EFFECTS OF EXTRACTING CONDITIONS USING CALCIUM CHLORIDE-ETHANOL PROCESS ON CHARACTERISTICS OF THAI SILK FIBROIN

Mr. Supawich Chankow

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ผลของสภาวะการสกัดด้วยกระบวนการแคลเซียมคลอไรด์-เอทานอลต่อลักษณะของไฟโบรอินไหมไทย

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By	Mr. Supawich Chankow
Field of Study	Biomedical Engineering
Thesis Advisor	Assistant Professor Sorada Kanokpanont, Ph.D.
Thesis Co-Advisor	Professor Pornanong Aramwit, Pharm.D., Ph.D.

Accepted by the Faculty of Engineering, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....Dean of the Faculty of Engineering
(Associate Professor Supot Teachavorasinskun, D.Eng.)

THESIS COMMITTEE

.....Chairman
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.....Thesis Advisor
(Assistant Professor Sorada Kanokpanont, Ph.D.)
.....Thesis Co-Advisor
(Professor Pornanong Aramwit, Pharm.D., Ph.D.)
.....External Examiner
(Associate Professor Kwanchanok Pasuwat, Ph.D.)

ศุภวิชญ์ จันทน์ขาว : ผลของสภาวะการสกัดด้วยกระบวนการแคลเซียมคลอไรด์-เอทานอลต่อลักษณะของไฟโบรอินไหมไทย (EFFECTS OF EXTRACTING CONDITIONS USING CALCIUM CHLORIDE-ETHANOL PROCESS ON CHARACTERISTICS OF THAI SILK FIBROIN) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. โศรดา กนกพานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ศ. ภญ. ดร. พรอนงค์ อร่ามวิทย์, 103 หน้า.

การศึกษาผลของสภาวะการสกัดไฟโบรอินด้วยตัวทำละลายอาจิซาวา (แคลเซียมคลอไรด์-น้ำ-เอทานอล) โดยใช้อัตราส่วน 1: 30 - 1: 10 ในช่วงอุณหภูมิ 50 - 80 องศาเซลเซียส โดยเปรียบเทียบกับผลการสกัดด้วยตัวทำละลายลิเทียมโบรไมด์โดยใช้อัตราส่วนเดียวกันที่อุณหภูมิ 65 องศาเซลเซียส พบว่าร้อยละผลได้ของการสกัดเท่ากับ 63 ± 9 - 74 ± 8 โดยไฟโบรอินที่สกัดได้มีโครงสร้างโมเลกุล และสมบัติทางความร้อนแตกต่างกัน อย่างไรก็ตามปริมาณของกรดอะมิโน มวลโมเลกุล ค่าศักย์เซต้า และความหนืดของสารละลายไฟโบรอินดังกล่าวไม่แตกต่างกัน ไฟโบรอินที่สกัดด้วยตัวทำละลายอาจิซาวามีโครงสร้างแบบไม่เป็นระเบียบเพิ่มขึ้น เมื่ออัตราส่วนที่ใช้ในการสกัดลดลง และอุณหภูมิที่ใช้ในการสกัดสูงขึ้น ซึ่งไฟโบรอินดังกล่าวสูญเสียมวลจากการสลายตัวทางความร้อนเพิ่มขึ้น และมีค่าการคายพลังงานที่เกี่ยวข้องกับการระเหยของน้ำ และปริมาณของน้ำสูงขึ้นตามปริมาณของโครงสร้างที่ไม่เป็นระเบียบ โดยไฟโบรอินที่สกัดด้วยตัวทำละลายอาจิซาวามีอุณหภูมิการสลายตัวทางความร้อนของสูงกว่าไฟโบรอินที่สกัดด้วยตัวทำละลายลิเทียมโบรไมด์เล็กน้อย ไฟโบรอินที่สกัดได้จากตัวทำละลายทั้งสองชนิดมีปริมาณของไกลซีน อะลานีน เซอรีน ไทโรซีน และวาลีนเท่ากับร้อยละ 46.6 - 48.3, 29.2 - 32.1, 4.9 - 8.2, 4.5 - 5.1 และ 2.3 - 2.6 โดยโมล ตามลำดับ และมีมวลโมเลกุลเฉลี่ยโดยน้ำหนักและมวลโมเลกุลเฉลี่ยโดยจำนวนเท่ากับ 336 - 340 กิโลดาลตัน และ 161 - 167 กิโลดาลตัน ตามลำดับ โดยกระบวนการสกัดทั้งสองทำลายไฟโบรอินสายหนักแต่รักษาไฟโบรอินสายเบาที่มีมวลเท่ากับ 25 กิโลดาลตัน ไว้ได้ ฟิล์มไฟโบรอินที่ขึ้นรูปจากสารละลายที่สกัดได้ทั้งสองวิธีมีปริมาณของเบต้าชีตเท่ากับร้อยละ 33.8 – 35.0 และมีปริมาณของเบต้าชีตเพิ่มขึ้นเป็นร้อยละ 38.9 - 40.0 และ 42.8 - 46.7 หลังจากผ่านการอบด้วยไอน้ำ และแช่ด้วยเอทานอลตามลำดับ ฟิล์มไฟโบรอินทุกชนิดมีค่าพลังงานพื้นผิวใกล้เคียงกัน แต่พื้นผิวของตัวอย่างที่อบด้วยไอน้ำแสดงความมีขั้วมากกว่าพื้นผิวของตัวอย่างที่แช่ด้วยเอทานอล ฟิล์มไฟโบรอินที่สกัดจากตัวทำละลายลิเทียมโบรไมด์ และอบด้วยไอน้ำหลุดออกหลังจาก 48 ชั่วโมง ฟิล์มไฟโบรอินที่อบด้วยไอน้ำมีค่าการยึดเกาะเริ่มต้นของเซลล์ NIH-3T3 สูงกว่าฟิล์มที่แช่ด้วยเอทานอล โดยเซลล์ที่ถูกเพาะเลี้ยงบนฟิล์มไฟโบรอินมีอัตราการเจริญเติบโตจำเพาะ และระยะเวลาการเพิ่มจำนวนทวีคูณใกล้เคียงกับเซลล์ที่ถูกเพาะเลี้ยงบนถาดเลี้ยงเซลล์ และพื้นผิวแก้ว กระบวนการเตรียมไฟโบรอินจากไหมไทยมีผลต่อโครงสร้างโมเลกุล ซึ่งส่งผลกระทบต่อสมบัติอื่นของไฟโบรอินรวมทั้งความเข้ากันได้ทางชีวภาพกับเซลล์

สาขาวิชา วิศวกรรมชีวเวช (สหสาขาวิชา) ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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SUPAWICH CHANKOW: EFFECTS OF EXTRACTING CONDITIONS USING CALCIUM CHLORIDE-ETHANOL PROCESS ON CHARACTERISTICS OF THAI SILK FIBROIN. ADVISOR: ASST. PROF. SORADA KANOKPANONT, Ph.D., CO-ADVISOR: PROF. PORNANONG ARAMWIT, Pharm.D., Ph.D., 103 pp.

Effects of extracting conditions using calcium chloride-ethanol (Ajisawa's reagent) and lithium bromide (LiBr) process on characteristics of Thai silk fibroin (SF) were studied using 1: 30 - 1: 10 (w/v) dissolving ratios at 50 - 80 °C. Yields of Thai SF extractions were at 63 ± 9 - 74 ± 8 %. The Thai SFs were different in their molecular structures and thermal properties although they were similar in their amino acid compositions, molecular weights, zeta potentials, and viscosities. The Thai SFs extracted with the Ajisawa's reagent had their random coil contents increased with decreases in the dissolving ratios and increases in the dissolution temperatures. They had their thermal decomposition masses, water evaporation enthalpies, and water contents increased with the random coil contents. Thermal decomposition temperatures of the Ajisawa-derived Thai SFs were slightly higher than those of the LiBr-derived Thai SFs. Relative contents in glycine, alanine, serine, tyrosine, and valine of the Thai SFs were at 46.6 - 48.3 mol %, 29.2 - 32.1 mol %, 4.9 - 8.2 mol %, 4.5 - 5.1 mol % and 2.3 - 2.6 mol %, respectively. Weight average and number average molecular weights of the Thai SFs were at 336 - 340 kilo Daltons and 161 - 167 kilo Daltons, respectively. Heavy-chain was degraded while light-chain was preserved at 25 kilo Daltons by the two dissolution processes. As-casted Thai SF films had their β-sheet contents at 33.8 - 35.0 %. The β-sheet contents of the SF films increased to 38.9 - 40.0 % and 42.8 - 46.7 % after treated with water vapor annealing and ethanol immersion, respectively. All SF films had similar total surface energies. Water vapor annealed SF films exhibited more surface polarities than ethanol treated SF films. The water vapor annealed film fabricated from the LiBr-derived SF was detached after 48 h. Early cell attachments of NIH-3T3 mouse fibroblasts cultured on the water vapor annealed films were higher than those cultured on the ethanol treated films. Both of them had their specific growth rates and population doubling times comparable to the cells cultured on tissue culture plates and glass substrates. The preparation processes affected the molecular structures of the Thai SFs. The SF's molecular structures further influenced other properties including biocompatibilities with cells.

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Student's Signature .....
Advisor's Signature .....
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LIST OF ABBREVIATIONS AND SYMBOLS

	Description
%	Percent
$\overline{M_n}$	Number average molecular weight
$\overline{M_w}$	Weight average molecular weight
M_i	Mass of molecule
N_1	Number of cells at specified time point t_1
N_2	Number of cells at specified time point t_2
N_i	Number of molecule
γ_L	Total surface free energy of liquid
γ_L^d	Surface energy due to dispersive component of liquid
γ_L^p	Surface energy due to polar component of liquid
γ_{SF}	Total surface free energy of silk fibroin film
γ_{SF}^d	Surface energy due to dispersive component of silk fibroin film
γ_{SF}^p	Surface energy due to polar component of silk fibroin film
°	Degree
°C	Degree Celsius
°C min^{-1}	Degree Celsius per minute
μ	Specific growth rate
μg ml^{-1}	Microgram per milliliter
μl	Microliter
μm	Micrometer
μm^2	Square micrometer
A	α-helix
A1	Silk fibroin extracted with Ajisawa's regent using a dissolving ratio of 1: 30 weight by volume at 50 °C
A2	Silk fibroin extracted with Ajisawa's regent using a dissolving ratio of 1: 30 weight by volume at 65 °C
A3	Silk fibroin extracted with Ajisawa's regent using a dissolving ratio of 1: 30 weight by volume at 80 °C
A4	Silk fibroin extracted with Ajisawa's regent using a dissolving ratio of 1: 15 weight by volume at 50 °C
A5	Silk fibroin extracted with Ajisawa's regent using a dissolving ratio of 1: 15 weight by volume at 65 °C
A6	Silk fibroin extracted with Ajisawa's regent using a dissolving ratio of 1: 15 weight by volume at 80 °C
A7	Silk fibroin extracted with Ajisawa's regent using a dissolving ratio of 1: 10 weight by volume at 50 °C
A8	Silk fibroin extracted with Ajisawa's regent using a dissolving ratio of 1: 10 weight by volume at 65 °C
A9	Silk fibroin extracted with Ajisawa's regent using a dissolving ratio of 1: 10 weight by volume at 80 °C

ATR-FTIR	Attenuated total reflectance-Fourier transform infrared spectroscopy
B	β-sheet
$CaCl_2$	Calcium chloride
Cells cm^{-2}	Cells per square meter
cm^{-1}	Reciprocal centimeter
DI	Deionized
dl g^{-1}	Deciliter per gram
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DS	Degummed silk fibers
DSC	Differential scanning calorimetry
EtOH	Ethanol/ ethanol treated silk fibroin film
FBS	Fetal bovine serum
FSD	Fourier self deconvolution
G	Gram
g l^{-1}	Gram per liter
g mol^{-1}	Gram per mole
GA	Glycine-alanine
GAGAGS	Glycine-alanine-glycine-alanine-glycine-serine
GAGAGY	Glycine-alanine-glycine-alanine-glycine-tyrosine
H	Hour
h^{-1}	Reciprocal hour
H2O	Water vapor annealed silk fibroin film
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
inHg	Inch of mercury
J g^{-1}	Joule per gram
K1	Thai-Japanese hybrid silk race
K8	Thai-Chinese hybrid silk race
kDa	Kilo Dalton
kg cm^2	Kilogram per square centimeter
kPa	Kilopascal
L	Liter
L2	Silk fibroin extracted with 9.3 molar lithium solution using a dissolving ratio of 1: 30 weight by volume at 65 °C
L5	Silk fibroin extracted with 9.3 molar lithium solution using a dissolving ratio of 1: 15 weight by volume at 65 °C
L8	Silk fibroin extracted with 9.3 molar lithium solution using a dissolving ratio of 1: 10 weight by volume at 65 °C
LiBr	Lithium bromide
M	Molar
mg ml^{-1}	Milligram per milliliter
Min	Minute
Ml	Milliliter
ml min^{-1}	Milliliter per minute
mN m^{-1}	Millinewton per meter
mol %	Mole percent

mPa·s	Millipascal second
MSCs	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
mV	Millivolt
MW	Molecular weight
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NN	Nangnoi Sisaket 1 silk race
Non	No treatment/ as-casted silk fibroin film
PBS	Phosphate buffer saline
PDI	Polydispersive index
PDT	Population doubling time
PES	Polyethersulfone
R	Random coil
Rad	Radian
Rpm	Revolution per minute
SC	Tyrosine side chain
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SEM	Scanning electron microscope/microscopy
SF	Silk fibroin
T	β-turn
T_c	Crystallization temperature
TCP	Tissue culture plate
T_d	Decomposition temperature
T_{d1}	First decomposition temperature
T_{d2}	Second decomposition temperature
T_{evap}	Water evaporation temperature
T_g	Glass transition temperature
TGA	Thermogravimetry analysis
Tris-HCl	Tris(hydroxymethyl)aminomethane-hydrochloride
w/v	Weight by volume
wt. %	Weight percent
ΔH_c	Enthalpy of crystallization
ΔH_d	Enthalpy of decomposition
ΔH_{evap}	Enthalpy of water evaporation
θ	Contact angle of liquid on silk fibroin film

CHAPTER I

INTRODUCTION

Silk fibroin (SF), a native protein from spider silks or silk cocoons has been studied as a promising biomaterial due to its excellent biocompatibility and high mechanical properties compared to other naturally-derived materials. Many studies report feasibility of developing the SF for tissue engineering and drug delivery purposes. SF biomaterials were demonstrated to support *in vitro* proliferations and differentiations of human MSCs, HUVEC, HCE-T, PBMC, rabbit osteoblasts, rat MSCs, NIH-3T3, MC3T3-E1, 3T3-L1, C3H10T1/2, M2-10B4, MG-63, CHO-k1, PC12, and human blood cells [1-26]. *In vivo* biocompatibilities of the SF was reported in Sprague Dawley and Wistar rats [20, 27] while promotion of bone differentiation was shown in a rabbit model [28]. The SF is made up to 66 - 75 % in *Bombyx mori* silk cocoons [25, 29-31]. It contains two units, heavy- (350 kilo Daltons) and light-chains (25 kilo Daltons), and a subunit, P25 (30 kilo Daltons) [13, 32-36]. These three components are formed together in a specific 6: 6: 1 molar ratio, respectively [33]. The heavy-chain SF is crystallizable and has high contents of glycine (~ 49 mol %), alanine (~ 30 mol %), and serine (~ 11 mol %). The non-crystallizable light-chain SF is built up mainly by the alanine (~ 17 mol %), aspartic acid (~ 15 mol %), and the glycine (~ 10 mol %) [11, 13, 19, 20, 32, 34, 35, 37-41]. Domestic Thai SF consists of more hydrophobic (82 mol %) and less hydrophilic (18 mol %) amino acids than Chinese- and Japanese-Thai hybrid SF (77 mol % and 23 mol %, respectively) [19]. These amino acids affect polarizabilities and molecular assemblies of the SF. Isoelectric point of the SF was reported at 3.5 - 4.4 [12, 42, 43]. Molecular structures of the Chinese SF in middle silk glands comprises of β-sheet (18.7 - 30.0 %), α-helix and random coil (27.7 - 35.5 %), and others (42.3 - 45.8 %) [44]. Degummed SF composes of the β-sheet (52 - 63 %), β-turn (12 - 15 %), and the α-helix and the random coil (25 - 29 %) [45, 46].

Processing of the SF methodically involves pre-treatments of silk cocoons (degummings), SF extractions with solvents (dissolutions), purifications (removal of solvents and impurities), fabrications (e.g. freeze-drying, film casting, and electrospinning), and post-treatments (β-sheet regenerations and sterilizations). These multi-step processes have been shown to influence SF's molecular weights (MW) and molecular structures. Alkaline solutions such as sodium carbonate (Na_2CO_3) solution, sodium bicarbonate solution, and urea are standard degumming solutions used to remove silk glue from the silk cocoons. Complete silk glue removal could be done with 8 M urea at 80 °C for 2 h without destroying heavy-chain Japanese SF [29]. However, degumming the silk cocoons with 0.02 M Na_2CO_3 solution has been used more frequently [1-3, 7, 8, 10-12, 14, 15, 18-22, 30, 41, 46-62], although the heavy-chain of Japanese SF was reported to be degraded [29]. Ionic salt solutions such as lithium halide solutions and calcium chloride ($CaCl_2$) solutions are common solvents used to extract the SF. Disruptions of hydrogen bonds (largely in the β-sheets) together with molecular disassembly caused by the ionic salt solutions, resulted in a

temporary change from water insoluble SF to water soluble-random coil SF [34]. Twenty percent of SF extraction with 9.3 M lithium bromide (LiBr) solution at 60 °C for 4 h has been widely reported in literatures [1, 3, 4, 7, 8, 10, 12, 14, 18, 19, 21, 30, 47-57, 59, 63-65]. However, the $CaCl_2$ extraction is more environmental friendly, economical, and of our interests. Ajisawa, A. (1968) discovered that the Japanese SF could be easily extracted with a ternary mixture of $CaCl_2$, water, and ethanol (EtOH) at 1: 8: 2 molar ratios [66, 67]. Ajisawa extractions were reported using 1: 30 - 1: 10 (w/v) of dissolving ratios at 55 - 85 °C for various dissolution times [6, 9, 23-25, 27, 29, 30, 41, 43, 45, 58, 60, 66-73]. The MW of the SF extracted with the Ajisawa's reagent (252.5 - 16 kilo Daltons) was generally lower than the SF extracted with the LiBr (376 - 82.7 kilo Daltons) [11, 15, 20, 24, 27, 43, 60, 74-76]. Prolong dissolution times of the Ajisawa's process resulted in decreases in the MW of the SF [41, 58, 68]. The Ajisawa's process could preserve the light-chain SF [9, 29, 60]. In contrast, two studies reported that the SF extracted with the Ajisawa's reagent had higher MW than the SF extracted with the LiBr [9, 69]. Beta-sheet contents of the SF extracted with the Ajisawa's reagent and the LiBr were ~ 30 % and 14.7 - 31.7 %, respectively [10, 27, 46, 56]. Random coil contents increased to ~ 60 % after the extractions [46]. It was proposed that Chinese SF extracted with the Ajisawa's reagent had a compactly coil, a readily β-sheet forming structure while the one extracted with the LiBr was in a free random coil form [77].

Two dimensional SF films were fabricated for physico-chemical and biological characterizations in several studies. LiBr-derived SF films had 12.5 - 28.4 % of the β-sheet contents [46, 54, 63, 78]. Physical and chemical treatments are intended to regenerate the β-sheet structure in order to improve stabilities and mechanical properties of the SF films. Ethanol immersion was applied to the SF films for insolubilization and sterilization [4, 6, 15, 17, 26, 79-82]. Water vapor annealing, a chemical-free process was applied alternatively to achieve water-insoluble/less-crystalline SF films for different applications [1, 2, 12, 53-55, 63, 82]. Ethanol treated SF films typically had higher β-sheet contents (18 - 51.0 %) than water vapor annealed SF films (14 - 47.3 %) [12, 46, 54, 55, 63, 80-82].

The molecular weights and the molecular structures of the SF play crucial roles in other properties. Viscosity of LiBr-derived Korean SF solution which had a high MW was higher (915 mPa·s) than Ajisawa-derived SF solutions. The Ajisawa-derived Korean SF solutions had their viscosities decreased (300 mPa·s, 150 mPa·s, and 50 mPa·s) with increasing dissolution times (3 min, 30 min, and 180 min, respectively) [68]. High MW SF scaffolds had more human MSCs adhesions compared to low MW SF scaffolds due to their greater surface stiffness [24]. The Low MW SF interestingly exhibited activities inducing proliferations and differentiations of 3T3-L1, C3H10T1/2, and M2-10B4 cells [5, 16]. Decomposition temperatures of well-oriented SF in the degummed silk fibers were higher (~ 310 °C) than regenerated SF (228 - 299 °C) [2, 6, 11, 18, 19, 25, 37, 38, 40, 45, 46, 53-56, 73, 83, 84]. Light-chain Thai SF scaffolds which were hydrophilic had more NIH-3T3 cell adhesions than heavy-chain ones [9]. The EtOH treated SF films had high surface rigidities that improved attachments and growths of mammalian cells [4, 6, 26]. Degradation kinetics of EtOH treated LiBr-derived SF scaffolds corresponded to

long-term osteogenesis rate of human MSCs [8]. The EtOH treated LiBr-derived Indian SF films lost 50 % of their masses after 24 days due to their high β-sheet characteristics [82]. The water vapor annealed SF films which had less β-sheet contents had fast *in vitro* degradations. These SF films lost half of their masses in phosphate buffer saline incubation at 37 °C for 3 days [1]. They were half-mass degraded in Protease XIV incubations at 37 °C for 20 h [55]. The water vapor annealed SF films were usually more biocompatible than the EtOH treated SF films [1, 2, 12, 14, 18, 21, 55]. They are more applicable for short-term usages.

Previously, the SF extractions with the Ajisawa's reagent in comparison with the LiBr using different dissolving ratios and dissolution times have not yet been studied systematically. Impacts of the extractions, the fabrications, and the β-sheet regenerations on the MW and the molecular structures of the Thai SF are not well-characterized. Investigations of the Thai SF's MW and molecular structure using size exclusion chromatography (SEC) and quantitative attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) have not yet been reported elsewhere. In this work, Thai SF was extracted from domestic silk cocoons, *Bombyx mori*, Nangnoi Sisaket 1. The Ajisawa's reagent and the LiBr solution were used to extract the SF using 1: 30 (w/v), 1: 15 (w/v), and 1: 10 (w/v) dissolving ratios at 50 °C, 65 °C, and 80 °C. SF solutions were characterized for their molecular weights, zeta potentials, and viscosities. Lyophilized SF was prepared for characterizations of amino acid compositions, molecular structures, and thermal properties. The SF solutions were casted into SF films and were subjected to the EtOH treatment and the water vapor annealing. These SF films were investigated for their liquid contact angles, molecular structures, and NIH-3T3 biocompatibility test in terms of cell attachments and proliferations. This work provides fundamental information of Thai SF characteristics and properties influenced by the preparation processes. This would benefit uses of Thai SF for a biomaterial.

1.1 Research objective

To study effects of extraction conditions using Ajisawa's reagent and 9.3 M LiBr on physico-chemical and biological characteristics of Thai SF from domestic silk cocoons, *Bombyx mori*, Nangnoi Sisaket 1.

1.2 Research scopes

A. To degum Thai silk cocoons, *Bombyx mori*, Nangnoi Sisaket 1 with boiling 0.02 M Na_2CO_3 solution for 20 min

B. To extract SF from degummed silk fibers with Ajisawa's reagent and 9.3 M LiBr using dissolving ratio of 1: 30 (w/v), 1: 15 (w/v), and 1: 10 (w/v) at 50 °C, 65 °C, and 80 °C and to dialyze SF extractions to aqueous SF solutions

C. To freeze-dry the SF solutions into lyophilized SF

D. To cast the SF solutions into SF films and to apply EtOH treatment and water vapor annealing to the SF films

E. To investigate physico-chemical characteristics of the SF solutions, the lyophilized SF, and the SF films with respect to the degummed silk fibers

a. Amino acid compositions using high performance liquid chromatography
b. Molecular weights using SEC and sodium dodecyl sulfate-polyacrylamide gel electrophoresis
c. Molecular structures using ATR-FTIR
d. Zeta potentials by a zeta sizer
e. Thermal properties using differential scanning calorimetry and thermogravimetry
f. Viscosities by a vibrational viscometer
g. Contact angles by a contact angle meter

F. To evaluate *in vitro* biocompatibility of NIH-3T3 mouse embryonic fibroblast cell line against the SF films in terms of cell attachments and proliferations

CHAPTER II

THEORITICAL BACKGROUND AND LITERATURE REVIEWS

Silk fibroin (SF), a protein produced by arthropods including *Bombyx mori* silkworms has been studied as a promising biomaterial for tissue engineering and drug delivery purposes. Among other naturally-derived materials, the SF exhibits excellent biocompatibility and high mechanical properties compared to chitin, cellulose, and their derivatives. Physico-chemical and biological properties of the SF are influenced by SF's intrinsic characteristics as well as its multi-step processes. The SF is built up mostly by hydrophobic amino acids which include up to ~ 75 % of glycine and alanine. Polypeptides of the SF rearrange themselves into secondary structures with high extents in β-sheet conformations while heavy- and light-chains, and P25 glycoprotein organize themselves into large protein complexes. Preparations of SF films from silk cocoons generally involve degummings, dissolutions of degummed silk fibers, removal of solvents and impurities, film castings, β-sheet regenerations and sterilizations. This chapter reviews the SF's characteristics relating to its preparation processes.

2.1 Silk cocoons

Silk cocoons are produced by mulberry and non-mulberry silkworms including *Bombyx mori*, Tasar, Muga, and Eri. The *Bombyx mori* silk is harvested domestically while the rests are wild silks. Physical structure of raw silk filament is shown in **Figure 2.1**. The raw silk filament which consists of two SF bundles and silk glue is 14.7 - 30.0 µm in diameter [30, 41]. A single SF bundle has a size of 7 - 12 µm and is categorized into other microstructures [40, 41]. **Table 2.1** presents compositions of the *Bombyx mori* silk cocoons. The silk glue contains sericin, wax, minerals, ash, and others which made up to 25 - 32 % of the raw silk filament [25, 29, 31]. They could be removed from the silk cocoons by degumming in alkaline solutions. Complete silk glue removals without silk fiber deterioration is desirable for preparation of high-quality silk fibers.

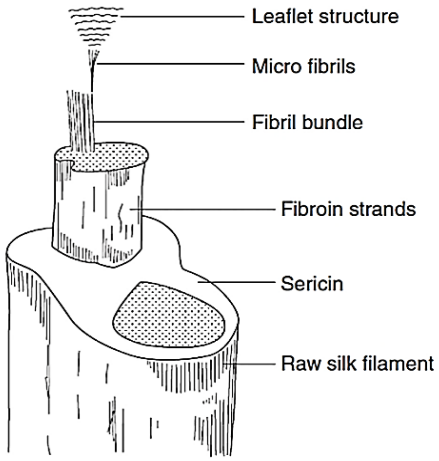

Figure 2. 1 Physical structure of raw silk filament portrayed by Babu, K.M. (2012) [85]

Table 2. 1 Compositions of *Bombyx mori* silk cocoons reported by Padaki, N.V. et al. (2015) [31]

Component	Weight (%)
Fibroin	66 - 72
Sericin	25 - 32
Wax	0.3 - 0.4
Minerals, ash, and others	0.7 - 0.8

2.2 Silk fibroin

2.2.1 Amino acid composition

Amino acid compositions of SF and their arrangements in polypeptide chains influence SF's molecular weights, molecular structures, and molecular assemblies. Three domains of the SF which are heavy- and light-chains and P25 glycoprotein are different in their amino acid characteristics. Amino acid sequences of the heavy-chain SF is shown in **Figure 2.2**. The heavy-chain is categorized into 12 units of crystalline blocks which are separated by 11 units of amorphous segments [86, 87]. The crystalline blocks are built up by GAGAGS, GAGAGY, and GAGYGA hexapeptides and GAAS tetrapeptide (G: glycine, A: alanine, S: serine, and Y: tyrosine) while the amorphous segments are made up by unrepeated hydrophilic amino acids [86, 87]. **Table 2.2** presents the amino acid compositions of the heavy- and the light-chain SF from Japanese posterior silk glands and of the SF from Chinese silk glands. **Table 2.3** presents residue masses, isoelectric points, and conformation preference parameters of the amino acids. Primary structure of the SF consists of up to 82.2 % of hydrophobic amino acids including 42.8 %, 30.0 %, 2.5 %, and 2.3 % of glycine, alanine, tyrosine, and valine, respectively [13]. Hydrophilic amino acids are at 17.8 % which include 11.0 % of serine [13]. The heavy-chain SF contains glycine, alanine, and serine at 49.4 mol %, 29.8 mol %, and 11.3 mol %, respectively. The light-chain SF mostly comprises of the alanine, aspartic acid, and the glycine at 16.9 mol %, 15.4 mol %, and 10.0 mol %, respectively [32]. Domestic Thai SF has its hydrophobic and hydrophilic amino acids at 82 mol % and 18 mol %, respectively, while Chinese- and Japanese-Thai hybrid SF had those contents at 77 mol % and 23 mol %, respectively. Therefore, the Thai SF was reported to be more hydrophobic than the other two [19].

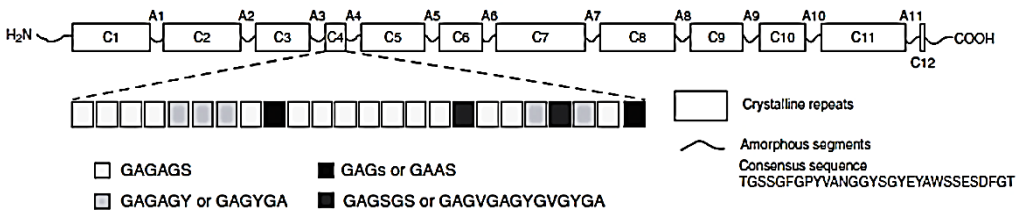

Figure 2. 2 Amino acid sequences in heavy-chain silk fibroin depicted by Murphy, A.R. et al. (2014) [87]

Table 2. 2 Amino acid compositions in mol % of heavy- and light-chain silk fibroin from Japanese posterior silk glands reported by Shimura, K. et al. (1982) [32] and of silk fibroin from Chinese silk glands reported by Yang, M. et al. (2012) [13]

	Japanese posterior silk gland		**Chinese silk gland**
	Heavy-chain	**Light-chain**	
Hydrophilic			
Acidic			
Aspartic acid (D)	0.6	15.4	2.1
Glutamic acid (E)	0.7	8.4	2.5
Basic			
Arginine (R)	0.2	3.8	0.6
Lysine (K)	0.1	1.5	0.4
Histidine (H)	0.1	1.6	0.2
Polar			
Serine (S)	11.3	7.9	11.0
Threonine (T)	0.4	2.8	1.0
Cysteine (C)	0.0	0.0	0.0
Total	13.4	41.4	17.8
Hydrophobic			
Non-polar			
Glycine (G)	49.4	10.0	43.8
Alanine (A)	29.8	16.9	30.0
Proline (P)	0.3	3.0	0.8
Valine (V)	2.0	7.4	2.3
Leucine (L)	0.1	7.2	0.6
Isoleucine (I)	0.1	7.3	0.6
Methionine (M)	0.0	0.4	0.0
Aromatic			
Tyrosine (Y)	4.6	3.4	2.5
Phenylalanine (F)	0.4	2.7	1.6
Tryptophan (W)	No data		No data
Total	86.7	58.3	82.2

Table 2. 3 Residue mass, isoelectric point, and conformation preference parameter of amino acids in silk fibroin

	Residue mass[a] (Dalton)	Isoelectric point[b]	Conformation preference parameter[c]		
			β-sheet	β-turn	α-helix
Hydrophilic					
Acidic					
Aspartic acid	115.09	3.0	0.5 - 0.7	1.4 - 1.5	0.9 - 1.1
Glutamic acid	129.12	3.2	0.5 - 0.8	0.7 - 1.0	1.4
Basic					
Arginine	156.19	10.8	0.7 - 1.0	0.9 - 1.0	0.9 - 1.4
Lysine	128.17	9.8	0.7 - 0.9	1.0	1.1 - 1.2
Histidine	137.14	7.6	0.8 - 1.1	0.7 - 1.0	1.0 - 1.2
Polar					
Serine	87.08	5.7	0.9 - 1.0	1.3 - 1.4	0.7 - 0.8
Threonine	101.11	6.5	1.2 - 1.3	1.0	0.7 - 0.8
Cysteine	103.14	5.0	0.8 - 1.2	0.9 - 1.2	0.9 - 1.0
Hydrophobic					
Non-polar					
Glycine	57.05	6.0	0.6 - 0.9	1.6	0.4 - 0.6
Alanine	71.08	6.0	0.8 - 0.9	0.7	1.3 - 1.5
Proline	97.12	6.3	0.4 - 0.6	1.5 - 1.9	0.5 - 0.6
Valine	99.13	6.0	1.5 - 1.7	0.5	0.9 - 1.0
Leucine	113.16	8.0	1.0 - 1.2	0.6	1.3
Isoleucine	113.16	6.1	1.5 - 1.8	0.5	1.0 - 1.1
Methionine	131.20	5.8	1.0 - 1.3	0.4 - 0.6	1.3 - 1.4
Aromatic					
Tyrosine	163.18	5.7	1.2 - 1.5	1.1	0.7 - 0.9
Phenylalanine	147.18	5.5	1.2 - 1.4	0.6	1.0 - 1.1
Tryptophan	186.21	5.9	1.2	0.8 - 1.0	1.0

[a]Reported by Lide, D.R. (1993), Creighton, T.E. (1993), and Coligan, et al. (1996) [88-90], [b]Reported by Graham Solomons, T.W. and Fryhle, C.B. [91], [c]Reported by Chou, P.Y. and Fasman, G.D. (1977, 1978a,b), Chou, J.Y. (1993), Creighton, T.E. (1993), and Thornton, J.M. et al. (1995) [89, 92-96].

2.2.2 Molecular weight

Residue masses of glycine, alanine, serine, tyrosine, and valine are at 57.05 Daltons, 71.08 Daltons, 87.08 Daltons, 163.18 Daltons, and 99.13 Daltons, respectively (**Table 2.3**). Amino acid compositions of SF together with the residue masses of its amino acids aggregate to yield SF's molecular weights. Heavy-chain SF which contains 5,263 amino acids has ~ 391 kilo Daltons of its molecular weight [86]. Light-chain SF and P25 glycoprotein have their molecular weights at 25 kilo Daltons and 30 kilo Daltons, respectively [13, 32-36]. These three domains assemble together in a specific 6: 6: 1 molar ratio, respectively [33]. The heavy- and the light-chains are connected to each other via disulfide bonds formed by two cysteines while the P25 is coupled to the heavy-chain SF via hydrophobic interactions [33].

2.2.3 Molecular structure and molecular assembly

Polypeptides of SF which are made of covalently bonded amino acids tend to assemble into higher ordered structures. Secondary structures of the SF are categorized into three types which are β-sheet, β-turn, and α-helix. These molecular conformations organize together in the SF to yield well-oriented raw silk fibers. Structural organizations of the SF in the raw silk fibers and the SF's secondary structures including the α-helix, the β-turn, and both antiparallel and parallel β-sheet are shown in **Figure 2.3** and **Figure 2.4**, respectively. The SF in the raw silk fibers contains both crystalline and amorphous regions. The β-sheet structures in the crystalline regions are stabilized by hydrogen bonds and Van der Waals forces. The α-helix is formed by the hydrogen bonds between carbonyl oxygens of the amino acids at positions *n* and amide groups of the amino acids at positions *n+4* while the β-turn is shaped by the hydrogen bonds between the carbonyl oxygens of the amino acids at the positions *n* and the amide groups of the amino acids at positions *n+3*. The β-sheet is stabilized by the hydrogen bonds between the carbonyl oxygens and the amide groups of the amino acids in adjacent strands. Silk II includes the β-sheet and the β-turn while silk I includes the α-helix and the unordered structures (random coils). Silk III is a 3-folded helix which is formed at aqueous-air interfaces. Each amino acid has different potentials to be arranged into each type of the secondary conformations, so-called preference parameters (**Table 2.3**). Hydrophobic amino acids such as isoleucine, valine, tyrosine, phenylalanine, and leucine have high propensities to be arranged into the β-sheet. Proline has the highest tendency to be arranged into the β-turn while glycine and serine have high tendencies to be formed into the same structure. The α-helix is mostly arranged by alanine and glutamic acid. The glycine and the alanine are the smallest amino acids which have their masses at 57.05 kilo Daltons and 71.08 kilo Daltons, respectively. They allow GA repeating units to arrange into the closest packing assemblies. As a result, the SF in silk fibers is high in β-sheet content which is at 52 - 63 %. The β-turn and the α-helix together with the unordered structures, random coils made up 12 - 15 % and 25 - 29 %, respectively of total molecular structures in the SF [45, 46]. The SF is therefore hydrophobic and water insoluble.

