**, 23, 4131.

[5] S. Hofmann, C.T. Wong Po Foo, F. Rossett, M. Textor, G. Vunjak-Novakovic, D.L. Kaplan, H.P. Merkle, L. Meinel, *J. Control. Rel.* **2006**, 111, 219.

[6] C. Chen, C. Chuanbao, M. Xilan, T. Yin, Z. Hesun, *Polymer* **2006**, 47, 6322.

[7] E. Marsano, P. Corsini, C. Arosio, A. Boschi, M. Mormino, G. Freddi, *Int. J. Biol. Macromol.* **2005**, 37, 179.

[8] D-H. Roh, S-K. Kang, J-Y. Kim, Y-B. Kwon, H.Y. Kweon, K-G. Lee, Y-H. Park, R-H. Baek, C-Y. Heo, J. Choe, J-H. Lee, *J. Mater. Sci. Mater. Med.* **2006**, 17, 547.

[9] L. Meinel, S. Hofmann, V. Karageorgiou, L. Zichner, R. Langer, D.L. Kaplan, G. Vunjak-Novakovic, *Biotechnol. Bioeng.* **2004**, 88, 379.

[10] L. Meinel, V. Karageorgiou, R. Fajardo, B. Snyder, V. Shinde-Patil, L. Zichner, D.L. Kaplan, R. Langer, G. Vunjak-Novakovic, *Ann. Biomed. Eng.* **2004**, 32, 112.

[11] L. Meinel, V. Karageorgiou, S. Hofmann, R. Fajardo, B. Snyder, C. Li, L. Zichner, R. Langer, G. Vunjak-Novakovic, D.L. Kaplan, *J. Biomed. Mater. Res. Part A* **2004**, 71A, 25.

[12] J. Nam, Y.H. Park, *J. Appl. Polym. Sci.* **2001**, 81, 3008.

[13] H-J. Jin, J. Park, V. Karageorgiou, U. Kim, R. Valluzzi, P. Cebe, D.L. Kaplan, *Adv. Funct. Mater.* **2005**, 15, 1241.

[14] H. Saitoh, K. Ohshima, K. Tsubouchi, Y. Takasu, H. Yamada, *Int. J. Biol. Macromol.* **2004**, 34, 259.

[15] J. Magoshi, M. Mizuide, Y. Magoshi, *J. Polym. Sci. Polym. Phys. Ed.* **1979**, 17, 515.

[16] T. Asakura, A. Kuzuhara, R. Tabeta, H. Saito, *Macromolecules* **1985**, 18, 1841.

[17] L. Meinel, S. Hofmann, V. Karageorgiou, C. Kirker-Head, J. McCool, G. Gronowicz, L. Zichner, R. Langer, G. Vunjak-Novakovic, D.L. Kaplan, *Biomaterials* **2005**, 26, 147.

[18] M. Li, M. Ogiso, N. Minoura, *Biomaterials* **2003**, 24, 357.

[19] T. Arai, G. Freddi,C. Innocenti, M. Tsukada, *J. Appl. Polym. Sci.* **2004**, 91, 2383.

[20] S. Tokura, S. Nishimura, N. Sakairi, N. Nishi. *Macromol. Symp.* **1996**, 101, 389.

[21] P. Wongpanit, N. Sanchavanakit, P. Pavasant, P. Supaphol, S. Tokura, R. Rujiravanit, *Macromol. Biosci.* **2005**, 5, 1001.

[22] J. Ragnhild, N. Hjerde, K.M. Varum, H. Grasden, S. Tokura, O. Smidsrod, *Carbohydr. Polym.* **1997**, 34, 131.

[23] A. Baxter, M. Dillon, K.D.A. Taylor, G.A.F. Roberts, *Int. J. Biol. Macromol.* **1992**, 14, 166.

[24] A. Domb, G.W.R. III Davidson, L.M. Sanders, *J. Control. Rel.* **1990**, 14, 133.

[25] F.G. Pearson, R.H. Marchessault, C.Y. Liang, *J. Polym. Sci.* **1960**, XLIII, 101.

[26] A. Garton, *"Infrared Spectroscopy of Polymer Blends, Composites and Surfaces"*, Hanser, Munich 1992.

[27] H-J. Jin, D. Kaplan, *Nature* **2003**, 424, 1057.

[28] K. Mita, S. Ichimura, T.C. James, *J. Mol. Evol.* **1994**, 38, 583.

Table 4.1 Spectral shifts of the amide I, amide II, and amide III of silk fibroin/CM-chitin blend films at various blend ratios.

