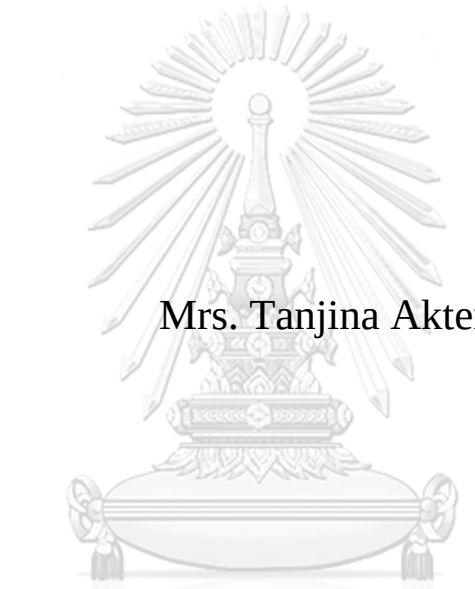
EFFECTS OF HEAT AND UV-C CURING ON PROPERTIES OF SOY PROTEIN FILM

Mrs. Tanjina Akter



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ผลของการบ่มด้วยความร้อนและยูวีซีต่อสมบัติของฟิล์มโปรตีนถั่วเหลือง

นางแทนจิน่า อัคทาร์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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Thesis Title	EFFECTS OF HEAT AND UV-C CURING ON PROPERTIES OF SOY PROTEIN FILM
By	Mrs. Tanjina Akter
Field of Study	Food Science and Technology
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แทนจิน่า อัคทาร์ : *ผลของการบ่มด้วยความร้อนและยูวีซีต่อสมบัติของฟิล์มโปรตีนถั่วเหลือง.* (EFFECTS OF HEAT AND UV-C CURING ON PROPERTIES OF SOY PROTEIN FILM) *อ.ที่ปรึกษาหลัก* : *ธนจันทร์ มหาวนิช*

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของการบ่มด้วยความร้อนและการบ่มด้วยยูวีซีต่อสมบัติของฟิล์มโปรตีนถั่วเหลือง การบ่มด้วยความร้อนและการบ่มด้วยยูวีซีทำต่อสารละลายฟิล์มหรือฟิล์มที่ขึ้นรูปแล้ว ในส่วนแรกของงานวิจัย การบ่มสารละลายฟิล์มหรือฟิล์มที่ขึ้นรูปแล้วทำที่ 60, 70 *หรือ* 80 *องศาเซลเซียส เป็นเวลา* 2, 4 *หรือ* 6 *ชั่วโมง พบว่าการบ่มด้วยความร้อนไม่มีผลต่อความหนาของฟิล์ม* (p>0.05) *แต่ทำให้ความต้านทานแรงดึงขาดเพิ่มขึ้น ฟิล์มที่ขึ้นรูปแล้วที่บ่มที่* 70 *องศาเซลเซียส เป็นเวลา* 4 *ชั่วโมง มีความต้านทานแรงดึงขาดสูงสุด* (3.49 *เมกะพาสคาล*) *ซึ่งเท่ากับ* 1.8 *เท่าของตัวอย่างควบคุม การเพิ่มขึ้นของความเข้มรามานในช่วงการยืดของ* S-S *ยืนยันการเกิดของพันธะเชื่อมข้ามไดซัลไฟด์ที่เหนี่ยวนำโดยความร้อน การเพิ่มขึ้นของพันธะเชื่อมข้ามนี้ยังส่งผลต่อการลดลงของการยืดตัวถึงจุดขาด นอกจากนี้พบว่าตัวอย่างฟิล์มที่บ่มด้วยความร้อนมีความเข้มของสีเหลืองเพิ่มขึ้น การบ่มสารละลายฟิล์มด้วยความร้อนทำให้ฟิล์มที่ได้มีความโปร่งใสเพิ่มขึ้น แต่การบ่มฟิล์มที่ขึ้นรูปแล้วด้วยความร้อนกลับทำให้ความโปร่งใสลดลง ฟิล์มที่บ่มด้วยความร้อนมีความสามารถในการละลายน้ำลดลงและมีความไม่ชอบน้ำของผิวฟิล์มเพิ่มขึ้น การบ่มสารละลายฟิล์มด้วยความร้อนทำให้ฟิล์มที่ได้มีสภาพซึมผ่านได้ของไอน้ำเพิ่มขึ้น ในขณะที่สภาพซึมผ่านได้ของไอน้ำของฟิล์มที่ขึ้นรูปแล้วที่บ่มความร้อนมีค่าใกล้เคียงกับตัวอย่างควบคุม* (p>0.05) *การศึกษาด้วยกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดแสดงให้เห็นความไม่เป็นเนื้อเดียวกันของโครงสร้างของฟิล์มที่บ่มด้วยความร้อน งานวิจัยส่วนที่สองนำฟิล์มที่บ่มด้วยความร้อนที่* 70 *องศาเซลเซียส เป็นเวลา* 4 *ชั่วโมง แล้วมาบ่มด้วยด้วยยูวีซีอีกครั้งแปรระดับรังสีเป็น* 4, 8, 12 *และ* 16 *จูล/ตารางเซ็นติเมตร พบว่าการบ่มด้วยยูวีไม่มีผลต่อความหนาของฟิล์มแต่ทำให้ความต้านทานแรงดึงขาดเพิ่มขึ้น ฟิล์มที่ขึ้นรูปแล้วที่บ่มด้วยความร้อนและบ่มด้วยยูวีที่ระดับรังสี* 12 *จูล/ตารางเซ็นติเมตร มีความต้านทานแรงดึงขาดสูงสุด* (6.37 *เมกะพาสคาล*) *เท่ากับ* 1.8 *เท่าของฟิล์มที่บ่มด้วยความร้อนอย่างเดียว และ* 3.4 *เท่าของตัวอย่างควบคุม การเกิดพันธะเชื่อมข้ามไดไทโรซีนที่เหนี่ยวนำด้วยยูวียืนยันได้โดยใช้เทคนิคฟลูออเรสเซนส์สเปกโทรสโกปี อย่างไรก็ตามการบ่มด้วยยูวีมีผลน้อยมากต่อการยืดตัวถึงจุดขาด ในทำนองเดียวกับการบ่มด้วยความร้อน การบ่มด้วยยูวีทำให้ความเข้มของสีเหลืองเพิ่มขึ้น ในขณะที่ความโปร่งใสลดลงเล็กน้อย ฟิล์มที่บ่มด้วยยูวีซีมีความสามารถในการละลายน้ำลดลง การบ่มสารละลายฟิล์มที่ให้ความร้อนแล้วด้วยยูวีไม่มีผลต่อสภาพให้ซึมผ่านได้ของไอน้ำ แต่การบ่มฟิล์มที่ขึ้นรูปแล้วและให้ความร้อนแล้วด้วยยูวีทำให้สภาพให้ซึมผ่านได้ของไอน้ำมีค่าเพิ่มขึ้น การบ่มด้วยยูวีซีที่ระดับรังสีต่ำๆ ทำให้ความไม่ชอบน้ำของผิวฟิล์มลดลง แต่เมื่อบ่มที่ระดับรังสีสูงๆ ความไม่ชอบน้ำของผิวฟิล์มกลับเพิ่มขึ้นอีกครั้งหนึ่ง ภาพจากกล้องจุลทรรศน์อิเล็กตรอนแบบส่องกราดแสดงให้เห็นถึงรอยแตกและรูขนาดเล็กในเนื้อฟิล์มที่บ่มด้วยความร้อนแล้วบ่มด้วยยูวี จากงานวิจัยนี้สามารถสรุปได้ว่าการบ่มด้วยความร้อนและการบ่มด้วยความร้อนร่วมกับการบ่มด้วยยูวีเป็นเทคนิคที่มีประสิทธิภาพในการปรับปรุงความแข็งแรงเชิงกลของฟิล์มโปรตีนถั่วเหลืองโดยการส่งเสริมให้เกิดพันธะโควาเลนต์เชื่อมข้ามระหว่างสายโซ่โปรตีน

สาขาวิชา	*วิทยาศาสตร์และเทคโนโลยีทางอาหาร*	*ลายมือชื่อนิสิต* .....
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6478014923 : MAJOR FOOD SCIENCE AND TECHNOLOGY
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Tanjina Akter : EFFECTS OF HEAT AND UV-C CURING ON PROPERTIES OF SOY PROTEIN FILM. Advisor: Asst. Prof. THANACHAN MAHAWANICH, Ph.D.

This study aimed to investigate the effects of heat curing and UV-C curing on properties of soy protein film. Both the heat and UV-C treatments were applied to either film-forming solution or pre-formed film. In the first part, film-forming solution or pre-formed film was cured at 60, 70, or 80°C for 2, 4, or 6 h. Heat curing had no effect on film thickness (p>0.05), but it did improve tensile strength of the films. Pre-formed film cured at 70°C for 4 h exhibited the highest tensile strength (3.49 MPa), which was 1.8 times higher than the control. Increasing Raman intensity in the S-S stretching region confirmed the formation of heat-induced disulfide cross-links. This increasing degree of cross-linking may also account for a decrease in elongation at break of the film samples. An increase in yellowness intensity was observed in heat-cured samples. Heat curing of film-forming solution significantly increased film transparency but posed the opposite effect on pre-formed films. Heated films exhibited a decrease in water solubility and an increase in surface hydrophobicity. Heat curing of film-forming solution produced a film with increasing water vapor permeability. Meanwhile, water vapor permeability of heat-cured pre-formed films was similar to the control (p>0.05). SEM revealed structural inhomogeneity of heat-cured films. In the second part, the effect of UV-C curing of film formerly heat-treated at 70°C for 4 h was investigated. The radiation doses were varied as 4, 8, 12, and 16 J/cm^2. UV-curing did not affect film thickness but did cause a significant increase in tensile strength. Heat-treated pre-formed film undergoing UV-curing at 12 J/cm^2 possessed the greatest tensile strength (6.37 MPa), 1.8-fold higher than the film heat-treated alone and 3.4-fold higher than the control. UV-induced dityrosine cross-linking was confirmed using fluorescence spectroscopic technique. In spite of that, UV-C treatment minimally affected elongation at break. Similar to heat treatment, UV-curing also induced an increase in yellowness intensity and a slight decrease in transparency. UV-C treatment produced a film with lower water solubility. UV-curing of heat-treated film-forming solution had no effect on water vapor permeability of the resulted film, but the treatment significantly increased water vapor permeability of heat-treated pre-formed film. UV-C curing at lower doses tended to result in a film with lower surface hydrophobicity. At higher UV-C doses, however, surface hydrophobicity became increasing again. SEM micrographs revealed cracks and pinholes in UV-cured heat-treated film matrices. In conclusion, heat curing and combined heat/UV-C curing were demonstrated as effective techniques for enhancing mechanical strength of soy protein film by promoting the formation of covalent cross-links between protein chains.

Field of Study:	Food Science and Technology	Student's Signature .....
Academic Year:	2022	Advisor's Signature .....

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Tanjina Akter

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CHAPTER 1
INTRODUCTION

Packaging is an important part of food products owing to its role in protecting the food from its surroundings, extending the shelf-life while maintaining the product quality, and offering convenience to the consumers. Packaging made of synthetic polymers comes with many advantages, for example, being lighter in weight, lower in cost, and better in mechanical and barrier characteristics (Isobe, 2003). These superior properties are responsible for the popularity of these petroleum-based plastics. The production and use of petroleum-derived plastic packaging have been increasing, with its global production exceeding 400 metric tons/year (Saratale et al., 2021). However, one major drawback of these plastics is that they generate waste management problems and environmental threats due to their non-biodegradable trait (Tharanathan, 2003). After their intended uses, these non-biodegradable petroleum-based packaging materials are often released or discarded into the environment as garbage, producing a huge amount of solid waste, resulting in environmental and soil pollution (Jang et al., 2020). The food and packaging industries are now collaborating to develop environmentally friendly biodegradable and edible packaging to replace their petroleum-based counterparts.

Packaging made from biopolymers is an alternative solution because of its biodegradability and renewability, which also fulfills the functional characteristics of packaging without negatively affecting the environment. Plant and animal-derived renewable biopolymers such as proteins (e. g. soy protein, whey protein, egg albumen, gelatin, and gluten) and polysaccharides (e. g. starch, cellulose derivatives, chitosan, pectin, plant gums, and seaweed polysaccharides) have been explored as potential alternatives for petroleum-derived synthetic plastics (Jiang et al., 2016). Plant-based biopolymers, like plant proteins, have drawn much attention due to their good film-forming ability, high production volume, and capability to be modified using various techniques (Coltelli et al., 2015). Among various protein sources, soy protein is considered one of the most promising raw materials because of its advantages, such as rich resources, reasonable price, good film-forming ability, and biodegradability (Wang et al., 2021). Soy protein isolate is produced from defatted

soy flour as a by-product of the soybean oil industry. It contains about 90% protein on a dry basis, the highest purity among all soy protein products. Globulins are the major protein of soy protein isolate, which can be divided into four fractions based on their sedimentation coefficients, namely 2S, 7S, 11S, and 15S globulins (Krochta et al., 1994). 7S globulin (β-conglycinin) and 11S globulin (glycinin) are the principal fractions, accounting for more than one-third of the total extractable proteins (Cho & Rhee, 2004). Both fractions were reported to have film-forming ability (Shin, 1998; Yong & Chul 2004). Like all proteins, soy protein also contains polar and non-polar side chains capable of forming various interactions, such as hydrogen bonding, van der Waals forces, electrostatic interactions, and hydrophobic interactions. Apart from non-covalent interactions, the side groups may also undergo different types of reactions and subsequent formation of covalent cross-links. These all together result in a cohesive matrix (Dhall, 2013) and restrict molecular mobility, leading to enhanced stiffness, yield point, and tensile strength (Zhang et al., 2001). Despite that, the mechanical strength of soy protein isolate film is still inferior to existing petroleum-based films (Pérez-Gago et al., 1999). These properties must be enhanced in order to make soy protein films able to compete with those plastic ones. Improving or modifying the properties of protein films via protein cross-linking can be achieved using various techniques such as heat treatment, chemical cross-linking, enzymatic cross-linking, irradiation, mechanical reinforcement, and fabrication into a composite film (Kim et al., 2019; Chiralt et al., 2018).