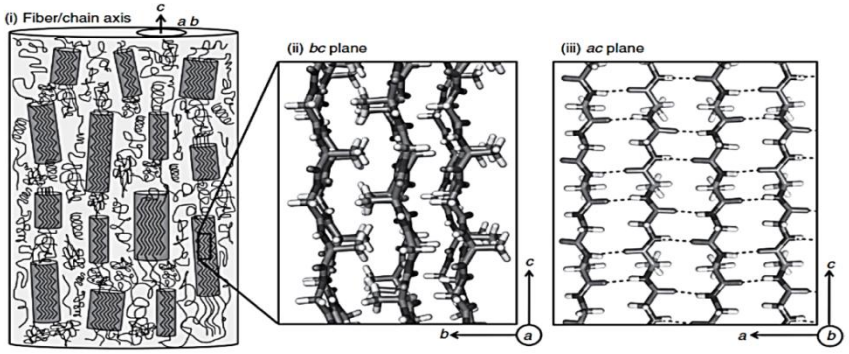

Figure 2. 3 Structural organizations of silk fibroin in raw silk fiber portrayed by Murphy, A.R. et al. (2014) [87], crystalline and amorphous regions are aligned in silk fiber, the β-sheet structures interact together in bc plane via Van der Waal forces, hydrogen bondings are formed in the antiparallel β-sheet in ac plane

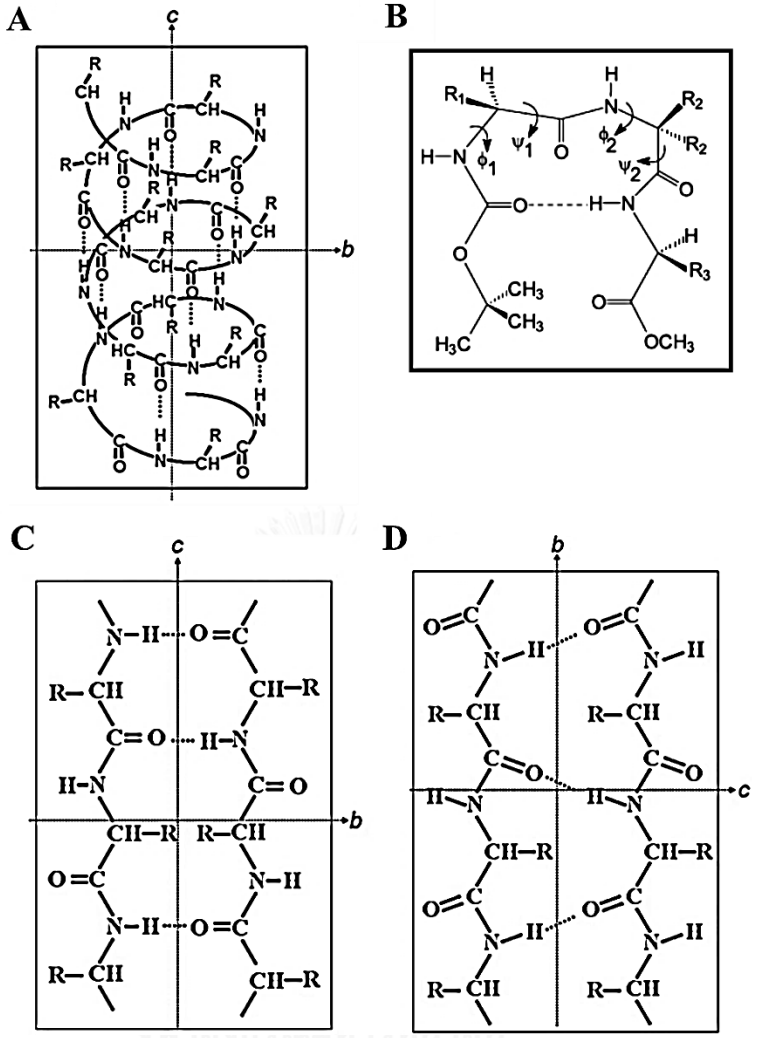

Figure 2. 4 Secondary structures of proteins: (A) α-helix, (B) β-turn, (C) antiparallel β-sheet, and (D) parallel β-sheet adapted from Ye, S. et al. (2013) and Dutt, A. et al. (2005) [97, 98]

2.2.4 Isoelectric point

Isoelectric points of glycine, alanine, serine, tyrosine, and valine are at 5.7 - 6.0. Acidic amino acids such as aspartic acid and glutamic acid exhibit low isoelectric points at 3.0 - 3.2 while those of arginine, lysine, and Histidine which are basic are at 7.6 - 10.8 (**Table 2.3**). SF contains ~ 5 mol % of highly charged amino acids while the rests are almost neutral. The isoelectric point of the SF was reported differently at 3.5 - 4.4 [12, 42, 43].

2.2.5 Thai silk fibroin

Thai SF from *Bombyx mori* silk cocoons, especially, Nangnoi races have been studied as a biomaterial in few studies. The Nangnoi Sisaket is a pure Thai silk race which has a natural yellowish color while others are normally white. Pure and hybrid Thai silk cocoons and their fibers' microstructures are shown in **Figure 2.5** and **Figure 2.6**, respectively. **Table 2.4** and **Table 2.5** present physico-chemical and biological characteristics of the pure and the hybrid Thai SF reported in other studies. The Thai SF exhibited physico-chemical characteristics comparable to SF from other silk races including molecular structures and thermal properties [19, 99, 100]. However, amino acid compositions which further influenced hydrophilicities and biocompatibilities of SF films were different. The Thai SF had more hydrophobic amino acids than Japanese- and Chinese-Thai hybrid SF. As a result, the Thai SF films were more hydrophobic and they had fewer early attachments of L929 fibroblasts and rat MSCs [19]. The molecular weights of the Thai SF were reported at 350 kilo Daltons for heavy-chain and 25 kilo Daltons for light-chain [9]. The Thai SF was fabricated into various types of biomaterials including films, electrospun mats, hydrogels, nanoparticles, and salt-leached scaffolds [3, 9, 99-106]. They supported attachments and growths of human gingival fibroblasts, rat MSCs, NIH-3T3 fibroblasts, and MC3T3-E1 osteoblasts [3, 9, 101, 103, 105, 106].

Figure 2. 5 Pure and hybrid Thai *Bombyx mori* silk cocoons: (A) Nangnoi Sisaket 1, (B) Japanese-Thai hybrid race, and (C) Chinese-Thai hybrid race studied by Kaewprasit, K. et al. (2014) [19]

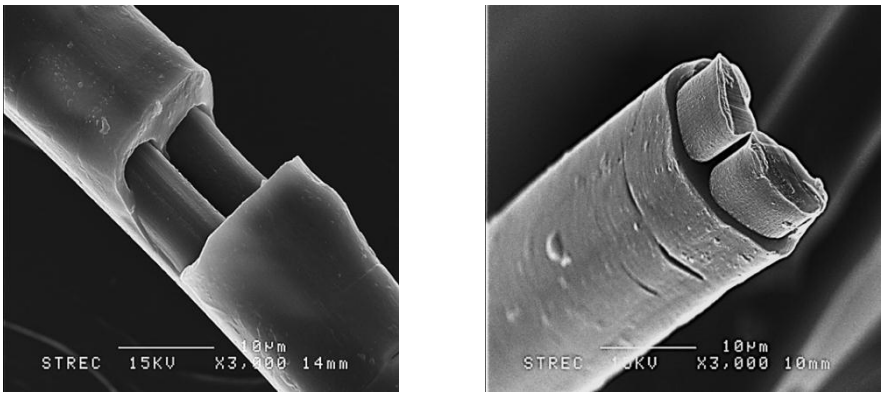

Figure 2. 6 Physical microstructures of silk fibers from Thai silk cocoons, *Bombyx mori*, Nangnoi Sisaket 1, scanning electron micrographs were kindly given by Rungnapa Yamdech, the Multidisciplinary Laboratory in Biomedical Engineering, Chulalongkorn University, Thailand (2016)

Table 2. 4 Physico-chemical and biological characteristics of methanol treated silk fibroin films prepared from pure Thai (Nangnoi Sisaket 1), Japanese-Thai hybrid, and Chinese-Thai hybrid *Bombyx mori* silk cocoons reported by Kaewprasit, K. et al. (2014) [19]

		Nangnoi Sisaket 1	Japanese-Thai hybrid	Chinese-Thai hybrid
Amino acid composition (mol %)	**Hydrophilic**	18.3	22.9	22.8
	Hydrophobic	81.7	77.1	77.2
Molecular structure		Mainly beta sheet		
Thermal decomposition temperature		277 - 279 °C		
Water contact angle (°)		68.3 ± 0.5	64.8 ± 1.2	66.0 ± 0.3
Early attachment of L929 cells (%)		60.6 ± 6.2	79.4 ± 10.8	66.9 ± 6.3
Early attachment of rat MSCs (%)		68.9 ± 1.9	81.1 ± 5.1	78.9 ± 6.9

Silk cocoons were degummed in boiling 0.02 M Na_2CO_3 solution for 20 min for 2 cycles. Degummed silk fibers were dissolved in 9.3 M LiBr using a dissolving ratio of 1: 5 (w/v) at 60 °C for 4 h

Table 2. 5 Physico-chemical and biological characteristics of *Bombyx mori* Thai silk fibroin reported in other studies

	Damrongrung, T. et al. (2007) [101]	**Wadbua, P. et al. (2010) [9]**	**Srisawasdi, T. et al. (2015) [99]**	**Wongkrongsak, S. et al. (2016) [100]**
Silk race	Nangnoi	Nangnoi Sisaket	No data	Nangnoi
Degumming procedure	Boiled in 0.02 M Na_2CO_3 solution, 30 min, twice	Boiled in 0.5 % (w/v) $NaHCO_3$ solution, 15 min	Boiled in 0.02 M Na_2CO_3 solution, 30 min	Boiled in 0.02 M Na_2CO_3 solution, 30 min
Extraction method	Ajisawa, 1: 20 (w/v), 70 °C, 6 h	Ajisawa, 1: 30 (w/v), 80 °C, 15 min & 9.3 M LiBr, 1: 30 (w/v), 60 °C, 40 min	9.3 M LiBr, 1: 5 (w/v), 60 °C, 4 h	Ajisawa, 80 °C, 2 h
Fabrication	Electrospun mats	Electrospun mats	Hydrogels	Nanoparticles
Molecular weight (kilo Daltons)	No data	350 (heavy-chain) & 25 (light-chain)	No data	250
Molecular structure	No data	Mainly β-sheet after methanol treatment	Mainly β-turn	Mainly β-sheet
Thermal decomposition temperature (°C)	No data	No data	270	No data
Biocompatibility	Supported human gingival fibroblast attachments at 24 h	Supported NIH-3T3 fibroblast attachments at 2 - 24 h	No data	No data

2.3 Processing of silk fibroin

2.3.1 Degumming procedures

Step by step preparations of SF solutions are described in **Figure 2.7** and preparations of SF films from silk cocoons are illustrated in **Figure 2.8**. Aqueous sodium carbonate (Na_2CO_3) and urea are common degumming solutions used to remove silk glue from the silk cocoons. **Table 2.6** presents percentages of silk glue removal after degumming of Japanese silk cocoons in boiling 0.05 % Na_2CO_3 solution for various times. **Table 2.7** presents the percentages of silk glue removal after the degumming of Japanese silk cocoons in 8M urea solution at different temperatures for 2 h. Complete silk glue removals of the Japanese silk cocoons could be done with the 0.05 % Na_2CO_3 solution at 100 °C for at least 15 - 20 min and the 8 M urea solution at 80 - 90 °C for 2 h [29]. The degumming of Korean silk cocoons in 1 % (w/v) sodium oleate mixed with the Na_2CO_3 solution removed 28.5 % of the silk glue [25]. [29]. The silk glue could be completely removed by the degumming either in the 0.05 % Na_2CO_3 solution at 100 °C for 5 min or the 8 M urea at 80 °C for 2 h without destroying heavy-chain Japanese SF [29].

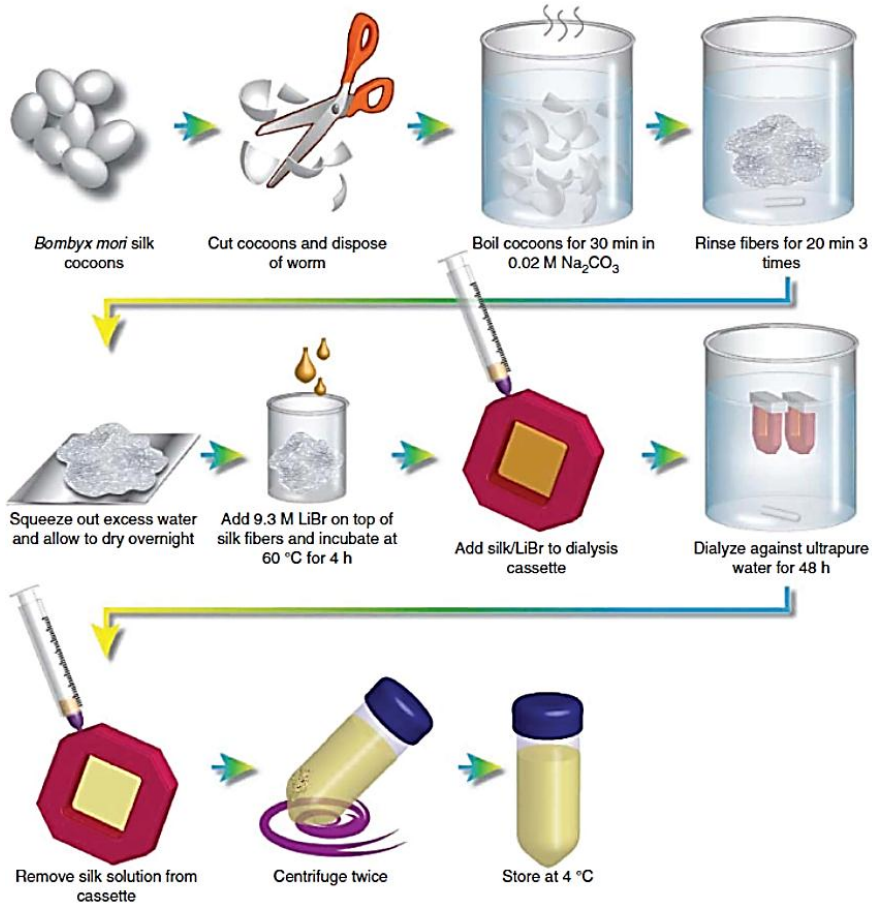

Figure 2. 7 Step by step preparations of aqueous silk fibroin solutions described by Rockwood, D.N. et al. (2011) [57]

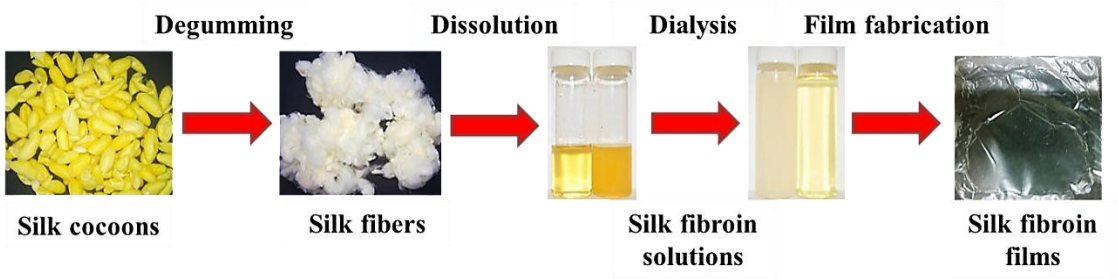

Figure 2. 8 Preparations of silk fibroin film from silk cocoons

Table 2. 6 Silk glue removal in % after degumming of Japanese silk cocoons in 0.05 % sodium carbonate solution at 100 °C for various times reported by Yamada, H. et al. (2001) [29]

Degumming time (min)	Silk glue removal (%)
5	22.5
10	24.2
15	27.3
20	25.9
60	22.6

Table 2. 7 Silk glue removal in % after degumming of Japanese silk cocoons in 8 M urea solution at various temperatures for 2 h reported by Yamada, H. et al. (2001) [29]

Degumming temperature (°C)	Silk glue removal (%)
50	10.6
60	10.1
70	15.2
80	25.3
90	27.4

2.3.2 Dissolution processes

Beta-sheet structures in crystalline regions as well as other molecular assemblies provide hydrophobic characteristics to silk fibers. The β-sheet was made up to 52 - 63 % in degummed silk fibers while the rests were β-turn, α-helix, and random coil. [45, 46]. The silk fibers which are water insoluble could be dissolved in aqueous salt solutions via partially disruptions of hydrogen bonds in SF's secondary structures. The higher ordered structures of the SF are therefore disassembled into the random coil structures which are water soluble. Beta-sheet content of the water soluble-SF decreased to ~ 30 % while silk I structures of that increased to ~ 60 % [10, 27, 46, 56]. Dissolutions of the SF were demonstrated in ionic solvents such as aqueous salt solutions of lithium bromide (LiBr), calcium chloride ($CaCl_2$), lithium thiocyanate, zinc chloride, ammonium thiocyanate, and calcium nitrate [34]. Solvency powers of cations of the aqueous salt solutions increase in the following order;

calcium < strontium < barium < lithium < zinc while those of anions increase in the following order; sulfate < citrate < tartrate < acetate < chloride < nitrite < bromide < iodide < thiocyanate < dichloroacetate [34]. The solvency power of the LiBr solution is therefore higher than that of the $CaCl_2$ solution. As a result, the SF extracted with the $CaCl_2$ solution was proposed to have a compactly coil conformation which is a β-sheet forming structure while that extracted with the LiBr was proposed to be free random coil [77]. The silk fibers could be easily dissolved in a mixture of the $CaCl_2$, water, and ethanol at 1: 8: 2 molar ratios, a so-called Ajisawa's reagent which corresponds to low surface energy of the solvent [66, 67]. The SF is extracted with the Ajisawa's reagent and the LiBr solution using different conditions. **Table 2.8** and **Table 2.9** present the dissolution conditions of the *Bombyx mori* SF extracted with the Ajisawa's reagent and the LiBr solution, respectively. SF-Ajisawa extractions were reported using 1: 30 - 1: 10 (w/v) dissolving ratios at 55 - 85 °C for various dissolution times [6, 9, 23-25, 27, 29, 30, 41, 43, 45, 58, 60, 66-73]. SF-LiBr extraction using 1: 5 (w/v) at 60 °C for 4 h was the most used conditions reported in literatures [1, 3, 4, 7, 8, 10, 12, 14, 18, 19, 21, 30, 47-57, 59, 63-65]. The dissolution times of the SF regularly decreased with increases in dissolution temperatures since the disruptions of SF's hydrogen bonds occur more easily at high temperatures. After the dissolution processes, the SF solutions are normally purified by dialysis in order to remove the solvents and to produce aqueous SF solutions.

Table 2. 8 Dissolution conditions of *Bombyx mori* silk fibroin extracted with Ajisawa's reagent reported in other studies

Dissolving ratio (w/v)	Dissolution temperature (°C)	Dissolution time	Reference
1: 30	55	1 h	[66, 67]
	80	15 min	[9]
1: 20	65	No data	[69]
	70	No data	[30]
	78	2 h	[43]
	85	3 min, 30 min, and 3 h	[25, 41, 58, 68]
1: 15	75	No data	[29]
1: 10	75	3 h	[23]
	80	40 min	[71-73]
	85	No data	[6]
No data	70 - 72	1 - 6 h	[27, 45, 60]
	80	10 - 120 min	[24, 70]

Table 2. 9 Dissolution conditions of *Bombyx mori* silk fibroin extracted with 9.3 M LiBr reported in other studies

Dissolving ratio (w/v)	Dissolution temperature (°C)	Dissolution time	Reference
1: 30	60	25 - 40 min	[9]
1: 20	25	6 h	[58, 68]
	60	3 h	[60]
1: 10	60	4 h	[15, 62]
1: 10 - 1: 5	65	2 - 4 h	[2, 11, 20, 46, 61]
1: 5	60	4 h	[1, 3, 4, 7, 8, 10, 12, 14, 18, 19, 21, 30, 47-57, 59, 63-65]
	80	30 min	[24]
1: 4	60	4h	[107]
No data	60	2 h	[27]

2.3.3 Film castings

Water soluble SF in aqueous solutions has temporary stable conformations with tendencies to assemble back to its original higher ordered structures. Molecular assemblies of the SF in aqueous solutions including globular structures, protofibers, and micelles are shown in **Figure 2.9**. Changes in the SF's molecular assemblies during film fabrications are shown in **Figure 2.10**. The SF which composes of numerous repeating crystalline regions made of hydrophobic amino acids is less stable in water which is polar. As a result, the SF rearranges itself by hidings its hydrophobic blocks within and facing its hydrophilic blocks towards the water as well as having its higher ordered structures, particularly, β-sheet structures regenerated. Film castings from aqueous SF solutions allow more β-sheet regenerations when concentrated SF solutions were prepared at high temperatures in humid conditions. Chinese SF films casted using high concentrated SF solutions at 1.5 mg ml^{-1} had more β-sheet characteristics than those casted using diluted SF solutions at 300 µg ml^{-1} [71, 72]. The SF films casted at 50 - 60 °C had more silk II characteristics than the SF films casted at room temperature and 37 °C [73, 78]. Silk I-dominated SF films could be prepared in < 55 % relative humidity while the SF films prepared in > 55 % relative humidity had more silk-II structures [62, 108].

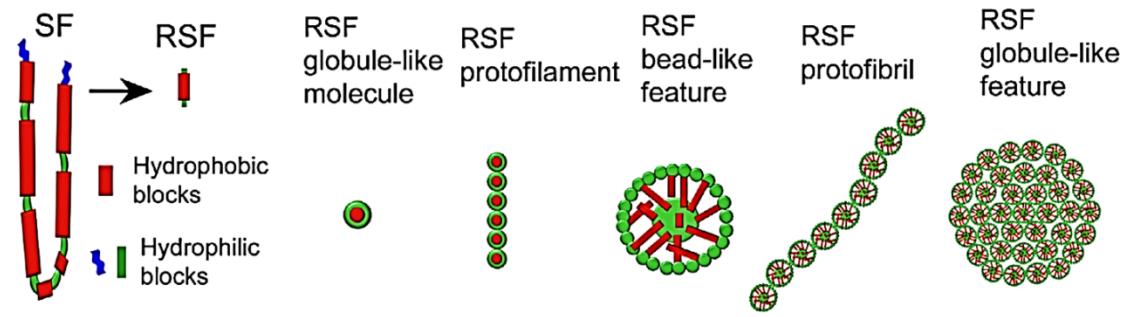

Figure 2. 9 Molecular assemblies of silk fibroin in aqueous solutions proposed by Zhong, J. et al. (2015) [72], RSF is regenerated silk fibroin

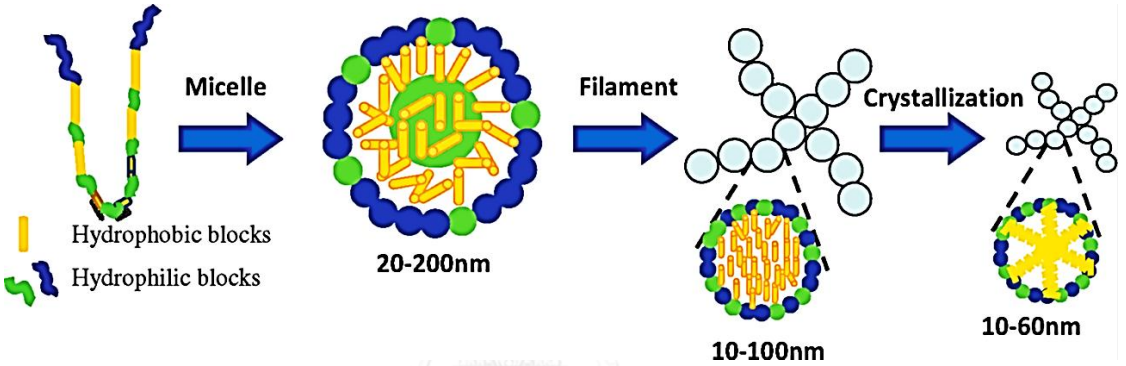

Figure 2. 10 Changes in molecular assemblies of silk fibroin during film fabrication proposed by Lu, Q. et al. (2012) [59]

2.3.4 Regenerations of β-sheet structure

As-casted SF films generally had moderate β-sheet crystallinity ~ 40 % of total molecular structures [12, 46, 54, 55, 63, 78]. Regenerations of β-sheet structures of SF are intended to increase SF's stabilities which include mechanical properties and degradabilities. Water vapor annealing, immersions in polar organic solvents such as methanol and ethanol solutions, and steam autoclaving are techniques used to regenerate the β-sheet structures. Rearrangements of SF molecules treated by the water vapor annealing and the immersions in polar organic solvents are shown in **Figure 2.11**. Conformational transitions of GAGAGS and GAGAGY hexapeptides during fabrication processes are depicted in **Figure 2.12**. During the β-sheet regeneration processes, water and polar organic molecules penetrate into SF's polypeptide chains and plasticize the SF molecules. Less ordered SF molecules assemble themselves into higher ordered structures (i.e. transitions of silk I and silk III into silk II) as solvated polypeptide chains have their mobilities increased. The SF molecules which are mostly hydrophobic tend to form into the higher ordered structures since they are less stable when surrounded by water and polar solvents. Alternatively, the water and the polar organic molecules bring glass transition temperature of the SF which is ~ 180 °C to room temperature [50, 51, 55]. As a result, the SF films have their hydrogen bonds regenerated and the β-sheet structures are

partially increased. The ethanol immersion could be applied to the SF films for insolubilization and sterilization [4, 6, 15, 17, 26, 79-82]. The water vapor annealing could be applied as an alternative method to achieve water insoluble with less crystalline SF films [1, 2, 12, 53-55, 63, 82]. The methanol and ethanol immersions regularly induce more β-sheet structures than the water vapor annealing. The SF films treated with the ethanol immersion partially regenerated their β-sheet structures up to 18 - 51.0 % while those treated with the water vapor annealing partially induced these structures up to 14 - 47.3 % [12, 46, 54, 55, 63, 80-82].

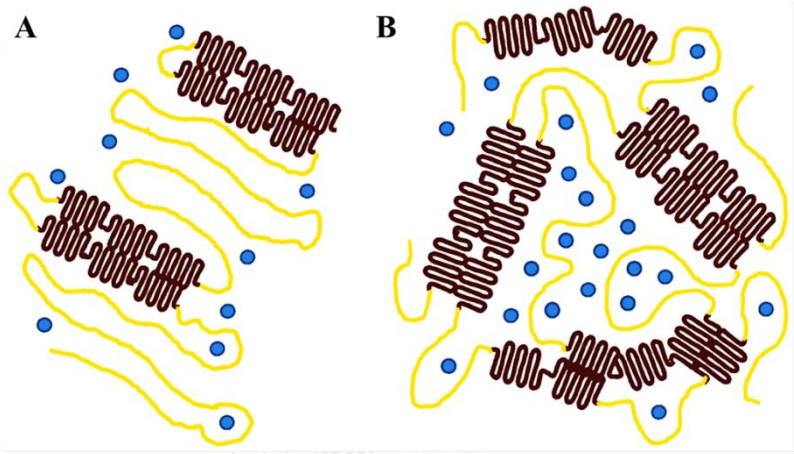

Figure 2. 11 Rearrangements of silk fibroin molecules via (A) water vapor annealing and (B) immersion in polar organic solvents proposed by Lawrence, B.D. et al. (2010) [53], red stacks are crystalline regions, yellow lines are amorphous regions, blue dots are water molecules

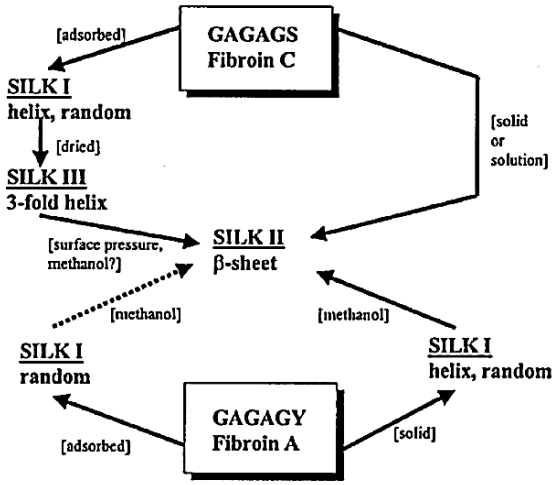

Figure 2.10 Conformational transitions of silk fibroin peptides depicted by Wilson, D. et al. (2000) [64]

2.3.5 Sterilizations

Sterilizations of SF films include γ-irradiation, steam autoclaving, ethanol immersion, ethylene oxide gas treatment, and other techniques. The SF films treated with the γ-irradiation both in air and in nitrogen gas and the 70 % aqueous ethanol immersion had high transparency. The SF films treated with the steam autoclaving were less transparent and had their microstructures modified by the sterilization. The γ-irradiated SF films had reduced tensile properties. The ethylene oxide gas had minor effects on the SF films although residual ethylene oxide gas could be toxic to mammalian cells [15].

2.4 Biomedical applications

Biomedical applications including tissue engineering and drug delivery systems with uses of SF have been shown in many studies. *In vivo* biocompatibilities in terms of proliferations were reported in Sprague Dawley and Wistar rats [20, 27]. Promotion of bone differentiation was shown in a rabbit model [28]. *In vitro* biocompatibilities were demonstrated in various types of mammalian cells including human's (MSCs, HUVEC, HCE-T, and PBMC), rabbit's osteoblasts, rat's MSCs, and mouse's (NIH-3T3, MC3T3-E1, 3T3-L1, C3H10T1/2, M2-10B4, MG-63, CHO-k1, and PC12) [1-26]. SF scaffolds which had different molecular weights and secondary structures resulted from their preparation processes exhibited dissimilar potentials in supporting cellular growths. The scaffolds fabricated from the SF with high molecular weights had greater surface stiffness which supported more human MSCs adhesions compared to those fabricated from low molecular weight SF [24]. The SF which had low molecular weights or SF hydrolysates exhibited activities inducing proliferations and differentiations of 3T3-L1, C3H10T1/2, and M2-10B4 cells [5, 16]. The scaffolds fabricated from heavy-chain of Thai SF which were hydrophobic had fewer attachments of NIH-3T3 fibroblasts than hydrophilic scaffolds fabricated from light-chain SF [9]. Ethanol treated SF films had high surface rigidities which improved cellular attachments and growths [4, 6, 26]. Water vapor annealed films fabricated from LiBr-derived SF which had less β-sheet contents supported more human MSCs attachments and proliferations than the ethanol treated SF films [55]. The SF films which had silk I dominated structures had less immunogenic responses than the three dimensional SF scaffolds with high silk II contents [14]. Lyophilized SF with high β-sheet content was less biocompatible with rat MSCs [21]. The SF scaffolds which were high in their molecular weights and β-sheet contents supported attachments and proliferations of mammalian cells physically. The mammalian cells were supported biochemically by the SF scaffolds which had less molecular weights and β-sheet contents.

2.5 Related patents

1) Yang, J. Ch., et al. [109]

Silk cocoons were degummed in de-ionized water using a degumming ratio of 1: 100 (w/v) by autoclaving at 121 °C at 1.2 kg cm^2 for 1 h. Degummed silk fibers were dried at 50 °C then dissolved in Ajisawa's reagent using a dissolving ratio of 1: 10 (w/v) at 70 °C for 4 - 6 h. SF solution was filtered and centrifuged at 5,000 rpm at 25 °C for 20 min. The SF solution was further used for fabrications of bone substitute SF scaffolds. The SF solution had its intrinsic viscosities of 1.22 - 1.27 dl g^{-1}.

2) Kaplan, D.L., et al. (2015) [110]

Silk cocoons were degummed in 0.02 M Na_2CO_3 solution at 100 °C for 20 - 30 min. Degummed silk fibers were air-dried at room temperature then dissolved in 9.3 M LiBr using a dissolving ratio of 1: 5 (w/v) at room temperature and 60 °C. SF solution was dialyzed against de-ionized water for 48 h using a dialysis membrane (MWCO: 2,000 Daltons) to produce water soluble SF at 8 wt. % of concentration. The SF solution was concentrated in 25 - 50 wt. % polyethylene glycol (MW: 8,000 - 10,000 g mol^{-1}) solution for 2 - 12 h using a dialysis membrane (MWCO: 3,500 Daltons) to yield the SF solutions at 10 - 30 wt. % of concentrations. The SF solutions were further used for fabrications of electrospun, salt-leached, and hydrogel SF scaffolds. The SF was mainly random coil in solutions while the SF scaffolds had mostly β-sheet structures. The salt-leached SF scaffolds supported osteogenic differentiations of human bone MSCs. Degradation kinetics of ethanol treated LiBr-derived SF scaffolds corresponded to rates of the osteogenic differentiations.

CHAPTER III

EXPERIMENTAL PROCEDURES

Thai domestic silk cocoons, *Bombyx mori*, Nangnoi Sisaket 1 were used as raw materials in this work. They were degummed using a single protocol to remove silk glue. Degummed silk fibers were extracted with either calcium chloride-ethanol solution (Ajisawa's reagent) or 9.3M lithium bromide (LiBr) solution using dissolving ratios of 1: 30 (w/v), 1: 15 (w/v), and 1: 10 (w/v) at designated temperatures followed by dialysis. Silk fibroin (SF) solutions were characterized for their molecular weights using size exclusion chromatography (SEC) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Viscosities and zeta potentials of the SF solutions were examined by a vibrational viscometer and a zeta-sizer, respectively. The SF solutions were freeze-dried into lyophilized SF and casted into SF films. High performance liquid chromatography (HPLC), attenuated totally reflectance-Fourier transform infrared spectroscopy (ATR-FTIR), differential scanning calorimetry (DSC), and thermogravimetry (TGA) were used for investigations of amino acid compositions, molecular structures, and thermal properties of the lyophilized SF, respectively. Ethanol (EtOH) immersion and water vapor annealing were applied to the as-casted SF films in order to regenerate β-sheet structure. Ethanol treated and water vapor annealed SF films were analyzed for their molecular structures and surface properties by the ATR-FTIR and a contact angle meter, respectively. ATR-FTIR spectra of both the lyophilized SF and the SF films were additionally deconvoluted and curve-fitted in their Amide I regions to quantify amounts of protein structures in these samples. Attachments and proliferations of NIH-3T3 mouse embryonic fibroblast cell line cultured on the SF films were evaluated by performing MTT assay for biocompatibility test. The overall research methodologies are clarified in **Figure 3.1** and **Figure 3.2**.