Fibroin/CM-chitin blend ratio	Spectral shift of as-cast films[a]			Spectral shift of methanol-treated films[a]		
	Amide I (cm^{-1})	Amide II (cm^{-1})	Amide III[b] (cm^{-1})	Amide I (cm^{-1})	Amide II (cm^{-1})	Amide III[b] (cm^{-1})
10/0	0	0	0	0	0	0
8/2	1	0	1	1	2	4
6/4	2	-2	2	-1	-4	-1
5/5	1	-3	2	0	-4	0
4/6	1	-3	2	0	-9	0
2/8	0	-4	-	-1	-6	2
0/10	0	0	-	0	0	-

[a] = the analysis was based upon differences between the spectrum of a blend and the weighted sum of the spectra of the pure polymers.

[b] = the analysis was calculated from the amide III of silk fibroin present in the blend films.

Table 4.2 Percent weight of films after enzymatic degradation for 6 days at 37°C (changing media daily)

Fibroin/CM-chitin blend ratio[a]	Weight of films after degradation (%)	
	PBS with protease	PBS without protease
10/0[b]	82.85±1.01	96.77±0.95
10/0[c]	83.06±0.56	96.46±0.25
9/1[c]	51.20±5.61	95.19±1.24
7/3[c]	19.70±1.85	95.58±0.04

[a] = Initial weight of films was ~6 mg.

[b] = films were immersed in 90% (v/v) methanol aqueous solution for 10 minutes (without crosslinking) to induce conformation transition of silk fibroin before enzymatic hydrolysis. A control was done in PBS solution without enzyme.

[c] = films were crosslinked with 0.0075% glutaraldehyde and subsequently immersed in 90% (v/v) methanol aqueous solution for 10 minutes to induce conformation transition of silk fibroin before enzymatic hydrolysis. A control was done in PBS solution without enzyme.

