Journal of Applied Sciences

ISSN 1812-5654

science alert

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Journal of Applied Sciences 11 (14): 2592-2598, 2011
ISSN 1812-5654 / DOI: 10.3923/jas.2011.2592.2598

Silk Fibroin/Chitosan Blend Films Loaded Methylene Blue as a Model for Polar Molecular Releasing: Comparison between Thai Silk Varieties

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Abstract: The aims of this study were to prepare Silk Fibroin (SF)/Chitosan (CS) blend films loaded Methylene Blue (MB) and characterize their related properties. The SF was obtained from various Thai silk varieties locally called "Nang Lai, Mo and Kaki". The blend films were prepared by mixing the SF, CS and MB solutions before pouring on polystyrene plates. They were then taken to an oven at 40°C for 3 days to obtain the films. In addition, each native SF silk variety and CS films were also prepared as control. SEM micrographs showed that native Nang Lai film appeared the bead like particles while other film types were smooth. Comparison between the blend films, Mo variety film has smooth surfaces while Nang Lai and Kaki films composed of flat particles covered their surfaces. FTIR results indicated that all of native SF has similar absorption bands at amide regions which coexisted of α-helix and β-sheet structures. Moreover, SF blended with CS showed strong bands at amide II, exhibit β-sheet structure. The blend film of Mo variety rapidly decomposed whereas Nang Lai was the lowest. *In vitro* releasing study indicated that MB released more quickly at the initial 30 min which Mo variety was the slowest.

Key words: Silk fibroin, surface area, controlled release, transitional change, maximum temperature of decomposition

INTRODUCTION

Silk is a natural fiber produced by some Lepidoptera insects such as silkworm, spiders, scorpions, mites and flies (Altman *et al*., 2003). Generally, silks are classified into mulberry (domesticated) and non-mulberry (wild) silks (Acharya *et al*., 2009). Each silk fiber consists of two insoluble fibroin fibers which coated by sericin glue-like protein (Takasu *et al*., 2010; Kim *et al*., 2005). Silk has been used in textile for long history. Recently, various applications from silk were reported such as cosmetics, food and drink additives or biomedical materials (Meinel *et al*., 2005; Acharya *et al*., 2008; Fang *et al*., 2009). Silk Fibroin (SF) shows excellent properties including high strength, biodegradability, biocompatibility and minimum inflammatory response (Schneider *et al*., 2009; Nogueira *et al*., 2010). SF could be prepared into different forms such as film, hydrogel, powder, sponge, fiber or mat depending on application (Zhang *et al*., 2007).

Chitosan (CS) is one kind of natural polymer. It is a derivative of chitin by deacetylation reaction. CS composed of D-glucosamine unites (Rinaudo, 2006). Therefore, CS was classified as cationic molecules which could be attached with anionic substances. The CS shows some excellent properties such as biocompatibility (Jones *et al*., 2009), biodegradability (Yang *et al*., 2009), high strength with flexibility (Wang and Li, 2007) or anti-infection (Chung *et al*., 2003). At present, CS has been applied for many fields including agriculture and animal stuff (Martinez-Camacho *et al*., 2010), biomedical (Kim *et al*., 2008; Bangoura *et al*., 2009), drug delivery system (Ramachandran *et al*., 2011). Moreover, CS can be used by its only or blend with other polymers.

In general, SF film was fragile and hard to transfer (Lu *et al*., 2010). There are many studies to improve this limit point. However, the simple method by blending SF with polar molecules such as gelatin (Fan *et al*., 2008), hyaluronic acid (Garcia-Fuentes *et al*., 2009), collagen (Zhou *et al*., 2010), sugar (Acharya *et al*., 2008; Baimark *et al*., 2009), starch (Srihanam, 2011) was popularly performed. Therefore, this study was aimed to improve the properties of SF by blending with CS for loading polar molecule MB. The effect of different silk varieties on the blend film properties were investigated and described.

MATERIALS AND METHODS

This study was performed for 4 months from December 1, 2010 to March 1, 2011. Most of the experiment was done at Department of Chemistry, Faculty of Sciences, Mahasarakham University, Thailand.

Silk cocoons: Three Thai silk varieties cocoons called Nang Lai, Mo and Kaki were kindly supplied by Silk Innovation Center (SIC), Mahasarakham University, Maha Sarakham, Thailand. Chitosan powder (MW 15,000 Da) was purchased from Seafresh Chitosan Lab Co., Ltd., Thailand. All of chemical used were analytical grade.