Physical modification, such as heat curing and irradiation (ultraviolet and gamma radiation), has been reported as an efficient method to improve protein film properties (Gennadios et al., 1996; Rhim et al., 2000; Kim et al., 2002; Insaward et al., 2014). Heat curing is a widely used physical modification treatment for protein-based films (Gennadios et al., 1996). During heating, the protein unfolds to expose its hydrophobic core, like sulfhydryl group. Heating further induces thiol-disulfide exchange reactions, forming inter- and intra-molecular disulfide linkages (Chiralt et al., 2018). Heat curing has been reported to help increase tensile strength and elongation at break while decreasing water solubility and water vapor permeability of protein films. An enhancement in soy protein film properties was induced by heat

curing at 60, 72.5, or 85°C for 24 h (Kim et al., 2002). It was also reported that heat curing at 80 and 95°C for 2, 6, 14, and 24 h could modify the properties of soy protein film (Gennadios et al., 1996). Gallic acid-incorporated soy protein film demonstrated enhanced properties upon being treated at 50, 70, and 90°C for 5, 10, and 15 h (Insaward et al., 2014). Heat curing of amaranth protein isolate film at 70 and 90°C enhanced the film functionality by inducing disulfide and hydrogen bonds (Condes et al., 2013). Heat curing of film-forming solution of cuttlefish (*Sepia pharaonis*) skin gelatin at 60 and 70°C was also reported to increase the film tensile strength and decrease water vapor permeability. However, heating the film-forming solution beyond 70°C was reported to cause a decrease in tensile strength due to protein degradation (Hoque et al., 2010). Al-Saadi et al. (2014) reported that heat treatment effectively reduced solubility of whey protein films. Heat curing was generally shown to improve tensile strength, decrease water vapor permeability and water solubility, and alter color of protein films.

Irradiation is another physical treatment that has been explored as a modification method for protein films. Ultraviolet (UV) radiation can be absorbed by the side group of aromatic amino acids, such as tyrosine (Tyr). The amino acids then undergo oxidation, producing amino acid free radicals, such as tyrosine radical (Tyr·). Subsequent recombination of these free radicals results in dityrosine cross-links (Tyr-Tyr), which exerts an effect on protein film properties (Wihodo & Moraru, 2013). However, it should be noted that besides inducing covalent cross-link, irradiation may alter protein film properties by instigating molecular degradation (Gennadios et al., 1998). Gennadios et al. (1998) reported that exposure of soy protein film to UV radiation resulted in an increase in tensile strength and yellow coloration with a decrease in elongation at break. Díaz et al. (2016) explored the effect of UV irradiation on whey protein film properties. UV was applied to either film-forming solution or pre-formed film at varying doses (0.12, 4.0, and 12.0 J/cm^2). The film-forming solution exposed to the highest UV dose yielded a film with significantly improved tensile strength, puncture strength, puncture deformation, with decreasing water solubility as compared to the untreated control. Shakil & Mahawanich (2022) assessed the impact of UV-C treatment (0.32, 1.56, 4.00, 12.00 J/cm^2) on either pre-

formed film or film-forming solution of ferulic acid-fortified soy protein film. It was revealed that the films treated with the highest radiation dose displayed an increase in both tensile strength and elongation at break as compared to the untreated controls.

To the best of our knowledge, no prior study has been conducted on the combined effect of heat and UV-C curing on properties soy protein film. Therefore, this study aimed to investigate the effects of heat and UV-C curing of film-forming solution or pre-formed film on properties of soy protein film.

CHAPTER 2
LITERATURE REVIEW

2.1 Food packaging

Packaging is a crucial part of a food product to protect the food from contamination, maintain hygiene, ensure organoleptic and nutritional attributes, and reduce product spoilage during handling, commercialization, and storage (Sharma et al., 2021). Selection of packaging is therefore important, particularly in the case of mass-produced commercial products (Cunha et al., 2021; Shao et al., 2021). Among various packaging materials used in the market today, petroleum-based non-biodegradable packaging is dominating in terms of volume. This is due to its availability, cost-competitiveness, as well as durability with superior mechanical properties, and excellent gas and liquid barrier properties (Haosagul et al., 2019). Because of its numerous advantages, the production and use of petroleum-derived plastic packaging have been continually increasing with its global production exceeds 400 metric tons/year (Pan, 2020; Saratale, 2021). After their intended uses, the majority of these petroleum-based packaging materials are released or discarded into the environment as garbage, contributing to a huge proportion of solid waste. Moreover, petroleum itself is non-renewable resource and petroleum-based packaging materials are non-biodegradable resulting in environmental pollution at their extreme uses (Jang et al., 2020). Disposal of petroleum-based packaging has prompted waste management challenge due to their non-biodegradability and environmental threat which is referred to as “white pollution” (Haosagul et al., 2019). Incineration and landfilling are the common way to deal with solid waste but these practices are not sustainable. Incineration generates large amount of heat, emits toxic gases which are the cause of global warming (Swain et al., 2004), as well as produces particulate matter 2.5 ($PM_{2.5}$) which is small enough to travel deeply into the respiratory tract causing short-term and long-term health effects to human and animals (Yan et al., 2016). Regarding landfilling, synthetic plastics breakdown and form microplastics by various natural and anthropogenic forces and these microplastics are known to pose a serious threat to aquatic animals. Through these animals, the microplastics eventually enter the human food chain, causing several diseases and injuries, such as skin

diseases, DNA damage, oxidative injury, asthma, infertility, and cardiovascular disease (Mujtaba et al., 2019). Therefore, the need for packaging materials from cheap, renewable, biodegradable, and readily available raw materials is currently on the rise from the view of both manufacturers and consumers. Biopolymers have emerged as the top materials for fabricating food packaging due to their processability, biodegradability, combination potential, and low level of contamination (Tharanathan, 2003).

2.2 Composition of biodegradable/edible packaging

Biodegradable packaging is an alternative way to replace petroleum-based packaging materials which may fulfill functional characteristics of the packaging without affecting the environment. In addition to being biodegradable, packaging made from biopolymers is also fabricated from renewable resources as their base components (Chen et al., 2019). Based on the biopolymers, biodegradable or edible packaging could be categorized into three types: hydrocolloids, lipids, and composites (Velickova et al., 2015). Hydrocolloids, composed of hydrophilic polymers, including proteins and polysaccharides (Shit & Shah, 2014). Among all biopolymers, protein-based biopolymers are becoming attractive raw materials for food packaging because of their low-cost, availability, and remarkable barrier against non-polar substances and UV light (Confente et al., 2020). Thus, developing eco-friendly biodegradable packaging from renewable resources to substitute petroleum-based materials is essential for the packaging sector (Zhao 2021).

Biodegradable or edible packaging has at least two components: a biopolymer base which provides integrity to the film matrix, and a solvent which is usually water. Nevertheless, varieties of additives and plasticizers are often incorporated to improve functional, mechanical, barrier, organoleptic, and nutritional properties of the film formulation (Vieira et al., 2011). The biopolymers used can be polysaccharides, proteins, or lipids (Otoni et al., 2017). Uniformity of the films can be controlled by adjusting the alkaline or acidic conditions of the film-forming solutions, particularly in the case of protein films. Gennadios et al. (1993) reported that tensile strength of wheat gluten and soy protein films produced under alkaline condition is significantly higher than that of the films produced under acidic condition. Additives such as

plasticizers, active compounds, prebiotics, probiotics, vitamins, and minerals may be included in the formulation to improve mechanical, functional, organoleptic, and nutritional characteristics.

2.3 Soy protein film

With its different amino acid subunits sequencing and arranging in a specific fashion, each protein possesses a unique primary, secondary, tertiary, and quaternary structures, which, in turn, pose a great effect on the protein functionality (Silva et al., 2014). Several proteins exhibit high biodegradability with extraordinary film-forming ability (Hadidi et al., 2022), with satisfactory mechanical and gas barrier properties compared to polysaccharide- and lipid-based films (Chen et al., 2019). In addition, each amino acid subunit carries side group with diverse properties, allowing the protein readily modified using various techniques. This makes proteins more attractive to the researchers in developing biodegradable films (De Graaf et al., 1998). Protein-based biodegradable films can be developed from both plant and animal sources such as amaranth, corn, cottonseed, collagen, casein, whey, egg albumen (egg white), gelatin, myofibril, peanut, rice bran, soybean, sunflower seed, and wheat (Kumar & Gupta, 2012).

Soy protein, a byproduct of soybean oil industry, is commercially available in three different forms, specifically, soy flour, soy protein concentrate, and soy protein isolate (Li et al., 2008). Among these soy protein products, soy protein isolate is of the highest purity, with ≥ 90% protein content. Commercial soy protein isolate is produced by isoelectric precipitation which may later be neutralized to improve its solubility (Tian et al., 2018). This highly refined soy product has been widely explored in preparation of protein film due to its outstanding film-forming ability. Glycinin and β-conglycinin are the two major fractions of soy globulins which are commonly referred to as 7S and 11S globulins, respectively (Cho & Rhee 2004). Different soy protein fractions produce a film with different characteristics. For example, film made from 11S fraction is smooth and opaque, with higher tensile strength presumably because 11S fraction has a higher tendency to form disulfide bonds as compared to 7S fraction. Meanwhile, film of 7S fraction is transparent and wrinkled (Shin, 1998; Yong & Chul 2004). Due to its polar nature, soy protein films

are superior barrier against non-polar molecules, such as oxygen, carbon dioxide, lipids, and organic volatiles. Regarding thermal stability, soy protein films were reported to show initial degradation at 292ºC. However, soy protein films still have limitation in terms of mechanical strength and water vapor barrier property.

2.4 Plasticizers

Apart from the basic ingredients, additional substances are often added and this could affect functional, mechanical, barrier, and nutritional properties of protein-based films (Hamed et al., 2022). Many protein films, without added plasticizer, are brittle and fragile. This is due to density of proteins themselves as well as extensive interactions among the protein chains, for instance, disulfide bond, hydrophobic and electrostatic interactions (Suhag et al., 2020). This brittleness and fragility place a limitation in protein film usage (Vieira et al., 2011). Plasticizer is one of the important additives commonly incorporated to protein films to make them more processable and maintain film integrity. The Council of the International Union of Pure and Applied Chemistry (IUPAC) defines plasticizer as "a substance or material incorporated in a material (usually a plastic or elastomer) to increase its flexibility, workability, or distensibility" (Jenkins, 1982). Plasticizers plays their role by interfering the formation of chemical interactions among polymeric chains, and hence increases flexibility of the polymer matrix (Ananey-Obiri et al., 2018; Suhag et al., 2020).

Plasticizers are liquid or solid substances with low volatility commonly added during film preparation (Swain et al., 2004). Molecular weight as well as number and position of hydroxyl groups affect plasticizing ability of the molecule (Bourtoom, 2009). Plasticizers can be divided into three broad groups, being polyols (e. g., glycerol, sorbitol, glyceryl derivatives, propylene glycol, and polyethylene glycol), organic esters (e. g., phthalate esters, dibutyl sebacate, citrate esters, and triacetin), and oils and glycerides (e. g., phospholipids, fatty acids, oils and waxes) (Sothornvit & Krochta, 2005). In the case of protein films, polyols have been reported to be particularly effective plasticizers (Zhang et al., 2006).

2.5 Protein film fabrication

Solvent casting and extrusion are two main conventional methods that have been used for edible film fabrication from biopolymers (Suhag et al., 2020).

2.5.1 Solvent casting

Solvent casting process, also known as wet process or bench casting, is a popular and inexpensive film preparation method that is more usable for a laboratory scale but less adequate for commercial scale film production (De Moraes et al., 2013; Mellinas et al., 2016).

In this solvent-casting process, three main steps include preparation of film-forming solution, casting, and drying. The film-forming solution is usually prepared by solubilizing biopolymer in an appropriate solvent with an addition of suitable plasticizer and other ingredients, such as nutrients and bioactive compounds (Suhag et al., 2020). Solvent is an important element in film preparation to solubilize and uniformly distribute the biopolymer upon casting (Jensen et al., 2015; Koide et al., 2013).

Casting of the film-forming solution is conventionally done on a flat surface. Various surface types have been used for protein film casting, including acrylic, silicone, ceramic, polytetrafluoroethylene (PTFE), and glass (Suhag et al., 2020). After casting, the film-forming solution is dried at ambient or under controlled condition using hot air oven, microwave oven, tray dryer, or vacuum dryer to evaporate the solvent (Suhag et al., 2020). Upon solvent removal, the polymer chains interact via covalent bonds and non-covalent interactions to form stable film matrix (Shahidi & Hossain, 2020; Šuput et al., 2015). The conditions used during drying affect characteristics of the resulted film (Sherrington, 1993). Low drying temperature is normally used in biopolymer film preparation because there is lower risk of thermal degradation, particularly for protein films (Kumar et al., 2022).

2.5.2 Extrusion

Extrusion is based on the thermoplastic behavior of polymers when plasticized and heated above their glass transition temperature (Verbeek & van den Berg, 2010). This process is also known as dry process since it can work without water, or adding just a small amount of water, or any other solvent (Kamal, 2019). In general, the extrusion process can be separated into feeding zone, kneading zone, and heating zone. In the first zone, the biopolymer mixture is carried into the feeding zone and compressed with air, then the polymer and additives are added to the extruder.

Extruder screw sufficiently combines the film-forming materials in the second zone (kneading zone). The final zone is the heating zone, where heat is applied to melt and mix the biopolymer and additives. A die at the end of the extruder controls the shape and thickness of the extruded film (Cheng et al., 2021). The advantage of extrusion process is that it can produce a variety of forms with uniform quality and within a short time which makes this process preferable for industrial scale. However, the extrusion technique is restricted to certain polymers that are heat stable and have low moisture content (Kamal, 2019). Other processing methods such as injection, blow-molding, and thermo-pressing are often combined with extrusion to produce the final film (Mellinas et al., 2016). Moreover, co-extruder can also be used to make a multilayered film (Skurtys et al., 2010).

2.6 Factors affecting film characteristics

Several factors play a role on film properties, including the polymer used as the film base, other additives, as well as the condition of film formation.

2.6.1 Type of polymeric materials

Polymers that are used as the film base can be categorized into two types, hydrophilic and hydrophobic materials. Most proteins are hydrophilic so they provide good barrier against hydrophobic molecules such as oils and organic volatiles. Films from hydrophilic proteins, such as soy protein, whey protein, fish protein, and pea protein, exhibit low to moderate barrier property against moisture and may become degraded upon being exposed to high humidity condition (Pooja et al., 2019). Higher humidity increases the plasticizing impact of water, which reduces the tensile strength and increases the extensibility of hydrophilic films, and also renders them more susceptible to moisture absorption (Cho & Rhee, 2002). Soy protein film has drawbacks of low mechanical strength, high brittleness, and poor moisture resistance (Jin et al., 2020). Few proteins, like corn zein, are hydrophobic and soluble only in non-polar solvents, such as alcohol. Due to the high proportions of non-polar amino acid residues, zein-based film shows better water resistance as compared to other protein films (More et al., 2016).