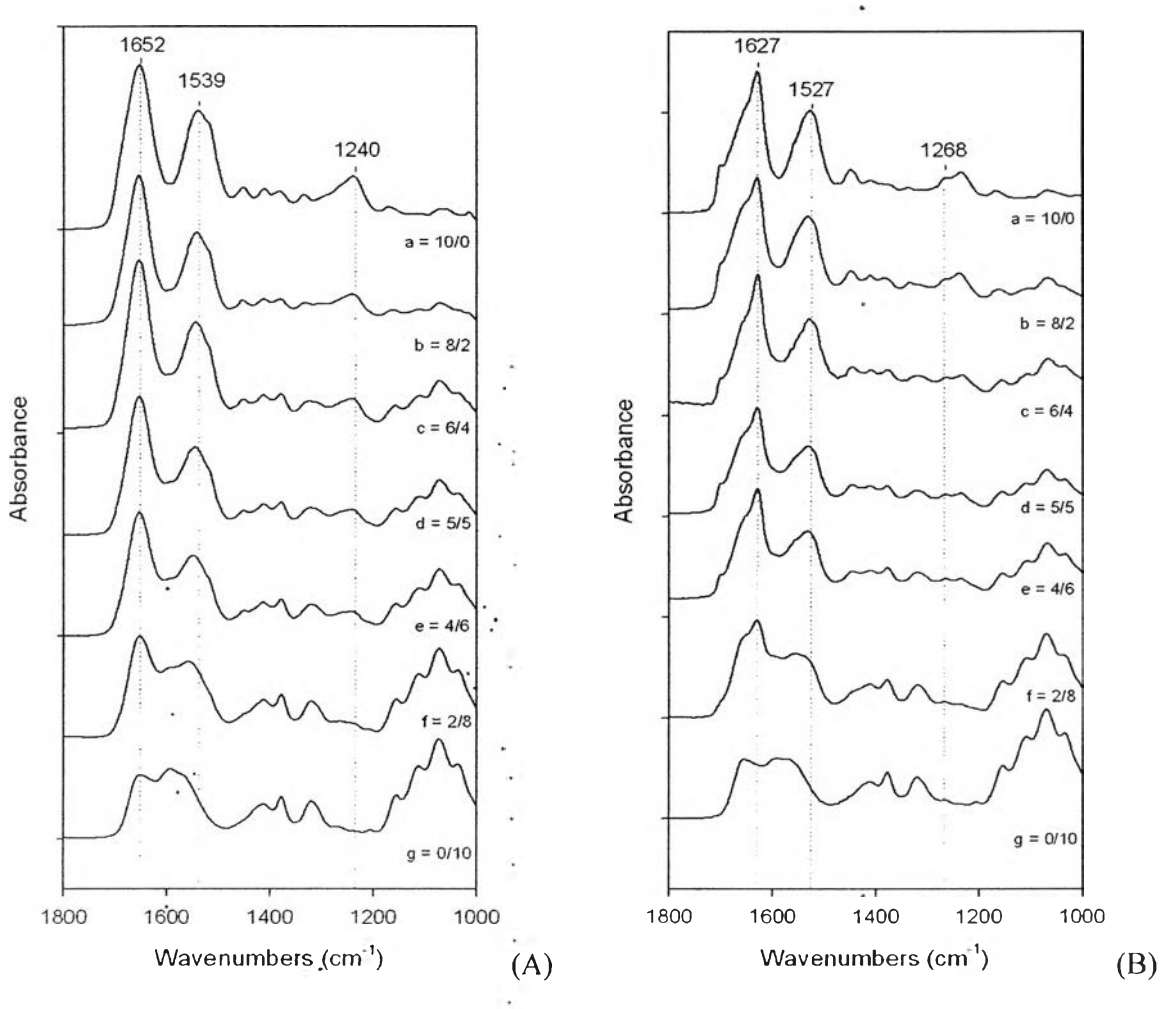


Figure 4.1 FTIR spectra of silk fibroin, CM-chitin, and the blend films at various silk fibroin/CM-chitin blend ratios (a to g) before methanol treatment (A) and after methanol treatment (B).