Methods

Preparation of SF solution: The cocoons of three varieties were boiled in 0.5% (w/v) $NaHCO_3$ solution at 90°C for 30 min and then rinsed twice with distilled water. The cocoons were dissolved using tertiary solvent system of $CaCl_2$-Ethanol-H$_2$O (1:2:8 by mole) with magnetic stirred at 90°C for 2 h. The SF solution was then dialyzed using dialysis bag against distilled water for 3 days to obtain pure SF solution.

Preparation of CS solution: Chitosan (CS) solution was prepared by weighing 1g of CS powder (MW 15,000 Da) and mixing with 100 mL of 2% (v/v) acetic acid. The mixture was stirred with magnetic stirrer until obtain CS solution.

Preparation of SF/CS blend films loaded methylene blue: SF/CS blend films could be prepared by mixing 10 mL of 1% (w/v) SF solution and 10 mL of 1% (w/v) CS solution with total of 20 mL per plate, then added 1% (w/v) methylene blue. The mixture was stirred before pouring into the polystyrene plates. They were left in an oven at 40°C for 3 days to obtain films.

Morphology observation: All of films were cut and observed their morphology under the Scanning Electron Microscope (SEM) (JEOL, JSM-6460LV, Tokyo, Japan). The samples were sputter coated with gold by double side of carbon for enhancing surface conductivity.

Secondary structure investigation: All of films were investigated their secondary structures using Fourier transform infrared (FTIR) spectroscopy (Perkin Elmer-Spectrum Gx, USA) in the spectral region of 4000-400 cm^{-1} at 4 cm^{-1} spectral resolution and 32 scans.

Thermal properties measurement: All of SF films (8-10 mg) were loaded in a platinum crucible. The thermogravimetric analysis (TGA) was then performed using TA instruments, SDT Q600 (Luken's drive, New Castle, DE). The samples were non-isothermal heated from 50°C to 600°C at a heating rate of 20°C min^{-1}. The TGA was carried out in nitrogen with the flow rate of 100 mL min^{-1}. The TG and DSC data were recorded with TA instrument's Q series explorer software. The analyses of the data were done using TA Instrument's Universal Analysis 2000 software (version 3.3B).

***In vitro* releasing study:** SF/CS blend films loaded methylene blue were cut and weighed for 0.15 g. They were then submersed in 20 mL Phosphate Buffer Saline (PBS) pH 7.4. The mixture was left for 2 h interval times. 10 mL of buffer solution was collected in each designation time points and measured at 640 nm using UV-Vis spectrophotometer. After collection, 10 mL of fresh PBS was added to the mixture. The study was extended for 72 h of submersion.

RESULTS

Morphology: With SEM micrographs, different kinds of films surfaces were observed. The native SF film of Nang Lai variety appeared bead-like particles while other varieties were smooth. The surfaces of blend films indicated slightly differed since the blend films of SF from Nang Lai and Kaki has flat particles covered their surface areas but Mo was not find as shown in Fig. 1.

Secondary structures: FTIR results showed that all of native SF and blend films have similar absorption bands, especially at amide regions. The native CS film shows absorption band differed from the SF. However, the blend films between SF/CS showed slightly differed comparing to both native film types. As shown in Fig. 2, the blend film of Nang Lai SF/CS showed transitional change of wave number into lower at amide I and amide II compare to SF film. In similar, the blend film of Kaki SF/CS showed slightly change of wave number to lower at amide II region but at amide I was higher (Fig. 3). In contrast, at amide II and III regions, Mo SF/CS blend film shows higher wave number (Fig. 4). Some specific absorption bands of all films were listed in Table 1.

Thermal behaviors: As shown in Fig. 5, films started to degrade at about 100°C and then gradually decomposed with the highest about 300-400°C. The native CS film was rapidly degraded than other. Comparison between native SF, Mo variety was the quickly decomposed but Nang Lai was the slowest. SF blended CS showed two points of $T_{d,max}$ as indicated in Fig. 6. Some $T_{d,max}$ characteristics of each films were summarized in Table 2. Considering from exo/endo-thermic peaks, different characteristics were found (Table 3). The result shows Nang Lai has the highest of temperature maximum of decomposition while Mo was the lowest.