3.1 Materials and Instruments

3.1.1 Materials

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Sigma Life Science, USA (catalog number M2128)

Absolute EtOH 99.9 %, Fluka, UK (catalog number 46139)

Absolute EtOH 99.9 %, RCI Labscan, Thailand (catalog number AR 1069)

AccQ·Flour reagent, Waters, USA (catalog number WAT052880)

AccQ·Tag Eluent A, Waters, USA (catalog number WAT052890)

Acetonitrile, Sigma Aldrich, USA (catalog number 270717)

Calcium chloride dihydrate ($CaCl_2 \cdot 2H_2O$), Sigma Life Science, USA (catalog number C3306)

Cellulose dialysis membrane, T1 series, molecular weight cut-off (MWCO): 3,500 Daltons, Cellusep, USA (catalog number 5015-46)

Cover glass (diameter: 15 mm), Thermo Scientific, USA (catalog number 22-031145)

Deionized (DI) water (pH = 5 - 6)

Dimethyl sulfoxide (DMSO), Sigma Aldrich, USA (catalog number 472301)

Dulbecco's modified eagle medium (DMEM)/high glucose with L-glutamine and phenol red, Hyclone, USA (catalog number SH30022.02)

Ethylene glycol, Sigma Aldrich, USA (catalog number 324558)

Fetal bovine serum (FBS), Hyclone, USA (catalog number SV30160.03)

Formic acid 98 - 100 %, Fisher Scientific, UK (catalog number 10141570)

Glass filter, Pore 1, pore size: 160 µm, Duran, Germany (catalog number 2472050 and 2520501)

HCl 36.5 - 38.0 %, J.T. Baker, USA (catalog number 9535)

Hydrochloric acid (HCl) $\geq$ 31.5%, Riedel-de Haën, USA (catalog number 07115)

LiBr, Sigma Aldrich, USA (catalog number 213225)

Minitab version 14 software

MOPS SDS running buffer, Invitrogen, USA (catalog number NP0001)

NIH-3T3 mouse embryonic fibroblast cell line (ATCC® CRL-1658)

Novex Colloidal Coomassie Blue staining solution, Invitrogen, USA (catalog number LC6025)

NuPAGE 4 - 12 % Bis-Tris Mini precast gel, Invitrogen, USA (catalog number NP0322BOX)

NuPAGE antioxidant, Invitrogen, USA (catalog number NP0005)

NuPAGE LDS sample buffer, Invitrogen, USA (catalog number NP0008)

NuPAGE sample reducing agent, Invitrogen, USA (catalog number NP0004)

Omnic version 8 software

Origin version 9 software

Penicillin-Streptomycin solution 10,000 units ml^{-1}, Hyclone, USA (catalog number SV30010)

Phosphate buffered saline (PBS) powder, Bio Basic, Canada (catalog number PD0100)

SeeBlue Plus2 pre-stained protein markers, Invitrogen, USA (catalog number LC5925)

Sodium carbonate (Na_2CO_3), Ajax Finechem, Australia (catalog number 463)

Sodium chloride (NaCl), Sigma Aldrich, USA (catalog number 73575)

Sodium hydroxide (NaOH), Loba Chemie Pvt., India (catalog number 0590001000)

Standard amino acid hydrolysates for HPLC, Waters, USA (catalog number WAT088122)

Standard proteins for SEC: albumin, amylase, apoferritin, carbonic anhydrase, cytochrome C, and thyroglobulin (MW: 12, 23, 66, 200, 443, and 669 kilo Daltons, respectively), Amersham Pharmacia biotech, UK (catalog number MWGF1000)

Syringe polyethersulfone (PES) membrane, pore size: 0.22 µm, Primo, Euroclone, Italy (catalog number EPSPV2230)

Thai domestic silk cocoons, *Bombyx mori*, Nangnoi Sisaket 1, Queen Sirikit Sericulture Center, Sisaket, Thailand

Tissue culture-treated culture dish (diameter: 10 cm), Corning, USA (catalog number CLS430167)

Tissue culture-treated flat bottom culture plate (24- and 96-well TCP), Costar, Corning, USA (catalog number CLS3526 and CLS3598)

Tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), Trizma, Sigma Aldrich, USA (catalog number T5941)

Trypan blue stain, Gibco, USA (catalog number 15250-061)

Trypsin 0.25% in sodium ethylenediaminetetraacetate 0.02% solution (trypsin-EDTA), Hyclone, USA (catalog number SH30042.02)

Urea, Riedel-de Haën, USA (catalog number U5378)

3.1.2 Instruments

AccQ·Tag column (column size: 3.9 x 150 mm), Waters, USA (catalog number WAT052885)

ATR-FTIR spectrometer, Precisely Spectrum One v5.0.1, Perkin Elmer, USA

Contact angle meter, JYSP-360 contact angle goniometer, China

DSC, STARe, DSC 20, Mettler Toledo, USA

Freeze-dryer, LIO-5P, Italy

HPLC equipment, Waters, USA

Inverted microscope, CKX41, Olympus, USA

Jasco UV/VIS-1570 detector, USA

Kodak Gel Logic 200 scientific imaging system, USA

Microplate reader, FLUOstar Omega, BMG Labtech, Germany

Microscopic imaging software, CellSens Standard, Olympus, USA

Nanodrop, ND-1000 Spectrophotometer v3.0.0, USA

pH meter, Mettler Toledo, USA

Phenomenex pre-column, USA

Scanning electron microscope (SEM), Inspect S50, FEI, USA

Shodex SB-805 HQ column (column size: 8.0 x 300 mm, exclusion limit: 4,000 kilo Daltons), Japan

TGA, STARe, TG 50, Mettler Toledo, USA

Vibrational viscometer, SV-10 sine-wave vibro viscometer, A&D, Japan

XCell SureLock Mini-Cell, Carlsbad, USA

Zeta sizer, Nano ZS, Malvern, UK

3.2 Research methodologies

3.2.1 Preparations and characterizations of Thai silk fibroin solutions and lyophilized Thai silk fibroin

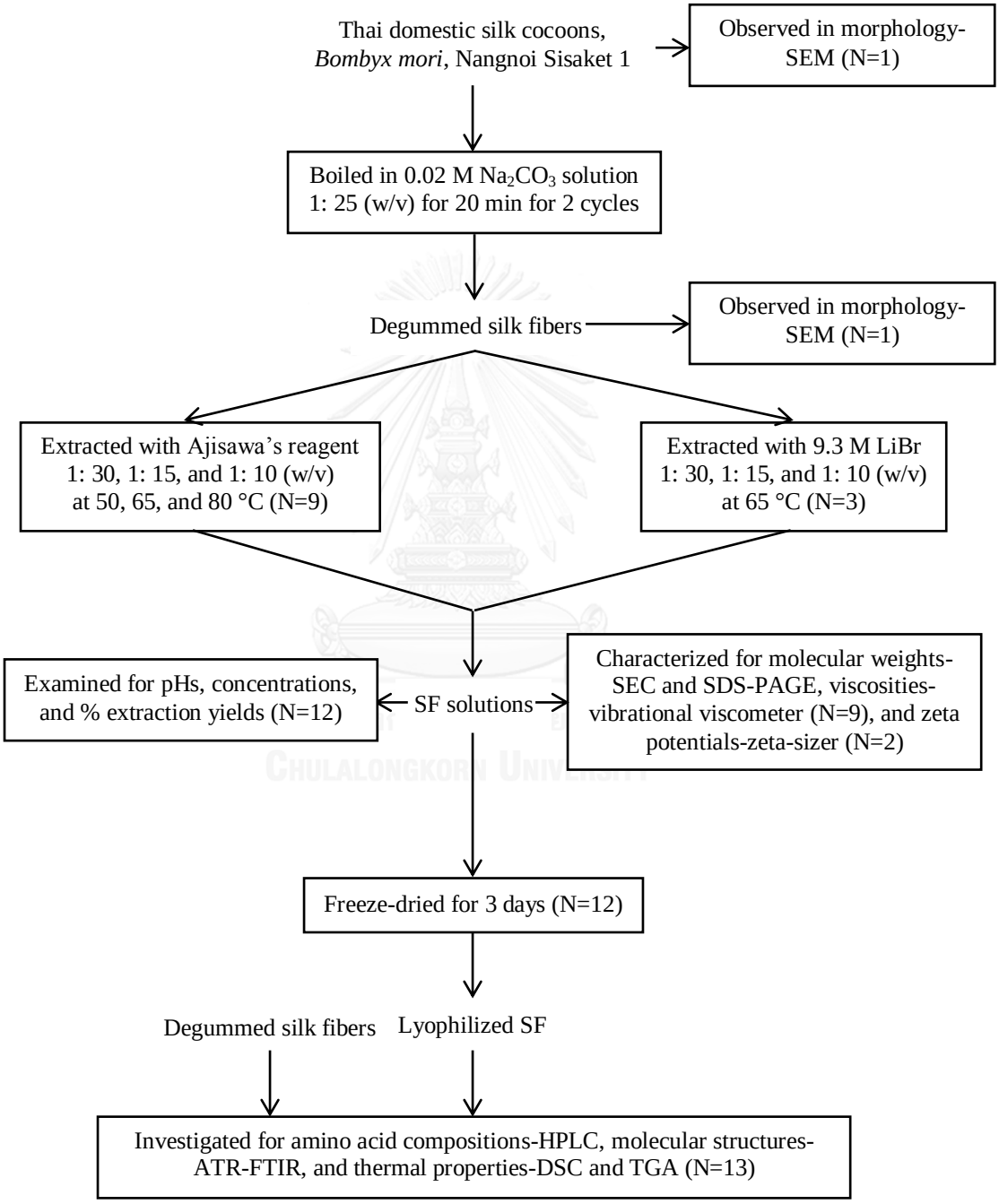

Figure 3. 1 Research scheme 1: Preparations and characterizations of Thai silk fibroin solutions and lyophilized Thai silk fibroin

3.2.2 Preparations and characterizations of Thai silk fibroin films

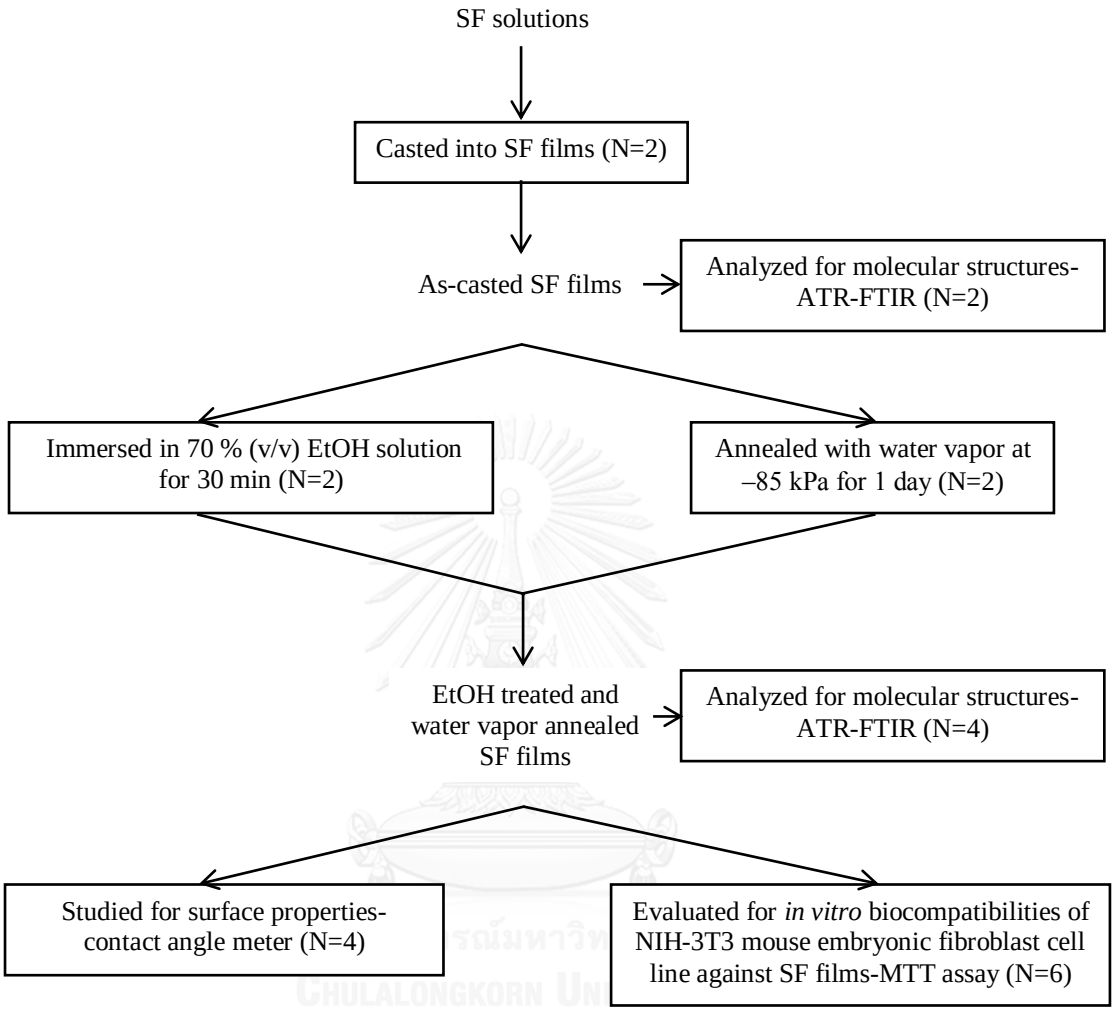

Figure 3. 2 Research scheme 2: Preparations and characterizations of Thai silk fibroin films

3.3 Preparations of Thai silk fibroin materials

3.3.1 Pre-treatment of Thai silk cocoons

Thai domestic silk cocoons, *Bombyx mori*, Nangnoi Sisaket 1 produced in standard sericulture and research facilities were degummed twice to remove silk glue [3, 47]. 40 g of the silk cocoons were cut into small pieces and boiled in a liter of 0.02 M Na_2CO_3 solution for 20 min. Degumming ratio of the silk cocoons to the degumming solution was 1: 25 (w/v). Degummed silk fibers were cleaned again with 5 l of DI water for 4 times and air-dried at room temperature for 3 days. They were hand-pulled and stored in dry condition.

Percentage of SF obtained from the degumming procedure was calculated by comparing weight of the degummed silk fibers to initial weight of the used silk cocoons in percentage while the rest was considered as silk glue. The silk cocoons and the degummed silk fibers were observed in their morphologies by SEM at the MEMS and Nanotechnology Laboratory, Department of Mechanical Engineering, Faculty of Engineering, Chulalongkorn University. Diameters of the silk fibers before and after degumming were measured and compared.

3.3.2 Extractions and purification of Thai silk fibroin solutions

Degummed silk fibers were extracted with Ajisawa's reagent and LiBr solution using different conditions. A mole fraction of 1: 6: 2 of $CaCl_2 \cdot 2H_2O$, DI water and EtOH was used to prepare the Ajisawa's reagent [66, 67]. The 9.3 M LiBr solution was prepared by dissolving LiBr in DI water.

The degummed silk fibers were either mixed with the Ajisawa's reagent or the 9.3 M LiBr solution using dissolving ratios of 1: 30 (w/v), 1: 15 (w/v), and 1: 10 (w/v). Ajisawa's reagent-mixed silk fibers were incubated at 50 °C, 65 °C, and 80 °C in an oven. LiBr solution-mixed silk fibers were only subjected to 65 °C. They were left at these designated temperatures until the silk fibers were completely dissolved. SF solutions were purified by dialysis against de-ionized water at room temperature for 3 days using a cellulose dialysis membrane. They were filtered again through a glass filter to eliminate undissolved silk fibers and silk precipitates. All SF samples studied in this work were summarized in **Table 3.1**.

Total protein concentrations of the SF solutions were examined by a Nanodrop spectrophotometer. Percent yield of each extraction condition was calculated by comparing total weight of the extracted SF to initial weight of the used degummed silk fibers in percentage. Acidities/Alkalinities of the SF solutions were measured by a pH meter.

Table 3. 1 Sample codes of Thai silk fibroin solutions studied in this work

Sample code	Solvent	Dissolving ratio (w/v)	Temperature (°C)
A1	Ajisawa's reagent	1: 30	50
A2			65
A3			80
A4	Ajisawa's reagent	1: 15	50
A5			65
A6			80
A7	Ajisawa's reagent	1: 10	50
A8			65
A9			80
L2	9.3 M LiBr	1: 30	65
L5		1: 15	
L8		1: 10	

Dissolving ratios are of degummed silk fibers to solvents

3.3.3 Freeze-drying

As-prepared SF solutions were subjected to a rapid cooling in liquid nitrogen prior to freeze-drying for 3 days. Lyophilized SF was stored in a desiccator for future analyses.

3.3.4 Film casting

As-prepared SF solutions prepared with Ajisawa's reagent and 9.3 M LiBr using a dissolving ratio of 1: 15 (w/v) at 65 ℃ (A5 and L5) were diluted with DI water to have the same concentration at 8 mg ml^{-1}. A hundred microliters of the SF solutions were casted into films on cover glasses and air-dried at room conditions (27 ºC and ~ 70 % relative humidity) for 2 days [19].

3.3.5 Ethanol immersion and water vapor annealing

As-casted SF films (A5 and L5) were either immersed in 70 % (v/v) EtOH solution for 30 min [6, 26] or water vapor annealed in a water-filled/vacuumed desiccator at –85 kPa (–25 inHg) for 24 h [54, 55] in order to regenerate β-sheet structure. Ethanol treated and water vapor annealed SF films were air-dried at room conditions (27 ºC and ~ 70 % relative humidity) for 2 days. They were stored in a desiccator for future analyses. The SF films studied in this work were summarized in **Table 3.2**.

Table 3. 2 Sample codes of Thai silk fibroin films studied in this work

Sample code	Treatment
A5-NON	As-casted
A5-EtOH	Ethanol treated
A5-H2O	Water vapor annealed
L5-NON	As-casted
L5-EtOH	Ethanol treated
L5-H2O	Water vapor annealed

A5 and L5 were SF extracted with Ajisawa's reagent and 9.3 M LiBr, respectively, using a dissolving ratio 1: 15 (w/v) at 65 ℃

3.4 Physico-chemical characterizations

3.4.1 Amino acid composition

Amino acid compositions of lyophilized SF and degummed silk fibers were investigated using reverse phase-HPLC by Lorenzo Moschini at the Biotech-Center for Biomedical Technologies, University of Trento Italy [11, 20]. The SF samples were hydrolyzed in 6 N HCl at 116 - 124 ℃ for 24 h. SF hydrolysate solutions were diluted with DI water to adjust their concentrations to 0.1 µM. The SF hydrolysate solutions were mixed with AccQ·Flour reagent to yield stable amino acid derivatives and filtered with a syringe PES membrane. They were injected into an

AccQ·Tag column with a flow rate at 1 ml min^{-1} at 37 °C. AccQ·Tag Eluent A mixed with water and acetonitrile was used as mobile phase. Standard amino acid hydrolysates were carried out in the same condition. The amino acid derivatives were detected by a Jasco UV/VIS-1570 detector at 254 nm.

3.4.2 Molecular weight

Molecular weight distributions of SF samples prepared by different extraction conditions were characterized using SEC by Lorenzo Moschini at the Biotech-Center for Biomedical Technologies, University of Trento, Italy [11, 20]. As-prepared SF solutions were diluted with an eluent (4 M urea, 0.02 M Tris-HCl, and 0.15 M NaCl solutions, pH = 7.5) to adjust their concentrations to 0.5 - 0.8 mg ml^{-1} and filtered with a syringe PES membrane. The SF solutions were injected into a Phenomenex pre-column and a Shodex SB-805 HQ column with a flow rate at 1 ml min^{-1} at 27 °C. Cytochrome C, carbonic anhydrase, albumin, amylase, apoferritin and thyroglobulin were used as standard proteins. The SF samples and the standard proteins were detected by a Jasco UV/VIS-1570 detector at 224 nm. Weight average and number average molecular weights of the SF samples were calculated (equation 3.1 and equation 3.2). Polydispersive indexes of the SF samples were calculated by dividing the weight average molecular weights to the number average molecular weights.

$$\overline{M_w} = \frac{\sum N_i {M_i}^2}{\sum N_i M_i} \qquad (3.1)$$

$$\overline{M_n} = \frac{\sum N_i M_i}{\sum N_i} \qquad (3.2)$$

where;

$\overline{M_w}$ is weight average molecular weight (Daltons)
$\overline{M_n}$ is number average molecular weight (Daltons)
N_i is number of molecules
M_i is mass of each molecule (Daltons)

Molecular weights of SF samples prepared by different extraction conditions were characterized using SDS-PAGE (NuPAGE Electrophoresis System, Invitrogen) at the Biotech-Center for Biomedical Technologies, University of Trento, Italy. As-prepared SF solutions were diluted with DI water to adjust their concentrations to 2.5 - 5 mg ml^{-1}. The SF solutions were mixed with NuPAGE reducing agent and NuPAGE LDS sample buffer prior to heating up at 70 °C for 10 min. They were loaded into NuPAGE 4 - 12% Bis-Tris Mini precast gel. MOPS SDS running buffer mixed with NuPAGE antioxidant was used as running buffer and SeeBlue Plus2 pre-stained standard was used as protein markers. The gel was run at 200 V for 50 min by an XCell SureLock Mini-Cell. It was stained with Novex Colloidal Coomassie Blue for 24 h and pictured by a Kodak Gel Logic 200 scientific imaging system using a transluminescent (black and white) mode.

3.4.3 Molecular structure

Molecular structures of lyophilized SF, SF films, and degummed silk fibers were investigated and quantified by ATR-FTIR at the Laboratory of Polymers and Composites, University of Trento, Italy. Quantification protocol of the SF's molecular structures from ATR-FTIR spectra was adapted from Hu, X. et al. (2006) [48]. The SF samples were scanned in 4000 - 650 cm^{-1} with 16 scans and a resolution of 4 cm^{-1}. The Amide I peak (1700 - 1590 cm^{-1}) of ATR-FTIR spectra was deconvoluted using the Omnic 8 software (bandwidth: 25 cm^{-1} and enhancement: 2.5). Deconvoluted spectra were curve-fitted with Gaussian profile using the Origin 9 program. Curve-fitted peaks were area normalized to the deconvoluted spectra to quantify relative contents of protein conformations and side chain. Peak positions of the curve-fitted peaks were assigned to their possible conformations and side chain as described in **TABLE 3.3**.

Table 3. 3 FTIR vibrational band assignments in the Amide I region

Peak assignment	Wavenumber (cm^{-1})	Reference
Tyrosine side chain	1590 - 1605	[54]
Beta-sheet	1608 - 1637, 1697 - 1700	[54, 64, 77-79, 111-119]
Random coil	1638 - 1653	[64, 77, 78, 111, 112, 118, 119]
Alpha-helix	1654 - 1662	[54, 78, 111-115, 119]
Beta-turn	1663 - 1696	[54, 64, 78, 112-116, 118-120]

3.4.4 Zeta potential

As prepared SF solutions were diluted with DI water to 8 mg ml^{-1}. Their pHs were adjusted to 3.5, 4.5, and 7.5 either by adding 0.1 M HCl or 0.1 M NaOH solutions. Zeta potentials of the SF solutions were characterized using a zeta sizer at 25 °C at the Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand [42, 43, 56].

3.4.5 Thermal property

Thermal properties of lyophilized SF and degummed silk fibers were investigated using DSC and TGA at the Laboratory of Polymers and Composites, University of Trento, Italy [2, 11, 20]. The SF samples (~ 10 mg) were placed into aluminium pans and alumina crucibles for the DSC and the TGA analyses, respectively. They were heated in 30 - 340 °C with a heating rate at 10 °C min^{-1} and a nitrogen gas flow rate at 100 ml min^{-1} in case of the DSC. In case of the TGA, they were heated in 30 - 650 °C with heating rates at 2.5 °C min^{-1} (250 - 350 °C) and 10 °C min^{-1} and the same nitrogen gas flow rate. First-order differentiations of TGA curves were done using the Origin 9 program. They were smoothed using 100-points of the Savitsky-Golay filter.

3.4.6 Viscosity

Silk fibroin solutions were diluted with DI water to 8 mg ml^{-1}. Their viscosities were characterized using a vibrational viscometer at a vibrational frequency of 30 Hz at 27 °C. The DI water was used as a reference. They were performed at the Multidisciplinary Laboratory in Biomedical Engineering, Chulalongkorn University, Thailand.

3.4.7 Contact angle

Contact angles of water and ethylene glycol on ethanol treated and water vapor annealed SF films (A5-EtOH, L5-EtOH, A5-H2O, and L5-H2O) were studied using a static sessile drop technique adapted from Tretinnikov, O.N. (2001) [78]. DI water and ethylene glycol (5 µl) were dropped onto the SF films by a micropipette and their contact angles were measured by a goniometer at 30 s (25 °C). They were performed at the King Mongkut's Institute of Technology Ladkrabang, Thailand.

Surface free energies of the SF films were calculated by the *Owen–Wendt* formula (equation 3.3) [70, 78, 121, 122]. Surface tensions due to dispersive and polar components of the water are 21.8 mN m^{-1} and 51.0 mN m^{-1}, respectively. These values in case of the ethylene glycol are 29.0 mN m^{-1} and 19.0 mN m^{-1}, respectively [121]. Surface polarities (equation 3.4) of the SF films were calculated.

$$\gamma_L(1+\cos\theta)/2 = \sqrt{\gamma_{SF}^d \gamma_L^d} + \sqrt{\gamma_{SF}^p \gamma_L^p} \qquad (3.3)$$

$$surface\ polarity = \gamma_{SF}^p / \gamma_{SF} \times 100\% \qquad (3.4)$$

where;

θ is contact angle of liquid on SF film (rad)
γ_L is total surface free energy of liquid (mN m^{-1})
γ_{SF}^d is surface energy due to dispersive component of SF film (mN m^{-1})
γ_L^d is surface energy due to dispersive component of liquid (mN m^{-1})
γ_{SF}^p is surface energy due to polar component of SF film (mN m^{-1})
γ_L^p is surface energy due to polar component of liquid (mN m^{-1})
γ_{SF} is total surface free energy of SF film (mN m^{-1})

3.5 Biological characterization: *In vitro* NIH-3T3 mouse embryonic fibroblast cell line-silk fibroin film biocompatibility test

In vitro NIH-3T3 mouse embryonic fibroblast cell line-SF film biocompatibility test was evaluated in terms of attachments and proliferations [22, 123, 124]. The NIH-3T3 cells were cultured in DMEM supplemented with 10 % FBS and 1 % penicillin-streptomycin in an incubator at 37 °C, 5 % carbon dioxide, and 95

% relative humidity. They were sub-cultured with trypsin-EDTA solution when they reached 70 % confluence. Ethanol treated and water vapor annealed SF films coated onto cover glasses were placed into 24-well TCP and sterilized with ethylene oxide gas. The NIH-3T3 cells were seeded onto the EtOH treated and the water vapor annealed SF films (A5-EtOH, L5-EtOH, A5-H2O, and L5-H2O), the cover glasses, and empty wells in the 24-well TCP at 2 x 10^4 cells per well (seeding density: 1 x 10^4 cells cm^{-2}). Cell spreading at 6 h was estimated using the CellSens Standard software. The NIH-3T3 were pictured and counted at 6 h for the cell attachment study and 1 day, 2 day, 3 day, 4 day, 5 day, and 6 day for the proliferation study using the MTT assay [125]. At the specified time points, the culture medium was discarded from the TCP. The NIH-3T3 cells were washed with PBS twice. MTT solution (0.5 mg ml^{-1}) was added into each well and the NIH-3T3 cells were incubated again in the same condition for 30 min. The MTT solution was replaced with DMSO to dissolve formazan crystals. Absorbance of formazan solution was measured at 570 nm by a microplate reader. Numbers of viable cells were considered from a standard curve plotting absorbance against known numbers of cells (1.56 x 10^4, 3.12 x 10^4, 6.25 x 10^4, 1.25 x 10^5, 2.50 x 10^5 and 5.00 x 10^5 cells). Percentage of early cell attachment was calculated by comparing numbers of the viable cells at 6 h to numbers of the seeded cells in percentage. Specific growth rate (μ) (equation 3.5) and population doubling time (PDT) (equation 3.6) were calculated.

$$\mu = \frac{lnN_2 - lnN_1}{t_2 - t_1} \tag{3.5}$$

where;

μ is specific growth rate (h^{-1})
N_1 is numbers of cells at specified time point t_1(cells)
N_2 is numbers of cells at specified time point t_2 (cells)

$$PDT = \frac{ln(2)}{\mu} \tag{3.6}$$

where;

PDT is population doubling time (h)

3.6 Statistical consideration

At least 4 replications of quantitative characterizations were done in this work. Average values ± standard deviations were calculated. The analysis of variance (ANOVA) at 95 % confidence level was analyzed by the Minitab version 14 software. Significant differences were considered when p-values were less than 0.05.

CHAPTER IV

RESULTS AND DISCUSSION

Degummed silk fibers, silk fibroin (SF) solutions, lyophilized SF, and ethanol (EtOH) treated and water vapor annealed SF films were successfully prepared from Thai domestic silk cocoons, *Bombyx mori*, Nangnoi Sisaket 1. Physico-chemical and biological characteristics of the SF resulted from degumming procedure, extraction methods, dialysis, film fabrication, and β-sheet regenerations were studied. Aqueous SF solutions extracted with calcium chloride-EtOH solution (Ajisawa's reagent) and 9.3 M lithium bromide (LiBr) using 1: 30 (w/v), 1: 15 (w/v), and 1: 10 (w/v) dissolving ratios at 50 °C, 65 °C, and 80 °C were characterized for their molecular weights, zeta potentials, and viscosities. The lyophilized SF was investigated for their amino acid compositions, molecular structures, and thermal properties together with the degummed silk fibers as a reference material. The EtOH treated and the water vapor annealed SF films were analyzed for their molecular structures and surface properties. Biocompatibility test in terms of cell attachments and proliferations was performed against NIH-3T3 mouse embryonic fibroblast cell line. Their results are presented and discussed in this chapter.

4.1 Degummed Thai silk fibers

Thai silk cocoons, *Bombyx mori*, Nangnoi Sisaket 1 were degummed in boiling 0.02 M sodium carbonate (Na_2CO_3) solution for 20 min for 2 cycles. Silk fibers of outer layer of the silk cocoons and degummed silk fibers are illustrated in **Figure 4.1**. Average sizes of undegummed and the degummed silk fibers were evaluated (**Appendix A**). **Table 4.1** presents diameters of the undegummed and the degummed silk fibers in this work in comparisons with silk fibers prepared in other studies. The silk cocoons and the degummed silk fibers were weighed before and after degumming. **Table 4.2** shows comparisons in percentages of SF and silk glue (which contains sericin, wax, minerals, ash, and others) resulted from the degumming procedure in this work with degumming results from other studies.

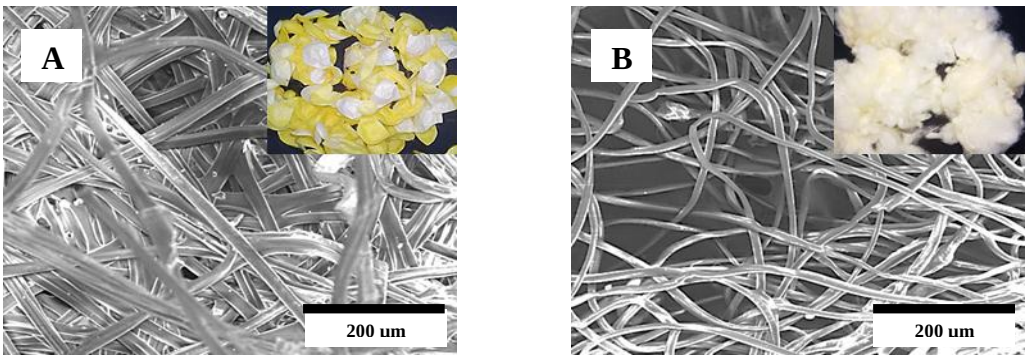

Figure 4. 1 Scanning electron micrographs of (A) outer layer of Thai silk cocoons, Nangnoi Sisaket 1 and (B) silk fibers degummed in boiling 0.02 M sodium carbonate solution for 20 min for 2 cycles (magnification = 500x)

Table 4. 1 Diameters of undegummed and degummed silk fibers in this work in comparisons with silk fibers prepared in other studies

	This work	**Mhuka, V. et al. (2013) [40]**	**Kundu, B. et al. (2014) [30]**	**Chung, D.E. et al. (2015) [41]**
Silk race	Nangnoi Sisaket 1 silk cocoons	South African silk cocoons	Indian silk cocoons	Korean silk cocoons[b]
Degumming procedure	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles	Boiled in 1.1 g l^{-1} Na_2CO_3 solution, 1 h	Boiled in 0.02 M Na_2CO_3 solution, 1 h	Boiled in 0.3 % (w/v) sodium oleate + 0.2 % (w/v) Na_2CO_3 solution, 1 h
Undegummed (µm)	20.7 ± 1.0[a]	No data	~ 28	14.7 - 30.0
Degummed (µm)	11.8 ± 1.0[a]	7 - 12	11 ± 2	No data

[a]Data are reported in average ± standard deviation (n = 5). These values are significantly different.
[b]Different races of *Bombyx mori* silk cocoons were studied.

Table 4. 2 Percentages of silk fibers and silk glue resulted from degumming procedure in this work in comparisons with degumming results from other studies

	This work	**Yamada, H. et al. (2001) [29]**		**Lee, J.H. et al. (2016) [25]**
Silk race	Nangnoi Sisaket 1 silk cocoons	Japanese silk cocoons	Japanese silk cocoons	Korean silk cocoons
Degumming procedure	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles	8 M urea, 80 °C, 2 h	Boiled in 0.05 % Na_2CO_3 solution, 20 min	Boiled in 0.6 % (w/v) sodium oleate + 0.4 % (w/v) Na_2CO_3 solution, 1 h
Silk fibers (%)	75.1 ± 0.4[a]	74.7	74.1	71.5
Silk glue (%)	24.9 ± 0.4[a]	25.3	25.9	28.5

[a]Five batches of silk fiber preparation were studied.

The undegummed silk fibers appeared in a ribbon-like shape whose fibers were joined by two SF bundles and non-uniformly cemented with silk glue cover. The degummed silk fibers appeared differently in a smooth and cylindrical single fiber form without the silk glue cover. Diameters of the silk fibers after the degumming (11.8 ± 1.0 µm) were about twice as less than the undegummed silk fibers (20.7 ± 1.0 µm) which showed disintegration of the two SF bundles. The silk fibers lost 24.9 ± 0.4 % of their weights after the degumming which indicated an effective removal of the silk glue.

Our degumming results were comparable to other studies. Complete silk glue removals without silk fiber deterioration is desirable for preparation of high-quality silk fibers. The 0.02 M Na_2CO_3 solution is the most used degumming solution reported in other studies [1-3, 7, 8, 10-12, 14, 15, 18-22, 30, 41, 46-62]. This alkaline solution was found to be more effective than sodium hydroxide solution, sodium chloride solution and urea [30]. The silk cocoon degumming in boiling Na_2CO_3 solution resulted in decrease in the silk fiber diameters by half (from 14.7 - 30.0 µm to 7 - 12 µm) since the two SF bundles lost their integrations [30, 40, 41]. Amount of the silk glue in the *Bombyx mori* silk cocoons is varied in 25 - 32 % depending on silk races [25, 29-31]. At least 15 - 20 min was required for the Na_2CO_3 degumming procedure to effectively remove the silk glue, although heavy-chain Japanese SF was found to be degraded [29]. The silk cocoons could alternatively be degummed with 8 M urea at 80 °C for 2 h to preserve the heavy-chain of Japanese SF [29]. However, the urea was found to be less effective for the degumming of Indian silk cocoons [30]. In this work, the degummed silk fibers were prepared with complete silk glue removal while the silk fiber deterioration was hardly noticed.

4.2 Thai silk fibroin solutions

Degummed Thai silk fibers were dissolved in Ajisawa's reagent and 9.3 M LiBr using 1: 30 (w/v), 1: 15 (w/v), and 1: 10 (w/v) dissolving ratios at 50 °C, 65 °C, and 80 °C. SF extractions were dialyzed against de-ionized water at room temperature for 3 days to obtain water soluble-SF solutions. The SF extractions and the aqueous SF solutions prepared in this work are shown in **Figure 4.2**. Total protein concentrations of aqueous SF solutions were used to calculate their yields. **Table 4.3** summarizes dissolution results of the aqueous SF solutions extracted using the two solvents in different conditions.