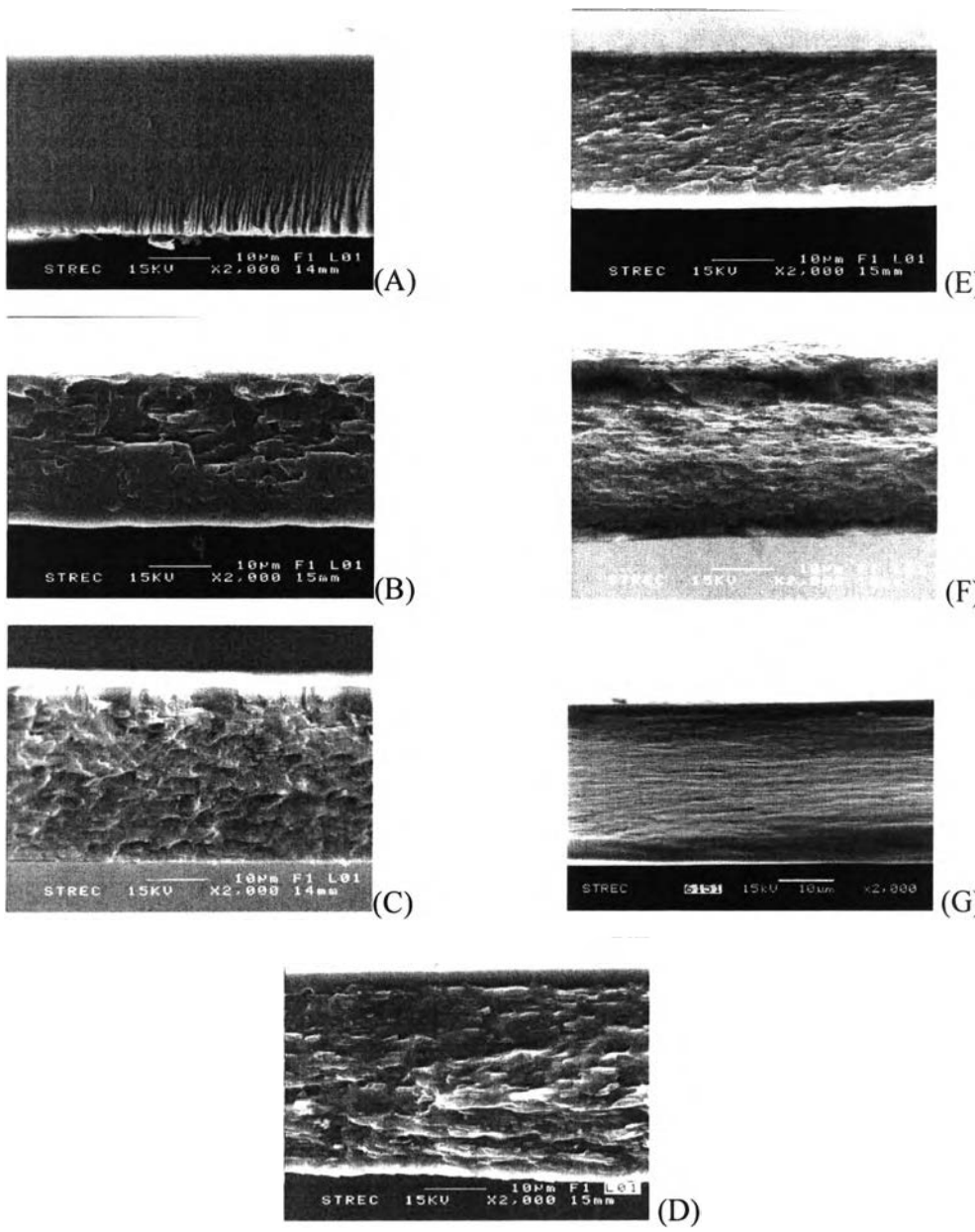


Figure 4.2 SEM micrographs of the fractured cross-sections of the methanol-treated silk fibroin/CM-chitin blend films at various blend ratios; 10/0 (A), 8/2 (B), 6/4 (C), 5/5 (D), 4/6 (E), 2/8 (F), and 0/10 (G).