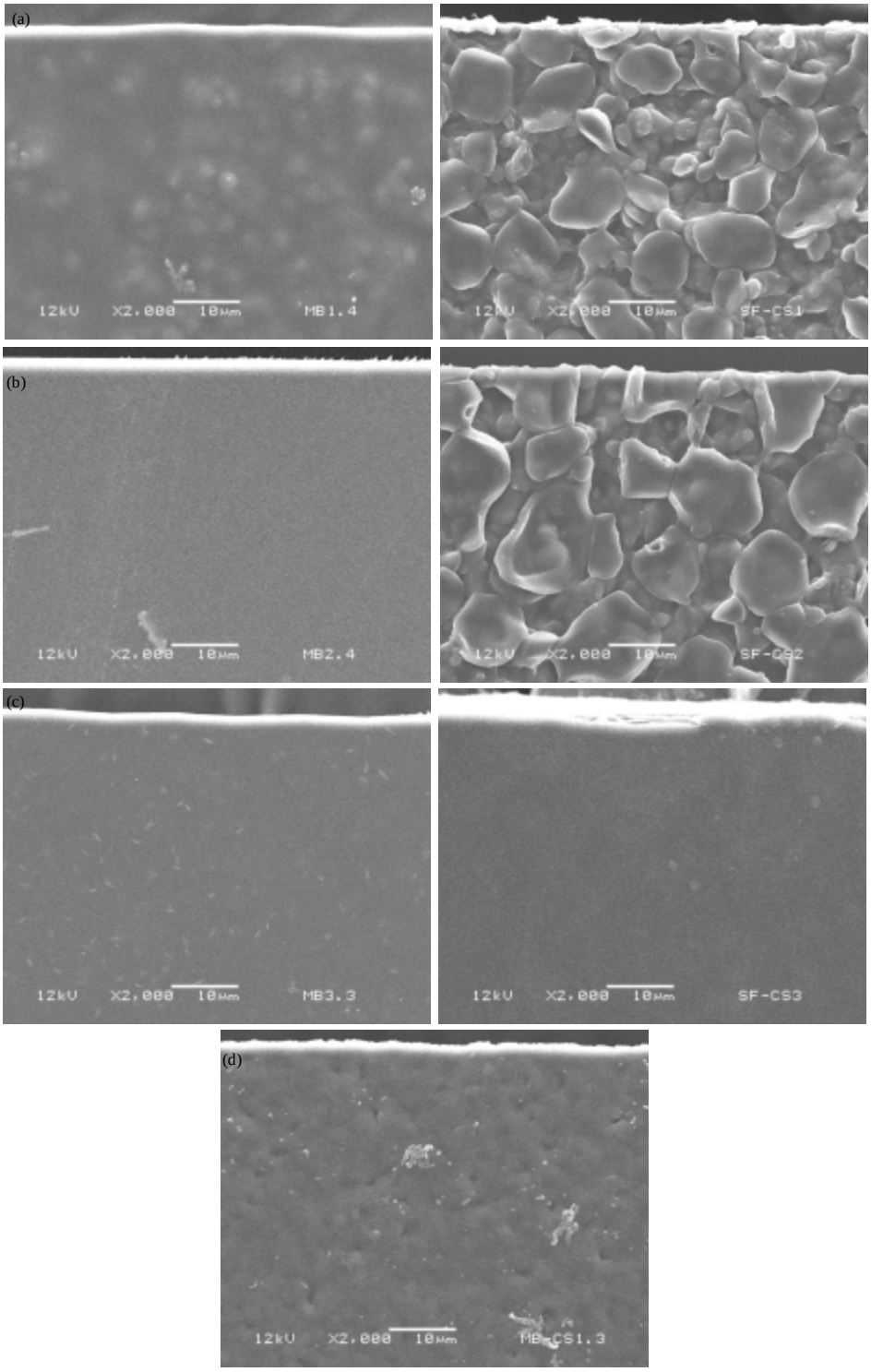

Fig. 1(a-d): SEM micrographs of different films; (a) Nang Lai (b) Kaki (c) Mo and (d) chitosan. Notes native films presented in left column and blend films presented in right column

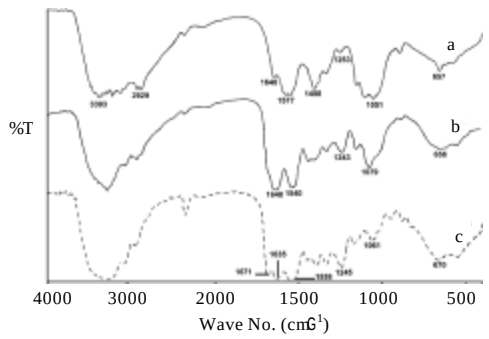

Fig. 2: FTIR spectra of chitosan (a) silk fibroin/chitosan blend (b) and silk fibroin and (c) films (Nang Lai variety)