Figure 4. 2 Silk fibroin solutions extracted with Ajisawa's reagent 1: 30 (w/v) at 50 °C, 65 °C, and 80 °C (A1, A2, and A3, respectively), 1: 15 (w/v) at 50 °C, 65 °C, and 80 °C (A4, A5, and A6, respectively), and 1: 10 (w/v) at 50 °C, 65 °C, and 80 °C (A7, A8, and A9, respectively) and 9.3 M LiBr 1: 30 (w/v), 1: 15 (w/v), and 1: 10 (w/v) at 65 °C (L2, L5, and L8, respectively) (Upper row) before and (Lower row) after dialysis, yellowish color of the Nangnoi Sisaket 1 cocoons stayed in the SF solutions

Table 4. 3 Dissolution results of aqueous silk fibroin solutions extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 (w/v), 1: 15 (w/v), and 1: 10 (w/v) at 65 °C

Sample code	Dissolution time (h)	Appearance	Concentration (mg ml^{-1})	Volume (ml)	Yield (%)
A1	18	Clear	8.15 ± 0.47^{a}	47 ± 5	73 ± 9
A2	1.5	Clear	7.94 ± 0.46^{a}	47 ± 5	72 ± 5
A3	0.5	Clear	8.03 ± 0.58^{a}	48 ± 4	74 ± 7
A4	18	Clear	15.90 ± 1.30^{b}	45 ± 5	70 ± 4
A5	1.5	Clear	15.85 ± 0.82^{b}	47 ± 4	70 ± 2
A6	0.5	Clear	15.90 ± 1.13^{b}	47 ± 4	71 ± 4
A7	18	Cloudy	21.28 ± 3.18^{c}	44 ± 4	63 ± 9
A8	1.5	Cloudy	21.31 ± 2.90^{c}	46 ± 4	64 ± 9
A9	0.5	Cloudy	21.07 ± 3.09^{c}	46 ± 4	64 ± 9
L2	3	Clear	9.86 ± 0.13^{d}	40 ± 5	74 ± 8
L5	3	Clear	17.53 ± 1.86^{b}	42 ± 5	71 ± 9
L8	3	Clear	23.56 ± 2.45^{c}	44 ± 5	68 ± 9

Appearances were of aqueous SF solutions which are presented in **Figure 4.2**, All quantitative data are reported in average ± standard deviation (n = 4). [a-d]These values are significantly different ($P<0.05$).

The degummed silk fibers were completely dissolved in the Ajisawa's reagent for shorter times (18 h, 1.5 h, and 30 min) when higher dissolution temperatures (50 °C, 65 °C, and 80 °C, respectively) were used. The aqueous SF solutions extracted with the Ajisawa's reagent had clear appearances except for the SF solutions extracted with the Ajisawa's reagent using the 1: 10 (w/v) dissolving ratios (A7 - A9). The aqueous SF solutions extracted with the LiBr (L2, L5, and L8) were all clear in appearances. The protein concentrations of the aqueous SF solutions increased with the dissolving ratios. The aqueous SF solutions extracted with the Ajisawa's reagent using the 1: 30 (w/v) dissolving ratio (A1 - A3) had significantly lower concentrations than the aqueous SF solutions extracted with the LiBr using the same dissolving ratio (L2) (7.94 ± 0.46 - 8.15 ± 0.47 mg ml^{-1} and 9.86 ± 0.13 mg ml^{-1}, respectively). The yields of the SF extracted with the Ajisawa's reagent using the 1: 10 (w/v) dissolving ratio (A7 - A9) (63 ± 9 - 64 ± 9 %) were lowers than those of other groups (68 ± 9 - 74 ± 8 %), although their numbers are not significantly different. All SF solutions had their pHs ~ 5.5.

Extractions of the SF using the Ajisawa's reagent were reported using 1: 30 - 1: 10 (w/v) dissolving ratios at 55 - 85 °C [6, 9, 23-25, 27, 29, 30, 41, 43, 45, 58, 60, 66-73]. The SF extracted with the 9.3 M LiBr was generally reported to use the dissolving ratios of 1: 30 - 1: 5 (w/v) dissolving ratios mostly at 60 - 65 °C for 3 - 4 h [1-4, 7-12, 14, 15, 18-21, 24, 27, 30, 46-65, 68, 107]. In this work, the SF-Ajisawa extractions were studied systematically in designated conditions reported from those studies. Since the Ajisawa's reagent has less solvency power to the SF than the LiBr [34], the SF-Ajisawa extractions are rather prepared to dissolve the SF at low concentrations at high temperatures. Ajisawa, A. (1998) reported that homogeneous Japanese SF extractions could be prepared at 20 - 30 °C and 55 °C for 720 h and 1 h,

respectively [66, 67]. Dissolution times of the SF-Ajisawa extractions decreased from 1 - 3 h (at 70 - 78 °C) to 3 - 40 min (at 80 - 85 °C) [6, 9, 23-25, 27, 41, 43, 45, 58, 68, 71-73]. Although SF-Ajisawa extractions were successfully prepared in some other studies [6, 23, 71-73], Cho, H.J. et al. (2012) found that the Korean SF solutions extracted with the same solvent using a 1: 20 (w/v) dissolving ratio at 85 °C for 3 - 180 min had cloudy appearances [58]. This suggests that SF's hydrogen bonds forming in β-sheet structures were partially de-stabilized. Chen, X. et al. (2001) proposed that the Chinese SF had a compactly coil, a readily β-sheet forming structure when the SF was extracted with the Ajisawa's reagent while the SF extracted with the LiBr had mostly random coil structures [77]. Moreover, presence of ethanol in the Ajisawa's reagent might have induced the β-sheet structures of the SF [12, 26, 80-82]. SF solubility resulted in its solution concentrations and yields. The yields Ajisawa extractions (50 - 66 %) were reported to be lower than those of the LiBr extractions (68 - 98 %) [9, 30]. In this work, Thai SF, Nangnoi Sisaket 1 extracted with the Ajisawa's reagent and the LiBr solution using our studied conditions had no significant differences in their yields. The SF extracted with the Ajisawa's reagent using the 1: 10 (w/v) dissolving ratio had slightly lower yields than others probably because some amounts of regenerated SF was removed by filtering step.

4.3 Amino acid composition

Degummed Thai silk fibers and lyophilized Thai SF extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C were investigated for their amino acid compositions using high performance liquid chromatography by Lorenzo Moschini. **Table 4.4** presents the amino acid compositions of the degummed silk fibers and the lyophilized SF prepared in this work. **Table 4.5** and **Table 4.6** show comparisons in the amino acid compositions of the SF prepared in this work and the SF prepared from silk glands and silk cocoons in other studies, respectively.

The degummed silk fibers and the lyophilized SF prepared in this work had similar amino acid compositions. Amounts of glycine, alanine, serine, tyrosine and valine which are the most abundant amino acids in the SF were at 46.6 - 48.3 mol %, 29.2 - 32.1 mol %, 4.9 - 8.2 mol %, 4.5 - 5.1 mol %, and 2.3 - 2.6 mol %, respectively. Hydrophobic and hydrophilic amino acids were at 86.5 - 90.0 mol % and 10.0 - 13.5 mol % in total, respectively. Our results suggest that different SF extraction conditions using the Ajisawa's reagent and the LiBr hardly affected the SF's amino acid compositions of the Thai SF. The amino acid compositions of the SF prepared in this work were comparable to the SF prepared from the silk glands and the silk cocoons in other studies except for some results. The heavy-chain Japanese SF extracted from posterior silk glands was high in glycine (49.4 mol %), alanine (29.8 mol %), and serine (11.3 mol %) contents while the light-chain domain was high in alanine (16.9 mol %), aspartic acid (15.4 mol %), and glycine (10.0 mol %) contents [32]. The Chinese SF extracted from silk glands had high contents in the glycine (43.8 mol %), alanine (30.0 mol %), and serine (11.0 mol %). The Italian SF degummed in Na_2CO_3 solution and extracted with formic acid reported by Motta, A. et al. (2014) [20] and the Korean SF degummed in the same solution and extracted

with the Ajisawa's reagent reported by Chung, D.E. et al. (2015) [41] had almost the same amino acid compositions as in ours. This suggests that silk races had no effect on the SF's amino acid compositions. However, the Thai SF degummed in Na_2CO_3 solution and extracted with the LiBr by Kaewprasit, K. et al. (2014) had more hydrophobic (82 mol %) and less hydrophilic (18 mol %) amino acids than the Chinese- and the Japanese-Thai hybrid SF (77 mol % and 23 mol %, respectively) [19]. The SF investigated in this work had more glycine and tyrosine contents by ~ 15 mol % and ~ 3 mol %, respectively, and less serine and glutamic acid contents by ~ 12 mol % and ~ 2 mol %, respectively, than in other studies. This might have due to different analysis conditions used among these studies. For instance, the amino acid compositions of the SF investigated in this work were calculated relatively from 15 amino acids while those reported in other studies were obtained from 15 - 18 amino acids.

Table 4. 4 Amino acid compositions in mol % of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 ℃ and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 ℃ with respect to degummed silk fibers (DS)

	A1	A2	A3	A4	A5	A6	A7	A8	A9	L2	L5	L8	DS
Hydrophilic													
Acidic													
Aspartic acid	1.3	1.4	1.5	1.6	1.8	2.1	1.7	1.8	1.4	1.9	2.0	2.0	2.7
Glutamic acid	1.6	1.2	0.8	0.8	1.2	1.2	1.1	1.3	1.1	1.4	1.6	1.4	1.6
Basic													
Arginine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.6	0.5	0.5	0.4	0.5	0.6
Lysine	0.4	0.4	0.4	0.4	0.4	0.4	0.5	0.6	0.5	0.4	0.4	0.4	0.4
Histidine	0.4	0.3	0.4	0.6	0.4	0.4	0.0	0.0	0.0	0.3	0.6	0.2	0.5
Polar													
Serine	5.1	6.9	6.9	6.8	6.4	8.1	7.4	8.4	8.2	7.4	7.3	7.4	4.9
Threonine	0.7	0.8	0.7	0.8	0.8	0.8	0.7	0.8	0.8	0.8	0.8	0.8	0.7
Total	10.0	11.5	11.2	11.5	11.5	13.5	11.9	13.5	12.5	12.7	13.1	12.7	11.4
Hydrophobic													
Non-polar													
Glycine	48.3	47.2	47.6	46.9	46.6	46.9	48.0	47.3	48.2	46.6	47.0	47.4	47.2
Alanine	32.1	31.3	31.0	31.5	31.4	29.8	30.6	29.2	29.7	30.5	30.0	30.3	31.5
Proline	0.4	0.4	0.4	0.5	0.5	0.4	0.5	0.4	0.5	0.4	0.4	0.4	0.5
Valine	2.5	2.5	2.4	2.6	2.6	2.4	2.4	2.4	2.4	2.6	2.5	2.3	2.5
Leucine	0.5	0.6	0.6	0.6	0.6	0.5	0.5	0.6	0.5	0.5	0.5	0.6	0.5
Isoleucine	1.0	1.0	1.0	1.1	1.0	1.0	0.9	1.0	0.9	0.9	1.0	0.9	0.8
Aromatic													
Tyrosine	4.5	4.8	5.0	4.8	5.0	4.8	4.8	5.1	4.8	5.0	4.8	4.7	5.0
Phenylalanine	0.7	0.7	0.7	0.7	0.7	0.7	0.6	0.7	0.6	0.7	0.7	0.7	0.7
Total	90.0	88.5	88.7	88.7	88.4	86.5	88.3	86.7	87.6	87.2	86.9	87.3	88.7

Table 4. 5 Amino acid compositions in mol % of lyophilized silk fibroin extracted with Ajisawa's reagent and 9.3 M LiBr 1: 15 (w/v) at 65 °C (A5 and L5, respectively) with respect to degummed silk fibers (DS) in comparisons with silk fibroin extracted from silk glands

	This work			**Shimura, K. et al. (1986) [32]**		**Yang, M. et al. (2012) [13]**
	A5	**L5**	**DS**			
Silk race	Nangnoi Sisaket 1 silk cocoons			Japanese posterior silk glands		Chinese silk glands
				Heavy-chain	Light-chain	
Degumming procedure	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles		None	None		None
Extraction method	Ajisawa and 9.3 M LiBr, 1: 15 (w/v), 65 °C		None	None		None
Hydrophilic						
Acidic						
Aspartic acid	1.8	2.0	2.7	0.6	15.4	2.1
Glutamic acid	1.2	1.6	1.6	0.7	8.4	2.5
Basic						
Arginine	0.5	0.4	0.6	0.2	3.8	0.6
Lysine	0.4	0.4	0.4	0.1	1.5	0.4
Histidine	0.4	0.6	0.5	0.1	1.6	0.2
Polar						
Serine	6.4	7.3	4.9	11.3	7.9	11.0
Threonine	0.8	0.8	0.7	0.4	2.8	1.0
Cysteine		No data		0.0	0.0	0.0
Total	11.5	13.1	11.4	13.4	41.4	17.8
Hydrophobic						
Non-polar						
Glycine	46.6	47	47.2	49.4	10.0	43.8
Alanine	31.4	30	31.5	29.8	16.9	30.0
Proline	0.5	0.4	0.5	0.3	3.0	0.8
Valine	2.6	2.5	2.5	2.0	7.4	2.3
Leucine	0.6	0.5	0.5	0.1	7.2	0.6
Isoleucine	1.0	1.0	0.8	0.1	7.3	0.6
Methionine		No data		0.0	0.4	0.0
Aromatic						
Tyrosine	5.0	4.8	5.0	4.6	3.4	2.5
Phenylalanine	0.7	0.7	0.7	0.4	2.7	1.6
Tryptophan		No data		No data		No data
Total	88.4	86.9	88.7	86.7	58.3	82.2

Table 4. 6 Amino acid compositions in mol % of lyophilized silk fibroin extracted with Ajisawa's reagent and 9.3 M LiBr 1: 15 (w/v) at 65 °C (A5 and L5, respectively) with respect to degummed silk fibers (DS) in comparisons with silk fibroin prepared in other studies

	This work			**Yang, M. et al. (2012) [13]**	**Kaewprasit, K. et al. (2014) [19]**			**Motta, A. et al. (2014) [20]**	**Chung, D.E. et al. (2015) [41]**
	A5	**L5**	**DS**						
Silk race	Nangnoi Sisaket 1 silk cocoons			Chinese silk glands	NN[a]	K1[b]	K8[c]	Italian silk cocoons	Korean silk cocoons
Degumming procedure	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles		None	None	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles			Boiled in 1.1 and 0.4 g l^{-1} Na_2CO_3 solution, 1 h	Boiled in 0.3 % (w/v) sodium oleate + 0.2 % (w/v) Na_2CO_3 solution, 1 h
Extraction method	Ajisawa and 9.3 M LiBr, 1: 15 (w/v), 65 °C		None	None	9.3 M LiBr, 1: 5 (w/v), 60 °C, 4 h			Formic acid, 1.5 % (w/v), room temperature	Ajisawa, 1: 20 (w/v), 85 °C, 3 min
Hydrophilic									
Acidic									
Aspartic acid	1.8	2.0	2.7	2.1	1.6	2.1	2.2	0.8	1.5
Glutamic acid	1.2	1.6	1.6	2.5	1.2	1.5	1.5	0.5	1.2
Basic									
Arginine	0.5	0.4	0.6	0.6	0.3	0.3	0.4	0.5	0.4
Lysine	0.4	0.4	0.4	0.4	0.2	0.3	0.3	0.6	0.2
Histidine	0.4	0.6	0.5	0.2	0.8	0.8	1.0	No data	0.3
Polar									
Serine	6.4	7.3	4.9	11.0	13.4	16.9	16.3	8.5	6.6
Threonine	0.8	0.8	0.7	1.0	0.8	1.1	1.1	0.8	0.9
Cysteine		No data		0.0	0.0	0.0	0.0	No data	2.9
Total	11.5	13.1	11.4	17.8	18.3	22.9	22.8	11.7	14.0
Hydrophobic									
Non-polar									
Glycine	46.6	47	47.2	43.8	38.3	33.0	35.8	46.7	44.5
Alanine	31.4	30	31.5	30.0	34.3	31.3	29.4	31.5	31.8
Proline	0.5	0.4	0.5	0.8	0.4	0.6	0.6	0.5	0.1
Valine	2.6	2.5	2.5	2.3	1.2	1.7	1.5	2.4	2.5
Leucine	0.6	0.5	0.5	0.6	0.3	0.4	0.4	0.5	0.3
Isoleucine	1.0	1.0	0.8	0.6	0.2	0.3	0.3	1.0	0.6
Methionine		No data		0.0	0.1	0.1	0.1	0.1	0.2
Aromatic									
Tyrosine	5.0	4.8	5.0	2.5	5.8	7.7	7.2	4.9	5.3
Phenylalanine	0.7	0.7	0.7	1.6	1.0	1.7	1.4	0.7	0.7
Tryptophan		No data		No data	0.2	0.3	0.4	No data	No data
Total	88.4	86.9	88.7	82.2	87.7	77.1	77.2	88.3	86.0

[a]Thai silk cocoons, Nangnoi Sisaket 1 produced from the Queen Sirikit Sericulture Center, Nakhon Ratchasima, Thailand, [b]Thai-Japanese hybrid silk cocoons, [c]Thai-Chinese hybrid silk cocoons

4.4 Molecular weight

Aqueous Thai SF solutions extracted with Ajisawa's reagent 1: 30 - 1: 15 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C were characterized for their molecular weights (MW) using size exclusion chromatography (SEC) by Lorenzo Moschini and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The SF was denatured and reduced for the SDS-PAGE while it was in its natural arrangements for the SEC. Therefore, the SF's MW analyzed using the SDS-PAGE depended solely on its sizes (masses) while that analyzed using the SEC depended on both its sizes and shapes (assemblies). The 1: 10 (w/v) SF-Ajisawa solutions were not studied since they had cloudy appearances and were less homogeneous. The MW of the SF samples characterized using the SDS-PAGE is shown (**Appendix B**). Chromatograms of the SF samples prepared in this work were used to calculate their MW (**Appendix C**). **Table 4.7** presents weight average MW ($\overline{M_w}$), number average MW ($\overline{M_n}$), and polydispersive indexes (PDI) of the SF samples. **Table 4.8** and **Table 4.9** present the MW characterized using the SDS-PAGE and molecular weight distributions characterized using the SEC, respectively, of the SF samples prepared in this work in comparisons with the SF prepared in other studies.

Table 4. 7 Molecular weight distributions of silk fibroin samples extracted with Ajisawa's reagent 1: 30 - 1: 15 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C characterized using SEC

Sample code	Weight average molecular weight (kilo Dalton)	Number average molecular weight (kilo Dalton)	Polydispersive index
A1	336	161	2.09
A2	340	164	2.07
A3	340	166	2.05
A4	337	161	2.09
A5	337	161	2.09
A6	340	165	2.06
L2	341	167	2.04
L5	336	161	2.09
L8	337	162	2.08

All SF samples had smear heavy-chain bands (> 250 - 100 kilo Daltons; kDa) and clear-cut light-chain bands (25 kDa) according to the SDS-PAGE. The heavy-chain SF extracted with the Ajisawa's reagent (> 250 - 100 kDa) had less MW than the SF extracted with the LiBr (> 250 - 150 kDa). The SF extracted with the Ajisawa's reagent and the LiBr in different conditions had similar molecular weight distributions. The $\overline{M_w}$ and the $\overline{M_n}$ of the SF samples were at 336 - 340 kDa and 161 - 167 kDa, respectively. The PDI of the SF samples were at 2.05 - 2.09.

The heavy- and the light-chain SF had their MW literally at 350 kDa and 25 kDa, respectively [13, 32-36]. The heavy-chain SF was degraded to 300 - 37 kDa after being degummed in Na_2CO_3 solution and extracted with either the Ajisawa's reagent or the LiBr perhaps due to hydrolysis of the SF [15, 27, 29, 60, 69]. Incomplete denaturation of the SF's β-sheets during sample preparations for the SDS-PAGE could cause poor separation of the SF's heavy-chain. The light-chain SF was preserved mostly by the SF-Ajisawa extractions [9, 29, 60] though some other studies suggested that it was removed by the degumming procedures and the dissolution processes [15, 27, 69].

The Ajisawa-derived SF and the LiBr-derived SF had their molecular weight distributions reported at 252.5 - 16 kDa and 376 - 82.7 kDa, respectively [11, 15, 20, 24, 27, 43, 60, 74-76]. Pawcenis, D. et al. (2014) reported viscosity average MW of the SF extracted with the Ajisawa's reagent at 260 kDa [76]. Cho, H.J. et al. (2012) and Chung, D.E. et al. (2015) reported the SF's MW characterized by the SEC at 450 kDa [41, 58, 68]. The SF's MW decreased from 450 kDa to 100 kDa when dissolution times of the SF in the Ajisawa's reagent increased from 3 min to 180 min. They additionally reported that SF samples extracted with the Ajisawa's reagent from various races of Korean *Bombyx mori* silk were all similar in their MW. All of the results suggest that the SF's MW was affected by mostly the extraction conditions. The SF tended to be more degraded by the Ajisawa's process. The PDI of the SF samples prepared in this work suggested that they had low molecular weight distributions. The PDI of the SF were reported in other studies at 1.4 - 8.63 depending on silk races, degumming procedures, and extraction methods [11, 20, 24].

Table 4. 8 Molecular weights of silk fibroin samples extracted with Ajisawa's reagent and 9.3 M LiBr 1: 15 (w/v) at 65 °C (A5 and L5, respectively) in comparisons with silk fibroin prepared in other studies

		Silk race	Degumming procedure	Extraction method	Heavy-chain (kilo Dalton)	Light-chain (kilo Dalton)
This work	**A5** **L5**	Nangnoi Sisaket 1 silk cocoons	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles	Ajisawa or 9.3 M LiBr, 1: 15 (w/v), 65 °C	> 250 - 100	25
Yamada, H. et al. (2001) [29]		Japanese silk cocoons	Boiled in 0.05 % (w/v) Na_2CO_3 solution, 60 min, 2 cycles	Ajisawa, 1: 15 (w/v), 75 °C	300 - 100	25
Wadbua, P. et al. (2010) [9]		Nangnoi Sisaket silk cocoons	Boiled in 0.5 % (w/v) $NaHCO_3$ solution, 15 min	Ajisawa, 1: 30 (w/v), 80 °C, 15 min and 9.3 M LiBr, 1: 30 (w/v), 60 °C, 25 - 40 min	350 (Ajisawa) Degraded (LiBr)	25

	Silk race	Degumming procedure	Extraction method	Heavy-chain (kilo Dalton)	Light-chain (kilo Dalton)
Zhang, J. et al. (2012) [69]	Chinese silk cocoons	0.25 % (w/v) SDS + 0.25 % (w/v) Na_2CO_3 solution, 98 °C, 30 min	Ajisawa, 1: 20 (w/v), 65 °C, 1 h	300 - 100	Not observed
You, R. et al. (2013) [27]	Chinese silk cocoons	Boiled in 0.05 % (w/v) Na_2CO_3 solution, 30 min, 3 cycles	Ajisawa, 72°C, 1h and 9.3 M LiBr, 60 °C, 2h	300 - 20	Not observed
George, K.A. et al. (2013) [15]	Japanese silk cocoons	Boiled in 0.02 M Na_2CO_3 solution, 1 h	9.3 M LiBr, 1: 10 (w/v), 60 °C, 4 h	250 - 37	Not observed
Aznar-Cervantes, S.D. et al. (2014) [60]	Spanish silk cocoons	Boiled in 0.02 M Na_2CO_3 solution, 45 min, 2 cycles	Ajisawa, 1: 10 (w/v), 70 °C, 6 h and 9.3 M LiBr, 1: 5 (w/v), 60 °C, 3 h	220 - 60	25 (Ajisawa) Not observed (LiBr)

Table 4. 9 Molecular weight distributions of silk fibroin samples extracted with Ajisawa's reagent and 9.3 M LiBr 1: 15 (w/v) at 65 °C (A5 and L5, respectively) in comparisons with silk fibroin prepared in other studies

		Silk race	Degumming procedure	Extraction method	Molecular weight (kilo Dalton)	Polydispersive index
This work	**A5**	Nangnoi Sisaket 1 silk cocoons	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles	Ajisawa and 9.3 M LiBr, 1: 15 (w/v), 65 °C	$\overline{M_w}$ = 337 $\overline{M_n}$ = 161	2.09
	L5				$\overline{M_w}$ = 336 $\overline{M_n}$ = 161	2.09
Malay, Ö. et al. (2007) [43]		Turkish silk cocoons	Boiled in 0.05 % Na_2CO_3 solution, 30 min, 3 cycles	Ajisawa 1: 20 (w/v), 78 °C, 2h	90	No data
Cho, H.J. et al. (2012) [58, 68]		Korean silk cocoons	Boiled in 0.3 % (w/v) sodium oleate + 0.2 % (w/v) Na_2CO_3 solution, 1 h	Ajisawa, 1: 20 (w/v), 85 °C, 3 min, 30 min, and 180 min 9.3 M LiBr, 1: 20 (w/v), 25 °C, 6h	$\overline{M_w}$ = 450 (3 min and 30 min) and 110 (180 min) $\overline{M_w}$ = 450	No data

	Silk race	Degumming procedure	Extraction method	Molecular weight (kilo Dalton)	Polydispersive index
Chung, D.E. et al. (2015) [41]	Korean silk cocoons	Boiled in 0.3 % (w/v) sodium oleate + 0.2 % (w/v) Na_2CO_3 solution, 1 h	Ajisawa, 1: 20 (w/v), 85 °C, 3 min	$\overline{M_w}$ = 450	No data
Kim, H.H. et al. (2016) [24]	Korean silk cocoons	Boiled in 0.3 % (w/v) sodium oleate + 0.2 % (w/v) Na_2CO_3 solution, 1 h	Ajisawa, 1: 5 (w/v), 80 °C, 5 min 9.3 M LiBr, 1: 5 (w/v), 80 °C, 30 min	$\overline{M_n}$ = 252 (Ajisawa) $\overline{M_n}$ = 263 (LiBr)	~ 1.4
Motta, A. et al. (2011) [11]	Italian silk cocoons	Boiled in 1.1 and 0.4 g l^{-1} Na_2CO_3 solution, 1 h	9.3 M LiBr, 1: 10 (w/v), 65 °C, 2 h	$\overline{M_w}$ = 304 $\overline{M_n}$ = 36.4	8.63
Motta, A. et al. (2014) [20]	Italian silk cocoons[a]	Boiled in 1.1 and 0.4 g l^{-1} Na_2CO_3 solution, 1 h	9.3 M LiBr, 1: 10 (w/v), 65 °C, 5 h	$\overline{M_w}$ = 358 and 376[a]	6.3 and 6.6[a]
Pawcenis, D. et al. (2014) [74-76]	Chinese silk fabric	None	9.3 M LiBr, 60 °C, 3 h	$\overline{M_w}$ = 359 - 270	No data

$\overline{M_w}$ is weight average MW and $\overline{M_n}$ is number average MW. [a]Pure race and polyhybrid silk were studied, respectively.

4.5 Molecular structure

Degummed Thai silk fibers and lyophilized Thai SF extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C were investigated for their molecular structures using attenuated totally reflectance-Fourier transform infrared spectroscopy (ATR-FTIR). ATR-FTIR spectra of the degummed silk fibers and the lyophilized SF prepared in this work are shown in **Figure 4.3**. The ATR-FTIR spectra of the SF samples prepared in this work were all similar except for the degummed silk fibers. The lyophilized SF had high intensities in their Amide I, Amide II, Amide III, and Amide IV at 1646 - 1645 cm^{-1}, 1517 - 1515 cm^{-1}, 1235 - 1232 cm^{-1}, and 1059 - 1056 cm^{-1}, respectively. These Amide bands in case of the degummed silk fibers were at 1620 cm^{-1}, 1513 cm^{-1}, 1228 cm^{-1}, and 1064 cm^{-1}, respectively. The degummed silk fibers were mainly β-sheet characterized by its Amide I, Amide II, and Amide IV bands and an Amide III shoulder at 1260 cm^{-1}. The lyophilized SF samples were mostly random coil identified by their Amide I, Amide III, and Amide IV bands. Although the lyophilized SF had their Amide II bands assigned to the β-sheet, their Amide II shoulders at 1540 cm^{-1} corresponded to the random coil structure.

The Amide I bands of the SF samples were deconvoluted and curve-fitted to quantitatively analyze their β-sheet, β-turn, α-helix, random coil, and tyrosine side chain contents (**Appendix D**). Relative contents in these molecular structures of each SF sample are presented in **Table 4.10**. The β-sheet and the β-turn contents were summarized to silk II-like structures which are considered as highly ordered structures of the SF. The α-helix and the random coil were summarized to silk I-like structures which are considered to be less ordered. **Table 4.11** presents the relative contents in the silk I- and the silk II-like structures found in the degummed silk fibers and the lyophilized SF. **Table 4.12** shows comparisons in relative β-sheet contents of the SF samples prepared in this work and the SF prepared in other studies.

The degummed silk fibers had its relative contents in the β-sheet, the β-turn, the α-helix, the random coil, and the tyrosine side chain at 59.1 %, 15.8 %, 8.5 %, 12.4 %, and 4.2 %, respectively. Its silk I- and the silk II-like structures were at 20.9 % and 74.9 %, respectively. The relative contents of the lyophilized SF were different only in their β-sheet and random coil while the β-turn, the α-helix, and the tyrosine side chain contents of these were similar. The Ajisawa-derived SF (A1 - A9) had their β-sheet/silk II-like contents decreased and random coil/silk I-like contents increased with increases in dissolution temperatures and decreases in dissolving ratios. The 1: 30 (w/v) SF-Ajisawa extractions (A1 - A3) had similar β-sheet and random coil contents (13.3 - 15.0 % and 43.0 - 47.3 %, respectively) and silk I- and silk II-like contents (55.5 - 59.0 % and 37.4 - 40.7 %, respectively) to the LiBr-derived SF (L2, L5, and L8). The 1: 15 (w/v) SF-Ajisawa extractions (A4 - A6) had their β-sheet/silk II-like contents decreased (from 22.3 % to 12.1 % and from 47.2 % to 39.6 %, respectively) and random coil/silk I-like contents increased (from 40.5 % to 46.0 % and from 48.5 % to 57.0 %, respectively) with increases in dissolution temperatures (from 50 °C to 80 °C). The 1: 10 (w/v) SF-Ajisawa extractions (A7 - A9) had their β-sheet and random coil contents (19.0 - 20.2 % and 39.0 - 43.2 %, respectively) and silk I- and the silk II-like contents (50.4 - 52.4 % and 42.9 - 46.0 %, respectively) unchanged with the increases in dissolution temperatures.

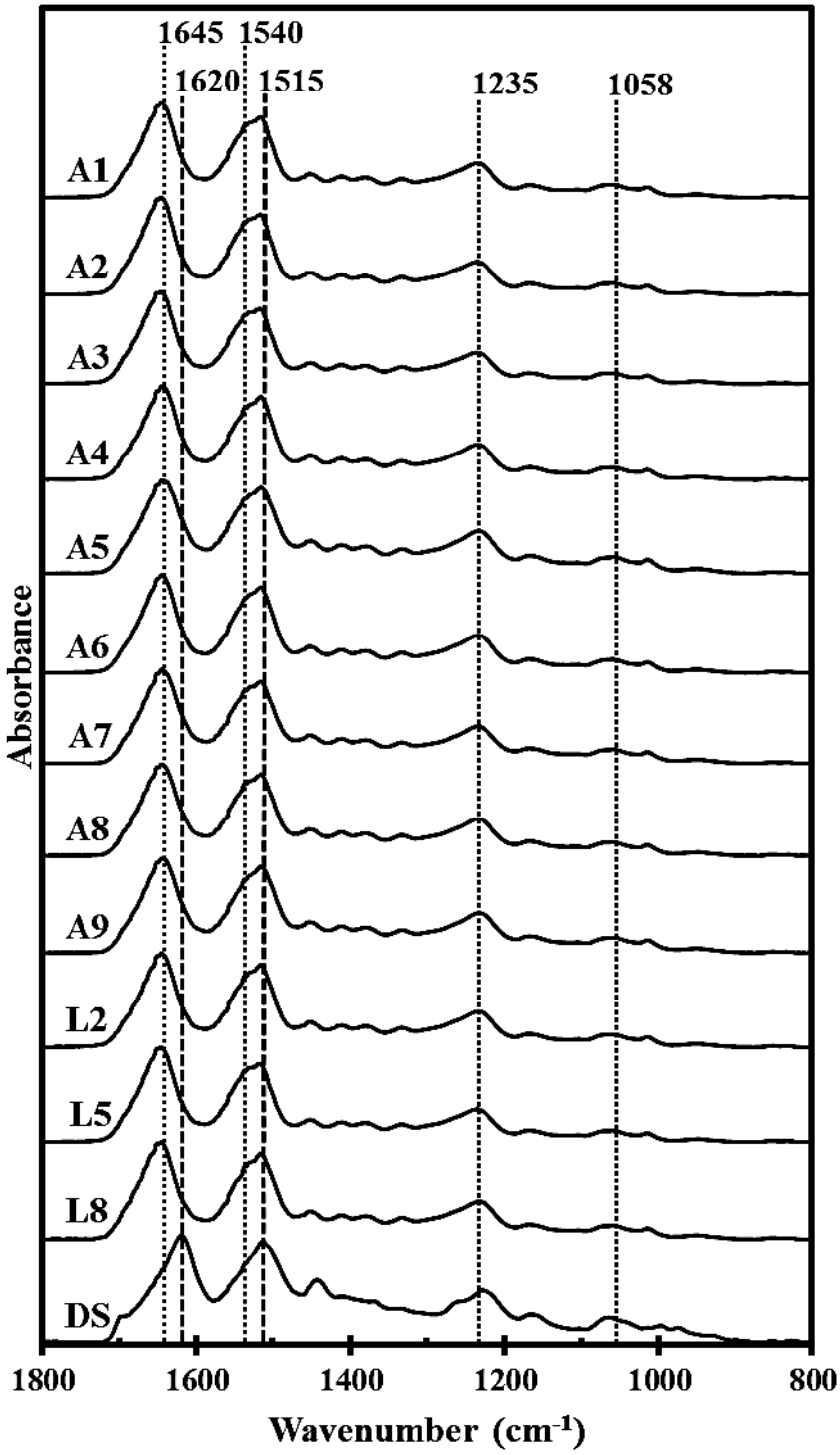

Figure 4. 3 ATR-FTIR spectra of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C with respect to degummed silk fibers (DS)

Table 4. 10 Relative contents in β-sheet, β-turn, α-helix, and random coil of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C with respect to degummed silk fibers (DS)

Sample code	β-sheet (%)	β-turn (%)	α-helix (%)	Random coil (%)
A1	14.9	24.2	11.9	45.3
A2	13.5	23.8	11.7	47.3
A3	13.3	24.5	11.8	46.9
A4	22.3	24.8	8.0	40.5
A5	18.2	24.9	11.5	41.5
A6	12.0	27.6	11.0	46.0
A7	22.2	23.8	11.4	39.0
A8	20.2	23.9	11.5	40.5
A9	19.0	23.9	9.7	43.2
L2	15.0	25.7	12.2	43.3
L5	14.4	24.6	11.5	45.8
L8	14.2	27.2	12.3	43.0
DS	59.1	15.8	8.5	12.4

Table 4. 11 Relative contents in silk I- and silk II-like structures of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C with respect to degummed silk fibers (DS)

Sample code	Silk I-like structures (%)	Silk II-like structures (%)
A1	57.2	39.1
A2	59.0	37.4
A3	58.7	37.8
A4	48.5	47.2
A5	53.0	43.1
A6	57.0	39.6
A7	50.4	46.0
A8	52.0	44.1
A9	52.4	42.9
L2	55.5	40.7
L5	57.3	39.0
L8	56.3	40.3
DS	20.9	74.9

Silk I-like structures include α-helix and random coil while silk II-like structures include β-sheet and β-turn.