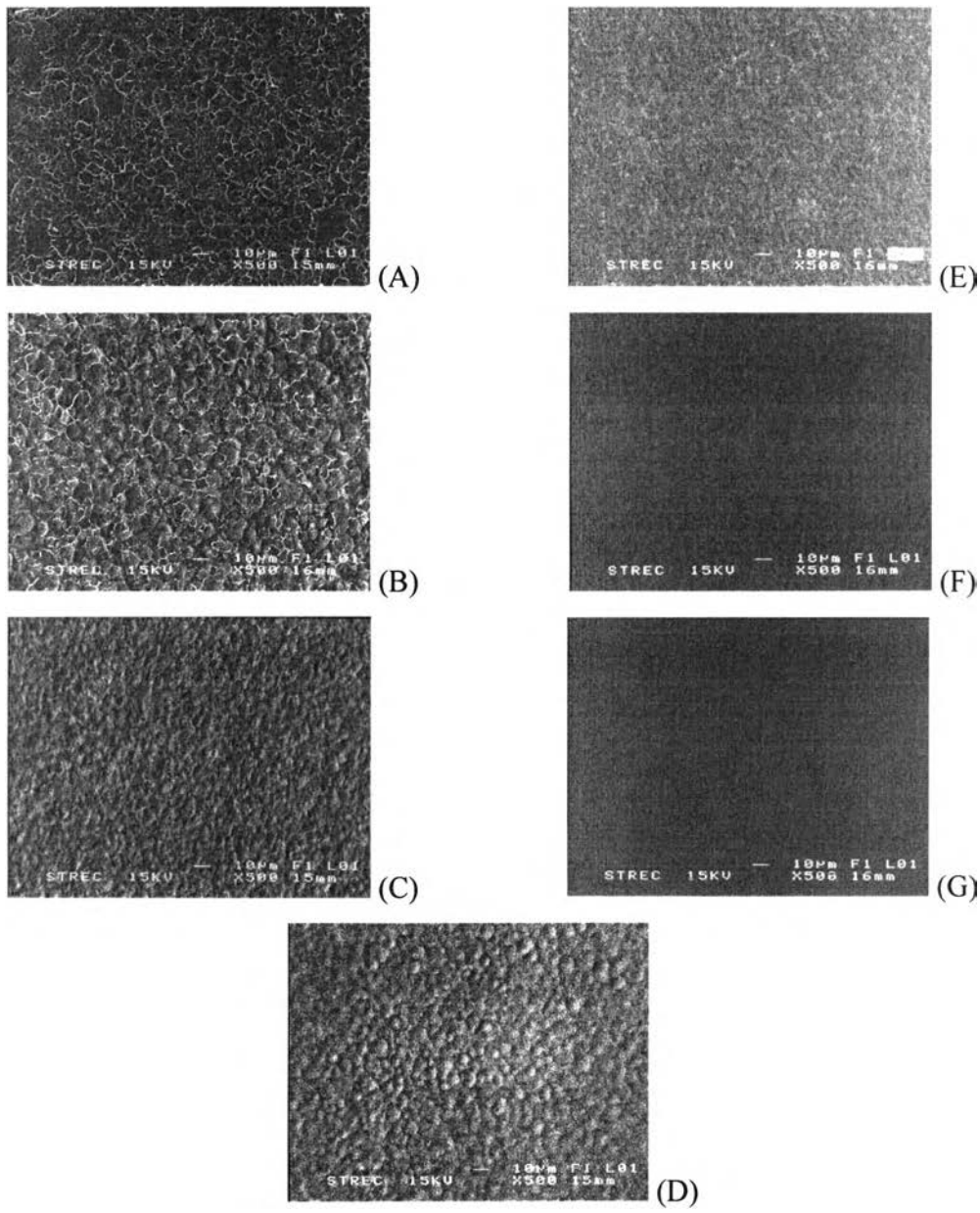


Figure 4.3 SEM micrographs of the surfaces of the methanol-treated silk fibroin/CM-chitin blend films at various blend ratios; 10/0 (A), 8/2 (B), 6/4 (C), 5/5 (D), 4/6 (E), 2/8 (F), and 0/10 (G).

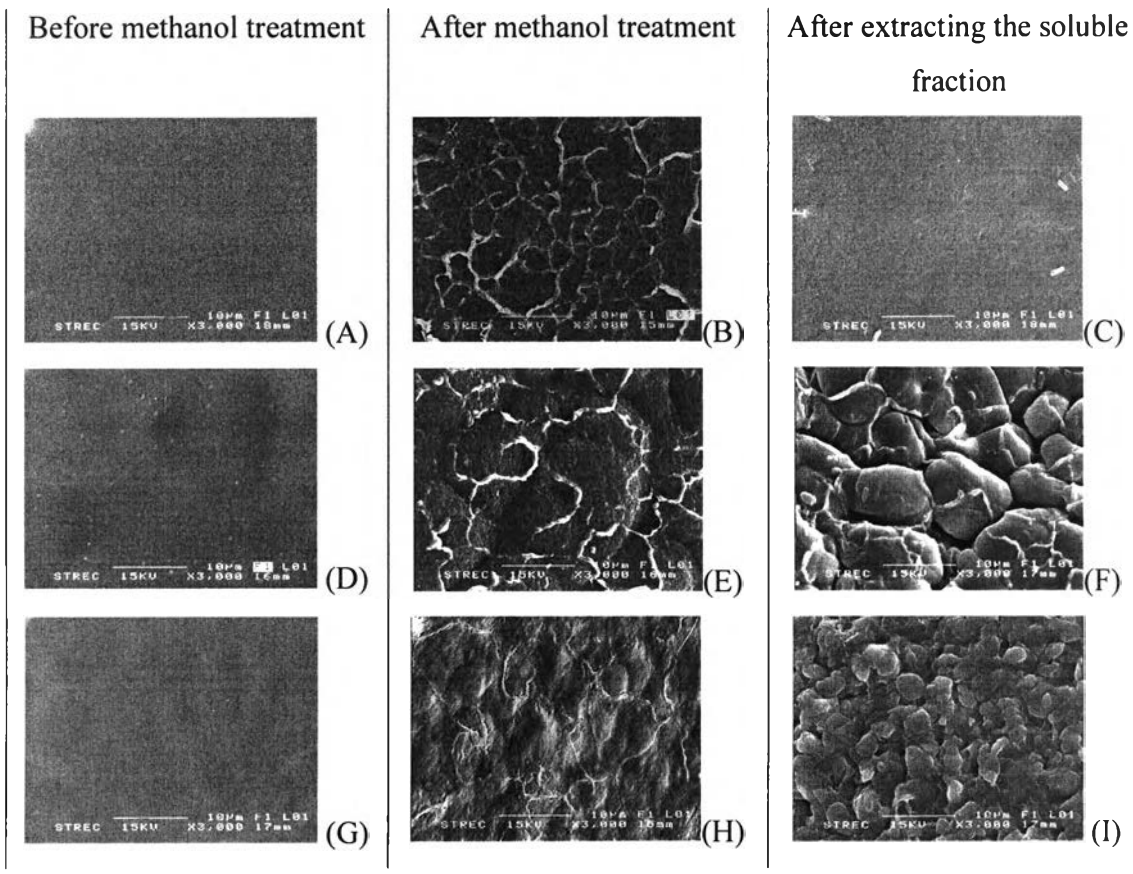


Figure 4.4 SEM micrographs of the surfaces of the silk fibroin/CM-chitin blend films at 10/0 (before methanol treatment (A), after methanol treatment (B), and after extraction of methanol-treated films in water (C)), 8/2 (before methanol treatment (D), after methanol treatment (E), and after extraction of methanol-treated films in water (F)), and 6/4 (before methanol treatment (G), after methanol treatment H, and after extraction of methanol-treated films in water (I)) blend ratios.