Molecules with regular structure are more diffusible than the ones with irregular stereochemical structure. Lower molecular weight fraction exhibits greater

cohesion. Being a highly polar polymer, self-adhesion by diffusion is insignificant in protein due to their less flexibility and fixed order structure. This is due to internal molecular forces that hold the polymer chains together. The film-forming ability of proteins is influenced by their polarity, amino acid profile, distribution, as well as interactions between side groups (Condés et al., 2013).

2.6.2 Protein concentration

Various types of protein-protein interactions, such as covalent bond, hydrogen bond, electrostatic and hydrophobic interactions, and Van der Waals force, can result in different types of cohesiveness which affect the protein mobility and film-forming ability (Wittaya, 2012). Films with higher protein concentration usually have higher moisture content and mechanical quality. Even though transparency of the films normally reduces with increasing protein concentration (Shroti & Saini, 2022). Chang & Nickerson, (2015) found a similar impact of protein concentration on canola protein-based film. Higher protein concentration influences protein-protein interactions that lead to protein aggregation, with a production of huge void space, resulting in an increase in moisture content of the film. A higher degree of protein-protein interactions results in increasing tensile strength (Shroti & Saini, 2022). According to Wittaya (2012), the self-adhesion of polymers and the extent of polymer matrix formation are both influenced by the film-forming solution concentration. Kaewprachu et al. (2016) demonstrated that as fish myofibrillar protein concentration increased, the film exhibited greater tensile strength, elongation at break, and water vapor permeability. Similar findings were also reported for gelatin films from beef, pork, and fish (Hanani et al., 2012), fish skin gelatin film (Jongjareonrak et al., 2006), and fish sarcoplasmic protein film (Iwata et al., 2000).

2.6.3 Plasticizers

Plasticizer is one of the fundamental components for the formulation of protein-based films (Kaewprachu & Rawdkuen, 2014). In general, biopolymer films are brittle due to the various interactions among the polymer chains, such as disulfide bond, hydrogen bond, hydrophobic interaction, and electrostatic interaction. Therefore, a plasticizer is usually added to facilitate the formation of uniform and flexible film by reducing chain-to-chain interactions and increasing free volume and

chain mobility (Vieira et al., 2011). Physical properties of protein films are strongly influenced by type, polarity, and amount of plasticizer used.

2.6.4 Other additives

Biopolymer films can also be used as a carrier of many types of additives. For example, the incorporation of antimicrobial agents into biopolymer films has been extensively studied (Cha & Chinnan, 2004; Rojas-Graü et al., 2009; Gómez-Guillén et al., 2009). Other additives, such as antioxidants, anti-browning agents, nutraceuticals, texture enhancers, as well as flavoring and coloring ingredients, can also be added to enhance the functional and organoleptic properties of the films and/or the packaged foods (Olivas & Barbosa-Cánovas, 2005; Lin & Zhao, 2007; Rojas-Graü et al., 2009). Addition of certain additives, like lipids and cross-linking agents, can significantly alter the film mechanical strength, extensibility, and barrier property (Ahammed et al., 2021).

With the development of nanotechnology, a variety of nano-sized filler materials, including nanoclays, nanometals, nanofibers, and nanoparticles, have been added to enhance mechanical and barrier properties of biopolymer films (Castro-Rosas et al., 2016). Moreover, incorporation of other biopolymers can also be done to produce a composite film with improving properties. Erickson et al. (2014) modified properties of zein-based film by adding other proteins. Formulation of composite film by combining gliadin with zein was reported to increase the film flexibility (Gu & Wang, 2013). Orliac et al. (2002) investigated the effects of various additives (aldehydes, plant tannins, alcohols, and fatty acids) on hydrophobicity, mechanical properties, and water uptake of thermo-molded sunflower protein isolate film. It was reported that octanoic acid yielded a film with the highest tensile strength while addition of octanol resulted in a significant increase in elongation. Incorporation of fatty alcohols reduced the film solubility while increasing surface hydrophobicity and mechanical properties. Addition of plant tannins gave a film with similar mechanical strength to those added with aldehydes.

2.6.5 pH of the film-forming solution

Functionality and structure of polymers are highly influenced by solution properties which further affect the film characteristics. At pH value above

their isoelectric point, proteins have a net negative charge, while a net positive charge appears below their isoelectric point. A protein has zero net charge at its isoelectric point, which leads to aggregation and precipitation. Protein solubility becomes increasing as pH moves away from its isoelectric point. (Wihodo & Moraru, 2013). Several studies explored the effect of pH on the film-forming ability of protein solutions (Brandenburg et al., 1993; Pérez-Gago & Krochta, 1999) and on the properties of the resulted films (Avena-Bustillos & Krochta, 1993). Gennadios et al. (1993) studied the effect of pH on properties of soy protein isolate film and found that highly acidic (pH<1) or alkaline (pH>12) conditions inhibit the formation of soy protein isolate film. Kinsella & Phillip (1979) reported that films formed near the isoelectric point of major proteins are more condensed and stronger. Jimenez et al. (2019) demonstrated that an increase in pH and the presence of additives improved water uptake capacity and increased both mechanical strength and water barrier property of wheat gluten film. High pH increased the film solubility, transparency, tensile strength, elongation at break, and puncture strength whereas swelling capacity, water activity, and water vapor permeability became decreasing. Shroti & Saini (2022) found that brewer's spent grain protein film formulated at higher pH condition contained more moisture than the films prepared at lower pH. Kumari et al. (2021) suggested that an increase in protein film solubility, tensile strength, and elongation at break at higher pH was due to the dissociation and reaggregation of protein subunits resulting in enhanced flexibility of the films.

2.6.6 Drying temperature

Besides heat and mass transfers, drying at elevated temperature also induces physicochemical changes that could alter the structure and physical characteristics of the materials (Jafari et al., 2016a,b). Proteins undergo conformational changes because of the continual evaporation of water during drying. Moreover, type and proportion of covalent bond (disulfide) and non-covalent interactions (hydrogen bond, ionic interaction, and hydrophobic interaction) between protein chains depend on the degree of changes in protein conformation (Dehnad et al., 2016). Water-soluble proteins, such as whey and soy proteins, require higher temperature and longer time to produce film than alcohol-soluble protein, like wheat

gluten and corn zein. However, too high temperature or too high solvent evaporation rate may result in a film with discontinuous matrix and inferior properties (Laovachirasuwan et al., 2010).

2.7 Modification of protein films

2.7.1 Heat treatment

Heating is among the common methods for modifying protein structural and functional properties. In respect of materials science, this treatment is often described as heat curing which is, according to Soroka, (2009), a process where a substrate is exposed to one or more heating cycles aimed at changing the molecular structure and rearranging the polymers. In terms of proteins, mild heating condition promotes protein unfolding, leading to an intermediate molten globule state with enhanced functionality. However, extreme thermal treatment causes irreversible changes in the protein structures, resulting in its denaturation and aggregation through different inter- and intra-molecular interactions including disulfide bond, hydrophobic interaction, and electrostatic interaction. During thermal treatment of a protein solution, as a consequence of the unfolding of polypeptide chains, the internal sulfhydryl groups and the hydrophobic side chains, previously buried in the core of the native-state structure, become more exposed (Aryee et al., 2018; Wang et al., 2017). The properties of the resulting protein network strongly depend on the temperature and also on the ionic strength and the presence of other molecules (Nicolai et al., 2011). However, the denaturation temperature strongly depends on protein concentration and solvent properties (Renkema et al., 2000). Thermal denaturation of gluten proteins begins at 90°C (Singh & MacRitchie, 2004), and for soy proteins, also at 90°C (Lakemond et al., 2000). In an extrusion process, soy protein was reported to denature at 120°C (Guo et al., 2015). Several studies examined the impact of heating on properties of the film-forming solutions (Stuchell & Krochta, 1994; Pérez-Gago et al., 1999; Pérez-Gago & Krochta, 2001; Liu et al., 2004), or on properties of the protein films (Micard et al., 2000). A handful of studies reported an adverse effect of heat treatment on the film functional properties (Chao et al., 2018; Lv et al., 2017). However, protein films processed under increased temperature generally show significantly increasing tensile strength (Zubeldía et al.,

2015; Kim et al., 2002; Micard et al., 2007; Liu et al., 2004; Sothornvit et al., 2007). There are also reports that heat curing of protein films increased their elongation at break. Heat treatment of soy protein prior to film formation was reported to produce a film which is smoother and more transparent, with reduced water vapor permeability (Kim et al., 2002; Rhim et al., 2000; Stuchell & Krochta, 1994). Stuchell & Krochta (1994) compared the properties of soy protein films made from uncured film-forming solution and those heat cured at 80°C. Although the differences were not statistically significant, their results showed that, as a trend, the heat-cured soy protein films exhibited greater tensile strength, lower water permeability, and higher elongation at break than their uncured counterpart. The study by Pérez-Gago & Krochta, (2001) revealed that oxygen permeability of whey protein isolate film made from film-forming solution which was heat-cured at 90°C for 30 min was significantly lower than that of the film made from uncured solution. Interestingly, water vapor permeability of the films was not affected by the heat treatment (Pérez-Gago et al., 1999). Pérez-Gago et al. (1999) and Pérez-Gago & Krochta (2001) also demonstrated that whey protein films made from film-forming solutions heated at 70-100°C for 5-20 min were stronger and more extendible. The ability of heat-treated whey protein films to withstand higher deformation may be due to the unfolding of the globular structure of whey protein, which exposes the sulfhydryl groups and enables the formation of strong covalent disulfide intermolecular bonds.

Apart from film-forming solution, heat curing could also be applied to pre-formed film. It was reported that water-holding capacity of faba bean protein was modified upon dry heat treatments at 75-175°C (Bühler et al., 2020). Thermal treatment was also reported to successfully improve gel-forming ability of cowpea protein (Peyrano et al., 2017), and album seed protein isolate (Mir et al., 2020). Micard et al. (2000) treated pre-formed wheat gluten films at 80, 95, 110, and 125°C for 15 min and at 140 °C for 1.5 and 15 min. It was demonstrated that heat- films had significantly higher tensile strength than untreated samples. Heated pre-formed films also had significantly lower elongation at break than the unheated films, which is contrary to the studies of Stuchell & Krochta, (1994), Pérez-Gago et al. (1999), and Pérez-Gago & Krochta (2001). Additionally, the wheat gluten films heated at 140°C

for 15 min were significantly stronger, but less extendible than those heated for 1.5 min. Micard et al. (2000) reported that applying heat to pre-formed films did not alter water vapor permeability of the final films.

2.7.2 High pressure treatment

High hydrostatic pressure (HHP) processing uses water as a medium to transfer pressure to the material under isothermal condition (Lorido et al., 2015). Among different purposes of using HHP, such as texture modification, emulsification, and microbial inactivation, another important application is its ability to modify food proteins by the breakage of hydrophobic and electrostatic interactions as well as the formation of new interactions which result in protein aggregation and subsequently gelation (Lv et al., 2020; Doost et al., 2019). The effect of pressure on proteins is often described by the principle of Le Chatelier, whereby a system reduces its free energy by minimizing the effect of the external factor. Consequently, a change in pressure is compensated by modification of the system volume (Mozhaev et al., 1996). HHP treatment typically increases the protein hydrophobicity and decreases its solubility due to the ability to expose buried sulfhydryl groups after unfolding and denaturation, which is followed generally by aggregation, gelation, or improvement of its techno-functional properties (Queiros et al., 2018). In terms of these structural changes, Lee et al. (2016) demonstrated an increased surface hydrophobicity and sulfhydryl group content of ginkgo seed protein as well as secondary structural changes after HHP treatment, which resulted in improved heat stability and emulsifying property. Although there are some studies showing decreased solubility of plant-based proteins after HHP, especially at higher applied pressures (>400 MPa) due to protein aggregation (Condes et al., 2015; Zhao et al., 2015), some other works indicated a positive effect of this process on protein solubility (Liu et al., 2020d). For instance, Cao et al., (2017) found an improvement in solubility, water holding capacity, and oil holding capacity of pine nut protein upon 200- and 400-MPa HHP treatments. In the case of kidney bean protein, HHP treatments at a pressure higher than 600 MPa had a significant effect on secondary structure of the protein as revealed by the FTIR spectroscopy. This change in protein structure significantly

improved water holding capacity, foaming capacity, and emulsifying property of the protein (Ahmed et al., 2018).

Piccini et al. (2019) compared HHP to thermal treatment for modification of calcium-added soy protein. It was revealed that HHP improved protein solubility and colloidal stability, as compared to the conventional thermal treatment. HHP-treated samples were also able to form transparent cold-set gels with excellent water holding capacity. Speroni et al. (2009) showed that soy β-conglycinin and glycinin formed hydrophobic interactions and disulfide bonds during HHP processing. Lee et al. (2007) suggested that high pressure affects protein conformation and dissociates large aggregates, exposing hydrophobic groups by unfolding and allowing the formation of inter- and intra-molecular disulfide bonds. To date, reports about HHP treatment on protein films are scarce. However, some researchers have investigated the effect of HHP treatment on similar systems, like protein gels. Camp et al. (1996) produced whey protein gels with comparable strength to those induced by heat and even stronger at high protein concentration. Compared to thermal processing, pressure-induced β-lactoglobulin gels appear to possess more porous and thicker stranded structure with weaker intermolecular interactions. The resulting gels have higher water exudation and water solubility, lower rigidity, and the proteins tend to aggregate during storage (Tedford & Schaschke, 2000).

In a different study, Zhao et al. (2018) evaluated the combined effect of salt addition and HHP on sweet potato protein and reported the improvement of the formed gels in terms of textural property and water-holding capacity. Similarly, the authors also observed a positive effect of sulfur-containing amino acids and HHP as a modification method on the textural properties of sweet potato protein gels.