Table 4. 12 Relative β-sheet contents of lyophilized silk fibroin extracted with Ajisawa's reagent and 9.3 M LiBr 1: 15 (w/v) at 65 °C (A5 and L5, respectively) with respect to degummed silk fibers (DS) in comparisons with silk fibroin prepared in other studies

		Silk race	Degumming procedure	Extraction method	β-sheet content (%)	Other structure (%)
This work	**A5**	Nangnoi Sisaket 1 silk cocoons	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles	Ajisawa or 9.3 M LiBr, 1: 15 (w/v), 65 °C	18.2	T = 24.9, A = 11.5, R = 41.5, and SC = 3.9
	L5				14.4	T = 24.6, A = 11.5, R = 45.8, and SC = 3.6
	DS			None	59.1	T = 15.8, A = 8.5, R = 12.4, and SC = 3.4
Cilurzo, A. et al. (2011) [45]		Italian DS	None	None	~ 52	T ~ 15, A+R ~ 29, and SC ~ 4
Lu, Q. et al. (2011) [10]		No data	Boiled in 0.02 M Na_2CO_3 solution, 20 min	9.3 M LiBr, 1: 5 (w/v), 60 °C, 4 h	17.1	T = 29.4, A = 8.5, and R = 12.4
Lu, Q. et al. (2011) [56]		No data	Boiled in 0.02 M Na_2CO_3 solution, 20 min	9.3 M LiBr, 1: 5 (w/v), 60 °C, 4 h	14.7	T and bend = 46.1, A = 11.0, and R = 26.1
Jin, Y. et al. (2013) [44]		Chinese middle silk glands	None	None	18.7 - 30.0	A+R = 27.7 - 35.5 and others 42.3 - 45.8
You, R. et al. (2013) [27]		Chinese silk cocoons	Boiled in 0.05 % (w/v) Na_2CO_3 solution, 30 min, 3 cycles	Ajisawa, 72°C, 1h and 9.3 M LiBr, 60 °C, 2h	30.0 (Ajisawa)	T = 23.0, A = 20.5, and R = 21.5 (Ajisawa)
					31.1 (LiBr)	T = 22.6, A = 22.3, and R = 15.9 (LiBr)
Callone, E. et al. (2016) [46]		Hybrid Japanese silk cocoons	Boiled in 1.1 and 0.4 g l^{-1} Na_2CO_3 solution, 1 h	9.3 M LiBr, 1: 10 (w/v), 65 °C, 2 h	19.9[a]	B+T = 36.5 and A+R = 63.5[a]

	Silk race	Degumming procedure	Extraction method	β-sheet content (%)	Other structure (%)
Callone, E. et al. (2016) [46]	Hybrid Japanese silk cocoons	Boiled in 1.1 and 0.4 g l^{-1} Na_2CO_3 solution, 1 h	None	63.3[a]	B+T = 75.5 and A+R = 24.5[a]

B: β-sheet, T: β-turn, A: α-helix, R: random coil, SC: tyrosine side chain, [a]These samples were characterized using solid state ^{13}C nuclear magnetic resonance.

The molecular structures of the Chinese SF in middle silk glands were 18.7 - 30.0 % of the β-sheet, 27.7 - 35.5 % of the α-helix and the random coil, and 42.3 - 45.8 % of other intermediates [44]. The SF becomes more crystallized and less amorphous as silkworms spin it to form silk cocoons. Italian and hybrid Japanese silk fibers degummed in Na_2CO_3 solutions contained 52 - 63 % of the β-sheet, 12 - 15 % of the β-turn, and 25 - 29 % of the α-helix and the random coil [45, 46] which were contributed to the degummed silk fibers prepared in this work. Partial de-stabilizations of the SF's hydrogen bonds using ionic salt solutions in certain conditions decrease the SF's crystallinity and resulted in water-soluble SF. The silk I-like contents were increased to 20.9 - 63.5 % after the extractions [10, 27, 46, 56]. The β-sheet contents of the SF extracted with the Ajisawa's reagent (30 %) were higher than those of the SF extracted with the LiBr (14.7 - 31.7 %) [10, 27, 46, 56]. This suggests that the SF extracted with the Ajisawa's process was less de-stabilized than that extracted with the LiBr process. Chen, X. et al. (2001) studied the SF's molecular structures using the FTIR spectroscopy and rheology. They proposed that the Chinese SF extracted with the Ajisawa's regent had a compactly coil, a readily β-sheet forming structure while the SF extracted with the LiBr was in a free random coil form [77]. In this work, the aqueous 1: 10 (w/v) SF-Ajisawa solutions were cloudy in their appearances as a result of less solubilized SF. These samples were high in β-sheet contents.

SF films were casted onto cover glasses and air-dried at room conditions (27 ºC and ~ 70 % relative humidity) for 2 days. As-casted SF films were either water vapor annealed at –85 kPa (–25 inHg) for 24 h or immersed in 70 % (v/v) EtOH solution for 30 min and air-dried in order to regenerate SF's β-sheet structures. The as-casted SF films, water vapor annealed samples, and EtOH treated samples prepared with the Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-NON, A5-H2O, and A5-EtOH, respectively) and the LiBr 1: 15 (w/v) at 65 °C (L5-NON, L5-H2O, and L5-EtOH, respectively) were investigated for their molecular structures using the same technique as in previous. The ATR-FTIR spectra of the SF films prepared in this work are shown in **Figure 4.4**. Their Amide I bands were deconvoluted and curve-fitted for molecular structure assessments (**Appendix E**). The relative contents in the β-sheet, the β-turn, the α-helix, the random coil and the tyrosine side chain of each SF film are shown in **Figure 4.5**. **Figure 4.6** shows the relative contents in the silk I- and the silk II-like structures found in the SF films. **Table 4.13** shows comparisons in relative β-sheet contents of the SF films prepared in this work and the SF films prepared in other studies.

The ATR-FTIR spectra of the SF films prepared with the Ajisawa's reagent and the LiBr using the same film treatments were similar. All SF films had high intensities in their Amide I, Amide II, Amide III, and Amide IV at 1620 cm^{-1}, 1515 cm^{-1}, 1235 cm^{-1} and 1058 cm^{-1}, respectively. Their Amide I and Amide II corresponded to the β-sheet while the Amide III and the Amide IV related to the random coil. Random coil shoulders at 1645 cm^{-1} (the Amide I) and 1540 cm^{-1} (the Amide II) became less intense after the as-casted SF films were treated with water vapor annealing and EtOH immersion, respectively. In contrast, β-sheet shoulders at 1260 cm^{-1} (the Amide III) gained more intensities as the random coil shoulders decreased. These suggest that the SF films were all mainly β-sheet with increases in their β-sheet characteristics in the following order, as-casted SF films < water vapor annealed SF films < EtOH treated SF films.

The as-casted SF films had their β-sheet, β-turn, α-helix, random coil, and tyrosine side chain contents at 33.8 - 35.0 %, 19.5 - 20.6 %, 9.6 - 9.8 %, 23.2 - 25.9 %, and 11.0 - 11.7 %, respectively. The water vapor annealing and the EtOH immersion influenced conformational transitions from the random coil to the β-sheet. The β-sheet and the random coil contents of the SF films were changed by ~ 5 % after the water vapor annealing and ~ 10 % after the EtOH immersion while the β-turn and the tyrosine side chain contents remained stable. The water vapor annealed SF film prepared with the LiBr (L5-H2O) had its α-helix content at 16.1 % while there were 7.7 - 10.3 % of the same structures in other samples. The silk I- and the silk II-like contents of the as-casted and water vapor annealed SF films were similar (30.5 - 39.9 % and 53.3 - 57.9 %, respectively). The EtOH treated SF films had ~ 10 % less silk I-like structures and more silk II-like structures than others.

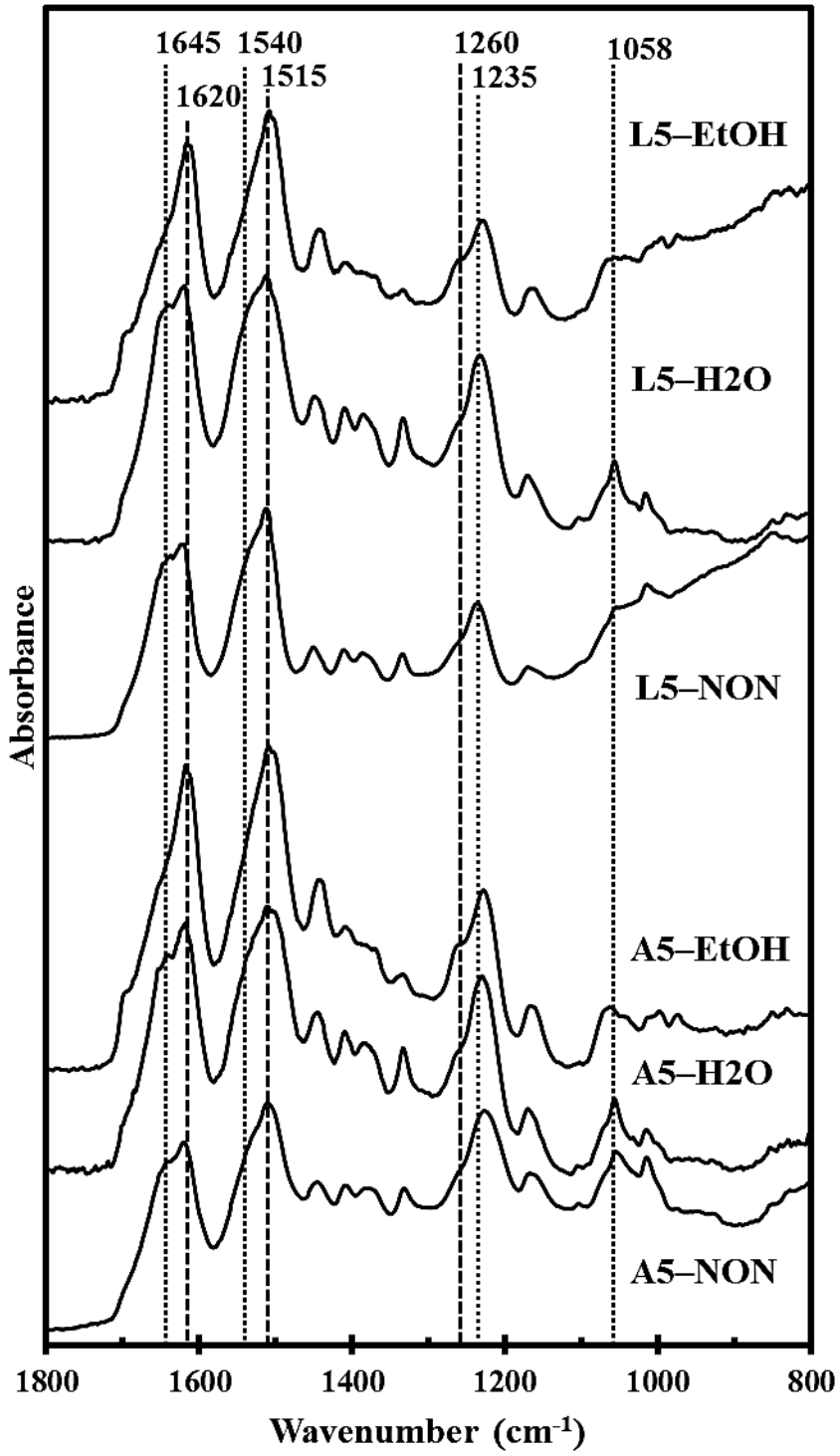

Figure 4. 4 ATR-FTIR spectra of as-casted silk fibroin films, water vapor annealed samples, and ethanol treated samples prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-NON, A5-H2O, and A5-EtOH, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-NON, L5-H2O, and L5-EtOH, respectively)

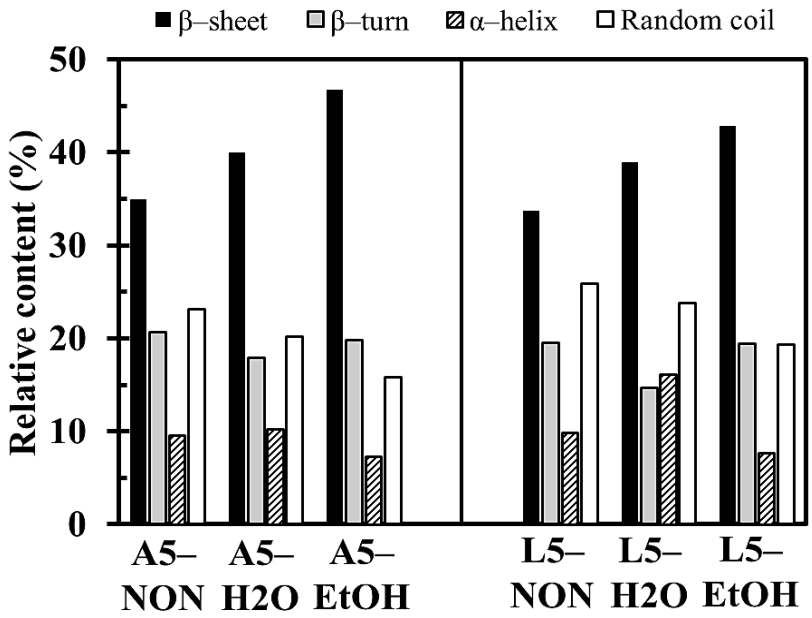

Figure 4. 5 Relative contents in molecular structures of as-casted silk fibroin films, water vapor annealed samples, and ethanol treated samples prepared with Ajisawa's reagent) and 9.3 M LiBr 1: 15 (w/v) at 65 °C

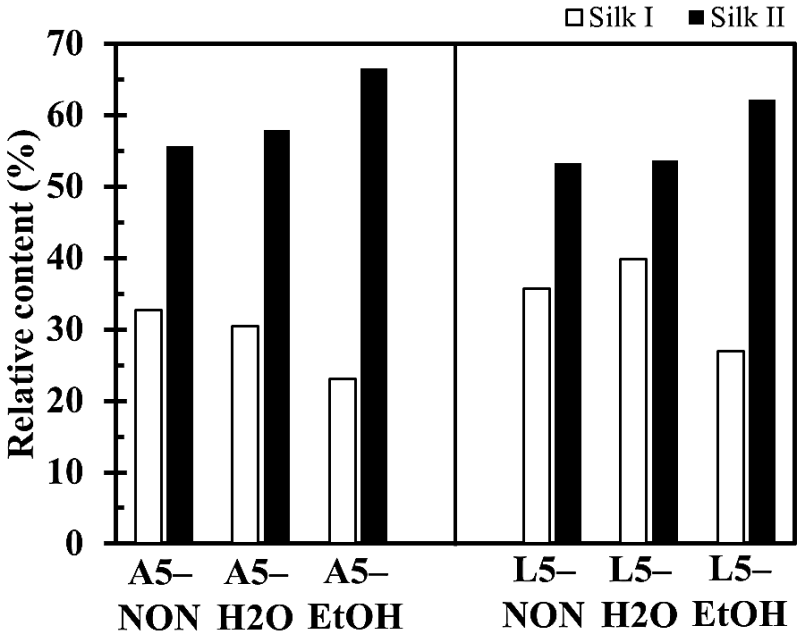

Figure 4. 6 Relative contents in silk I- and silk II-like structures of as-casted silk fibroin films, water vapor annealed samples, and ethanol treated samples prepared with Ajisawa's reagent and 9.3 M LiBr 1: 15 (w/v) at 65 °C, Silk I-like structures include α-helix and random coil while silk II-like structures include β-sheet and β-turn

Table 4. 13 Relative β-sheet contents of as-casted silk fibroin films, water vapor annealed samples, and ethanol treated samples prepared with Ajisawa's reagent and 9.3 M LiBr 1: 15 (w/v) at 65 °C in comparisons with silk fibroin prepared in other studies

		Silk race	Degumming procedure	Extraction method	Film casting/ treatment	β-sheet content (%)
This work	**A5-NON**	Nangnoi Sisaket 1 silk cocoons	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles	Ajisawa or 9.3 M LiBr, 1: 15 (w/v), 65 °C	As-casted films without treatment and with 24 h water vapor annealing and 70 % (v/v) EtOH immersion for 30 min	35.0
	A5-H2O					40.0
	A5-EtOH					46.7
	L5-NON					33.8
	L5-H2O					38.9
	L5-EtOH					42.8
Asakura, T. et al. (1985) [63]		Japanese silk glands	None	None	As-casted films, 20 - 27 °C	17 - 21[a]
					Water vapor annealing	30[a]
Tretinnikov, O.N. et al. (2001) [78]		Japanese silk cocoons	No data	9.0 M LiBr	As-casted film, 30 °C	12.5
Chen, X. et al. (2007, 2009) [80, 81]		Chinese silk cocoons	Boiled in 0.5 % (w/w) $NaHCO_3$ solution, 30 min, 2 cycles	9.5 M LiBr	70 % (v/v) EtOH immersion	18 - 33
Lu, Q. et al. (2010) [54]		Japanese silk cocoons	Boiled in 0.02 M Na_2CO_3 solution, 20 min	9.3 M LiBr, 1: 5 (w/v), 60 °C, 4 h	As-casted film	20.0
					Water vapor annealing, 12 h	30.3
Hu, X. et al. (2011) [55]		No data	Boiled in 0.02 M Na_2CO_3 solution, 20 min	9.3 M LiBr, 1: 5 (w/v), 60 °C, 4 h	Water vapor annealing, 4 °C, 12 h	14
					Water vapor annealing, 100 °C, 12 h	58

	Silk race	Degumming procedure	Extraction method	Film casting/ treatment	β-sheet content (%)
Seib, F.Ph. et al. (2012) [12]	No data	Boiled in 0.02 M Na_2CO_3 solution, 20 min	9.3 M LiBr, 1: 5 (w/v), 60 °C, 4 h	Water vapor annealing, 6 h	35.1
				70 % (v/v) EtOH immersion, 6h	36.2
Callone, E. et al. (2016) [46]	Hybrid Japanese silk cocoons	Boiled in 1.1 and 0.4 g l^{-1} Na_2CO_3 solution, 1 h	9.3 M LiBr, 1: 10 (w/v), 65 °C, 2 h	As-casted films	35.3 - 39.8[a]
				Water vapor annealing, 24 h	41.9 - 47.3[a]

B: β-sheet, T: β-turn, A: α-helix, R: random coil, SC: tyrosine side chain, [a]These samples were characterized using solid state ^{13}C nuclear magnetic resonance.

The Ajisawa-derived SF films had more β-sheet contents than the LiBr-derived SF films as a result of less de-stabilized SF presented in its as-prepared solutions. The as-casted SF films (fabricated using 8 mg ml^{-1} of as-prepared SF solutions at 27 ºC and ~ 70 % relative humidity) had ~ 20 % more β-sheet contents than the lyophilized SF. Rapid freezing in liquid nitrogen followed by freeze-drying could effectively preserved the molecular structures of as-prepared SF. Slow-dryings in film fabrications allow more spontaneous β-sheet regenerations, especially, when using concentrated SF solutions, high temperatures, and humid conditions. The as-casted SF films prepared from Japanese silk glands and silk cocoons had 12.5 - 39.8 % of the β-sheet contents while those of slow-dried SF films were at 22.7 - 42.2 % [12, 46, 54, 55, 63, 78]. Zhong, J. et al. (2014 and 2015) reported that the Chinese SF films casted using high concentrated SF solutions (1.5 mg ml^{-1}) prepared with Na_2CO_3 degumming procedure and the Ajisawa's process had more β-sheet characteristics than those casted using diluted SF solutions (300 µg ml^{-1}) [71, 72]. Tretinnikov, O.N. et al. (2001) and Zhou, J. et al. (2016) reported that the high temperature (50 - 60 °C) casted SF films had more silk II characteristics than the SF films casted at room temperature and 37 °C [73, 78]. Ming, J. et al. (2012 and 2015) reported that the Chinese SF solutions prepared with the Na_2CO_3 degumming procedure and the LiBr process could be casted into silk I-dominated films in < 55 % relative humidity condition [62, 108]. The SF films treated with EtOH partially regenerated their β-sheet structures up to 18 - 51.0 % while those treated with water vapor annealing partially induced the same structure up to 14 - 47.3 % [12, 46, 54, 55, 63, 80-82]. The EtOH treatment was more effective in the SF's β-sheet regenerations than the water vapor annealing since the former process could chemically regenerate the SF's hydrogen bonds and dehydrate the SF molecules. The regenerations of the SF's hydrogen bonds in the water vapor annealing process were physically controlled by temperatures. Hu, X. et al. (2011) found that the water vapor annealed SF film treated at 25 °C had its β-sheet contents at 30 % while that treated at 100 °C had 58 %

of the β-sheets [55]. Callone, E. et al. (2016) found that the water vapor annealed LiBr-derived/hybrid Japanese SF film had significant 3-folded (silk III) structure [46]. This might have contributed to the water vapor annealed LiBr-derived SF film prepared in our study (L5-H2O) which had ~ 6 % more helical content than other samples.

4.6 Zeta potential

Zeta potential is electrical potential differences between charges in diffuse layers of particles and surrounding environments. It is often used to estimate surface charges and isoelectric points of the particles (SF molecules in our case). As-prepared Thai SF solutions extracted with Ajisawa's reagent and 9.3 M LiBr using a 1: 15 (w/v) dissolving ratio at 65 °C (A5 and L5, respectively) were diluted to yield the same concentration at 8 mg ml^{-1} and their pHs were adjusted to 3.5, 4.5, and 7.5. They were characterized for their zeta potentials by a zeta sizer at 25 °C. The zeta potentials of the two SF solutions at different pHs are shown in **Figure 4.7**. Isoelectric points of the two SF samples were estimated at zeta potentials = 0. **Table 4.14** presents the zeta potentials and the isoelectric points of the two SF samples in comparisons with the SF prepared in other studies. The isoelectric points of the Ajisawa- and LiBr-derived SF were at 4.04 and 4.12, respectively. The Ajisawa-derived SF exhibited similar negative charges (–6.28 ± 0.38 mV and –7.86 ± 0.33 mV, respectively) to the LiBr-derived SF (–8.11 ± 0.25 mV and –11.96 ± 0.41 mV, respectively) at pH 5.5 and 7.5. Their zeta potentials (~ –10 mV) suggested that the SF was not stable in aqueous solutions and tended to aggregate and assemble into large particles [56]. These might have resulted from differences in their molecular structures since there was no difference in their amino acid compositions. Beta-sheet/silk II-like structures are compact conformations dominated in the Ajisawa-derived SF whereas random coil/silk I-like structures are free-form conformations dominated in the LiBr-derived SF [77]. The compact conformations might have limited their charge exhibitions in solutions while the free-form conformations might have exhibited their charges with less restriction.

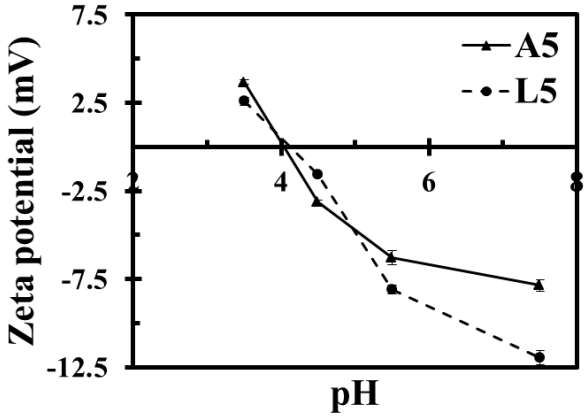

Figure 4. 7 Zeta potentials of aqueous silk fibroin solutions extracted with Ajisawa's reagent and 9.3 M LiBr 1: 15 (w/v) at 65 °C (A5 and L5, respectively) at different pHs at 25 °C

Table 4. 14 Zeta potentials and isoelectric points of aqueous silk fibroin samples extracted with Ajisawa's reagent and 9.3 M LiBr 1: 15 (w/v) at 65 °C (A5 and L5, respectively) in comparisons with silk fibroin prepared in other studies

	This work		**Ayub, Z.H. et al. (1993) [42]**	**Malay, Ö. et al. (2007) [43]**	**Lu, Q. et al. (2011) [56]**
	A5	**L5**			
Silk race	Nangnoi Sisaket 1 silk cocoons		Japanese waste silk	Turkish silk cocoons	No data
Degumming procedure	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles		Boiled in 0.5 % Na_2CO_3 solution, 20 min	Boiled in 0.05 % Na_2CO_3 solution, 30 min, 3 cycles	Boiled in 0.02 M Na_2CO_3 solution, 20 min
Extraction method	Ajisawa and 9.3 M LiBr, 1: 15 (w/v), 65 °C		Ajisawa, 16 % (w/v)	Ajisawa 1: 20 (w/v), 78C, 2h	9.3 M LiBr, 1: 5 (w/v), 60 °C, 4 h
Concentration (mg ml^{-1})	8		30	1	10
Temperature (°C)	25		20	20	25
Zeta potential at pH = 5.5 (mV)	–6.28 ± 0.38[a]	–8.11 ± 0.25[a]	No data	–3	–7.7
Isoelectric point	4.04	4.12	3.8 - 3.9	3.9	No data

[a]Data are reported in average ± standard deviation (n = 4). These values are significantly different ($P<0.05$).

Table 4. 15 Zeta potentials of water vapor annealed and ethanol treated silk fibroin films prepared in other studies

	Seib, F.Ph. et al. (2012) [12]	**Terada, D. et al. (2016) [26]**
Silk race	No data	Japanese silk threads
Degumming procedure	Boiled in 0.02 M Na_2CO_3 solution, 20 min	None
Extraction method	9.3 M LiBr, 1: 5 (w/v), 60 °C, 4 h	9 M LiBr, 6 % (w/w), RT, 6 h
Film casting/ treatment	Water vapor annealing, 6 h	70 % (v/v) EtOH immersion, 24 h
Zeta potential at pH = 7 (mV)	–75	–26.4

The zeta potentials and the isoelectric points of the SF solutions prepared in other studies were comparable to our results depending principally on their measured concentrations and temperatures. Lu, Q. et al. (2001) reported the zeta potential of the SF degummed in Na_2CO_3 solution and extracted with the LiBr at pH 5.5 (–7.7 mV) which was similar to our results [56]. The zeta potentials of water vapor annealed and EtOH treated SF films were reported differently. **Table 4.15** presents the zeta potentials of the water vapor annealed and the EtOH treated SF films prepared in other studies. The water vapor annealed SF film exhibited particularly more negative charges than the EtOH treated SF film at neutral pH by ~ 50 mV [12, 26]. This might have due to domination of the free random coil conformations found in the water vapor annealed sample.

4.7 Thermal property

Degummed Thai silk fibers and lyophilized Thai SF extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C were investigated for their thermal properties using differential scanning calorimetry (DSC) and thermogravimetry (TGA). DSC and TGA curves of the degummed silk fibers and the lyophilized SF prepared in this work are shown in **Figure 4.8** and **Figure 4.9**, respectively. The TGA curves were derived using first-order differentiations to determine their decomposition steps **(Appendix G)**. Thermal characteristics of the SF samples were obtained from the DSC and the TGA curves (**Appendix F** and **Appendix J**, respectively). **Table 4.16** presents the thermal characteristics of the SF samples characterized using the DSC and the TGA. **Table 4.17** shows comparisons in decomposition temperatures of the SF prepared in this work and the SF prepared in other studies. The SF samples were additionally characterized at 250 - 350 °C using the TGA with a heating rate at 2.5 °C min^{-1} to increase resolutions of the results (**Appendix H** and **Appendix I**).

The DSC curves of the SF samples prepared in this work were all similar except for the degummed silk fibers. The degummed silk fibers exhibited two large endothermic peaks at 84 °C and 311 ° C which could be assigned to water evaporation and decomposition of the SF. The lyophilized SF had those two endothermic peaks at different temperatures as well as their glass transitions and crystallizations at 178 - 180 °C and 221 - 225 °C, respectively. The Ajisawa-derived SF samples had their water evaporation enthalpy decreased (from –105 - –125 J g^{-1} to –57 - –76 J g^{-1}) with increases in dissolving ratios from 1: 30 (w/v) to 1: 10 (w/v). Decomposition temperatures of the Ajisawa-derived SF (287 - 290 °C) were higher than the LiBr-derived SF (283 - 284 °C). Weight losses of the SF samples analyzed using the TGA with a heating rate at 10 °C min^{-1} were all in three major steps which were results of the water evaporations and two decomposition steps of the SF. First and second decomposition temperatures of the degummed silk fibers (314 °C and 618 °C, respectively) were higher than those of the lyophilized SF (283 - 290 °C and 537 - 613 °C, respectively). Water contents of the 1: 10 (w/v) SF-Ajisawa extractions (A7 - A9) were less than other SF samples by ~ 5 %. The 1: 15 (w/v) SF-Ajisawa extractions (A4 - A6) had their weight losses after the first decompositions increased (from 41 % to 57 %) with increases in dissolution temperatures from 50 °C to 80 °C. Both the DSC and the TGA curves of some SF samples exhibited shoulders ~ 300 °C.

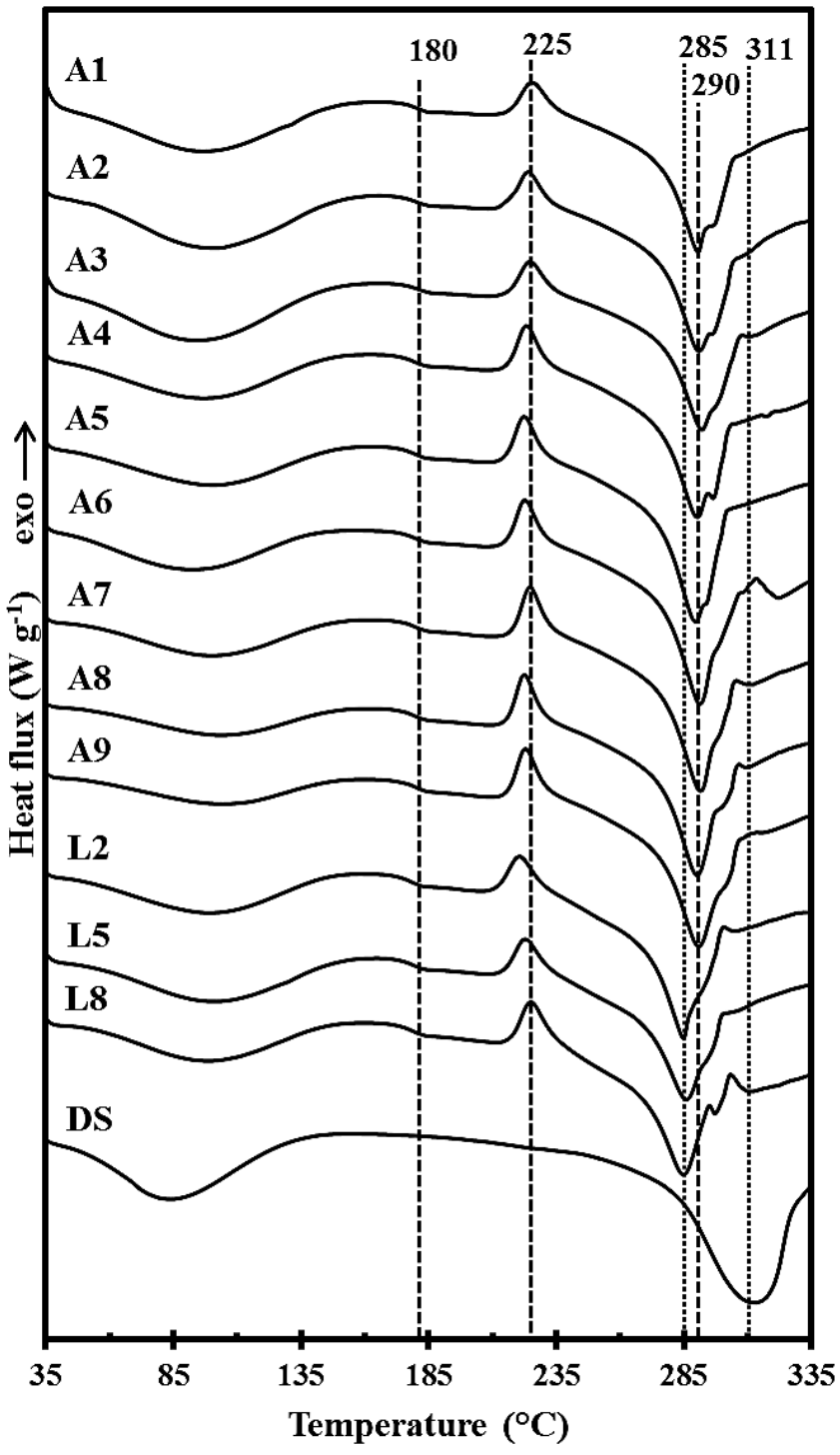

Figure 4. 8 DSC curves of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C with respect to degummed silk fibers (DS)

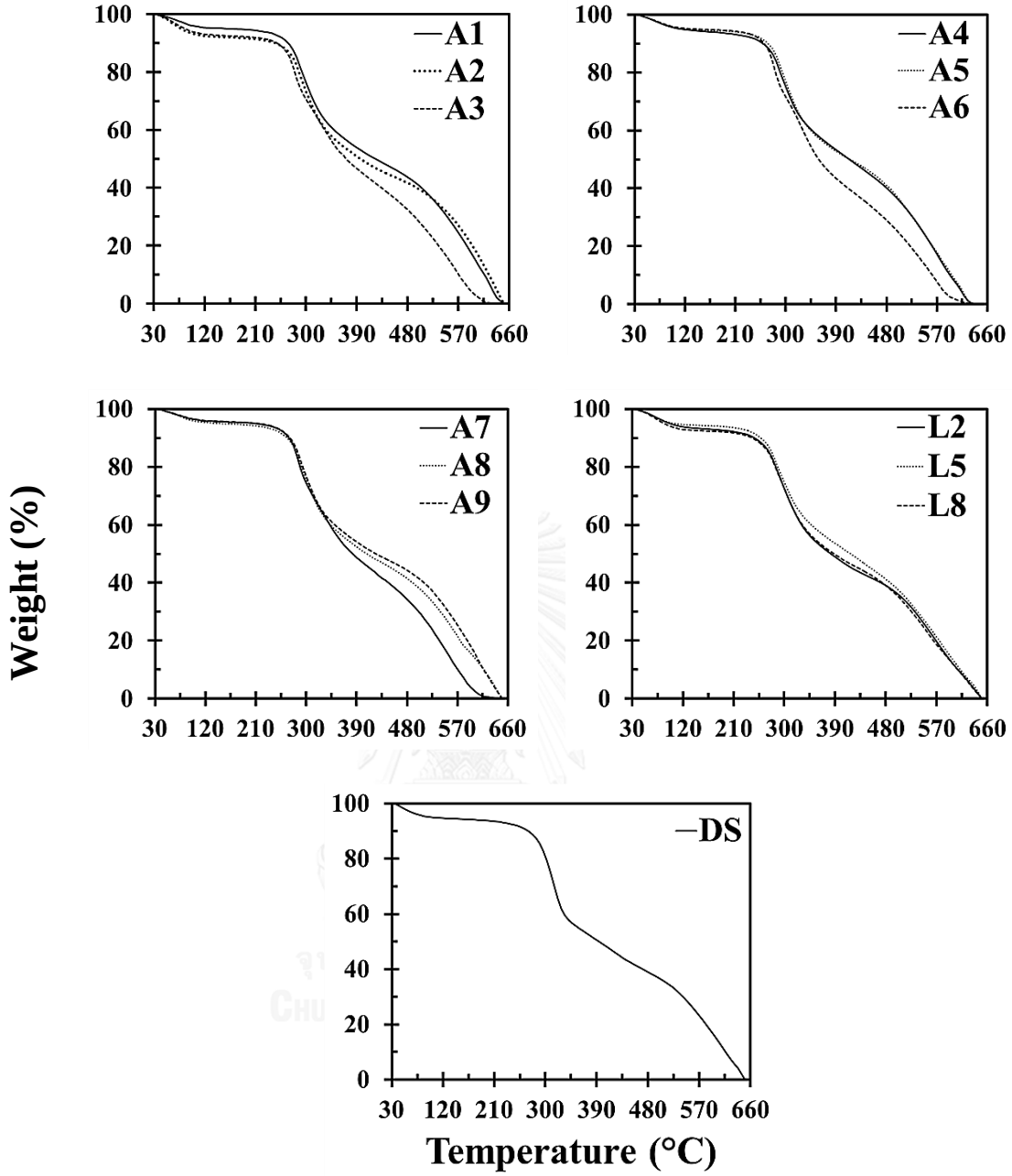

Figure 4. 9 TGA curves of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 ℃ and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 ℃ with respect to degummed silk fibers (DS), The samples were heated in 30 - 650 ℃ with a heating rate at 10 ℃ min^{-1}.