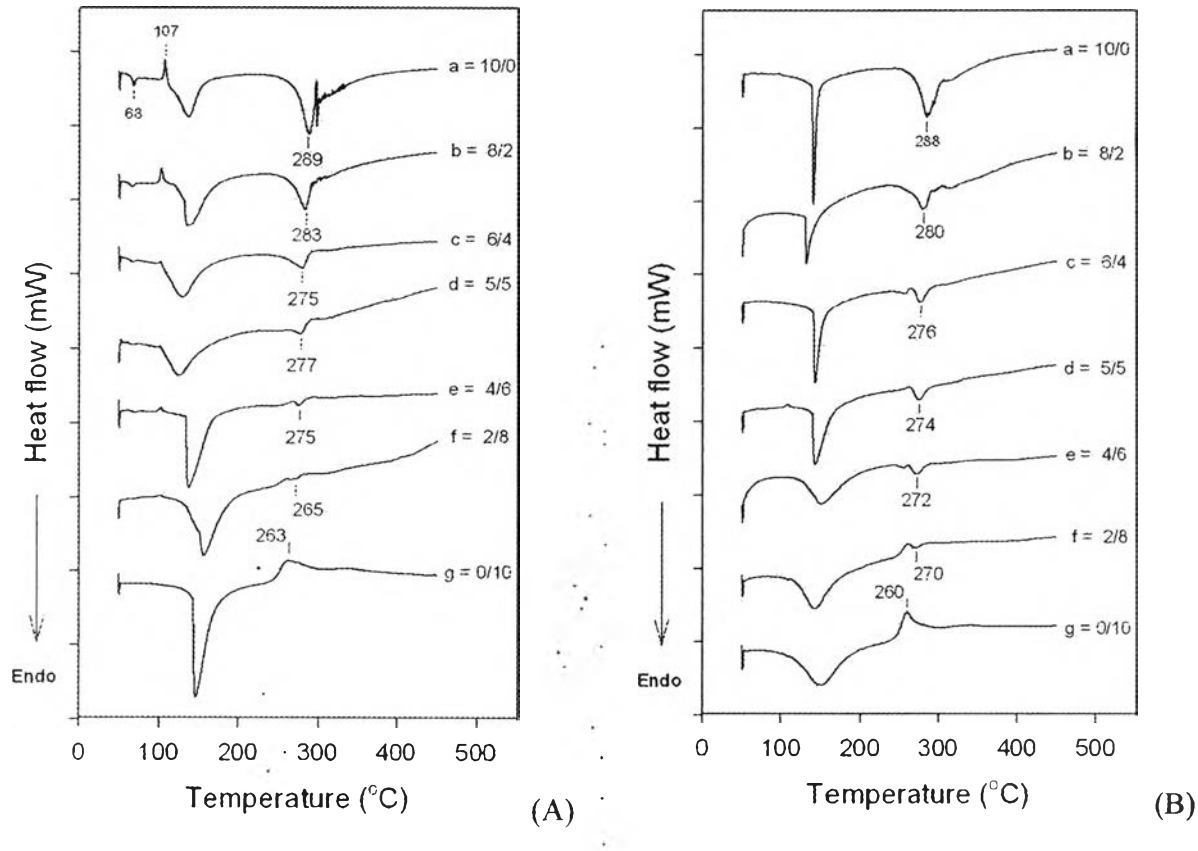


Figure 4.5 DSC thermograms of the silk fibroin/CM-chitin blend films at various blend ratios from pure silk fibroin film (a) to pure CM-chitin films (g) before methanol treatment (A) and after methanol treatment (B).