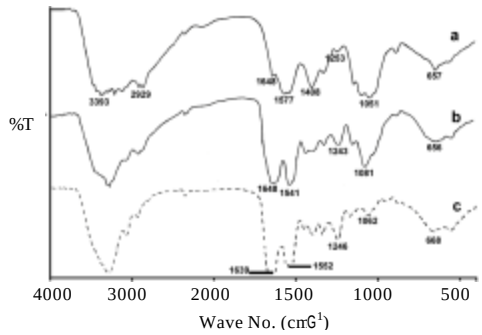

Fig. 3: FTIR spectra of chitosan (a) silk fibroin/chitosan blend (b) and silk fibroin (c) films (Kaki variety)

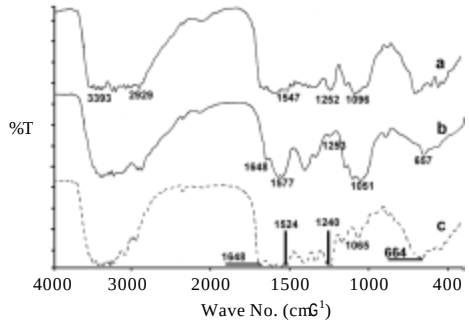

Fig. 4: FTIR spectra of chitosan (a) silk fibroin/chitosan blend (b) and silk fibroin and (c) films (Mo variety)

Methylene Blue (MB) released study: MB was released the highest in the initial 30 min of study. The released MB content was increased until 10 h and then gradually increased even the last time of 72 h. In addition, the releasing of MB from SF of Mo variety blended with CS film has lower rate than other about 3 times as shown in Fig. 7.

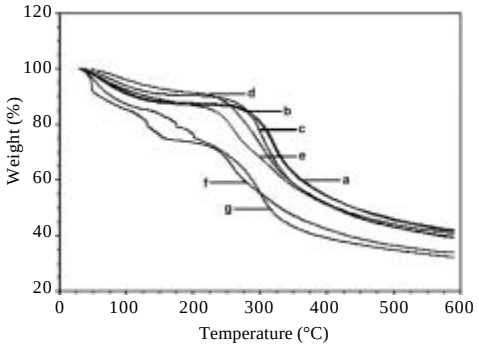

Fig. 5: TG curves of Nang Lai (a) Kaki (b) Mo (c) silk fibroin (Nang Lai)/chitosan blend (d) silk fibroin (Kaki)/chitosan blend (e) silk fibroin (Mo)/chitosan blend (f) and chitosan and (g) films

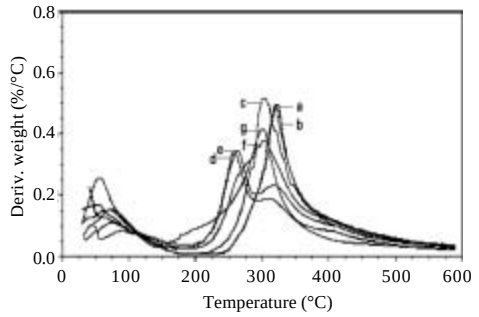

Fig. 6: TG curves of Nang Lai (a) Kaki (b) Mo (c) silk fibroin (Nang Lai)/chitosan blend (d) silk fibroin (Kaki)/chitosan blend (e) silk fibroin (Mo)/chitosan blend (f) chitosan and (g) films

Table 1: Absorption bands of different film types

	Absorption bands (cm^{-1})				
Type of films	Amide I	Amide II	Amide III	Amide IV	Amide V
Mo	1648	1524	1240	1065	664
Mo/CS	1667	1547	1252	1096	611
Kaki	1639	1552	1246	1062	668
Kaki/CS	1648	1541	1243	1081	656
Nang Lai	1671 1635	1558	1245	1061	670
Nang Lai/CS	1648	1540	1243	1079	655

Table 2: Temperature of maximum decomposition rate of different film types

Type of films	Temperature of maximum decomposition rate ($T_{d,max}$)
Chitosan (CS)	301
Nang Lai	323
Kaki	319
Mo	300
Nang Lai/CS	272 323
Kaki/CS	263 319
Mo/CS	278 306

DISCUSSION

Silk Fibroin (SF) is an interest material due to its excellent properties which are suitable for application.