2.7.3 Ultrasonic treatment

An acoustic wave with a frequency greater than 20 kHz, which is above human auditory detection, is referred to as an ultrasound (Corso et al., 1963). During ultrasound treatment, acoustic waves are transmitted through solid, liquid, or gaseous systems. Ultrasould treatment can be categorized into different types based on frequency, intensity, and application (Cárcel et al., 2012). At high intensity, ultrasound treatment cavitation occurs due to the compression and decompression

cycles of the sonic waves, which produce high shearing force through the formation and implosion of gas bubbles that are strong enough to break down polymer chains dissolved in solution (Maniglia et al., 2021). Gas bubbles cause microstreaming, which promotes the convection of reactive components and speeds up chemical reactions occurring in proteins (Coleman & Roy, 2014). As a side effect, local temperature increases contributing to the modification effect and thermal decomposition or sonolysis of water (Maniglia et al., 2021). Protein denaturation by high-intensity ultrasound is mostly caused by water sonolysis in conjunction with shear stress. For different carbohydrate- and protein-based films or coatings, ultrasound treatment has been shown to be able to improve gelling and tensile properties, as well as increase solubility and surface hydrophobicity, and reduce water vapor permeability (Wang et al., 2020; Brodnjak, 2017). Kadam et al. (2013) explored the ultrasound effect on nanoparticle- containing whey protein isolate films. Whey protein and nanoparticles were sonicated at different amplitudes prior to casting to get the nanoparticles homogeneously distributed in whey protein isolate films. It was shown that sonication improved nanoparticle distribution in the film matrix. It was also found that increasing sonication amplitudes significantly improved film strength, elasticity, and hydrophobicity, whereas water vapor permeability remained unchanged. The effect of ultrasound-treated whey protein coating on the frozen fish quality was inspected by Rodriguez et al. (2012). It was revealed that frozen fish with sonicated whey protein coating underwent lower lipid oxidation as compared to the untreated fish. Jambrak et al. (2009) investigated the effect of ultrasound treatment using either ultrasound probe at 20 kHz or ultrasound bath at 40 and 500 kHz on physical properties of soy protein isolate and concentrate. It was found that solubility of soy protein concentrate increased after ultrasonic treatment, which also caused significant changes in conductivity, with an increase in specific surface area and emulsion activity index. Wang et al. (2013) attributed the improvement of water and oxygen barrier properties of soy protein-based films to the formation of free hydroxy radicals upon sonication. Moreover, Wang et al. (2014) reported an improvement in surface hydrophobicity and film density of ultrasound-treated soy protein-based films. According to Hu et al. (2013), surface hydrophobicity, protein solubility, and free

sulfhydryl content of soy protein isolate solutions increased with time under high-frequency ultrasound.

2.7.4 Irradiation

2.7.4.1 Gamma irradiation

Gamma irradiation, a non-thermal process, aside from prolonging shelf-life by reducing microorganisms, has the capability to trigger a chemical alteration in proteins beyond physical aggregation, including crosslinking and fragmentation (Han et al., 2018). Upon interacting with ionizing radiation, such as gamma radiation, water molecules are transformed into free radicals and high energy electrons that could induce cross-linking and hydrolysis of the polymer chains (Bashir & Aggarwal, 2019). The hydroxy and superoxide anion radicals produced by gamma radiation can cause alterations in the protein primary, secondary, tertiary, and even quaternary structures (Han et al., 2018). Baccaro et al. (2018) noticed changes in secondary and tertiary structures as well as chemical composition of rice protein upon gamma irradiation. The authors suggested that, with increasing in absorbed dose, unfolding of protein chain and production of new molecules increased as a result of the interaction between amino acids, such as tyrosine and tryptophan, and the radical species. Xu et al. (2012) developed biodegradable molded material from soy protein isolate and starch and reported that gamma irradiation is a useful cross-linking measure to improve properties of this soy protein-starch mixture. Increased cross-linking was noted with increasing radiation dose, resulting in an increase in tensile strength and water resistance of the material.

Effect of gamma radiation on sunflower protein isolate was investigated by Malik et al. (2017). It was found that gamma radiation induced conformational changes due to crosslinking and aggregation of protein molecules with changes in α-helix and β-sheet contents. The altered protein functionality was a result of changes in the secondary and tertiary structures of the protein. Thermal stability and molecular weight of protein were found to increase due to radiation-induced protein crosslinking. Malik & Saini (2017) also applied gamma radiation on sunflower protein isolate and reported an improvement in surface hydrophobicity, antioxidant capacity, emulsifying property, foaming capabily, and oil binding

capacity, while a reduction in water binding ability was observed. In another study, Hassan et al. (2018) reported an improvement in emulsifying property of gamma-irradiated sesame proteins. Yao et al. (2022) examined the impact of radiation dose (0, 0.5, 1, 2, 3, 5 kGy) on functional and physicochemical attributes of gamma-irradiated rice protein. It was revealed that the physicochemical and functional characteristics of rice protein can be significantly improved by gamma radiation treatment. The rice protein was found to exhibit the greatest solubility, water- and oil-holding capacity, as well as emulsifying activity and emulsifying stability at a radiation dose of 2 kGy. The authors also noted that sensory quality of the rice protein was not affected if the radiation dose of lower than 5 kGy was used.

2.7.4.2 Ultraviolet (UV) irradiation

UV is an electromagnetic radiation with a wavelength in the range of 100-400 nm and a frequency in the range of 10^{15}-10^{18} Hz. There are three types of UV radiation, UV-A, UV-B, and UV-C.

UV-A or long-wave UV, generally known as black light, has a wavelength in the range of 315-400 nm, with a photon energy of 3.10-3.94 electron volts (eV) or 0.497-0.631 attojoules (aJ), the lowest energy among the three types of UV. Solar UV-A is not absorbed by the ozone layer of the atmosphere and it can penetrate to the dermis of the skin. UV-B or medium-wave UV, also known as sunburn radiation, has a wavelength in the range of 280-315 nm, with a photon energy of 3.94-4.43 eV or 0.631-0.710 aJ. Only 0.3% of the UV-B emitted by the sun could reach the ground. It is mostly absorbed by the skin epidermis and does not penetrate further. Both UV-A and UV-B are known as the cause of skin aging, melanoma and some other types of skin cancers. For UV-C, it is also called short-wave UV or germicidal UV. The radiation is of the wavelength of 100-280 nm and has photon energy of 4.43-12.4 eV or 0.710-1.987 aJ, the highest energy among the three. Solar UV-C is totally absorbed by the ozone layer and never reaches the ground. The radiation is widely used to kill microorganisms by disrupting their DNA and vital cellular functions.

A majority of the studies use UV-C to modify proteins because of its high energy. The advantages of UV treatment are that it is cheap, easy to use,

safe, and environmentally friendly. UV radiations can be absorbed by side group of aromatic amino acids, like tyrosine (Tyr), releasing the amino acid free radicals (Tyr·), which upon recombination resulting in a dityrosine cross-link (Tyr-Tyr) (Liu et al., 2019).

UV treatment is typically applied through a UV-penetrable tube using turbulent flow for liquid product or laminar flow for thin layer film. Fathi et al. (2018) examined the application of UV-A, UV-B, and UV-C on sesame protein isolate edible films. The UV radiation was applied to either film-forming solutions or pre-formed films. Solubility, water vapor permeability, and moisture content of irradiated films were found to decrease. On the other hand, film density, surface hydrophobicity, and mechanical properties were found to increase as compared to the uncured control. The dry film obtained from UV-C irradiated film-forming solution exhibited the highest tensile strength (8.29 MPa) and Young's modulus (118.35 MPa). This indicated that UV-C application on film-forming solution was more effective than other types of UV radiation and then the application on pre-formed films. In another study, Schmid et al. (2015) investigated the effect of different UV-C doses (1.2-42 J/cm^2) on properties of whey protein isolate-based film. It was found that tensile strength, Young's modulus, and yellowness increased with increasing radiation dose. Meanwhile, there were no significant changes in water vapor and oxygen barrier properties, as well as elongation at break of the irradiated films as compared to the untreated control.

2.7.5 Chemical modification

Structurally, proteins contain a wide range of functional groups that can serve as reactive sites for chemical modification. Chemical modification of food proteins has been widely used due to its efficiency, low cost, and ease of operation.

To improve the mechanical properties of protein films, various external cross-linking agents have been used, such as glutaraldehyde (Huang & Netravali, 2007), epichlorohydrins (Zhong et al., 2007), genipin (González et al., 2011), glutaric dialdehyde (Fang et al., 2012), and phenolic acids (Insaward et al., 2014). For example, Friesen et al. (2014) modified soy protein isolate edible films using phenolic compounds, rutin, and epicatechin as cross-linking agents. Rutin addition resulted in a

film with improved puncture strength (9.3 N) as compared to the control (6.4 N). On the other hand, addition of epicatechin was found to pose no effect on puncture strength of the film.

2.7.6 Enzymatic modification

Enzymes are proteins with catalytic activity that are produced by organisms and have the ability to catalyze a reaction. In general, enzymes are either unique to the substrate of the catalyzed reaction or the reaction itself. Changes in temperature and pH can affect enzyme activity. In the case of non-spontaneous reactions, enzymes lower the activation energy and accelerate the rate of reaction (Belitz & Grosch, 2013).

Different types of enzymes can be used for protein modification through cross-linking, e.g., peroxidase (EC1.11.1.7) and transglutaminase (EC2.3.2.13). Xu et al. (2021) modified whey protein film using transglutaminase. It was shown that transglutaminase could improve tensile strength and elongation of the film. Kouravand et al. (2020) investigated the effect of microbial transglutaminase addition (0, 5, 10, and 15 units/g protein) on properties of whey protein isolate film. Lower doses of transglutaminase (5 and 10 units/g protein) were found to significantly improve mechanical properties of the film as compared to the control. Meanwhile, a slight decrease in tensile strength was noticed at higher transglutaminase concentration (15 units/g protein). By increasing the enzyme concentration from 5 to 10 units/g protein, water vapor transferability and water-soluble fractions decreased significantly ($p \leq 0.05$). The films that were treated with transglutaminase exhibited a uniform, even surface with the exception of the film that was added with 15 units of transglutaminase/g protein.

Stuchell & Krochta (1994) monitored protein cross-linking in soy protein isolate film by peroxidase. It was found that the films added with peroxidase possessed higher Young's modulus. In spite of that, tensile strength and elongation at break were found to decrease. Peroxidase treatment was found to pose no effect on water vapor barrier property. As monitored using an SDS-PAGE, peroxidase treatment brought about protein cross-linking, but at the same time, it also induced protein degradation.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

Soy protein isolate, food-grade, 90% protein (wet basis), Krungthep Chemi (Bangkok, Thailand)

Glycerol, food-grade, Krungthep Chemi (Bangkok, Thailand)

3.2 Equipment

Chroma meter, model CR-400 (Konica Minolta Sensing, Osaka, Japan)

Confocal Raman microscope, model XploRA PLUS (Horiba, Loos, France)

Homogenizer, model X10/25 (Ystral, Ballrechten-Dottingen, Germany)

Hotplate magnetic stirrer, model MS-H280-Pro (Scilogex, Rocky Hill, CT, USA

Laboratory hot air oven, model 5200 (Kubota, Fujioka, Japan)

Laboratory shaker, Innova®, model 2050 (New Brunswick Scientific, Edison, NJ, USA)

Optical contact angle measuring and contour analysis systems, model OCA15EC (Data Physics Instrument, Filderstadt, Germany)

Scanning electron microscope, model JSM-IT300 (JEOL, Tokyo, Japan)

Spectrofluorometer, model FP-6200 (Jasco, Tokyo, Japan)

Texture analyzer, model TA.XTplus (Stable Micro Systems, Godalming, UK)

Thickness gauge, model 7301 (Mitutoyo, Tokyo, Japan)

Ultrasonic bath, model 136H (Fisher Scientific, Schwerte, Germany)

UV-C cabinet, model PIS-88C (P Inter Supply, Bangkok, Thailand)

UV-C light meter, model TM-218 (Tenmars Electronics, Taipei, Taiwan)

UV-Vis spectrophotometer (model GENESYS20, Thermo Scientific, Waltham, MA, USA)

Water bath, model SW23 (Julabolabortechnik, Seelbach, Germany)

3.3 Methodology

This study was divided into two parts. The first part dealt with exploring the effect of heat curing on soy protein film properties. The heat was applied to either film-forming solution or pre-formed film. In the second part, the effect of UV-C radiation on the properties of heat-cured soy protein film was investigated. Heat-cured film-forming solutions and heat-cured pre-formed films were subsequently treated with UV-C at varying doses.

3.3.1 Effect of heat curing on properties of soy protein film

Film samples were prepared according to the method described earlier (Shakil & Mahawanich, 2022) with some modifications. Soy protein film without heat curing treatment was used as a control. To prepare the control film, 5% (w/w) film-forming solution was prepared by dissolving 5 g of soy protein isolate and 2.75 g of glycerol in 92.25 g of phosphate buffer (pH7.4). The mixture was homogenized using a homogenizer (model X10/2, Ystral, Ballrechten-Dottingen, Germany) at 22,000 rpm for 2 min. The solution was then heated at 70°C for 30 min to partly denature the protein. After being cooled to room temperature (25°C), the solution was homogenized again at 22,000 rpm for 2 min. Air bubbles were removed using an ultrasonic bath (model 136H, Fisher Scientific, Schwerte, Germany). After that, the film-forming solution (45 mL) was casted on an acrylic mold (150 mm×150 mm) and dried at 40°C for 24 h. The film sample was subsequently removed from the mold and equilibrated at 50% RH for 48 h before being subjected to property analyses.

For heat-cured film samples, a combination of heating temperature (60, 70, and 80°C) and heating time (2, 4, and 6 h) was applied on either film-forming solution (FS) or pre-formed film (PF). In the case of film-forming solution, after protein denaturation step, heat was applied to the solution using a water bath at a specified temperature for a specified period. Then, the solution was cooled to room temperature (25°C) and homogenized at 22,000 rpm for 2 min. After air bubble

removal, an aliquot (45 mL) of heat-cured film-forming solution was transferred to an acrylic mold (150 mm×150 mm) and dried at 40°C for 24 h. The film sample was then removed from the mold and equilibrated at 50% RH for 48 h before further analyses.

For the pre-formed film samples, the films were prepared using the same protocol as the control. After being dried at 40°C for 24 h, the films were subjected to heat curing at a specified temperature and time. The film samples were later equilibrated at 50% RH and 25°C for 48 h and analysed for their properties. Table 3.1 summarizes the heat-curing conditions used in this study.