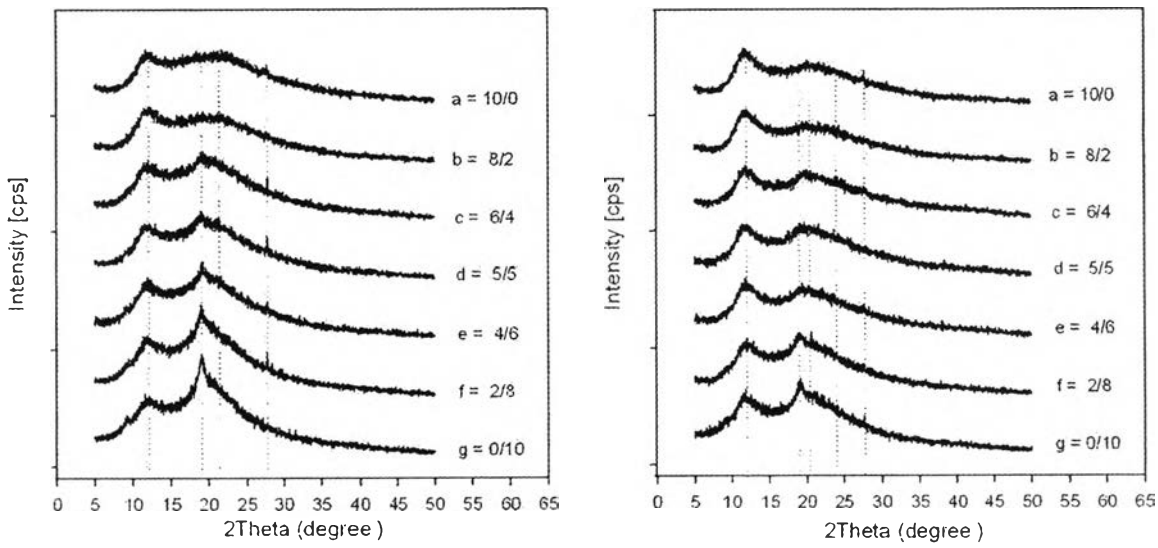
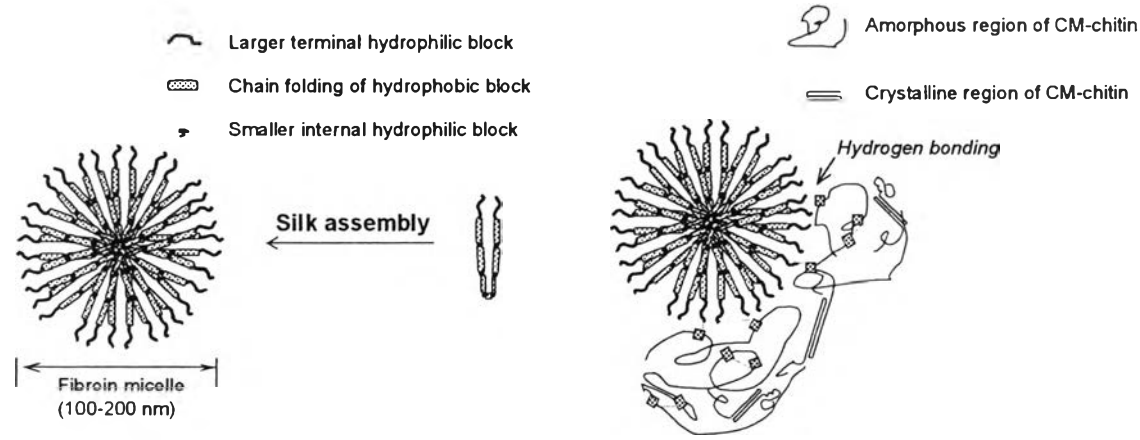


Figure 4.6 XRD patterns of the silk fibroin/CM-chitin blend films at various blend ratios from pure silk fibroin film (a) to pure CM-chitin films (g) before-methanol treatment (A) and after -methanol treatment (B).



(A) (B)

Figure 4.7 Formation of micellar structure of silk fibroin via self assembly (A). Interaction between silk fibroin micelle and CM-chitin chains (B).