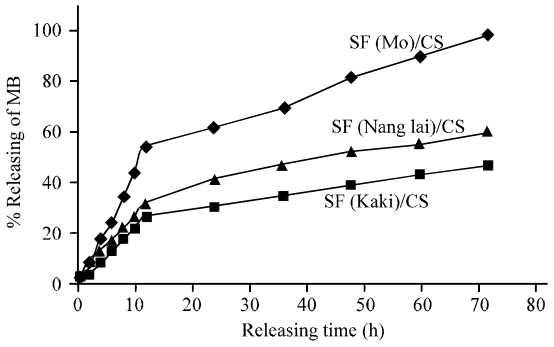

Fig. 7: Releasing contents (%) of Methylene Blue (MB) from different silk fibroin/chitosan blend films

Table 3: Exo/endo-thermic peaks of different film types

Type of films	Endo-thermic peaks (°C)	Exo-thermic peaks (°C)
Chitosan (CS)	225	159 312
Mo	225 301	223 285 380
Kaki	265 327	250 280
Nang Lai	260 328	218 285 380
Mo/CS	314	200 375
Kaki/CS	275 325	202 300 375
Nang Lai/CS	230 320	200 275 375

Many researchers who studied about SF are published (Vepari and Kaplan, 2007; Wang and Li, 2007; Lu *et al*., 2010). On the other hand, chitosan (CS) is also an interest material since it composes of many excellent properties too. Therefore, study and application of the CS were also reported (Park *et al*., 2004; Yuan *et al*., 2004; Griffon *et al*., 2006). Beside the good properties of both materials, some researchers have been focused on preparation of SF and CS blend material (Gil *et al*., 2007; Fan *et al*., 2008; Thein-Han *et al*., 2009).

Previously, there are reported about properties of SF which were affected by food, environment or silk variety. The result from this study showed that the films prepared from different silk varieties were differed in their surfaces and some properties. This might be suggested that internal composition such as amino acid and interaction between them of each silk variety were differences. Furthermore, the immiscibility between SF and CS might be caused from the polarity of materials. SF is an insoluble fiber composed of dominantly small hydrophobic amino acids (Acharya *et al*., 2009; Du *et al*., 2009) while CS is a polar molecule (Griffon *et al*., 2006).

The conformational structure is an important part and significantly affected on protein (Lee *et al*., 2003). The protein structures were analyzed by FTIR which indicated typical absorption bands sensitive to the molecular conformation of protein, especially SF (Kweon *et al*., 2000; Tasukada *et al*., 1995). The structures of the SF protein are indicated in amide I (1700-1600 cm^{-1}), amide II (1600-1500 cm^{-1}) and amide III (1300-1200 cm^{-1}) regions (Hino *et al*., 2003; Kweon *et al*., 2000). The present study found that all native SF films show similar absorption bands represent coexisted between α-helix and β-sheet structures. (Li *et al*., 2003; Mandal *et al*., 2009). The absorption bands were changed to lower wave number after SF blended with CS, especially amide II. It is suggested that SF can be formed the interaction to CS resulted to increase of β-sheet structure. Thermal properties of the films were confirmed that different silk varieties have different characteristics. The result indicated that SF of Nang Lai variety resisted decomposing in the highest temperature. This suggested that amino acid compositions of Nang Lai might be more hydrophobic than other. This point was related to lower interaction with polar molecule like MB which found in released study. On the other hand, Mo variety might be composed of high hydrophilic amino acids which helped to form interaction bond with MB in higher ratio (Ulubayram *et al*., 2001; Okhawilai *et al*., 2010).

CONCLUSION

The different characteristics of Thai silk varieties when blended with chitosan and loaded methylene blue were reported in this study. The morphology of each film was slightly differed; Nang Lai indicated bead-like particles on its surfaces while other has not. They are also appeared different characteristics of the blend films. Mo blended with chitosan has smoother surfaces than both Nang Lai and Kaki blend films. However, secondary structure of all blend films showed similar pattern which were α-helix mixed β-sheet structures. Thermal study indicated that Mo variety was rapidly degraded comparing to other Thai silks. It might be involved to the amino acid composition of its fiber. This result related to the interaction between silk fibroin and methylene blue since the releasing content of the methylene blue from Mo variety was the lowest.

ACKNOWLEDGMENTS

I would like to thank Miss Karnikar Phasai and Miss Nichawan Chernchom, my B.Sc students for their help to do my work. I also great fully thank Division of Research Facilitation and Dissemination, Department of Chemistry, Faculty of Science, Mahasarakham University and Center of Excellence for Innovation in Chemistry, Commission on Higher Education, Ministry of Education, Thailand for financial support of this study.

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