Table 3. 1 Heat-curing conditions of the film samples

Film samples*	Heat-curing conditions	
	Temperature (°C)	Time (h)
Control	-	-
FS 60/2 and PF 60/2	60	2
FS 60/4 and PF 60/4	60	4
FS 60/6 and PF 60/6	60	6
FS 70/2 and PF 70/2	70	2
FS 70/4 and PF 70/4	70	4
FS 70/6 and PF 70/6	70	6
FS 80/2 and PF 80/2	80	2
FS 80/4 and PF 80/4	80	4
FS 80/6 and PF 80/6	80	6

* Control: soy protein film without heat curing

FS *x*/*y*: film sample obtained by heat-curing of film-forming solution at *x*°C for *y* h

PF *x*/*y*: film sample obtained by heat-curing of pre-formed film at *x*°C for *y* h

The film samples were subjected to the following analyses:

3.3.1.1 Thickness

Film samples were cut into a 100 mm×30 mm strip. Thickness was measured using a thickness gauge (model 7301, Mitutoyo, Tokyo, Japan). To determine thickness of the film sample, measurements were taken at ten random

positions across the film strip. The measurements were averaged and taken as thickness of each replicate.

3.3.1.2 Mechanical properties

Mechanical properties of the film samples were analyzed using a tensile test following the ASTM D882 standard method (ASTM, 2018). The test was carried out using a Texture Analyzer (model TA.XTplus, Stable Micro Systems, Godalming, UK) equipped with tensile grips (A/TG) probe and initial clamp distance of 50 mm. A 30 mm×100 mm film strip was mounted onto both grips, ensuring that the film sample was held securely. The film sample was then pulled at a constant speed of 8.33 mm/s until failure. The maximum force required to pull the sample apart (in g_f) and the distance that the sample stretched to its maximum extent before failure (in mm) were used to calculate the tensile strength and elongation at break according to Equations (3.1) and (3.2):

$$\text{Tensile strength (MPa)} = \frac{F\,(0.009807\times10^{-6})}{w\,d} \quad \ldots(3.1)$$

where F is the maximum force applied before failure (g_f); w is the film width (m); and d is the film thickness (m).

$$\text{Elongation at break (\%)} = \frac{L_f}{L_i} \times 100 \quad \ldots(3.2)$$

where L_f is the distance that the sample stretched to its maximum extent before failure (mm); and L_i is the initial length of the film between the grips (mm).

3.3.1.3 Color

A chromameter (model CR400, Konica Minolta Sensing, Osaka, Japan) was used to determine the film color in the CIELAB system with 10° observer angle and standard illuminant D65 (daylight illuminant). Measurements were taken at ten random places on each film sample and averaged to represent the color values of each replicate. Total color differences ΔE, hue angle, and chroma were calculated from the CIE L*, a*, b* using Equations (3.3), (3.4), and (3.5):

$$\text{Total color difference } (\Delta E) = \sqrt{(L^* - L_0^*)^2 + (a^* - a_0^*)^2 + (b^* - b_0^*)^2} \quad \ldots(3.3)$$

where L^*_0, a^*_0, and b^*_0 are color parameters of the control; and L^*, a^*, b^* are color parameters of the treated film sample.

Hue angle = arctan (b*/a*) …(3.4)

Chroma = $(a^{*2} + b^{*2})^{1/2}$ …(3.5)

3.3.1.4 Transparency

Transparency of the film samples was expressed as %transmittance. A film sample was cut into an exact dimension of a side of a quartz cuvette (10 mm×40 mm) and then mounted onto the inside of the cuvette. Empty cuvette was used to set the spectrophotometer to 100% transmittance. %Transmittance was measured at 500 nm using a UV-Vis spectrophotometer (model GENESYS20, Thermo Scientific, Waltham, MA, USA).

3.3.1.5 Water vapor permeability

Water vapor permeability of the film samples was determined following the ASTM E96-95 method (ASTM, 2017). A film sample free of pinholes and scratches was cut into a 60 mm×60 mm piece. A glass permeation cup was filled with 20 g of dried silica gel. High vacuum silicone grease was sparingly applied to the rim of the cup. The film piece was mounted onto the cup and tightened with a rubber O-ring and Parafilm®. The sample cup was then weighed, placed in a chamber containing distilled water, and equilibrated at 25°C. The weight of the permeation cup was taken every 24 h for 7 days and calculated for water vapor permeability using equation (3.6):

$$\text{Water vapor permeability} = \frac{W\,d}{A\,t\,(P_2 - P_1)} \quad \ldots(3.6)$$

where W is weight gain of the permeation cup (g); d is the film thickness (m); A is exposed area of the film available for water permeation; t is the time to reach equilibrium (h); and (P_2-P_1) is the difference in partial pressure of water vapor across both sides of the film (Pa).

3.3.1.6 Water solubility

Water solubility of the film samples, expressed in terms of total soluble matter, was determined according to the method described by Insaward et al.

(2015). A 20 mm×20 mm piece of film sample, along with Whatman grade 4 filter paper, were first dried in a hot air oven at 70°C for 8 h. After being weighed for its initial weight, the film sample was transferred to a test tube containing 20 mL of distilled water. The test tube was then placed on a laboratory shaker (Innova®, model 2050, New Brunswick Scientific, Edison, NJ, USA) and continuously shaken at room temperature (25°C) for 24 h. After that, the suspension was filtered through a pre-dried and weighed Whatman grade 4 filter paper and washed with 10 mL of distilled water. The film residue, together with the filter paper, were dried at 70°C for 24 h and then weighed to obtain to weight of the film residue. Total soluble matter was calculated using Equation (3.7):

$$\%\text{Total soluble matter} = \frac{W_i - W_f}{W_i} \times 100 \quad \ldots(3.7)$$

where W_i is initial weight of the dried film; and W_f is weight of the film residue

3.3.1.7 Surface hydrophobicity

Film surface hydrophobicity is expressed in terms of contact angle between a water droplet and the film surface, which was evaluated using ooptical contact angle measuring and contour analysis systems (model OCA15EC, Data Physics Instrument, Filderstadt, Germany). After placing the film sample on the instrument sample table, a droplet (4 μL) of distilled water was dosed on the film surface and the contact angle of the water droplet to the film surface (θ) was measured, with θ<90° and θ>90° characterizing hydrophilic and hydrophobic surfaces, respectively.

3.3.1.8 Cross-sectional microstructure

Microstructure of the film samples were analysed using a scanning electron microscope (model JSM-IT300, JEOL, Tokyo, Japan). A film sample was cut using a sharp razor blade to expose a clean cross-sectional area. The sample was mounted on a sample stub where the cross section was in the upward direction. Then the sample was coated with gold and examined using 10 kV accelerating voltage with 1000× magnification.

3.3.1.9 Disulfide cross-linking

S-S bonding was monitored using a confocal Raman microscope (model XploRA PLUS, Horiba, Loos, France) with 100× objective lens and helium-neon (HeNe) laser at 785 nm. The acquisition time was 1.42194 s.

For this part, all experiments were done in three replicates. Data were analysed using Analysis of Variance (ANOVA). A Duncan's new multiple range test was used to determine the difference among sample means at p=0.05 using SPSS Statistics 22.0 (IBM, Armonk, NY, USA).

From the results of this part, based on tensile strength, one heat-curing condition (70°C for 4 h) was selected to be used in 3.3.2.

3.3.2 Effect of UV-C curing on properties of heat-treated soy protein film

Film samples prepared by heat-curing of either film-forming solution or pre-formed film at 70°C for 4 h were used as the base films for UV-C curing experiment. The control was soy protein film without heat- and UV-C curing.

UV-C treatment was applied on either film-forming solution or pre-formed films at different doses (Table 3.2). The irradiation treatment was carried out in a UV-C cabinet (model PIS-88C, P Inter Supply, Bangkok, Thailand) at 253.7 nm wavelength and 2500 μW/cm^2 radiation intensity.

For UV-C curing of the film-forming solution, the solution was first heat-cured at 70°C for 4 h and transferred to a mold according to the protocol in 3.3.1. The UV-C radiation was then applied on the thin-layer film-forming solution at a specified time, i. e. at a specified radiation dose. The solution was then dried at 40°C for 24 h. After removal from the mold, the film was equilibrated at 50% RH and 25°C for 48 h and analysed for its properties according to 3.3.1.1 to 3.3.1.8. The film was also monitored for dityrosine cross-linking as outlined in 3.3.2.1.

Table 3. 2 UV-C curing conditions of the film samples

Film samples*	Heat curing	UV-C curing	
		Radiation dose (J/cm^2)	Irradiation time (min)
Control	-	-	-
HTFS 70/4	70°C/4 h	-	-
HT+FSUV 4	70°C/4 h	4	26
HT+FSUV 8	70°C/4 h	8	53
HT+FSUV 12	70°C/4 h	12	80
HT+FSUV 16	70°C/4 h	16	106
HTPF 70/4	70°C/4 h	-	-
HT+PFUV 4	70°C/4 h	4	26
HT+PFUV 8	70°C/4 h	8	53
HT+PFUV 12	70°C/4 h	12	80
HT+PFUV 16	70°C/4 h	16	106

*Control: soy protein film without heat curing and UV-C curing

HTFS70/4: the film obtained from film-forming solution that was heat cured at 70°C for 4 h, but without UV-C curing

HT+FSUV*i*: the film obtained from film-forming solution that was heat cured at 70°C for 4 h, and subsequently UV-C cured at a radiation dose of *i* J/cm^2

HTPF70/4: the film obtained from pre-formed film that was heat cured at 70°C for 4 h, but without UV-C curing

HT+PFUV*i*: the film obtained from pre-formed film that was heat cured at 70°C for 4 h, and subsequently UV-C cured at a radiation dose of *i* J/cm^2

For UV-C curing of the pre-formed film, after drying, the film obtained was heat-cured at 70°C for 4 h as described in 3.3.1. The UV-C radiation was then applied on the pre-formed film at a specified radiation dose. The film was then equilibrated at 50% RH and 25°C for 48 h and analysed for its properties according to 3.3.1.1 to 3.3.1.8 and 3.3.2.1.

3.3.2.1 Dityrosine cross-linking

A spectrofluorometer (model FP-6200, Jasco, Tokyo, Japan) was used to monitor dityrosine cross-linking with an excitation wavelength of 320

nm. Emitted fluorescence, characteristic of dityrosine, can be detected in the emission wavelength of 340-500 nm (Al-Hilaly et al., 2016). The scanning speed of the spectrofluorometer was set at 125 nm/min.

For this part, all experiments were done in three replicates. Data were analyzed using ANOVA. A Duncan's new multiple range test was used to determine the difference among sample means at p=0.05 using SPSS Statistics 22.0 (IBM, Armonk, NY, USA).

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Effect of heat curing on properties of soy protein film

In this part of the study, the effect of heat curing of either film-forming solution (FS) or pre-formed film (PF) was investigated. Heating temperature was varied as 60, 70, and 80°C and heating time was varied as 2, 4, and 6 h. Properties of heat-cured soy protein films are as follow.

4.1.1 Thickness

All film samples had thickness in the range of 0.17-0.19 mm (Table 4.1), which were not significantly different (p>0.05). Galus et al. (2012) suggested that solid content and condition of film preparation are the two factors playing a role on film thickness. Since the film samples in this study were prepared using the same concentration of soy protein isolate and glycerol and were equilibrated to a final moisture content at the same %RH, their thicknesses were thus of similar values. Therefore, the difference in other properties, if any, will not be due to thickness of the films.

4.1.2 Mechanical properties

Tensile strength of the heat-cured film samples is depicted in Figure 4.1. Tensile strength of the films tended to increase with increasing curing temperature. Curing of film-forming solution at 80°C for 2 and 4 h (FS 80/2, FS 80/4) and of pre-formed film at 60°C for 4 h (PF 60/4), 70°C for 4 and 6 h (PF 70/4, PF 70/6), and 80°C for 2, 4, and 6 h (PF 80/2, PF 80/4, and PF 80/6) produced a film with significantly greater tensile strength than the untreated control (p≤0.05). Among all samples, PF 70/4 exhibited the greatest tensile strength (3.49 MPa), a 1.8-fold increase from that of the control (1.89 MPa). An increase in tensile strength upon heat curing is assumed to be due to heat-induced cross-linking of proteins. During heating, the protein unfolds to expose the buried sulfhydryl groups. Heating further induces thiol-disulfide exchange reaction, with the formation of inter- and intra-molecular disulfide linkages (Chiralt et al., 2018). These disulfide bonds are developed through a series of reactions between thiolates (mostly cysteine) and oxidizing sulfides (Chiralt et al., 2018). With higher bond energy than non-covalent interactions, these

covalent disulfide bonds resulted in a stronger protein network. The unfolded protein conformation also promotes non-covalent interactions, like hydrogen bonding and hydrophobic interaction, between polypeptide chains during the process of film formation (Condes et al., 2013). This increasing degree of protein interaction and bonding is responsible for the increase in tensile strength of the heated film.

Table 4. 1 Thickness of heat-cured soy protein films

Film samples[§]	Thickness (mm)[ns]
Control	0.18±0.02
FS 60/2	0.18±0.01
FS 60/4	0.19±0.01
FS 60/6	0.18±0.02
FS 70/2	0.19±0.01
FS 70/4	0.19±0.01
FS 70/6	0.18±0.02
FS 80/2	0.18±0.01
FS 80/4	0.19±0.01
FS 80/6	0.19±0.01
PF 60/2	0.19±0.01
PF 60/4	0.17±0.03
PF 60/6	0.18±0.01
PF 70/2	0.18±0.01
PF 70/4	0.18±0.01
PF 70/6	0.18±0.01
PF 80/2	0.19±0.02
PF 80/4	0.19±0.01
PF 80/6	0.19±0.01

[§] Control: soy protein film without heat curing

FS *x*/*y*: film sample obtained by heat-curing of film-forming solution at *x*°C for *y* h

PF *x*/*y*: film sample obtained by heat-curing of pre-formed film at *x*°C for *y* h

Mean±SD of three replicates

[ns] Means are not significantly different ($p>0.05$).

It should be noted that films heat-cured for 6 h generally demonstrated lower tensile strength than those heated for 4 h at the same temperature. Disruption of disulfide bond upon prolonged heating might be responsible for this decrease in tensile strength (Futami et al., 2017). Additionally, curing of PF usually resulted in a film with higher tensile strength than that of FS heated at the same condition. This could be due to that pre-formed film is more concentrated system with protein chains

existing in closer proximity, thus facilitating the interaction and bonding among the adjacent chains.