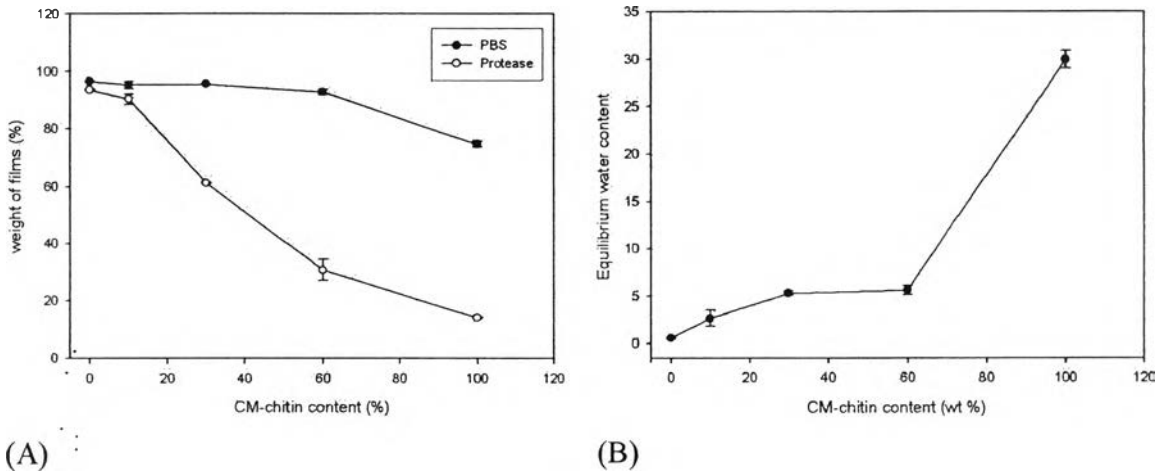


Figure 4.8 Percent weight of films after enzymatic degradation (A) and percent equilibrium water content (B) of the methanol-treated silk fibroin and silk fibroin/CM-chitin blend films crosslinked with 0.0075% glutaraldehyde as a function of CM-chitin content (●, films in PBS solution without protease; ○, films in PBS solution with protease). N = 3, bars represent standard deviation. The films were submerged in media for 2 days at 37°C.

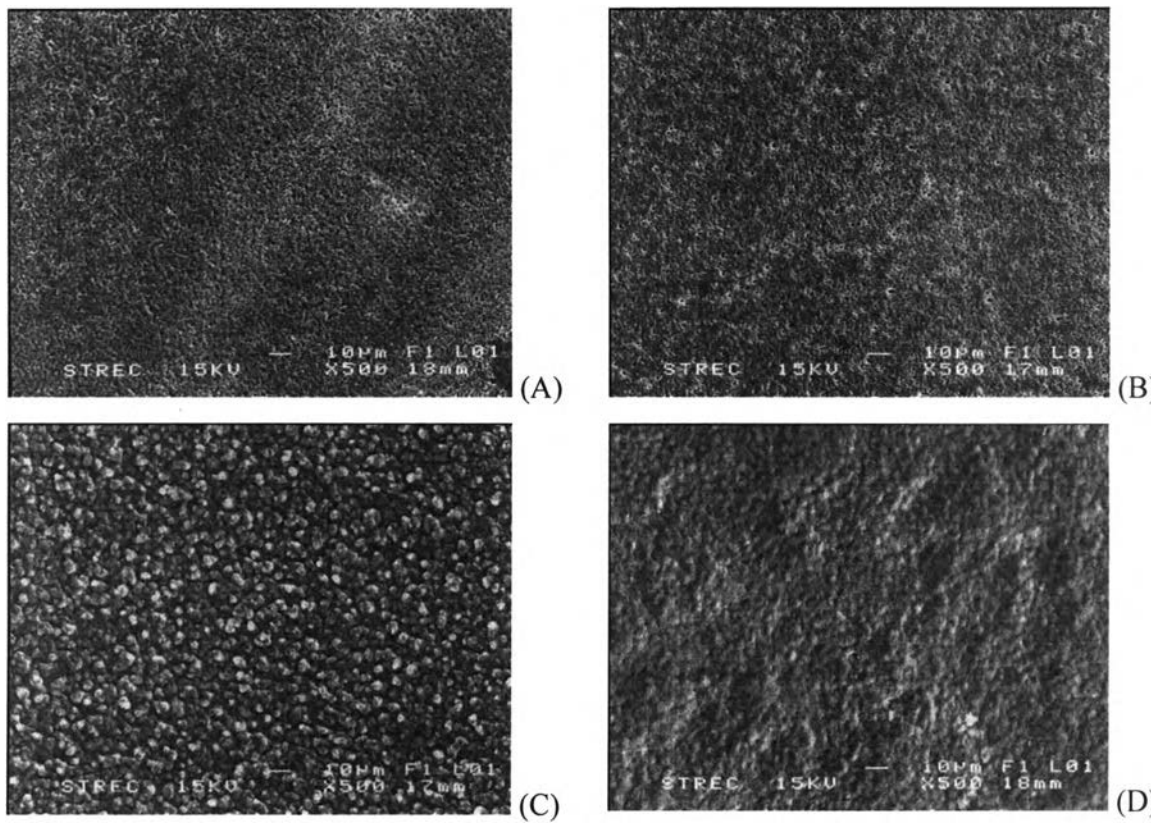


Figure 4.9 SEM micrographs of the methanol-treated films with and without crosslinking after enzymatic degradation for 6 days 37°C in protease solution; the methanol-treated silk fibroin film without crosslinking (A), the methanol-treated silk fibroin/CM-chitin blend films crosslinked with 0.0075% glutaraldehyde at 10/0 (B), 9/1 (C), and 7/3 (D) blend ratios.