Table 4. 16 Thermal characteristics of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 (w/v), 1: 15 (w/v), and 1: 10 (w/v) at 65 °C with respect to degummed silk fibers (DS) calculated from DSC and TGA curves

Sample code	Differential scanning calorimetry		Thermogravimetry analysis		
	ΔH_{evap} (J g^{-1})	T_d (°C)	Water content (%)	Weight loss after first decomposition (%)	Weight loss after second decomposition (%)
A1	–105	289	5	49	46
A2	–113	289	8	50	42
A3	–125	290	7	53	40
A4	–106	287	6	41	53
A5	–98	287	6	44	50
A6	–86	289	5	57	38
A7	–76	290	4	52	44
A8	–63	288	5	48	47
A9	–57	289	4	49	47
L2	–97	283	7	51	42
L5	–100	284	6	41	53
L8	–83	283	8	47	45
DS	–122	311	6	49	45

ΔH_{evap} refers to enthalpy of water evaporation; and T_d refers to decomposition temperature.

Table 4. 17 Decomposition temperatures of lyophilized silk fibroin extracted with Ajisawa's reagent and 9.3 M LiBr 1: 15 (w/v) at 65 °C (A5 and L5, respectively) with respect to degummed silk fibers (DS) in comparisons with silk fibroin prepared in other studies

		Silk race	Degumming procedure	Extraction method	Fabrication	Decomposition temperature (°C)
This work	**A5**	Nangnoi Sisaket 1 silk cocoons	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles	Ajisawa or 9.3 M LiBr, 1: 15 (w/v), 65 °C	Lyophilized sample	287
	L5				Lyophilized sample	284
	DS			None	None	311
Tsukada, M. et al. (1996) [37]		Japanese silk cocoons	5 g l^{-1} soap 2 g l^{-1} Na_2CO_3 solution, 98 °C, 45 min	None	None	306 - 312 (DSC)
Freddi, G. et al. (1999) [38]		No data	7 g l^{-1} soap solution, 95 °C, 1 h	None	None	310 (DSC)

	Silk race	Degumming procedure	Extraction method	Fabrication	Decomposition temperature (°C)
Freddi, G. et al. (1999) [83]	Posterior silk glands	None	None	As-casted film, 20 °C, 65 % RH	280 (DSC)
Lu, Q. et al. (2010) [54]	Japanese silk cocoons	Boiled in 0.02 M Na_2CO_3 solution, 20 min	9.3 M LiBr, 1: 5 (w/v), 60 °C, 4 h	As-casted film	257 (DSC)
Cilurzo, A. et al. (2011) [45]	Italian DS	None	None	None	300 (DSC)
Hu, X. et al. (2011) [55]	No data	Boiled in 0.02 M Na_2CO_3 solution, 20 min	9.3 M LiBr, 1: 5 (w/v), 60 °C, 4 h	As-casted film	260 (DSC)
			None	None	290 (DSC)
Mhuka, V. et al. (2013) [40]	South African silk cocoons	Boiled in 1.1 g l^{-1} Na_2CO_3 solution, 1 h	None	None	320 (TGA)
Farokhi, M. et al. (2014) [18]	No data	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles	9.3 M LiBr, 1: 5 (w/v), 60 °C, 4 h	Lyophilized sample	281 (DSC)
Mazzi, S. et al. (2014) [84]	Chinese silk cocoons	Boiled in 0.02 M $NaHCO_3$ solution, 2 h	None	None	289 (DSC) and 304 (TGA)
Motta, A. et al. (2014) [20]	Italian silk cocoons[a]	Boiled in 1.1 and 0.4 g l^{-1} Na_2CO_3 solution, 1 h	None	None	313 and 317[a] (DSC)
Callone, E. et al. (2016) [46]	Hybrid Japanese silk cocoons	Boiled in 1.1 and 0.4 g l^{-1} Na_2CO_3 solution, 1 h	9.3 M LiBr, 1: 10 (w/v), 65 °C, 2 h	Lyophilized sample	280 (DSC)
				As-casted film	290 (DSC)
			None	None	311 (DSC)
Lee, J.H. et al. (2016) [25]	Korean silk cocoons	Boiled in 0.6 % (w/v) sodium oleate + 0.4 % (w/v) Na_2CO_3 solution, 1 h	Ajisawa, 1: 20 (w/v), 85 °C, 30 min	As-casted film	276 (DSC)
Zhou, J. et al. (2016) [73]	Chinese silk cocoons	Boiled in 5 g l^{-1} Na_2CO_3 solution, 1 h	Ajisawa, 1: 10 (w/v), 80 °C, 40 min	As-casted film, 37 °C	252 (TGA)
				As-casted film, 60 °C	264 (TGA)

[a]Pure race and polyhybrid silk were studied, respectively.

Molecular structure orientations influenced the thermal characteristics of the SF. Well-oriented, less-oriented, and amorphous SF were reported to have their decomposition temperatures at > 300 °C, 295 - 290 °C, and < 290 °C, respectively [38]. The degummed silk fibers prepared in this work which had the most well-oriented molecular structures (β-sheet/silk II-like contents at 59.1 % and 74.9 %, respectively) exhibited different thermal characteristics from the lyophilized SF. They had their decomposition temperatures comparable with Chinese, Italian, Japanese, and South African silk fibers degummed in Na_2CO_3, $NaHCO_3$, and soap solutions which were reported at 289 - 320 °C [20, 37, 38, 40, 45, 46, 55, 84]. The lyophilized SF samples prepared in this work (random coil/silk I-like contents at 39.0 - 47.3 % and 48.5 - 59.0 %, respectively) had their decomposition temperatures comparable to those of lyophilized hybrid Japanese SF prepared with the Na_2CO_3 degumming procedure and LiBr process which were ~ 280 °C [18, 46]. SF films prepared with different silk races, degumming procedures, and extraction methods in other studies had their decomposition temperatures reported differently at 252 - 290 °C [25, 46, 54, 55, 73, 83]. The SF prepared by Callone, E. et al. (2016) had its decomposition temperatures when it was fabricated into film higher than that of its lyophilized form [46]. The SF film fabricated at high temperature (60 °C) was reported by Zhou, J. et al. (2016) to be more thermally stable than the one casted at low temperature (37 °C) [73]. Hu, X. et al. (2011) found that the decomposition temperatures of the water vapor annealed SF films increased with increases in β-sheet contents as a result of increasing casting temperatures [55]. In this work, the Ajisawa-derived SF had more β-sheet/silk II-like contents than the LiBr-derived SF by 5 %. This contributed more thermal stabilities to the SF extracted with the Ajisawa's reagent. The 1: 10 (w/v) SF-Ajisawa extractions had less water evaporation enthalpies and water contents as a result of high β-sheet/silk II-like contents (19.9 - 22.2 % and 42.9 - 46.0 %, respectively). The water vapor annealed Japanese SF film prepared with the Na_2CO_3 degumming procedure and the LiBr process by Lu, Q. et al. (2010) had no water evaporation due to its highly crystalline structures [54]. Callone, E. et al. (2016) reported that the water evaporation enthalpies of the degummed silk fibers < the water vapor annealed SF films < as-casted SF films < the lyophilized SF as the molecular structure orientations decreased [46]. Multiple degradation steps of the SF were results of its different molecular structure orientations. Less thermally stable molecules such as amino acid residues was reported to be decomposed at 230 °C followed by the SF decompositions at higher temperatures [40, 83]. In this work, the 1: 15 (w/v) SF-Ajisawa extractions had their weight losses at 283 - 290 °C increased with the dissolution temperatures since the random coil/silk I-like contents were increased (from 40.5 % to 46.0 % and from 48.5 % to 57.0 %, respectively). The silk I- and the silk II-like structures were reported to be decomposed at 247 - 252 °C and 257 - 259 °C, respectively [54]. Silk III (3-folded helical structure) was reported to be decomposed at 261 °C [46].

The Thai SF extracted with the Ajisawa's reagent and the LiBr using different dissolution conditions were different only in their molecular structures and thermal characteristics. The LiBr process was more effective in dissolving the SF than the Ajisawa's process. The LiBr-derived SF extracted using 1: 30 - 1: 10 (w/v) dissolving ratios had high random coil and silk I contents at 43.0 - 45.8 % and 55.5 - 57.3 %,

respectively, and low β-sheet and silk II contents at 14.2 - 15.0 % and 39.0 - 40.7 %, respectively. The LiBr-derived SF had their thermal decomposition temperatures (283 - 284 °C) lower than the Ajisawa-derived SF (287 - 290 °C) as a result of less β-sheet/silk II contents. In case of the SF extracted with the Ajisawa's reagent, the dissolving ratios had more effects on those characteristics than the dissolution temperatures. As the dissolving ratios increased from 1: 30 (w/v) to 1: 10 (w/v), the β-sheet and silk II contents of the Ajisawa-derived SF increased from 13.3 - 14.9 % to 19.0 - 22.2 % and from 37.4 - 39.1 % to 42.9 - 46.0 %, respectively, while the random coil and silk I contents of that decreased from 45.3 - 47.3 % to 39.0 - 43.2 % and from 57.2 - 59.0 % to 50.4 - 52.4 %, respectively. The SF extracted with the Ajisawa's reagent using 1: 10 (w/v) dissolving ratios had their water evaporation enthalpies and water contents (–57 - –76 J g^{-1} and ~ 5 %, respectively) lower than others (–86 - –125 J g^{-1} and 5 - 8 %, respectively) as a result of high β-sheet/silk II contents. As the dissolution temperatures increased from 50 °C to 80 °C, the β-sheet and silk II contents of the Ajisawa-derived SF decreased slightly from 14.9 - 22.2 % to 13.3 - 19.0 % and from 39.1 - 46.0 % to 37.8 - 42.9 %, respectively, while the random coil and silk I contents of that increased slightly from 39.0 - 45.3 % to 43.2 - 46.9 % and from 50.4 - 57.2 % to 52.4 - 58.7 %, respectively. The SF extracted with the Ajisawa's reagent using the 1: 15 (w/v) dissolving ratios had their weight losses after first decomposition increased from 41 % to 57 % as their random coil and silk I contents increased from 40.5 - 46.0 % to 48.5 - 57.0 %, respectively.

4.8 Viscosity

As-prepared Thai SF solutions extracted with Ajisawa's reagent 1: 30 - 1: 15 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C were diluted to yield the same concentration at 8 mg ml^{-1}. They were characterized for their viscosities by a vibrational viscometer at 26 °C. The 1: 10 (w/v) SF-Ajisawa solutions (A7 - A9) were not studied since they had cloudy appearances and less homogeneity. **Table 4.18** presents dynamic viscosities of the studied SF solutions. **Table 4.19** shows comparisons in the dynamic viscosities of the SF solutions studied in this work and the SF solutions prepared in other studies.

The Ajisawa- and the LiBr-derived SF solutions prepared in this work had their dynamic viscosities at 1.22 ± 0.11 - 1.39 ± 0.10 mPa·s with no significant differences. The dynamic viscosities of the SF solutions prepared in other studies were reported differently at 1 - 1000 mPa·s depending on mediums, concentrations, and temperatures [41, 52, 68, 77]. Matsumoto, M. et al. (2008) reported that the dynamic viscosity of the aqueous SF solution prepared with Na_2CO_3 degumming procedure and LiBr process was at 1 mPa·s which was contributed to our results [52]. The dynamic viscosity of the LiBr-derived Korean SF solution which had its molecular weight of 450 kilo Daltons was at 915 mPa·s while those of the Ajisawa-derived SF solutions decreased (300 mPa·s, 150 mPa·s, and 50 mPa·s) with their molecular weights (from 450 kilo Daltons to 150 kilo Daltons) as dissolution times increased (3 min, 30 min, and 180 min, respectively) [68]. However, the SF samples prepared in this work had no differences in their molecular weights and their zeta potentials in aqueous solutions at pH ~ 5.5 were almost similar. As a result, they are not different in their viscosity properties.

Table 4. 18 Dynamic viscosities of 8 mg ml^{-1} aqueous silk fibroin solutions extracted with Ajisawa's reagent 1: 30 - 1: 15 (w/v) at 50 - 80 ℃ and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 ℃ at 26 ℃

Sample code	Dynamic viscosity (mPa·s)
A1	1.22 ± 0.11
A2	1.24 ± 0.05
A3	1.23 ± 0.12
A4	1.26 ± 0.06
A5	1.24 ± 0.07
A6	1.25 ± 0.04
L2	1.32 ± 0.11
L5	1.39 ± 0.10
L8	1.29 ± 0.11

Data are reported in average ± standard deviation (n = 4).

Table 4. 19 Dynamic viscosities of aqueous silk fibroin solutions extracted with Ajisawa's reagent and 9.3 M LiBr 1: 15 (w/v) at 65 ℃ (A5 and L5, respectively) in comparisons with silk fibroin prepared in other studies

	This work		**Chen, X. et al. (2001) [77]**	**Matsumoto, M. et al. (2008) [52]**	**Cho, H.J. et al. (2012) [68]**	**Chung, D.E. et al. (2015) [41]**
	A5	**L5**				
Silk race	Nangnoi Sisaket 1 silk cocoons		Chinese silk cocoons	Bave silk	Korean silk cocoons	Korean silk cocoons[c]
Degumming procedure	Boiled in 0.02 M Na_2CO_3 solution, 20 min, 2 cycles		Boiled in 0.5 % (w/w) $NaHCO_3$ solution, 30 min, 2 cycles	Boiled in 0.02 M Na_2CO_3 solution, 1 h	Boiled in 0.3 % (w/v) sodium oleate + 0.2 % (w/v) Na_2CO_3 solution, 1 h	Boiled in 0.3 % (w/v) sodium oleate + 0.2 % (w/v) Na_2CO_3 solution, 1 h
Extraction method	Ajisawa and 9.3 M LiBr, 1: 15 (w/v), 65 ℃		Ajisawa, 2 % (w/w), 80 ℃	9.3 M LiBr, 1: 5 (w/v), 60 ℃, 4 h	Ajisawa, 1: 20 (w/v), 85 ℃, 3 min, 30 min, and 180 min and 9.3 M LiBr, 1: 20 (w/v), 25 ℃, 6 h	Ajisawa, 1: 20 (w/v), 85 ℃, 3 min
Medium	Water		Ajisawa	Water	Formic acid[b]	Formic acid[b]

	This work		Chen, X. et al. (2001) [77]	Matsumoto, M. et al. (2008) [52]	Cho, H.J. et al. (2012) [68]	Chung, D.E. et al. (2015) [41]
	A5	L5				
Concentration % (w/w)	1		2	1	14	10
Temperature (°C)	26		25.0	25	20	25
Dynamic viscosity (mPa·s)	1.24	1.39	70[a]	1[a]	300 (3 min), 150 (30 min) and 50 (180 min)[a] (Ajisawa) and 915[a] (LiBr)	200 - 1000[a]

[a]These samples were characterized by a rotational viscometer. [b]SF solutions were dried then re-dissolved again in 98 % (v/v) formic acid. [c]Different races of silk cocoons were studied.

4.9 Contact angle

Ethanol treated and water vapor annealed Thai SF films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively) were studied for their surface properties by a contact angle meter at 25 °C. Water and ethylene glycol contact angles on the SF films are shown in **Figure 4.10**. The surface properties of the SF films were calculated by the *Owen–Wendt* equation. **Table 4.20** presents surface energies and polarities of the SF films fabricated in this work. **Table 4.21** presents the surface properties of the SF films fabricated in other studies.

The EtOH treated SF films (A5-EtOH and L5-EtOH) had their water and ethylene glycol contact angles similar to each other's (58.6 ± 2.0 - 59.0 ± 0.9 ° and 25.6 ± 3.1 - 28.1 ± 1.3 °, respectively). The water vapor annealed SF films (A5-H2O and L5-H2O) had their ethylene glycol contact angles similarly (45.2 °) but significantly higher than those of the EtOH treated samples. The A5-H2O had its water contact angle (58.1 ± 1.8 °) similar to those of the EtOH treated samples. The L5-H2O had its water contact angle (51.4 ± 1.9 °) significantly less than other SF samples. All SF films had similar total surface energies (43.4 - 45.1 mN m^{-1}) although the surface polarities of the water vapor annealed samples (46.3 - 51.1 %) were considerably higher than those of the EtOH treated samples (13.1 - 16.9 %).

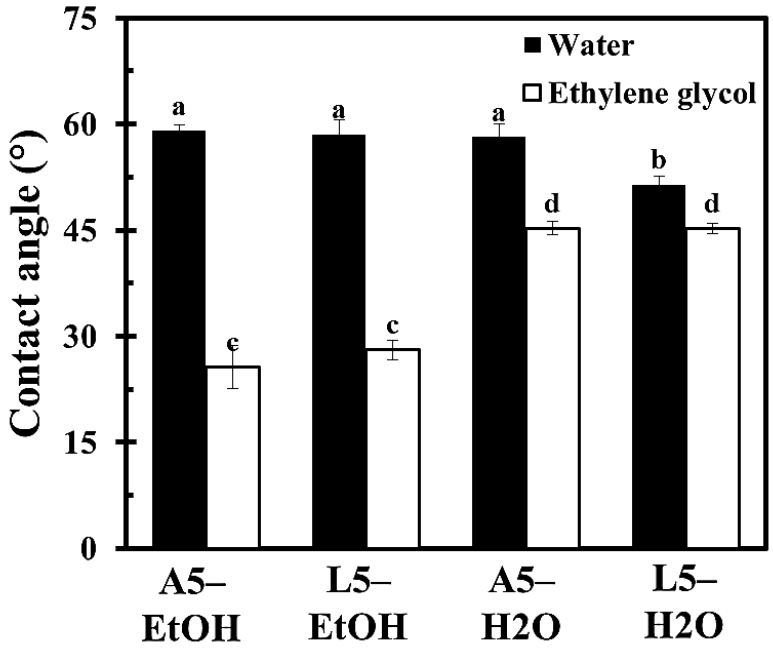

Figure 4. 10 Water and ethylene glycol contact angles on ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively), Liquid contact angles were studied at 25 °C. [a-d]Data are reported in average ± standard deviation (n = 4). These values are significantly different (P<0.05).

Table 4. 20 Surfaces energies and polarities of ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively)

	γ_{SF}^{d} (mN m^{-1})	γ_{SF}^{p} (mN m^{-1})	γ_{SF} (mN m^{-1})	Surface polarity (%)
A5-EtOH	36.4	7.4	43.8	16.9
L5-EtOH	39.2	5.9	45.1	13.1
A5-H2O	21.2	22.4	43.8	51.1
L5-H2O	23.3	20.1	43.4	46.3

γ_{SF}^{d} refers to surface energy due to dispersive component of SF film; γ_{SF}^{p} refers to surface energy due to polar component of SF film; and γ_{SF} refers to total surface free energy of SF film.

Surface wettability of the SF films was influenced by factors such as surface hydrophilicity and surface roughness [70, 122]. In this work, molecular structures were the main factor influencing the surface properties of the SF. Silk I-like contents of the water vapor annealed SF films (30.5 - 39.9 %) were more than those of the EtOH treated SF films (23.0 - 27.0 %) while silk II-like contents of the water vapor annealed samples (53.7 - 57.9 %) were less than those of the EtOH treated samples

(62.2 - 66.6 %). The EtOH treatment additionally dehydrated the SF films which resulted further in increases in their surface stiffness [26]. As a result, the A5-H2O and the L5-H2O were more hydrophilic than the A5-EtOH and the L5-EtOH. The L5-H2O had almost 10 % more helical structure content than other samples. This contributed more hydrophilic characteristics to the L5-H2O and resulted in a surface with the least water contact angle and the highest surface polarity. The water contact angles of the water vapor annealed and EtOH treated SF films (62.6 - 82 °) were generally higher than those of as-casted films (44 - 68 °) as crystallinity increased [1, 12, 14, 25, 26, 61, 78]. Tretinnikov, O.N. et al. (2001) reported the total surface energy of the as-casted SF film (39.5 - 39.7 mN m^{-1}) which was similar to our results [78]. Sionkowska, A. et al. (2013) found that the surface polarity of the SF film was only 10.3 % since it was characteristically hydrophobic [70]. Their results were similar to those of our EtOH treated samples.

Table 4. 21 Surface properties of silk fibroin prepared in other studies

	Film casting/ treatment	**Water contact angle (°)**	**Surface energy (mN m^{-1})**
Tretinnikov, O.N. et al. (2001) [78]	As-casted film, 22 °C	68	γ_{SF} = 39.5 - 39.7
Jin, H.J. et al. (2005) [1]	As-casted film	51.4	No data
	Water vapor annealed film, 24 h	62.6	No data
Seib, F.Ph. et al. (2012) [12]	Water vapor annealed film, 25 °C, 6 h	82	No data
Seib, F.Ph. et al. (2012) [12]	70 % (v/v) EtOH treated film, 6 h	64	No data
Bhattacharjee, M. et al. (2013) [14]	As-casted film, 40 °C	67.3	No data
Foss, C. et al. (2013) [61]	Water vapor annealed film, 24 h	70	No data
Sionkowska, A. et al. (2013) [70]	As-casted film	No data	γ_{SF}^{d} = 25.02 γ_{SF}^{p} = 2.87
Lee, J.H. et al. (2016) [25]	As-casted film, 25 °C	44	No data
Terada, D. et al. (2016) [26]	70 % (v/v) EtOH treated film, 24 h	48	No data

γ_{SF}^{d} refers to surface energy due to dispersive component of SF film; γ_{SF}^{p} refers to surface energy due to polar component of SF film; and γ_{SF} refers to total surface free energy of SF film.

4.10 *In vitro* NIH-3T3 mouse embryonic fibroblast cell line-silk fibroin film biocompatibility test

NIH-3T3 mouse embryonic fibroblasts were cultured on EtOH treated and water vapor annealed Thai SF films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively) for *in vitro* biocompatibility test in terms of cell attachments and proliferations. The NIH-3T3 cells were seeded onto the SF films, glass substrates, and empty wells in 24-well tissue culture plate (TCP) at 2 x 10^4 cells per well (seeding density: 1 x 10^4 cells cm^{-2}). Cell spreading was analyzed at 6 h (**Appendix K**) while cell attachments and proliferations were studied at 6 h, 1 day, 2 day, 3 day, 4 day, 5 day, and 6 day using the MTT assay. Growth curves of the NIH-3T3 cells cultured on each substrate are shown in **Figure 4.11**. The NIH-3T3 cells cultured on each substrate at each specific time point are shown in **Figure 4.12-4.18**. A standard curve plotting absorbance against known numbers of cells was done (**Appendix L**). Numbers of viable cells at each time point were considered from logarithmic scale of the growth curve (**Appendix M**). Specific growth rates (μ) and population doubling times (PDT) of the cells cultured on the SF films were calculated.

Table 4.22 summarizes attachment and proliferation characteristics of the NIH-3T3 cells cultured on the SF films, the TCP, and the glass substrates.

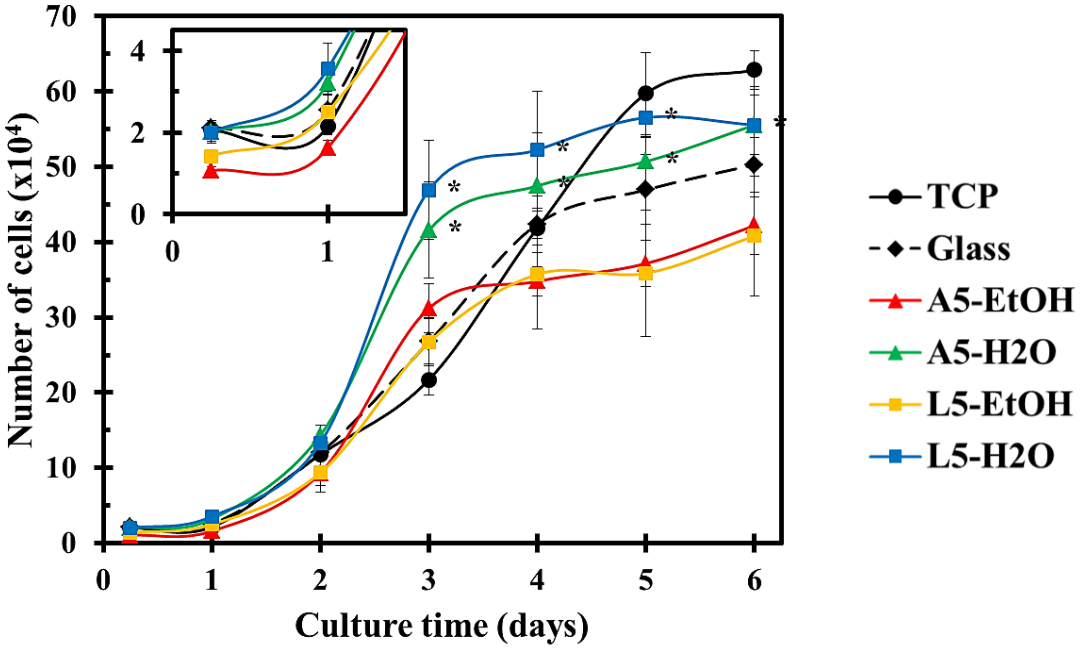

Figure 4. 11 Proliferations of NIH-3T3 mouse embryonic fibroblasts cultured on ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively) with respect to proliferations the cells cultured on tissue culture plate (TCP) and glass substrate, *These numbers were of the cells proliferated on detached films.

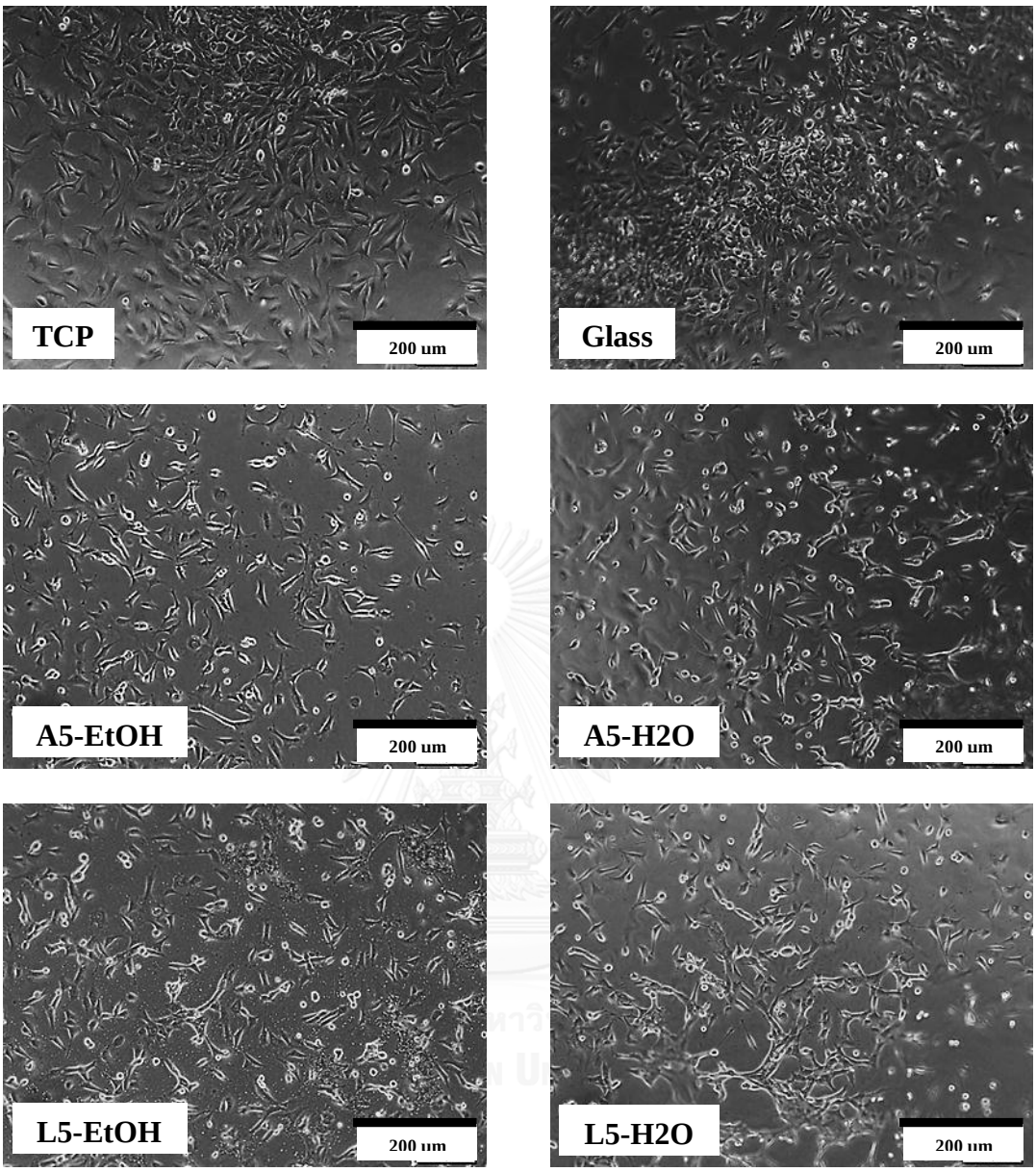

Figure 4. 12 NIH-3T3 mouse embryonic fibroblasts cultured on ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively) with respect to the cells cultured on tissue culture plate (TCP) and glass substrate for 6 h (magnification = 10x)

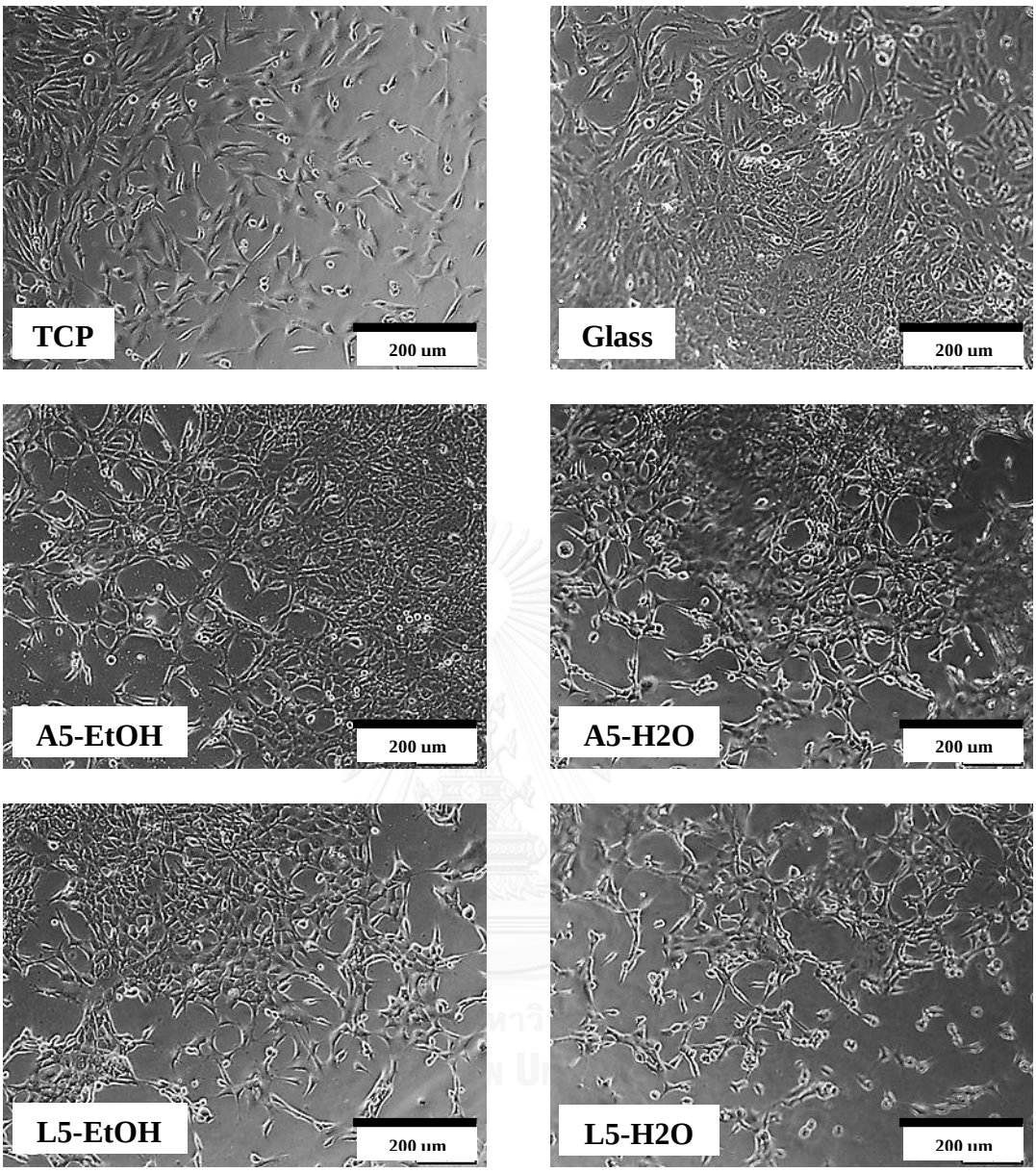

Figure 4. 13 NIH-3T3 mouse embryonic fibroblasts cultured on ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively) with respect to the cells cultured on tissue culture plate (TCP) and glass substrate for 1 day (magnification = 10x)

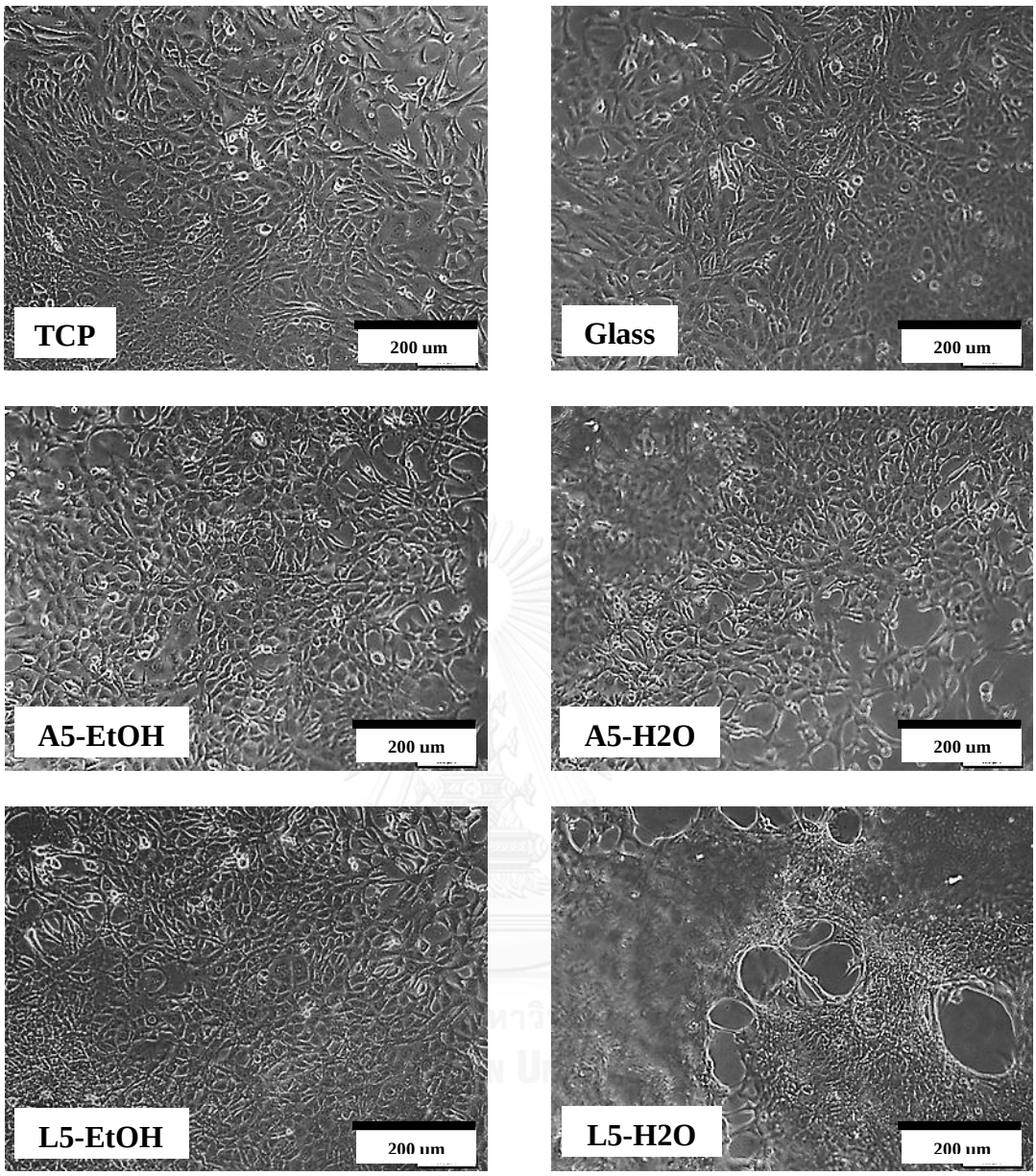

Figure 4. 14 NIH-3T3 mouse embryonic fibroblasts cultured on ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively) with respect to the cells cultured on tissue culture plate (TCP) and glass substrate for 2 days (magnification = 10x)