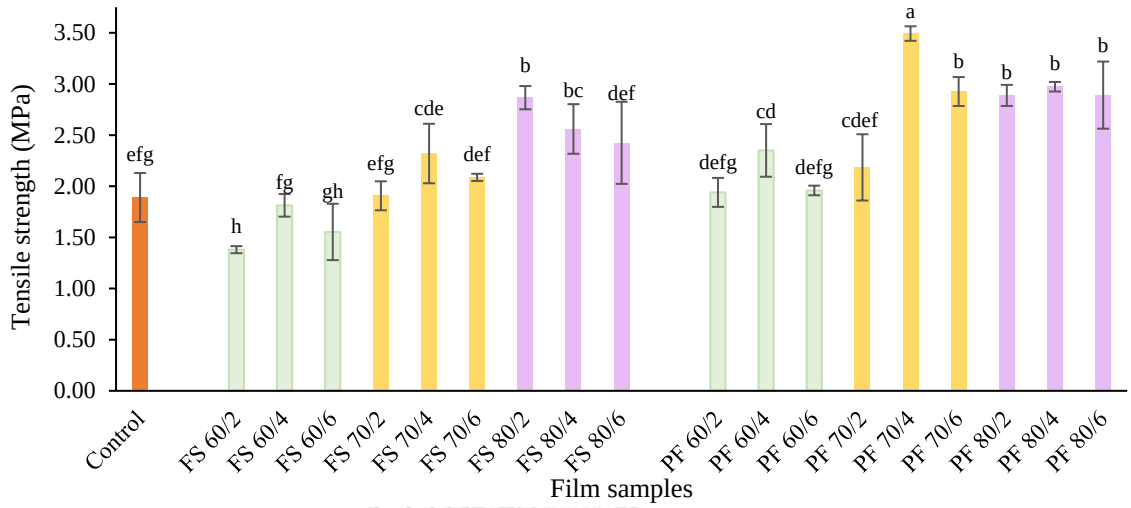

Figure 4. 1 Tensile strength of heat-cured soy protein films. Means with different letters are significantly different ($p \leq 0.05$).

Our findings are in line with Rhim et al. (2000), who found that heat-curing at 90°C for 24 h could improve tensile strength of pre-formed soy protein film. In another study, Perez-Gago & Krochta (2001) observed that heat-treated wheat protein films demonstrated increasing tensile strength which was attributed to the formation of disulfide cross-links. Choi & Han (2002) reported that heat-treated yellow field pea protein films exhibited increasing tensile strength and elongation, along with a decrease in Young's modulus, as compared to the films prepared without heat-curing. Similarly, Hoque et al. (2010) examined the effect of heat treatment (40-90°C) of film-forming solution on properties of cuttlefish skin gelatin film. They reported that tensile strength of the film increased with increasing heating temperature up to 70°C. The authors suggested that this increase in tensile strength was likely attributed to the formation more junction zones between gelatin molecules through hydrogen bonding. In the same study, a decrease in tensile strength was reported for the heating temperature above 70°C.

Raman microscopy was used to monitor disulfide bond formation in the heat-cured film samples. Raman spectra of the FS and PF films are depicted in

Figures 4.2 and 4.3, respectively. For heat-cured samples, an increase in Raman intensity was observed around 490 cm^{-1} which is in the S-S stretching region (El-Hag & Dahab, 2016). This indicates that S-S bond formation was induced upon heat-curing of FS and PF. Peak intensity was found to increase with increasing curing temperature and time. However, heat curing on FS at 70 and 80°C for 6 h resulted in a lower peak intensity than heat treatments at the same temperatures for 4 h (Figure 4.2). This is in good agreement with the lower tensile strength of the films (Figure 4.1). This may be due to the fact that too severe heating may induce disulfide bond disruption which can eventually lead to irreversible protein denaturation (Futami et al., 2017). For the heat-cured PF (Figure 4.3), the films cured at 70°C demonstrated higher peak intensity compared to the others, implying that these conditions favor the formation of S-S linkages, and this corresponds well with the greater tensile strength of the films (Figure 4.1).

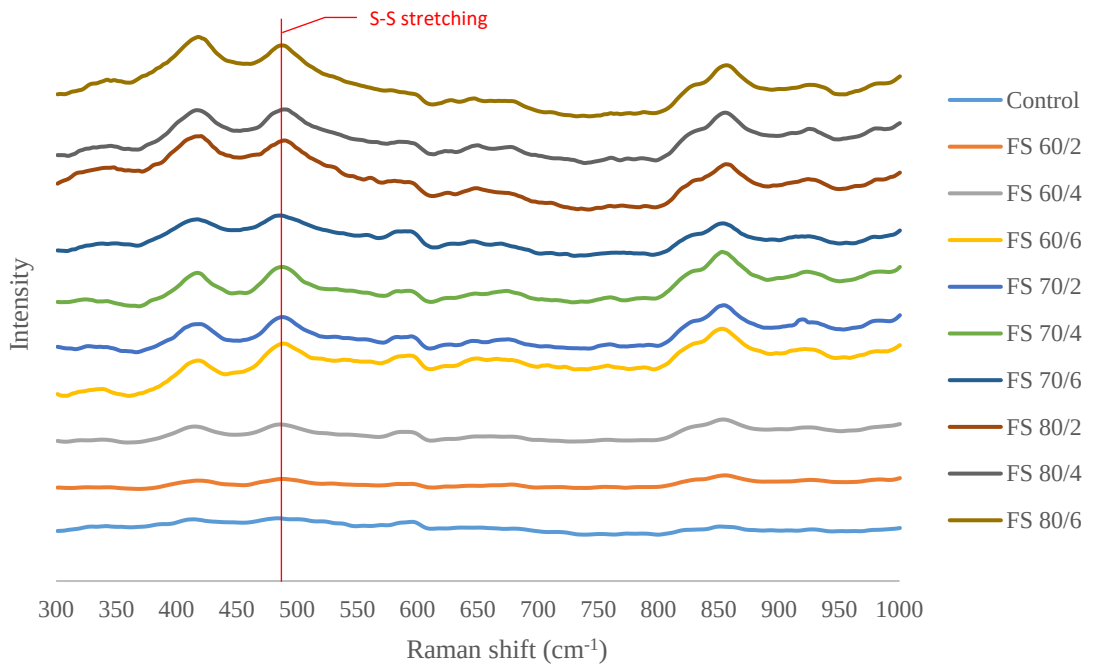

Figure 4. 2 Raman spectra of soy protein films obtained from heat-cured film-forming solution

In terms of elongation at break (Figure 4.4), all heat-cured films possessed lower elongation at break than the untreated control. This could be due to

that heat curing promotes the formation of inter- and intra-molecular disulfide cross-links as well as non-covalent interactions among the protein chains (Gennadios et al., 1996; Liu et al., 2004). Similar findings were also reported for whey protein films (Amin & Ustuno, 2007) and soy protein films (Kim et al., 2002). It should be noted that at extreme heating conditions, such as at 80°C for 6 h, disulfide bond breakage and protein denaturation might also be responsible for a remarkable decrease in elongation at break of the films.

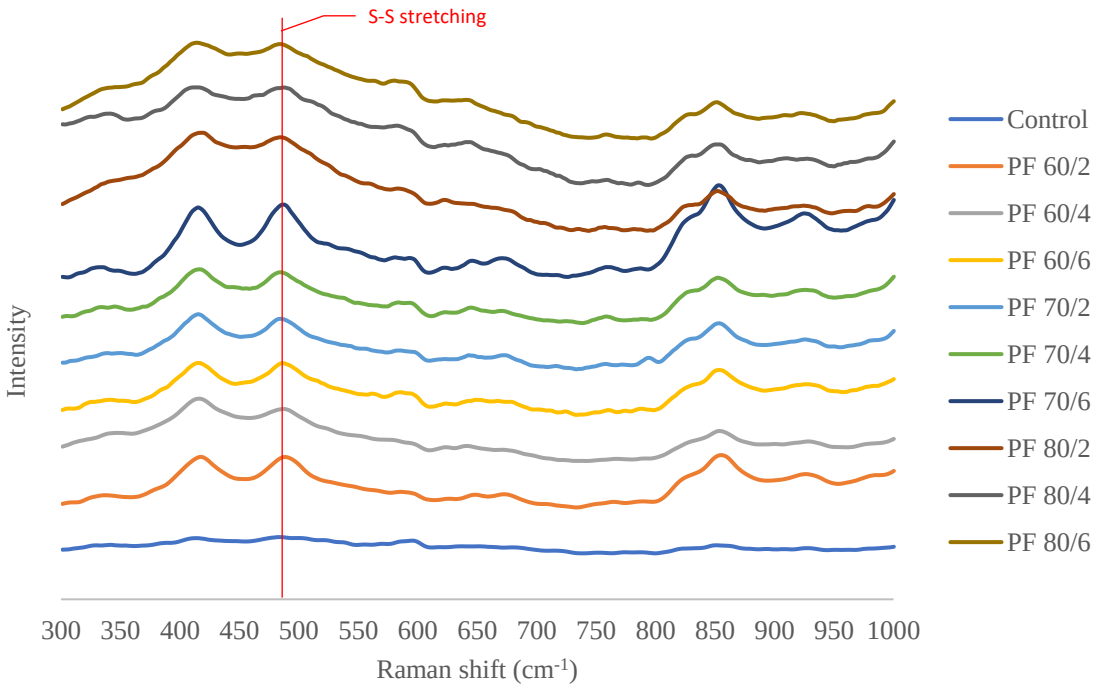

Figure 4. 3 Raman spectra of heat-cured pre-formed soy protein films

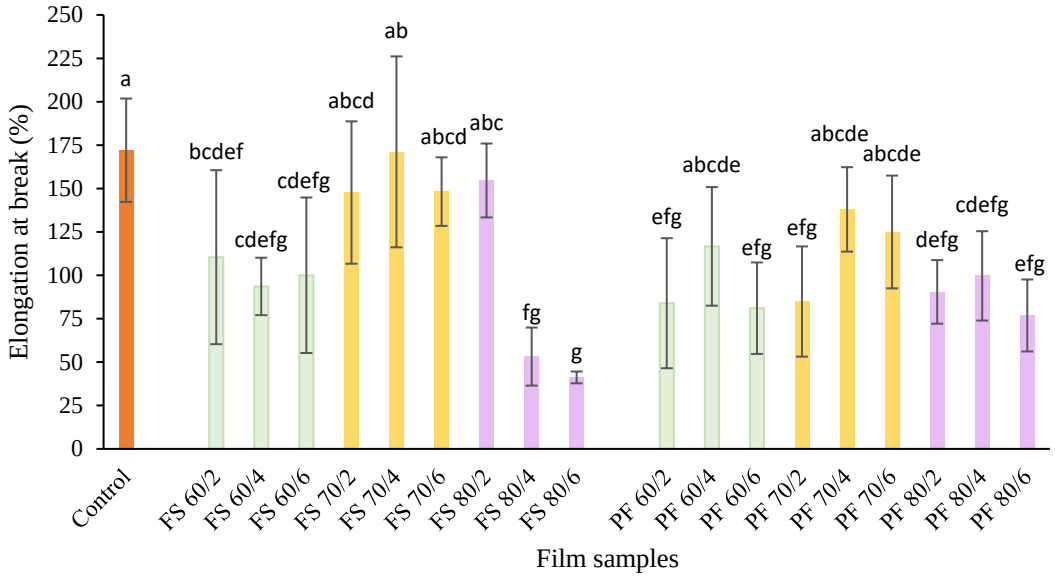

Figure 4. 4 Elongation at break of heat-cured soy protein films. Means with different letters are significantly different ($p \leq 0.05$).

4.1.3 Color

Film color holds significant importance in packaging application and influences consumer acceptance. Color parameters of heat-cured soy protein films are summarized in Table 4.2.

L* of the FS films was found to increase with increasing curing temperature and time, while that of the PF films remained almost constant. FS 80/6 possessed the highest L* of 90.05. This is in consistence with Liu et al. (2004) who reported that films obtained by heating the film-forming solution demonstrated and increase in L* with increasing temperature. Meanwhile, Micard et al. (2000) reported that heating temperature posed no effect on L* of pre-formed wheat gluten films.

While heat curing posed a minimal and inconsistent effect on a*, it was found to induce an increase in b* in all FS and PF films. Heat curing to FS and PF at a higher temperature for a longer time brought about a higher b* value. FS 80/6 exhibited the greatest b* of +21.83 and the lowest a* of -1.14. The increase in b* of the heat-cured films may be due to the formation of colored products of the Maillard reaction. The Maillard reaction-induced color changes are often encountered in a process involving heat treatment of a protein system which reducing sugar may also co-exist (Manzocco et al., 2000). An increase in yellowness of heat-cured soy protein

films was reported earlier by Rhim et al. (2000). In another study, Hoque et al. (2010) observed that heat treatment of film-forming solution of cuttlefish skin gelatin films resulted in an increase in b*. Kim et al. (2002) reported a similar finding for films made from heat-treated soy protein. Heating of amaranth protein films at 70 and 90°C also induced similar color changes (Condés et al., 2013). Contrastingly, Choi & Han (2002) did not observe any differences in terms of color between control and heat-treated pea protein films.

The higher degree of changes in b*, particularly for FS 80/4 and FS 80/6, resulted in a greater ΔE value. The average human eye cannot detect any color differences if $\Delta E \leq 3$. To the naked eyes, these two samples appeared intensely yellow and very different in terms of color from the control. All film samples were of yellow shade, conforming to a hue angle of around 90°, which is the angle of yellow color. Chroma value reflects the color intensity. In the case of FS 80/4 and FS 80/6, their high chroma indicates the high color intensity.