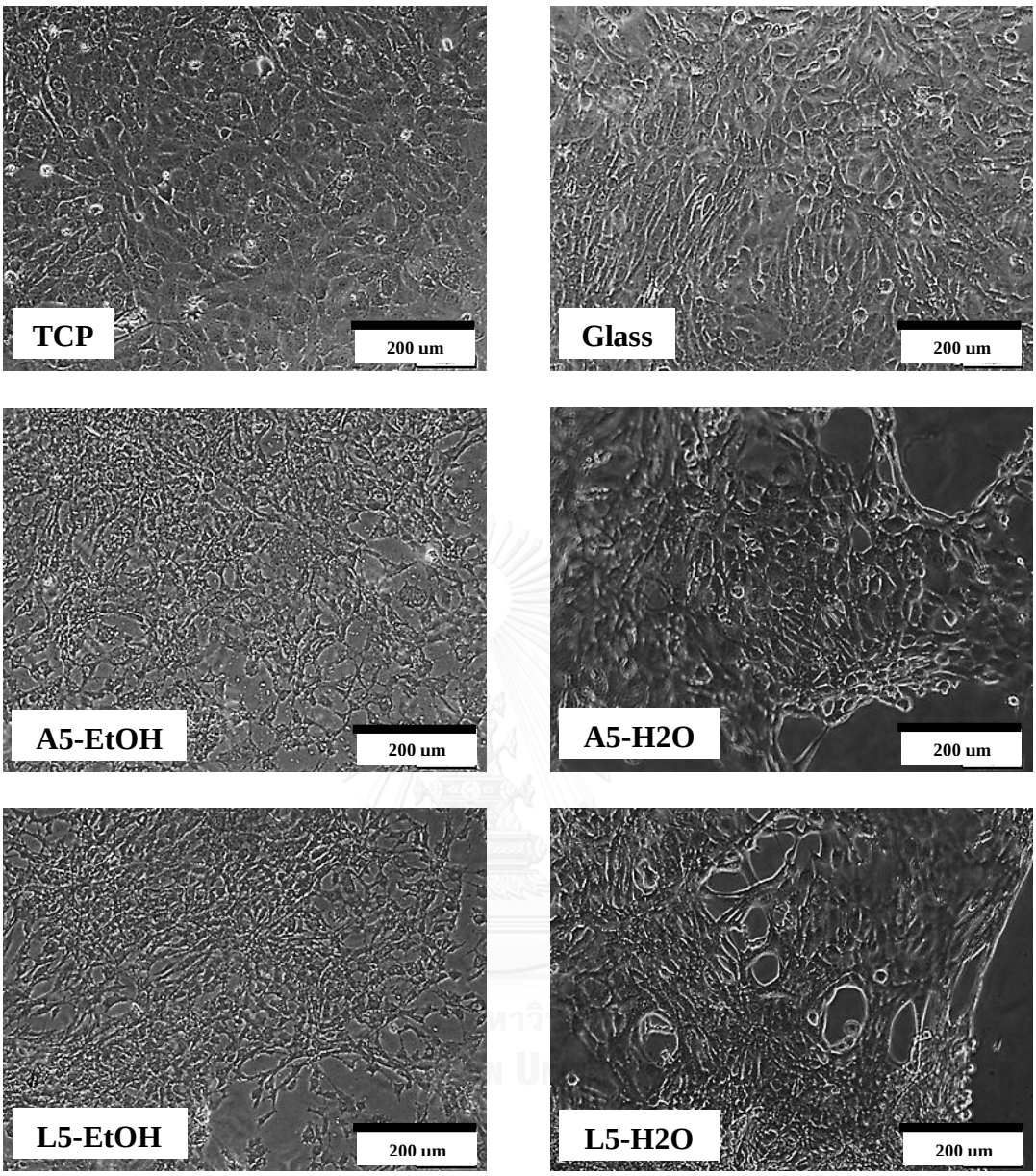

Figure 4. 15 NIH-3T3 mouse embryonic fibroblasts cultured on ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively) with respect to the cells cultured on tissue culture plate (TCP) and glass substrate for 3 days (magnification = 10x)

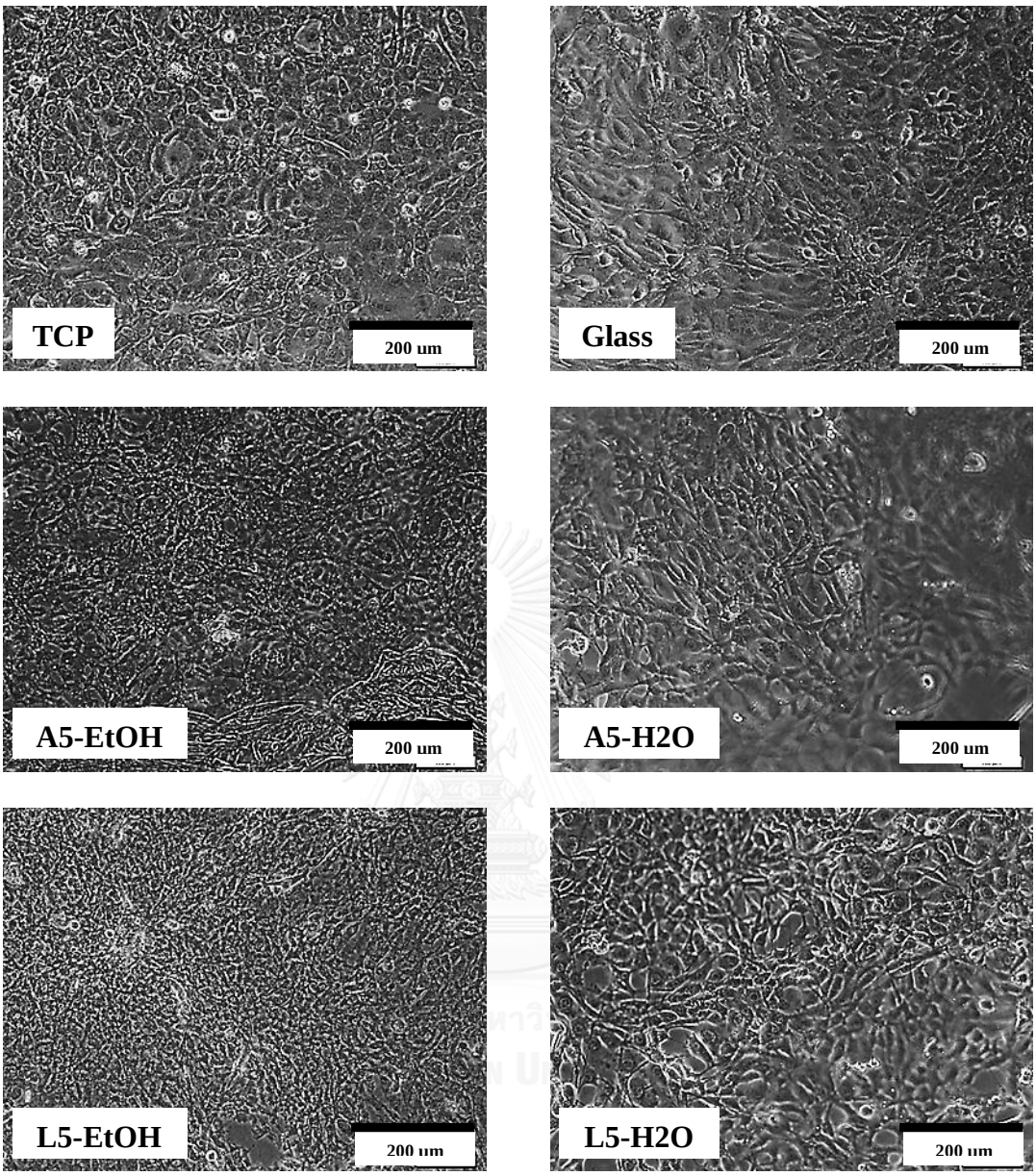

Figure 4. 16 NIH-3T3 mouse embryonic fibroblasts cultured on ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively) with respect to the cells cultured on tissue culture plate (TCP) and glass substrate for 4 days (magnification = 10x)

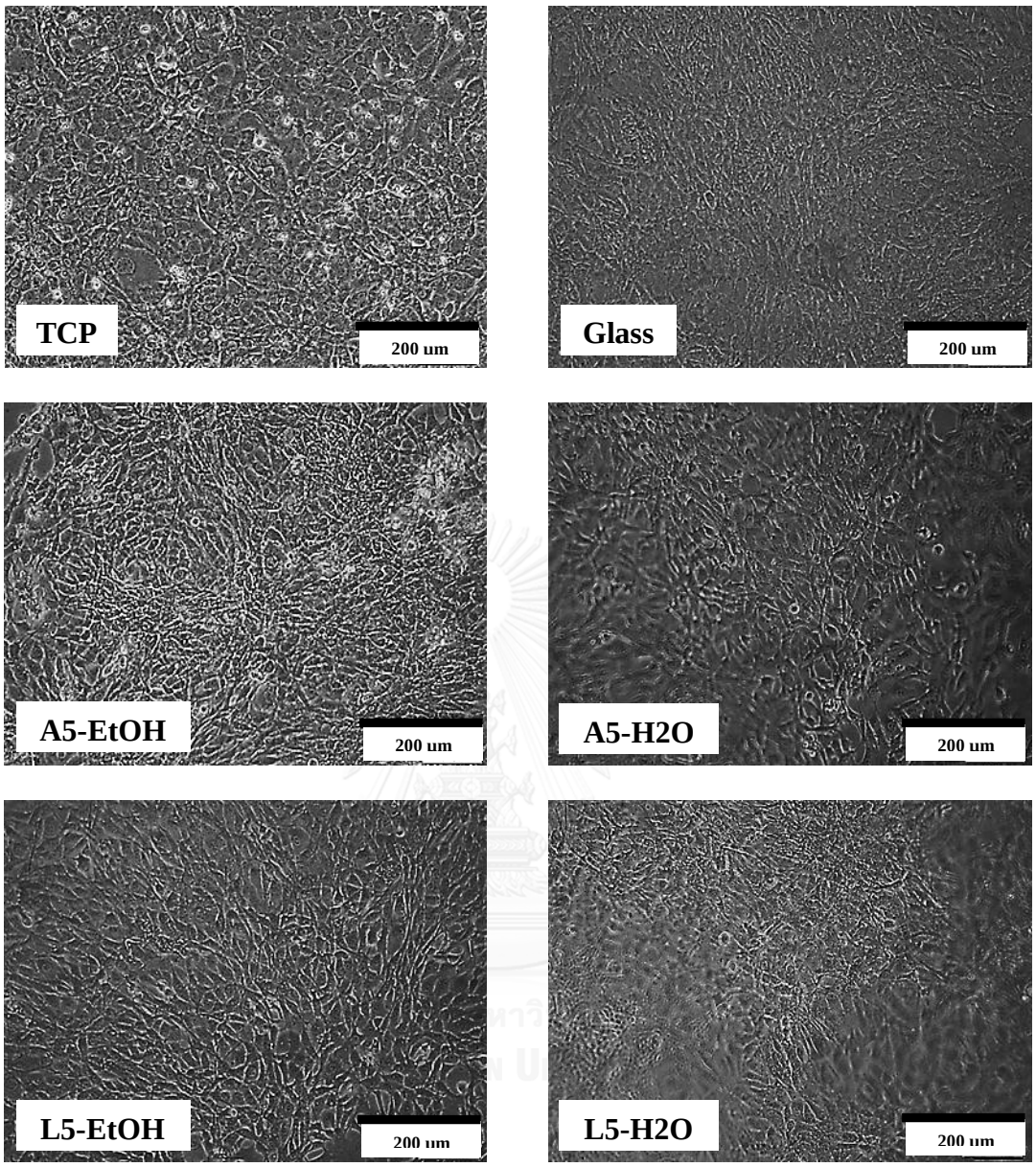

Figure 4. 17 NIH-3T3 mouse embryonic fibroblasts cultured on ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively) with respect to the cells cultured on tissue culture plate (TCP) and glass substrate for 5 days (magnification = 10x)

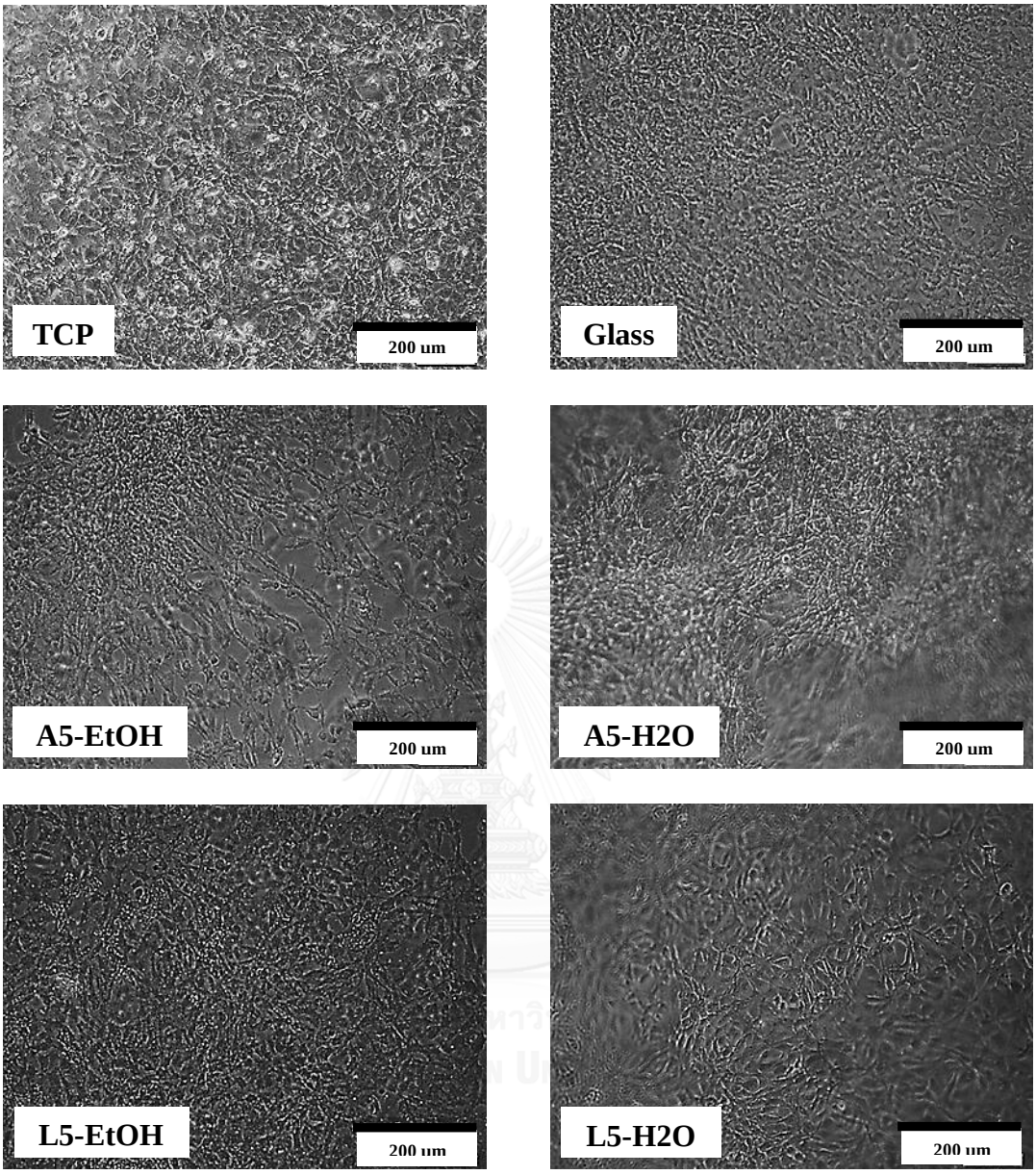

Figure 4. 18 NIH-3T3 mouse embryonic fibroblasts cultured on ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively) with respect to the cells cultured on tissue culture plate (TCP) and glass substrate for 6 days (magnification = 10x)

Table 4. 22 Attachment and proliferation characteristics of NIH-3T3 mouse embryonic fibroblasts cultured on ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively) with respect to proliferations the cells cultured on tissue culture plate (TCP) and glass substrate

	Cell attachment (%)	**Cell spreading (μm^2)**	**Lag time (h)**	**μ (10^{-2} h^{-1})**	**PDT (h)**	**Maximum density (10^4 cells cm^{-2})**
TCP	102.5 ± 7.4^{a}	743.6 ± 362.9^{c}	24	7.08	10.8	31.4 ± 2.6^{e}
Glass	105.2 ± 8.8^{a}	559.1 ± 236.1^{c}	24	6.47	10.7	25.1 ± 3.6^{g}
A5-EtOH	53.1 ± 10.0^{b}	401.5 ± 206.9^{d}	24	6.17	11.2	21.1 ± 3.8^{f}
A5-H2O	107.7 ± 11.4^{a}	445.8 ± 185.4^{d}	24	5.33	13.0	$27.8 \pm 4.0^{e,g}$
L5-EtOH	$71.0 \pm 5.4^{a,b}$	401.7 ± 176.3^{d}	24	4.93	14.0	20.4 ± 8.0^{f}
L5-H2O	99.8 ± 12.6^{a}	410.7 ± 173.3^{d}	24	5.37	12.9	$27.8 \pm 5.0^{e,g}$

μ refers to specific growth rate while PDT refers to population doubling time. All data are reported in average ± standard deviation (n = 4). Growth kinetics was calculated from day 1 to day 2 in case of cells cultured on TCP and glass and from day 1 to day 3 in case of the cells cultured on SF films. ^{a,b}Data were collected at 6 h. ^{c,d}Data were obtained from 50 cells in each group. $^{e-f}$Data were collected after 6 days of cell culture. In case of the A5-H2O and the L5-H2O, numbers of cells on day 2 used to calculate their μ and PDT, and their maximum densities were of the cells cultured on detached films (highlightened in gray). All superscript letters indicate significant differences ($P<0.05$).

The NIH-3T3 cells attached onto the TCP more uniformly than other substrates. The TCP and the glass substrates had significantly more cell spreading areas than the SF films (559.1 ± 236.1 - 743.6 ± 362.9 μm^2 and 401.5 ± 206.9 - 445.8 ± 185.4 μm^2, respectively). Percentages of cell attachment on the water vapor annealed SF films, the TCP, and the glass substrates were similar (99.8 ± 12.6 - 107.7 ± 11.4 %) while that of the A5-EtOH was significantly lower (53.1 ± 10.0 %). The L5-H2O was found to be detached after 48 h while detachment of the A5-H2O was observed after 72 h. The water vapor annealed SF films had their viable cells significantly higher than those of other substrates at day 3. The NIH-3T3 cells cultured on the SF films, the glass substrates, and the TCP reached their stationary phases at day 4, day 5, and day 6, respectively. The cells cultured on the SF films had their μ (5.50 - 6.30 x 10^{-2} h^{-1}) and PDT (11.1 - 12.6 h) similar to those of the TCP and the glass substrates (6.47 - 7.08 x 10^{-2} h^{-1} and 10.7 - 10.8). The TCP had significantly the highest maximum density of cells (31.4 ± 2.6 x 10^4 cells cm^{-2}). The EtOH treated SF films had their cell maximum densities (20.4 ± 8.0 - 21.1 ± 3.8 x 10^4 cells cm^{-2}) significantly lower than those of the TCP and the glass substrates (25.1 ± 3.6 x 10^4 cells cm^{-2}).

The *in vitro* biocompatibilities of the SF against mammalian cells were demonstrated in many studies [1-26]. Factors such as surface polarities and surface stiffness of the SF films influenced proliferations and differentiations of the 3T3 cells

[4, 9, 22, 26, 41]. Jones, J.L. et al. (2009) reported that the water vapor annealed films fabricated from LiBr-derived Italian SF had better results in osteoblast/osteoclast proliferations than chitosan, methanol treated SF, and poly (L-lactic acid) films [2]. Miyamoto, S. et al. (2013) reported that the EtOH treated SF films promoted MC3T3 osteoblast differentiations better than films made of bovine serum albumin [17]. The TCP (plasma treated-polystyrene) was known to promote the attachments of anchoring cells due to its excellent surface stiffness with highly negative charges. Hirata, M. et al. (2010) reported that the 3T3 cells cultured on the TCP spread uniformly with their actin stress fibers stretched while those cultured on the LiBr-derived Japanese SF films were round with dense actin fibers at 2 h [4].

Although Terada, M. et al. (2016) found that the EtOH treated films fabricated from LiBr-derived Japanese SF had the 3T3 cells attached to their surfaces comparable to the glass substrates at 24 h due to its high surface stiffness [26], other studies reported these biocompatibility results differently. The water vapor annealed SF films were reported to be more biocompatible than the EtOH treated samples [1, 2, 12, 14, 18, 21, 55]. Hu, X. et al. (2010) reported that the water vapor annealed films fabricated from LiBr-derived SF which had less β-sheet crystallinity supported more human MSCs attachments and proliferations than the EtOH treated samples [55]. Bhattacharjee, M. et al. (2013) reported that the SF films which had silk I dominated structures had less immunogenic responses than the three dimensional SF scaffold with high silk II contents [14]. Bai, Sh. Et al. (2015) reported that the SF scaffold with high β-sheet content was less biocompatible with rat MSCs [21]. Wadbua, P. et al. (2010) found that light-chain scaffolds fabricated from Ajisawa-derived Thai SF which were hydrophilic had more NIH-3T3 cell adhesions than heavy-chain ones [9]. In this work, the cell attachments and cell spreading areas depended on molecular structures of the SF films which were influenced mostly by SF film treatments (water vapor annealing and EtOH immersion). The A5-H2O and the L5-H2O had their silk I-like content (30.5 - 39.9 %) higher than those of the EtOH treated SF films (23.0 - 27.0 %). This contributed more hydrophilic characteristics to the water vapor annealed SF films. They had more surface polarities (46.3 - 51.1 %) than the EtOH treated samples (13.1 - 16.9 %). Zeta potentials at pH 7 of the water vapor annealed films fabricated from LiBr-derived SF (–75 mV) reported by Seib, F.Ph. et al. (2012) was highly negative than that of the EtOH treated films fabricated from LiBr-derived Japanese SF (–26.4 mV) reported by Terada, D. et al. (2016).

Detachments of the water vapor annealed SF films (possibly caused by dissolutions or degradations) were influenced by their molecular structures which further resulted in different cell responses. The L5-H2O which had almost 10 % more helical structure content than other SF films fabricated in this work was detached after 2 days. Park, S.H. et al. (2010) found that degradations of the EtOH treated LiBr-derived SF scaffolds corresponded to slow osteogenesis rate of human MSCs [8]. Jin, H.J. et al. (2005) reported that the water vapor annealed LiBr-derived Japanese SF films which had less β-sheet characteristics lost 50 % of their masses in Protease XIV incubation at 37 °C for 2 - 3 days [1]. Rajkhowa, R. et al. (2011) reported that the EtOH treated Indian SF films were half-mass degraded in the Protease XIV incubation at 37 °C after 24 days [82]. Degradation products from the water vapor

annealed SF films might have induced the SF's proliferations, especially, at earlier phases. Huang, G. et al. (2010) and Jung, S.R. et al. (2013) reported activities of low molecular weight fractions of Korean SF that induced proliferations and differentiations of 3T3-L1, C3H10T1/2, and M2-10B4 cells [5, 16]. The NIH-3T3 cells cultured on the SF films fabricated in this work had narrow spreading areas and their attachments on the EtOH treated films were less than others. However, they had comparable proliferation kinetics with to the cells cultured on the TCP and the glass substrates. Our results suggest that the water vapor annealed and the EtOH treated films fabricated from the Thai SF were biocompatible with mammalian cells.

CHAPTER V
CONCLUSION

Thai silk fibroin (SF) was prepared from Thai domestic silk cocoons, *Bombyx mori*, Nangnoi Sisaket 1. Degummed Thai silk fibers were extracted systematically with calcium chloride-ethanol (Ajisawa's reagent) and 9.3 M lithium bromide (LiBr) using well-described dissolution conditions, 1: 30 - 1: 10 (w/v) dissolving ratios at 50 - 80 °C. Yields of Thai SF extractions were at 63 ± 9 - 74 ± 8 %. The Thai SF extracted with the Ajisawa's reagent and the LiBr in different conditions were similar in their amino acid compositions and molecular weights. They had glycine, alanine, serine, tyrosine, and valine compositions at 46.6 - 48.3 mol %, 29.2 - 32.1 mol %, 4.9 - 8.2 mol %, 4.5 - 5.1 mol % and 2.3 - 2.6 mol %, respectively. Weight average and number average molecular weights of the Thai SF analyzed using size exclusion chromatography were at 336 - 340 kilo Daltons and 161 - 167 kilo Daltons, respectively, with narrow polydispersive indexes of 2.05 - 2.09. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that heavy-chain SF was degraded to ~ 150 - 100 kilo Daltons while light-chain was preserved at 25 kilo Daltons. The degummed Thai silk fibers had the highest β-sheet and silk II contents at 59.1 and 74.9 %, respectively. The Thai SF extracted with the Ajisawa's reagent had their β-sheet and silk II contents decreased with the dissolving ratios while the β-sheet and silk II contents decreased with increases in dissolution temperatures. The β-sheet and silk II contents of the Thai SF extracted with the LiBr were similar to those extracted with the Ajisawa's reagent at 1: 30 (w/v) dissolving ratio.

Dynamic viscosities of Ajisawa- and LiBr-derived Thai SF solutions did not changed with the extracting conditions used in this work. The Ajisawa-derived Thai SF exhibited almost similar zeta potentials in aqueous solutions at pH 5.5 and 7.5 to that extracted with the LiBr. Thermal decomposition temperatures of the Ajisawa-derived Thai SF were at 287 - 290 °C which was slightly higher than 283 - 284 °C of the LiBr extractions. The Ajisawa-derived Thai SF had their weight losses, water evaporation enthalpies, and water contents increased with random coil and silk I contents as the dissolving ratios decreased and the dissolution temperatures increased.

The β-sheet contents of the Thai SF films increased in the following order, as-casted films (33.8 - 35.0 %) < water vapor annealed film (38.9 - 40.0 %) < ethanol treated film (42.8 - 46.7 %). All SF films had similar total surface energies. The water vapor annealed samples had more surface polarities than the ethanol treated samples. The water vapor annealed film fabricated from the LiBr-derived SF had ~ 10 % more α-helix than other films. It had the least water contact angle compared to others.

Early cell attachments of NIH-3T3 mouse fibroblasts cultured on the water vapor annealed films were higher than those cultured on the ethanol treated films. Both of them had similar specific growth rates and population doubling times to the cells cultured on tissue culture plates and glass substrates. The water vapor annealed

film fabricated from the LiBr-derived SF which had extra helical structures was detached after 48 h while that fabricated from the Ajisawa's reagent was detached after 72 h.

The Thai SF extracted with the Ajisawa's reagent and the LiBr using different dissolving ratios and dissolution temperatures were different in their molecular structures which affected their thermal characteristics. Beta-sheet regeneration processes, water vapor annealing and ethanol immersion additionally affected molecular structures and hydrophilicities of the SF films which further influenced cellular attachments and proliferations. Our results suggest that the Thai SF extracted with the Ajisawa's reagent using 1: 30 - 1: 15 (w/v) dissolving ratios had comparable physico-chemical characteristics to the SF extracted with the LiBr. The Ajisawa's process is economical and environmental friendly. However, the LiBr process is required for the extractions of high concentrated SF solutions. The SF solutions extracted with the Ajisawa's process using dissolving ratios > 1: 15 (w/v) were less homogeneous which limit their shelf-lives and usages for further applications.

Recommendations for future work

1) The SF has high extents in hydrophobic amino acids which tend to assemble into higher order structures in aqueous solutions. It might have assembled into some highly ordered structures including the β-sheet and interrupted mass separation during sodium dodecyl sulfate-gel electrophoresis which resulted in smeared bands of the heavy-chains.

2) Intrinsic viscosities of the SF are characteristics relating to the SF's molecular weights. Viscosity average molecular weights of the SF could be additionally investigated.

3) The water vapor annealed SF films started to detached after 48 h of cell culture which discontinued cell proliferations on them. Denser water vapor annealed SF films are required to be fabricated in order to study cell proliferations for extended periods.

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APPENDIX

Appendix A

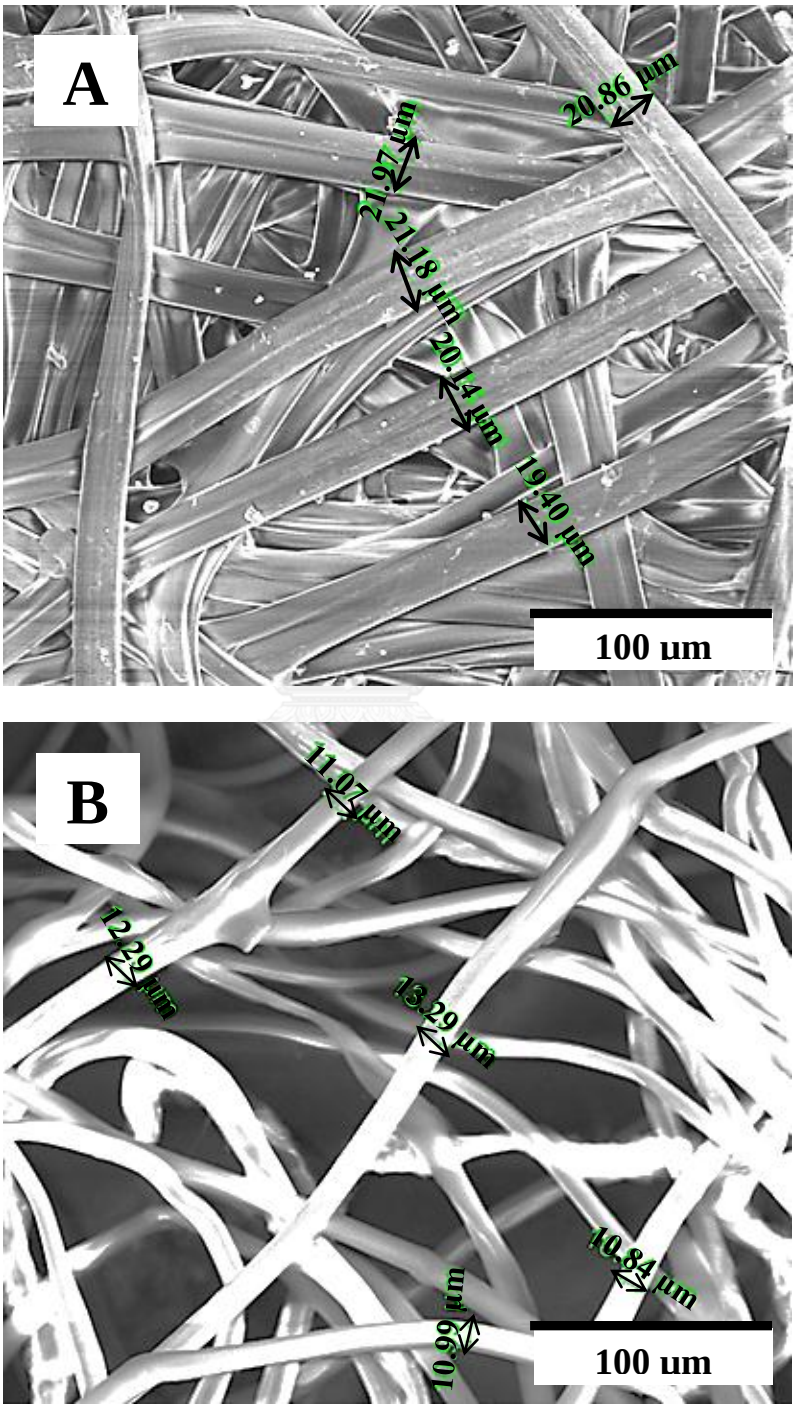

Figure I Measurements of silk fiber sizes of (A) outer layer of Thai silk cocoon, Nangnoi Sisaket 1 and (B) silk fibers degummed in boiling 0.02 M sodium carbonate solution for 20 min for 2 cycles (magnification = 1000x)

Appendix B

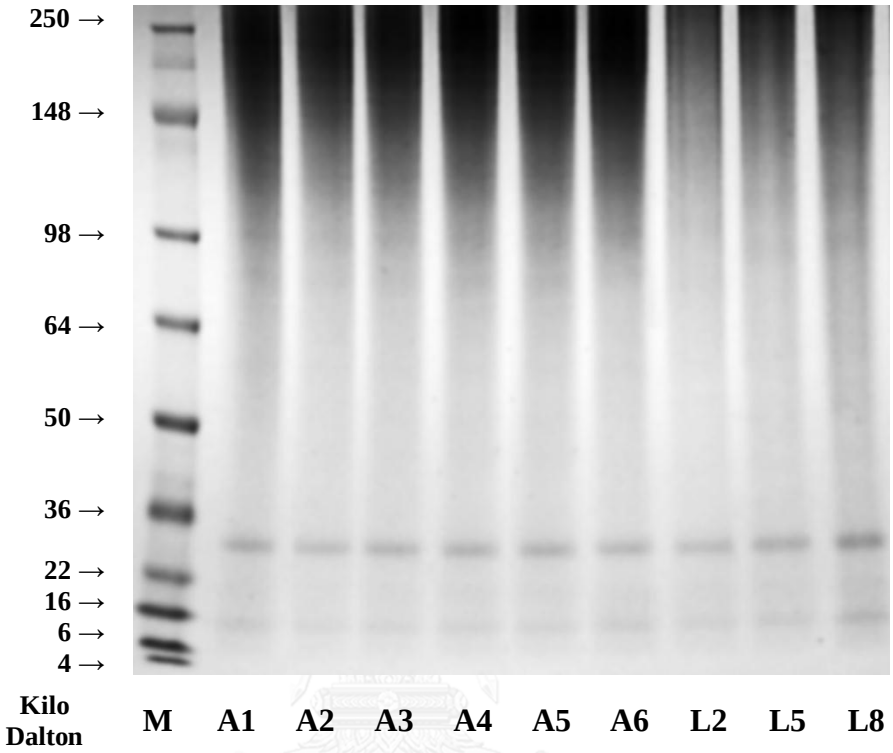

Figure II Molecular weights of silk fibroin samples extracted with Ajisawa's reagent 1: 30 - 1: 15 (w/v) at 50 - 80 °C (lane 2 - 7) and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C (lane 8 - 10) characterized using SDS-PAGE, M: markers

Appendix C

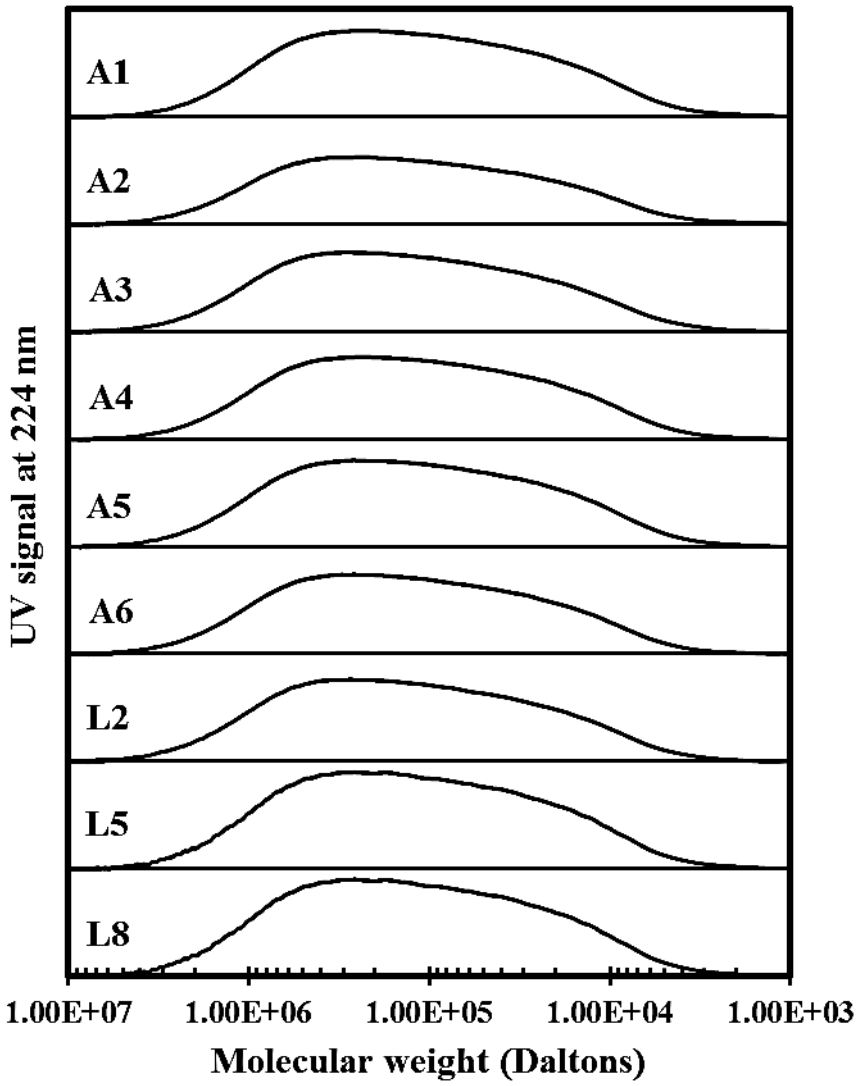

Figure III Molecular weight distributions of silk fibroin samples extracted with Ajisawa's reagent 1: 30 - 1: 15 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C characterized using size exclusion chromatography

Appendix D

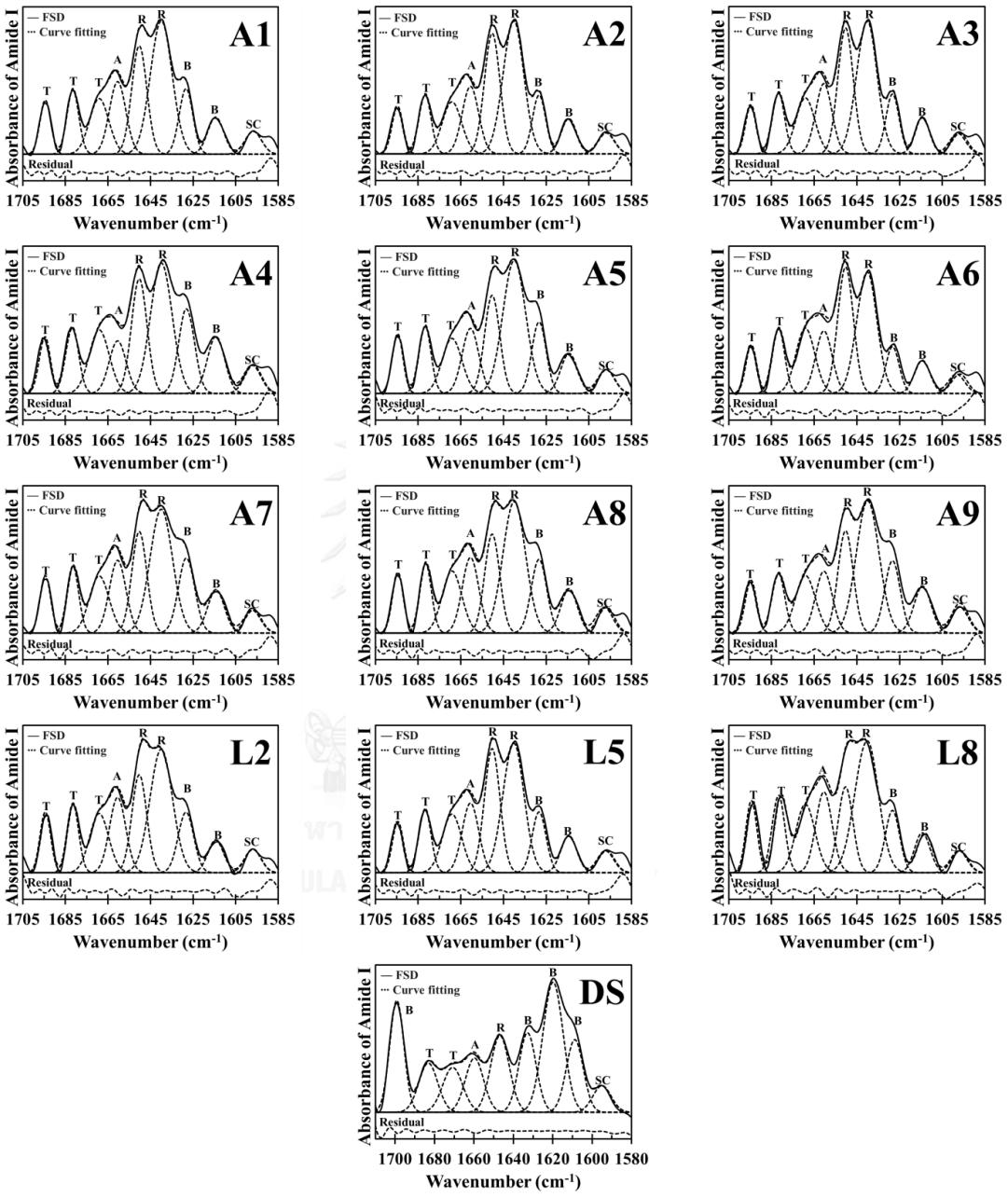

Figure IV Curve-fitting of Fourier self-deconvolution (FSD) Amide I spectra of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C with respect to degummed silk fibers (DS), B: β-sheet, T: β-turn, A: α-helix, R: random coil, SC: tyrosine side chain

Appendix E

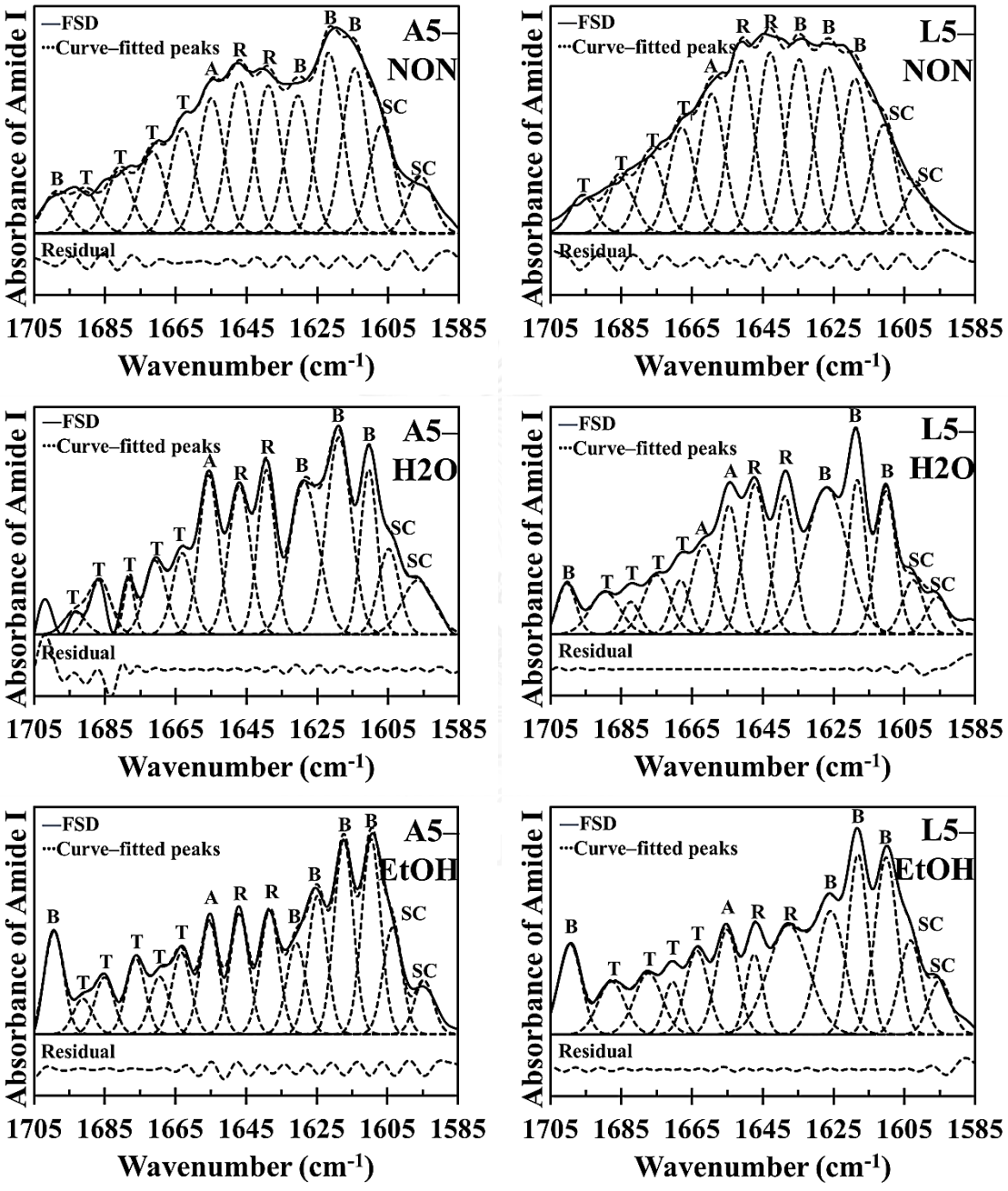

Figure V Curve-fitting of Fourier self-deconvolution (FSD) Amide I spectra of as-casted silk fibroin films, water vapor annealed samples, and ethanol treated samples prepared with Ajisawa's reagent and 9.3 M LiBr 1: 15 (w/v) at 65 °C, B: β-sheet, T: β-turn, A: α-helix, R: random coil, SC: tyrosine side chain

Appendix F

Table I Thermal characteristics of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C with respect to degummed silk fibers (DS) calculated from DSC curves

Sample code	T_{evap} (°C)	ΔH_{evap} (J g^{-1})	T_g (°C)	T_c (°C)	ΔH_c (J g^{-1})	T_d (°C)	ΔH_d (J g^{-1})
A1	96	–105	180	225	21	289	–169
A2	101	–113	180	224	23	289	–189
A3	94	–125	180	225	23	290	–168
A4	95	–106	178	222	25	287	–167
A5	100	–98	179	222	28	287	–170
A6	92	–86	179	223	27	289	–167
A7	99	–76	179	225	27	290	–162
A8	102	–63	179	222	27	288	–164
A9	102	–57	179	223	26	289	–178
L2	98	–97	177	221	24	283	–178
L5	99	–100	178	223	22	284	–179
L8	97	–83	178	225	25	283	–146
DS	84	–122	none	none	none	311	–296

T_{evap} refers to water evaporation temperature; ΔH_{evap} refers to enthalpy of water evaporation; T_g refers to glass transition temperature; T_c refers to crystallization temperature; ΔH_c refers to enthalpy of crystallization; T_d refers to decomposition temperature; and ΔH_d refers to enthalpy of decomposition.

Appendix G

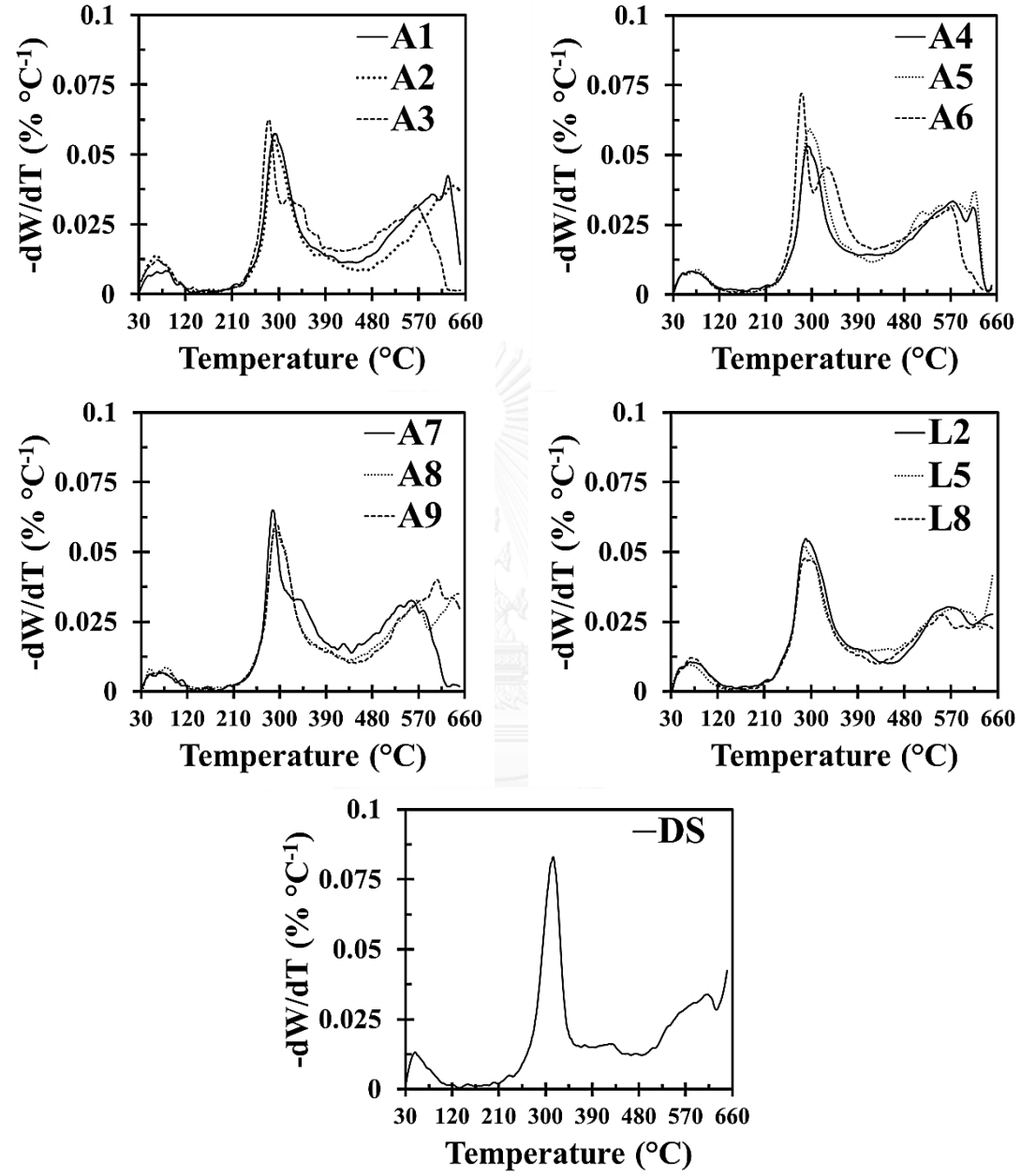

Figure VI First-order derivatives of TGA curves of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 ℃ and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 ℃ with respect to degummed silk fibers (DS), The samples were heated in 30 - 650 ℃ with a heating rate at 10 ℃ min^{-1}.

Appendix H

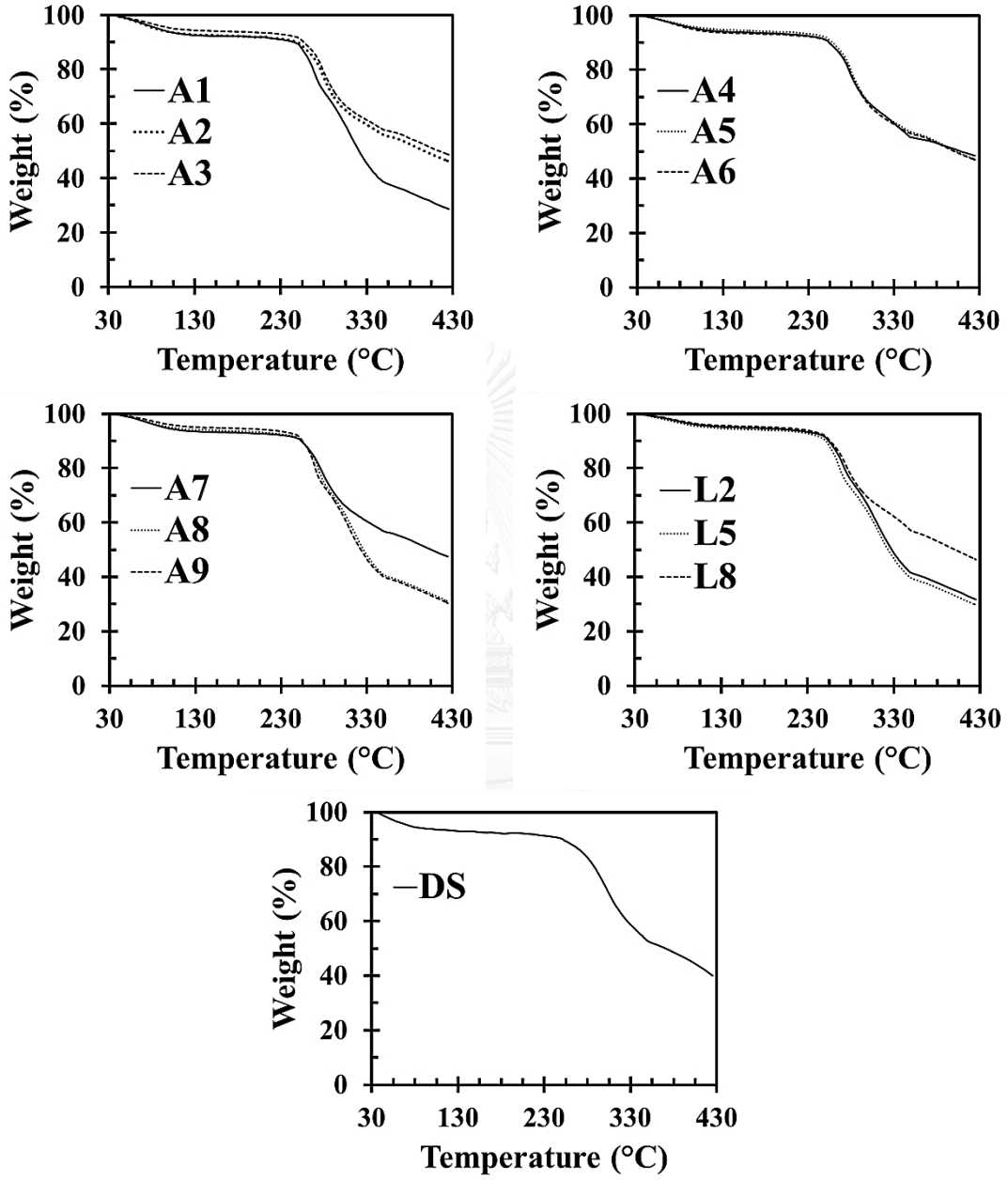

Figure VII TGA curves of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C with respect to degummed silk fibers (DS), The samples were heated in 250 - 350 °C with a heating rate at 2.5 °C min^{-1}.

Appendix I

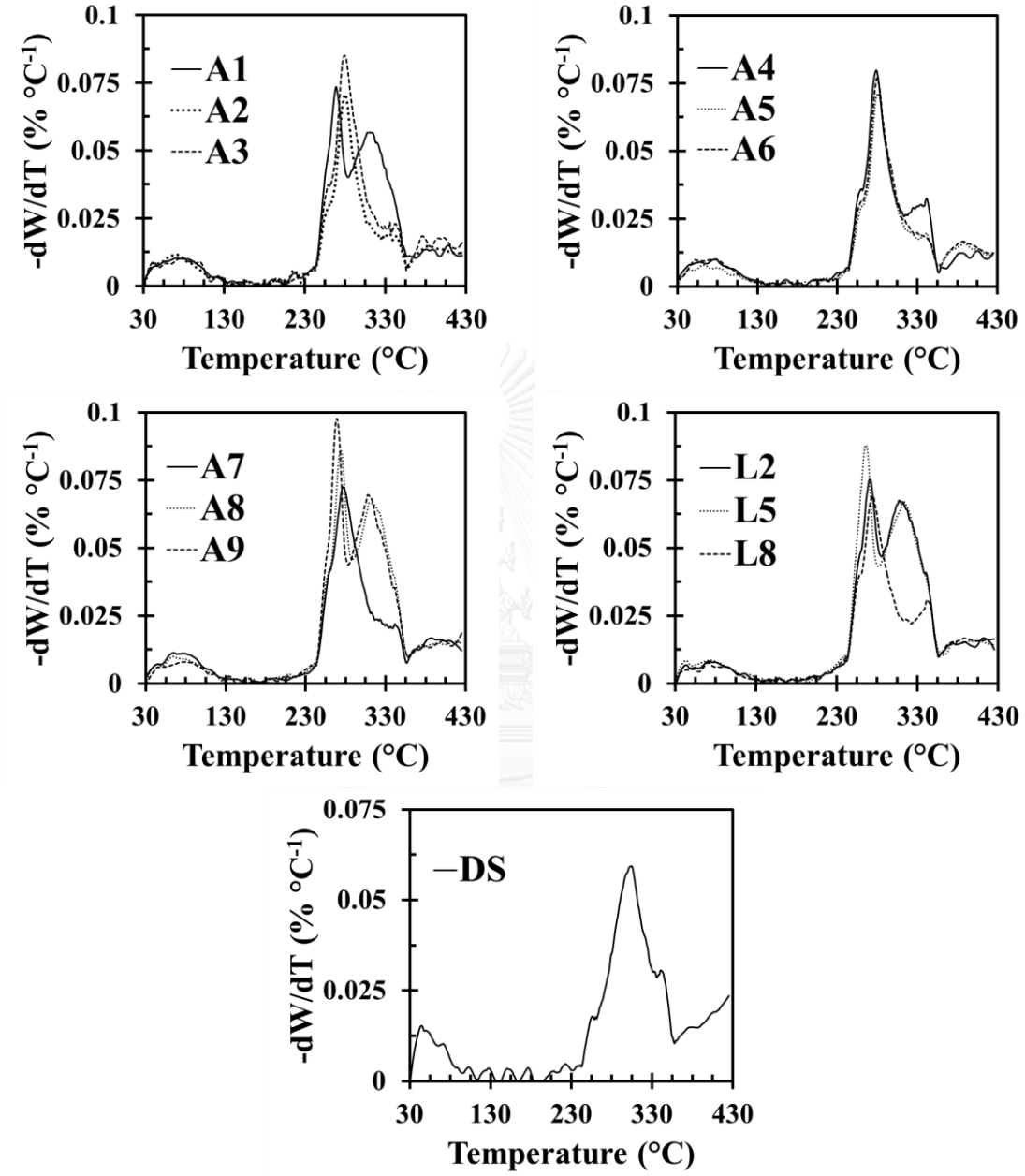

Figure VIII First-order derivatives of TGA curves of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C with respect to degummed silk fibers (DS), The samples were heated in 250 - 350 °C with a heating rate at 2.5 °C min^{-1}.

Appendix J

Table II Thermal characteristics of lyophilized silk fibroin extracted with Ajisawa's reagent 1: 30 - 1: 10 (w/v) at 50 - 80 °C and 9.3 M LiBr 1: 30 - 1: 10 (w/v) at 65 °C with respect to degummed silk fibers (DS) calculated from TGA curves

Sample code	T_{evap} (°C)	Weight loss after water evaporation (%)	T_{d1} (°C)	Weight loss after first decomposition (%)	T_{d2} (°C)	Weight loss after second decomposition (%)
A1	74	5	284	49	606	46
A2	56	8	284	50	537	42
A3	74	7	283	53	563	40
A4	56	6	284	41	569	53
A5	80	6	290	44	613	50
A6	67	5	283	57	580	38
A7	62	4	289	52	587	44
A8	86	5	284	48	574	47
A9	80	4	290	49	601	47
L2	62	7	289	51	569	42
L5	62	6	289	41	575	53
L8	69	8	289	47	550	45
DS	67	6	314	49	618	45

T_{evap} refers to water evaporation temperature; T_{d1} refers to first decomposition temperature; and T_{d2} refers to second decomposition temperature.

Appendix K

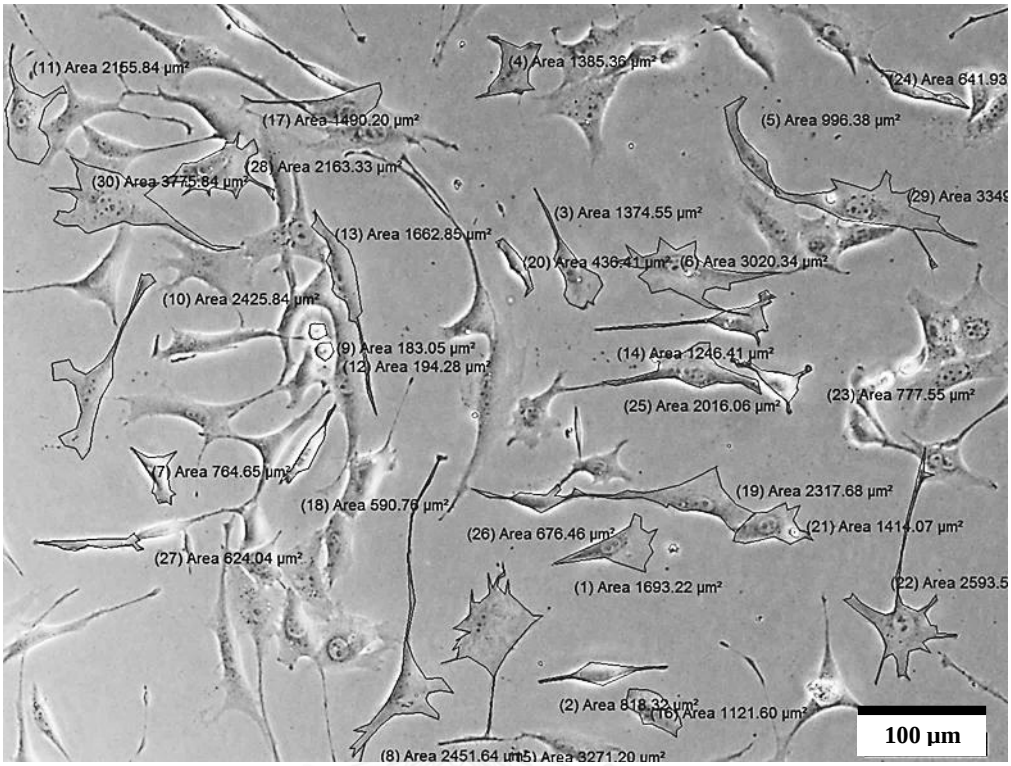

Figure IX An example of cell spreading estimation of NIH-3T3 mouse embryonic fibroblasts cultured on tissue culture plate for 24 h (magnification = 20x), cell attachment areas were estimated using closed-polygon dragging lines

Appendix L

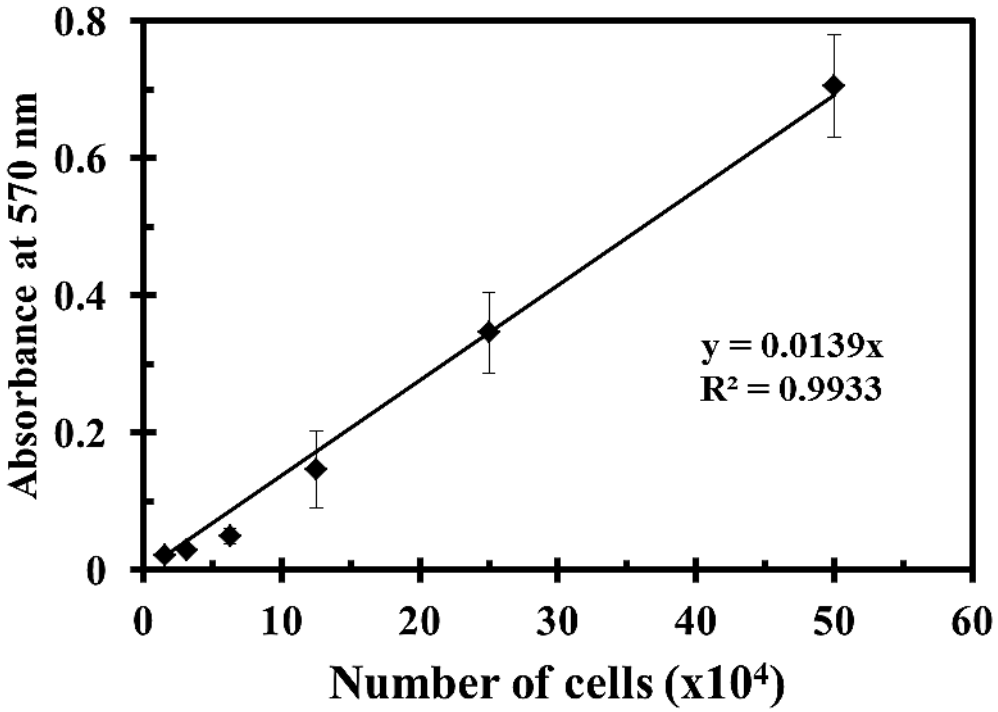

Figure X A standard curve of NIH-3T3 mouse embryonic fibroblasts cultured on tissue culture plate for 6 h, The cells were seeded at concentrations of $5.00x10^5$, $2.50x10^5$, $1.25x10^5$, $6.25x10^4$, $3.12x10^4$ and $1.56x10^4$ cells per well

Appendix M

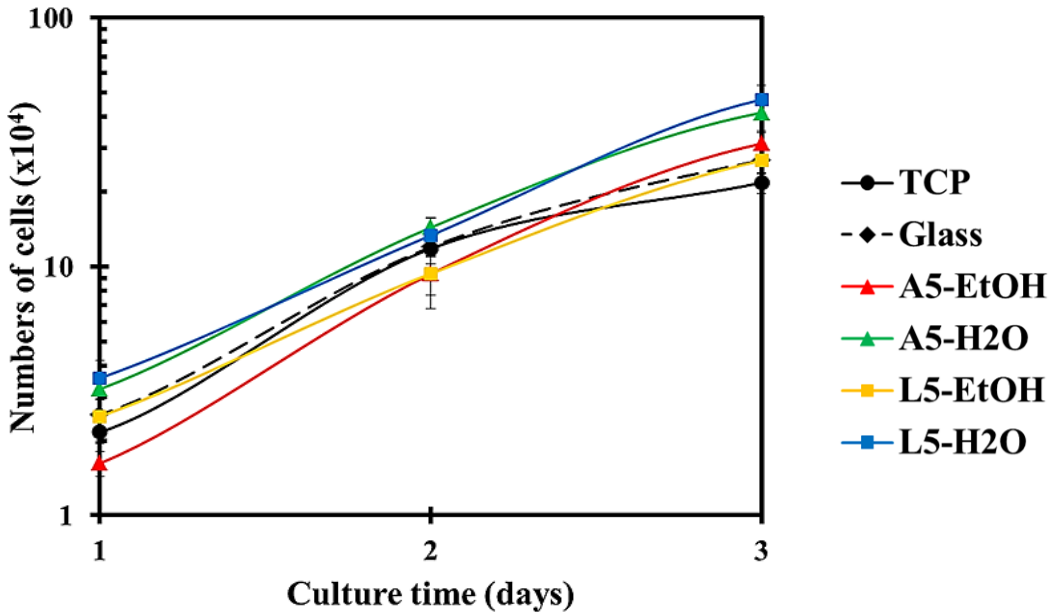

Figure XI Logarithmic scale of proliferations of NIH-3T3 mouse embryonic fibroblasts cultured on ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively), tissue culture plate (TCP), and glass substrate

Table III Numbers of NIH-3T3 mouse embryonic fibroblasts cultured on ethanol treated and water vapor annealed silk fibroin films prepared with Ajisawa's reagent 1: 15 (w/v) at 65 °C (A5-EtOH and A5-H2O, respectively) and 9.3 M LiBr 1: 15 (w/v) at 65 °C (L5-EtOH and L5-H2O, respectively) with respect to proliferations the cells cultured on tissue culture plate (TCP) and glass substrate from 6 h to 6 days

	6 h	1 day	2 days	3 days	4 days	5 days	6 days
TCP	2.0 ± 0.3^{a}	$2.2 \pm 0.4^{a,b}$	11.8 ± 0.8^{c}	$21.7 \pm 4.2^{c,d}$	41.9 ± 4.6^{e}	$59.7 \pm 10.9^{e,f}$	62.9 ± 5.1^{f}
Glass	2.1 ± 0.4^{a}	$2.5 \pm 1.2^{a,b}$	12.0 ± 3.5^{c}	26.8 ± 6.4^{d}	42.4 ± 7.5^{e}	46.9 ± 9.3^{e}	50.3 ± 7.2^{e}
A5-EtOH	1.1 ± 0.2^{b}	$1.6 \pm 0.4^{a,b}$	9.3 ± 5.2^{c}	31.2 ± 6.4^{d}	34.8 ± 3.9^{d}	37.2 ± 6.1^{d}	42.2 ± 7.7^{d}
A5-H2O	2.0 ± 0.5^{a}	3.2 ± 0.6^{b}	14.4 ± 2.6^{c}	41.6 ± 12.8^{e}	$47.5 \pm 14.0^{e,f}$	$50.7 \pm 6.4^{e,f}$	$55.6 \pm 7.9^{e,f}$
L5-EtOH	$1.4 \pm 0.1^{a,b}$	$2.5 \pm 1.0^{a,b}$	9.4 ± 3.4^{c}	26.7 ± 6.2^{d}	35.7 ± 14.6^{d}	35.8 ± 16.8^{d}	40.8 ± 15.9^{d}
L5-H2O	2.0 ± 0.5^{a}	3.6 ± 1.2^{a}	13.3 ± 4.7^{c}	46.9 ± 13.1^{e}	$52.3 \pm 15.5^{e,f}$	$56.6 \pm 5.0^{e,f}$	$55.6 \pm 10.1^{e,f}$

All data are reported in average ± standard deviation (n = 4). All superscript letters indicate significant differences ($P<0.05$). Data highlighted in gray were of the cells cultured on detached films.

VITA

Supawich Chankow is a half Filipino-Thai, who was born in Bangkok on March 30th, 1989. He went to the Chulalongkorn University Demonstration School and proceeded to study at the Chulalongkorn University for higher education. He has a bachelor's degree of Science in Chemistry with his senior project, "Preparation of (Ba0.5Sr0.5)1-xCaxCo0.8Fe0.2O3-δ-YSZ". He further joined the Multidisciplinary in Biomedical Engineering Program to study tissue engineering. In 2014-2015, he received the Erasmus–Mundus Scholarship to study at the University of Trento, Italy. He conducted a research on "Effects of extracting conditions using calcium chloride–ethanol process on characteristic of Thai silk fibroin" for his master's degree. Conferences and meetings he attended during his master's degree are listed below.

Supawich Chankow, Walter Bonani, Somchai Luemunkong, Pornanong Aramwit, Sorada Kanokpanont, and Antonella Motta, Molecular weights of Thai silk fibroin studied by size exclusion chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Oral presentation, Possibility for a fast track in the translation of naturally-derived biomaterials into the clinic (NDB2015), Chulalongkorn University, Bangkok, Thailand, November 2nd-3rd, 2015.

Supawich Chankow, Walter Bonani, Somchai Luemunkong, Pornanong Aramwit, Sorada Kanokpanont, and Antonella Motta, Effects of lithium bromide and calcium chloride-ethanol dissolution processes on molecular weights and secondary structures of Thai silk fibroin Nangnoi Sisaket-1 (Bombyx mori), Poster presentation, The 41st Congress on Science and Technology of Thailand (STT41), Suranaree University of Technology, Nakhon Ratchasima, Thailand, November 6th–8th, 2015.

Kanyaluk Kaewprasit, Supawich Chankow, Rungnapa Yamdech, Jutarat Jamkratoke, Sanong Ekgasit, Siriporn Damrongsakkul, and Sorada Kanokpanont, Quantitative analysis of Thai silk fibroin secondary structures using ATR-FTIR spectroscopy, Poster presentation, The 24th International Congress on Sericulture and silk industry (ISC24), Queen Sirikit Convention Center, Bangkok, Thailand, August 10th-14th, 2016.

Supawich Chankow, Somchai Luemunkong, and Sorada Kanokpanont, Conformational transitions of Thai silk fibroin secondary structures, Oral presentation, The 9th Biomedical Engineering International Conference (BMEiCON2016), Laung Prabang, Laos, December 7th-9th, 2016.