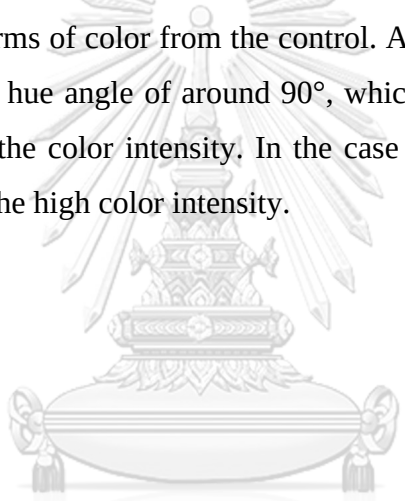

Table 4. 2 CIELAB color parameters of heat-cured soy protein films

Film samples[§]	L*	a*	b*	ΔE	Hue angle (°)	Chroma
Control	85.83±0.79^{c}	0.51±0.11^{bcd}	10.37±1.65^{ghi}	-	87.05±1.02^{j}	10.38±1.65^{ghi}
FS 60/2	85.23±0.48^{cd}	0.51±0.06^{bcd}	11.25±0.98^{fghi}	1.16±0.98^{fg}	87.37±0.47^{ij}	11.26±0.98^{bc}
FS 60/4	85.50±0.85^{c}	0.25±0.12^{fg}	11.51±2.09^{fghi}	2.08±1.40^{def}	88.62±.085^{efg}	11.51±2.08^{fghi}
FS 60/6	85.52±0.94^{cd}	0.19±0.12^{gh}	12.38±2.10^{efg}	2..47±1.86^{def}	89.02±0.74^{def}	12.38±2.09^{efg}
FS 70/2	86.13±0.65^{bc}	0.11±0.15^{hi}	10.34±1.61^{ghi}	1.52±0.87^{efg}	89.24±0.97^{cde}	10.34±1.16^{ghi}
FS 70/4	86.69±0.68^{b}	-0.03±0.22^{j}	10.95±2.22^{hi}	2.26±1.06^{def}	89.91±1.45^{c}	10.96±2.22^{hi}
FS 70/6	86.79±0.96^{b}	-0.01±0.25^{j}	10.86±2.95^{i}	2.78±1.64^{def}	89.62±1.82^{cd}	10.87±2.94^{i}
FS 80/2	87.36±0.73^{b}	0.03±0.13^{ij}	12.98±2.74^{def}	3.14±2.27^{de}	89.77±0.74^{cd}	12.98±2.73^{ef}
FS 80/4	89.53±1.32^{a}	-0.81±0.14^{k}	21.22±2.92^{a}	11.73±2.35^{a}	92.26±0.60^{b}	21.23±2.91^{a}
FS 80/6	90.05±1.62^{a}	-1.14±0.20^{l}	21.83±4.05^{a}	12.49±3.16^{a}	93.14±0.98^{a}	21.86±4.03^{a}
PF 60/2	84.87±0.83^{de}	0.39±0.17^{de}	11.98±1.77^{efghi}	2.26±1.43^{def}	88.04±1.03^{ghi}	11.99±1.77^{efgh}
PF 60/4	84.68±1.07^{def}	0.51±0.05^{bcd}	12.10±1.92^{efgh}	2.38±1.82^{def}	87.50±0.62^{ij}	12.11±1.92^{efgh}
PF 60/6	84.71±1.13^{def}	0.49±0.05^{bcd}	12.19±2.05^{efg}	2.66±1.64^{def}	87.61±0.68^{hij}	12.20±2.05^{efg}
PF 70/2	83.86±1.11^{fg}	0.47±0.08^{bcd}	13.70±1.97^{cde}	3.88±2.25^{cd}	88.03±0.21^{ghi}	13.71±1.9^{cde}
PF 70/4	83.46±0.74^{g}	0.64±0.04^{a}	14.99±1.58^{bcd}	5.20±1.74^{bc}	87.53±.22^{ij}	15.00±1.58^{bcd}
PF 70/6	83.35±0.48^{g}	0.54±0.04^{abc}	16.06±0.93^{b}	6.21±1.03^{b}	88.08±0.12^{ghi}	16.07±0.93^{b}
PF 80/2	84.59±0.39^{def}	0.34±0.07^{ef}	13.80±0.80^{cde}	3.66±0.89^{cd}	88.59±0.29^{efg}	13.80±0.80^{cde}
PF 80/4	83.32±1.20^{g}	0.56±0.09^{ab}	16.21±2.30^{b}	6.37±2.58^{b}	88.01±0.19^{ghi}	16.22±2.29^{b}
PF 80/6	84.20±0.70^{efg}	0.42±0.08^{cde}	15.30±1.41^{bc}	5.21±1.55^{bc}	88.43±0.25^{fgh}	15.30±1.41^{bc}

[§] Control: soy protein film without heat curing

FS *x*/*y*: film sample obtained by heat-curing of film-forming solution at *x*°C for *y* h

PF *x*/*y*: film sample obtained by heat-curing of pre-formed film at *x*°C for *y* h

Mean±SD of three replicates

Means with different superscript letters in a column are significantly different ($p \leq 0.05$).

4.1.4 Transparency

Transparency of materials is advantageous in many applications, including food packaging, as it enables customers to observe the item before purchasing the product. On the other hand, food should be shielded from the effects of light, especially UV radiation, by the materials used as packaging. Transparency of the heat-cured film samples, expressed in %transmittance, is shown in Table 4.3.

Transparency of the film samples was found to be affected by heat-curing treatments. Heat curing on FS significantly increased %transmittance of the resulted film. In addition to heating temperature, increasing heating time significantly increased %transmittance of FS films. On the other hand, PF samples exhibited lower %transmittance as compared to the control. Transparency of the PF films tended to decrease with increasing heating time. The highest transparency was recorded for FS 80/6 with %transmittance of 74.17.

Kowalczyk & Baraniak (2011) reported that thermal treatment of film-forming solution increased light transmission of pea protein film. In contrast, Choi & Han (2002) revealed that heating film-forming solution at 90°C for up to 50 min posed no effect on transparency of pea protein film.

Table 4. 3 Transparency (expressed as %transmittance) of heat-cured soy protein films

Film samples[§]	%Transmittance
Control	45.03±1.77^{g}
FS 60/2	41.7±0.62^{h}
FS 60/4	59.7±1.35^{d}
FS 60/6	56.1±0.78^{e}
FS 70/2	60.93±0.32^{cd}
FS 70/4	51.1±1.01^{f}
FS 70/6	62.43±0.68^{c}
FS 80/2	69.67±0.40^{b}
FS 80/4	61.47±2.99^{cd}
FS 80/6	74.17±0.21^{a}
PF 60/2	33.47±1.99^{j}
PF 60/4	31.90±2.08^{jk}
PF 60/6	29.73±0.42^{kl}
PF 70/2	28.43±0.49^{l}
PF 70/4	28.17±0.31^{l}
PF 70/6	27.07±1.10^{l}
PF 80/2	43.00±1.85^{gh}
PF 80/4	37.53±0.35^{i}
PF 80/6	32.23±2.08^{j}

[§] Control: soy protein film without heat curing

FS *x*/*y*: film sample obtained by heat-curing of film-forming solution at *x*°C for *y* h

PF *x*/*y*: film sample obtained by heat-curing of pre-formed film at *x*°C for *y* h

Mean±SD of three replicates

Means with different superscript letters are significantly different ($p \leq 0.05$).

4.1.5 Water solubility

Application of high temperature to a protein film was reported to induce the reorganization of protein, leading to protein cross-linking and film with lower water solubility (Gopalakrishnan et al., 2021). Water solubility of the heat-cured soy protein films, expressed as %total soluble matter, are tabulated in Table 4.4

Table 4. 4 Water solubility, expressed as %total soluble matter, of heat-cured soy protein films

Film samples[§]	%Total soluble matter
Control	59.42±3.70^{bc}
FS 60/2	68.59±7.35^{b}
FS 60/4	62.70±3.16^{ab}
FS 60/6	62.71±5.91^{ab}
FS 70/2	53.80±3.44^{cd}
FS 70/4	50.19±1.24^{de}
FS 70/6	47.86±1.21^{def}
FS 80/2	44.09±1.87^{efg}
FS 80/4	42.82±3.09^{efg}
FS 80/6	39.74±2.34^{gh}
PF 60/2	69.77±2.83^{a}
PF 60/4	64.56±1.96^{ab}
PF 60/6	58.06±5.88^{bc}
PF 70/2	46.55±1.56^{defg}
PF 70/4	41.12±0.72^{fgh}
PF 70/6	34.69±3.88^{h}
PF 80/2	27.34±5.82^{i}
PF 80/4	22.98±4.29^{i}
PF 80/6	23.84±7.49^{i}

[§] Control: soy protein film without heat curing

FS *x*/*y*: film sample obtained by heat-curing of film-forming solution at *x*°C for *y* h

PF *x*/*y*: film sample obtained by heat-curing of pre-formed film at *x*°C for *y* h

Mean±SD of three replicates

Means with different superscript letters are significantly different ($p \leq 0.05$).

Water solubility of the film samples was affected by heat-curing treatment. It was found to decrease with increasing curing temperature and time. Heat curing of both FS and PF at 70 and 80°C significantly lowered the film solubility as compared to the control. The lowest water solubility was demonstrated by PF samples cured at 80°C. This decrease in water solubility of the heat-cured films is most likely induced by the formation of covalent cross-links resulting in a network with higher molecular weight, thus reducing water solubility (Amin & Ustunol, 2007). Our findings are consistent with Gennadios et al. (1996) who heated pre-formed soy protein film at 85 and 95°C. They reported that the heat treatment significantly decreased the film solubility (Gennadios et al., 1996). Kim et al. (2002) noticed a reduction in solubility of heat-treated soy protein film. Pérez-Gago & Krochta (2001) also noted a significant decrease in solubility of heat-treated whey protein film. The

authors suggested that this decrease was due to formation of higher-energy intermolecular bonds among the unfolded proteins. Similarly, Al-Saadi et al. (2014) reported that heat treatment reduced solubility of goat whey protein film. The formation of high molecular weight cross-linked proteins, particularly through the development of disulfide bonds, was designated as the key factor in reducing film solubility. In another study, Weng et al. (2007) observed that surimi protein film cured at 70°C exhibited reducing water solubility with increasing curing time. However, curing at a higher temperature (100°C) significantly enhanced the film solubility. They suggested that the increase in water solubility of the film cured at 100°C was due to its weakened structure.

4.1.6 Water vapor permeability

Water vapor permeability indicates the amount of water permeating per unit area and time through the packaging material (Janjarasskul & Krochta, 2010). For food packaging, this property is important to the food shelf life since physicochemical and microbiological stability is closely related to water activity. Water vapor permeability of the heat-cured films is summarized in Table 4.5.

Heat curing of FS slightly, but significantly ($p \leq 0.05$), increased water vapor permeability of the films. All heat-cured FS films exhibited significantly higher water vapor permeability than the control ($p \leq 0.05$), but different treatments on FS resulted in a film with similar water vapor permeability ($p > 0.05$). The results of this study are in good agreement with Cruz-Diaz et al. (2019) who reported that heating whey protein film resulted in significantly higher water vapor permeability than the unheated film. Hoque et al. (2010) observed that heating the film-forming solution at 70°C increased water vapor permeability of cuttlefish skin gelatin film as compared to the control. However, heating at 90°C for 30 min caused a significant decrease in water vapor permeability. They proposed that the decrease in water vapor permeability of the film treated at severe heating condition was possibly due to the exposure of hydrophobic domains of gelatin. A decrease in water vapor permeability of zein/wheat gluten composite films prepared from heat-treated film-forming solution at 40-80°C was also reported by Guo et al. (2012). The authors suggested that this decrease in water vapor permeability could be due to the increase in cross-

linking of protein chains at higher, resulting in a restriction of polypeptide chain mobility and a reduction of water vapor diffusion through the film.

Table 4. 5 Water vapor permeability of heat-cured soy protein films

Film samples[§]	Water vapor permeability (g m/m^2 h Pa)
Control	0.72±0.017^{cd}
FS 60/2	0.80±0.010^{a}
FS 60/4	0.79±0.004^{a}
FS 60/6	0.81±0.004^{a}
FS 70/2	0.80±0.043^{a}
FS 70/4	0.80±0.004^{a}
FS 70/6	0.78±0.003^{a}
FS 80/2	0.78±0.074^{a}
FS 80/4	0.80±0.073^{a}
FS 80/6	0.78±0.026^{ab}
PF 60/2	0.75±0.033^{bc}
PF 60/4	0.74±0.034^{bc}
PF 60/6	0.75±0.033^{bc}
PF 70/2	0.71±0.067^{d}
PF 70/4	0.71±0.066^{d}
PF 70/6	0.71±0.065^{d}
PF 80/2	0.72±.0034^{d}
PF 80/4	0.72±0.075^{cd}
PF 80/6	0.72±0.038^{cd}

§ Control: soy protein film without heat curing

FS *x*/*y*: film sample obtained by heat-curing of film-forming solution at *x*°C for *y* h

PF *x*/*y*: film sample obtained by heat-curing of pre-formed film at *x*°C for *y* h

Mean±SD of three replicates

Means with different superscript letters are significantly different ($p \leq 0.05$).

Pertaining to heat-cured PF, water vapor permeability of the films treated at different conditions was similar to that of the control ($p>0.05$). Contrary to this study, Gennadios et al. (1996) reported that pre-formed films cured at 85 and 95°C demonstrated a decreasing trend in water vapor permeability with increasing curing temperature and time. The authors explained that heat-induced covalent bond formation within the films, together with the decrease in protein hydrophilicity, were responsible for the decrease in water vapor permeability of the heat-treated films.

4.1.7 Surface hydrophobicity

Contact angle between a water droplet and the film surface is an indicator of surface hydrophobicity. A contact angle of less than 90° signifies a

hydrophilic surface while that of greater than 90° is characteristic of a hydrophobic surface. The contact angle of the heat-cured film samples is summarized in Table 4.6. From the current study, it was found that all film samples possessed a contact angle of lower than 90°, indicating that the film samples had a hydrophilic surface. This was due to the hydrophilic nature of soy protein and glycerol, the two main components of the films. Heat curing of either FS or PF was found to induce an increase in contact angle, implying that the film possesses a surface with increasing hydrophobicity upon heat treatment. However, it should be noted that prolonged heating time (6 h) may induce a decrease in contact angle as compared to the 2 and 4 h treatments. The increase in surface hydrophobicity upon heat curing is probably due to the development of protein cross-linking and a decrease in free hydrophilic groups (Fathi et al., 2018).

Table 4. 6 Surface hydrophobicity, expressed as contact angle between water droplet and surface of heat-cured soy protein films

Film samples[§]	Contact angle (°)
Control	31.87±2.66^{k}
FS 60/2	33.68±1.53^{j}
FS 60/4	43.05±1.40^{h}
FS 60/6	40.83±0.44^{i}
FS 70/2	41.45±0.91^{i}
FS 70/4	45.90±1.49^{fg}
FS 70/6	44.85±1.10^{fg}
FS 80/2	61.30±1.63^{b}
FS 80/4	54.68±0.48^{e}
FS 80/6	46.20±1.46^{f}
PF 60/2	44.27±1.61^{gh}
PF 60/4	44.86±0.81^{fg}
PF 60/6	44.28±0.83^{gh}
PF 70/2	45.97±1.24^{fg}
PF 70/4	52.45±0.94^{e}
PF 70/6	61.99±3.31^{b}
PF 80/2	59.35±1.24^{c}
PF 80/4	59.52±1.21^{c}
PF 80/6	57.38±4.01^{d}

[§] Control: soy protein film without heat curing

FS *x*/*y*: film sample obtained by heat-curing of film-forming solution at *x*°C for *y* h

PF *x*/*y*: film sample obtained by heat-curing of pre-formed film at *x*°C for *y* h

Mean±SD of three replicates

Means with different superscript letters are significantly different ($p \leq 0.05$).

4.1.8 Cross-sectional microstructure

SEM micrographs of cross-section of heat-cured soy protein films were shown in Figures 4.5 and 4.6.

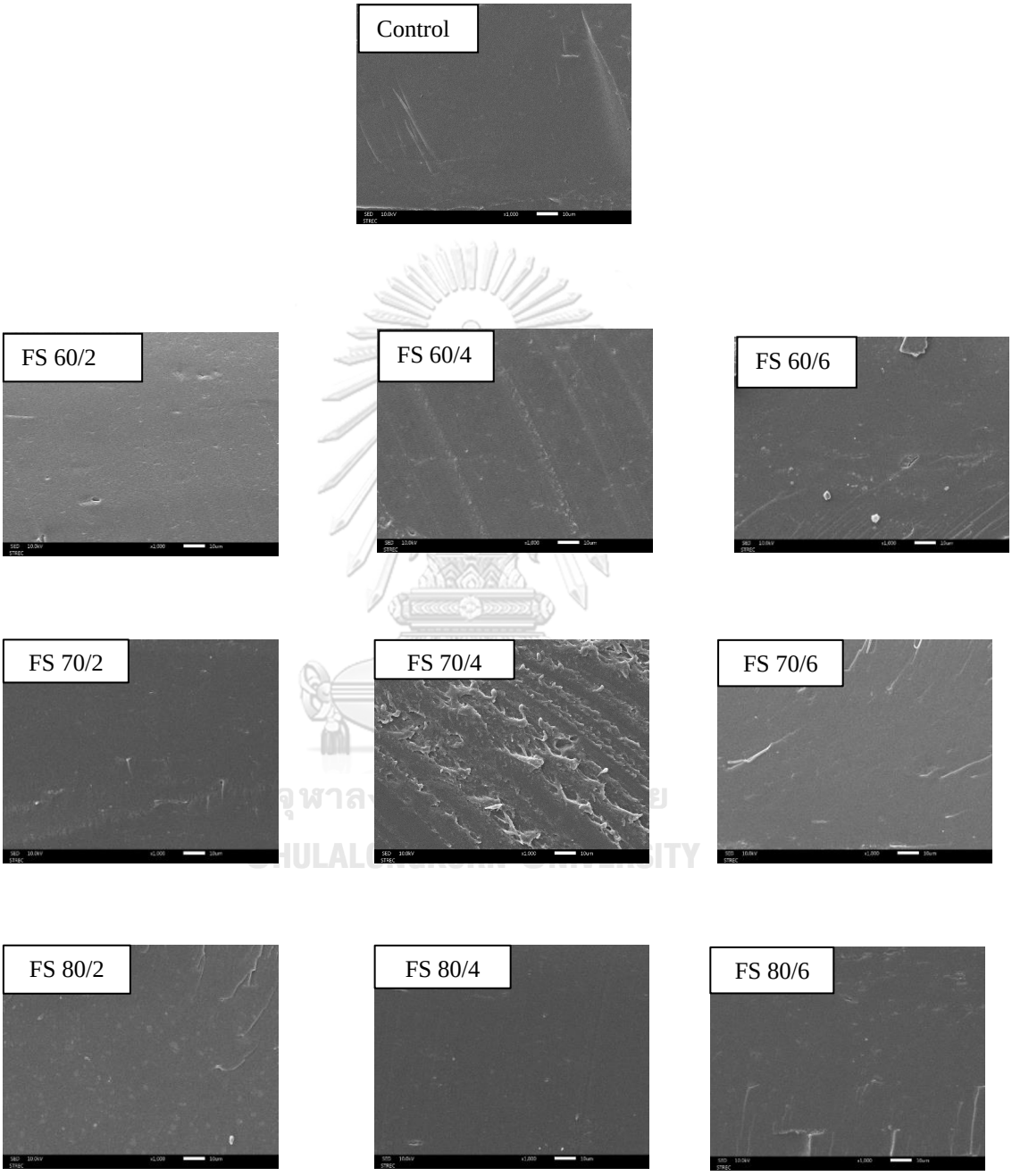

Figure 4. 5 Cross-sectional SEM micrographs of soy protein films prepared from heat-cured film-forming solution, taken at 1000× magnification

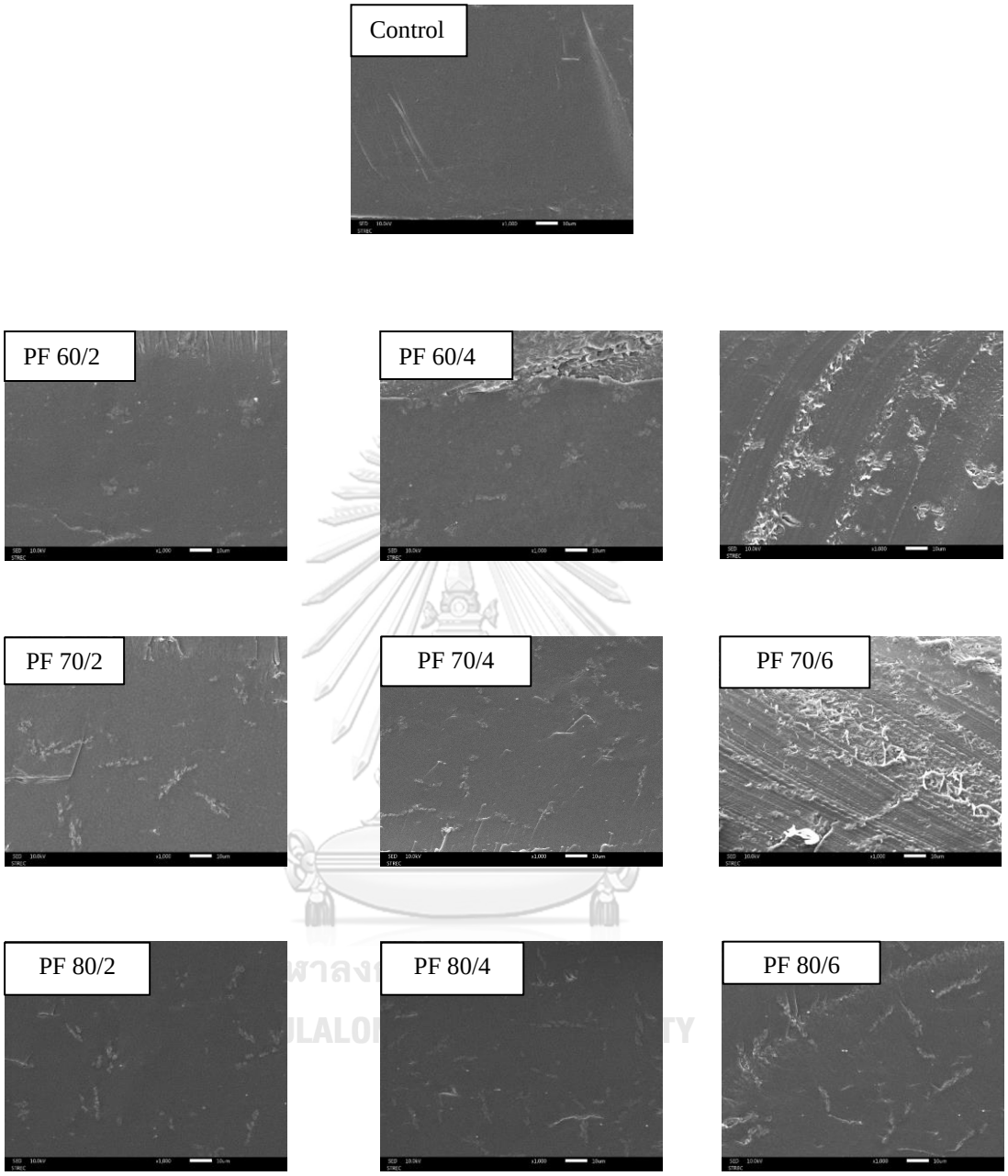

Figure 4. 6 Cross-sectional SEM micrographs of soy protein films prepared from heat-cured film-forming solution, taken at 1000× magnification

Cross-sectional morphology of the control film appeared compact and homogenous compared to the heat-cured films. With regards to FS films (Figure 4.5), only a slight increase in inhomogeneity was observed. Meanwhile, a higher degree of inhomogeneity was manifested in PF films (Figure 4.6). This structural inhomogeneity might be a result of protein cross-linking and aggregation upon heat treatment.

4.2 Effect of UV-C curing on properties of heat-treated soy protein film

From 4.1, since PF 70/4 demonstrated the highest tensile strength, the FS and PF samples treated at this heat-curing condition (70°C for 4 h) was selected as the base films for UV-C curing study in this part. UV-C dose, which is a function of radiation intensity and exposure time, was varied as 4, 8, 12, and 16 J/cm^2. UV-C radiation was applied to either film-forming solution (FSUV) or pre-formed film (PFUV). Properties of UV-C-cured heat-treated soy protein films are as follow.

4.2.1 Thickness

Thickness of the film samples is shown in Table 4.7. All film samples had a similar thickness in the range of 0.18-0.19 mm (p>0.05). This was due to the fact that the film samples were prepared using the same concentration of soy protein isolate and glycerol and were equilibrated to a final moisture content at the same %RH. Therefore, the difference in other properties, if any, will not be due to thickness of the films.

Table 4. 7 Thickness of UV-C-cured heat-treated soy protein films

Film samples[§]	Thickness (mm)[ns]
Control	0.18±0.02
HTFS 70/4	0.19±0.01
HT+FSUV 4	0.19±0.01
HT+FSUV 8	0.19±0.02
HT+FSUV 12	0.18±0.01
HT+FSUV 16	0.19±0.01
HTPF 70/4	0.18±0.01
HT+PFUV 4	0.18±0.01
HT+PFUV 8	0.18±0.01
HT+PFUV 12	0.19±0.01
HT+PFUV 16	0.18±0.02

[§] Control: soy protein film without heat curing and UV-C curing

HTFS 70/4: film sample obtained by heat-curing of film-forming solution at 70°C for 4 h, and without UV-C curing

HT+FSUV *i*: film sample obtained from film-forming solution heat-treated at 70°C for 4 h and later UV-C-cured at a radiation dose of *i* J/cm^2

HTPF 70/4: film sample obtained by heat-curing of pre-formed film at 70°C for 4 h, and without UV-C curing

HT+PFUV *i*: film sample obtained from pre-formed film heat-treated at 70°C for 4 h and later UV-C-cured at a radiation dose of *i* J/cm^2

Mean±SD of three replicates

[ns] Means are not significantly different (p>0.05).

4.2.2 Mechanical properties

Tensile strength of UV-C-cured heat-treated soy protein films are illustrated in Figure 4.7.

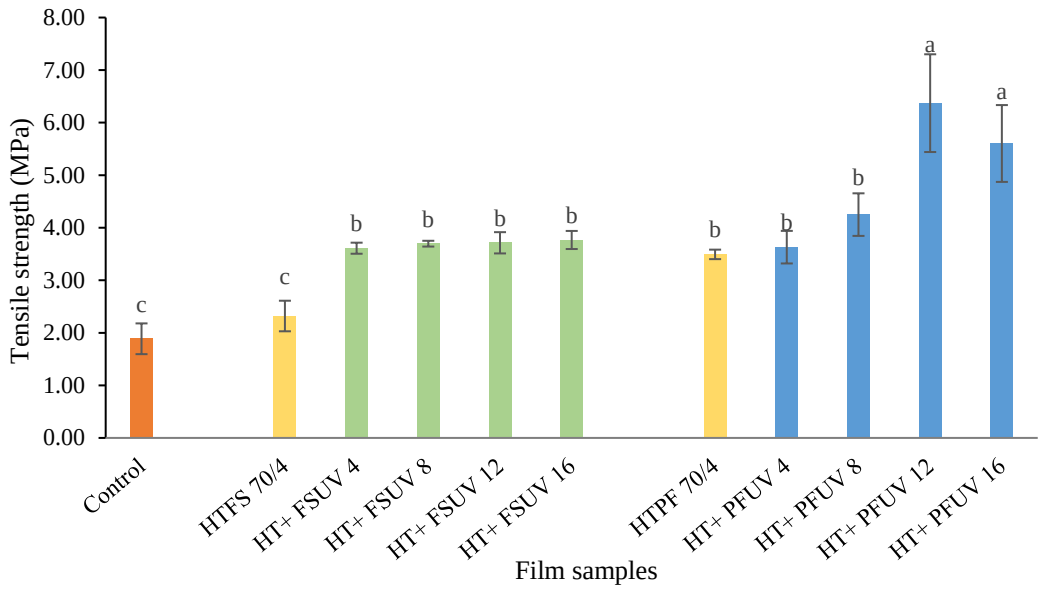

Figure 4. 7 Tensile strength of UV-C-cured heat-treated soy protein films. Means with different letters are significantly different ($p \leq 0.05$).

From Figure 4.7, it is evident that UV-C-curing further improved tensile strength of the heat-treated films, as compared to the corresponding film sample undergoing heat-curing alone. This was observed in the films obtained from UV-C-cured heat-treated film-forming solution (HT+FSUV) and the UV-C-cured heat-treated pre-formed films (HT+PFUV).

HT+FSUV cured at any UV-C dosed demonstrated a significantly greater tensile strength than the corresponding heat-treated film (HTFS 70/4). However, increasing the UV-C dose did not pose a significant effect on tensile strength of the films, since all HT+FSUV samples possessed a similar tensile strength ($p>0.05$). Meanwhile, UV-C-curing of heat-treated pre-formed film (HT+PFUV) showed a significant increase in tensile strength over the corresponding heat-treated sample (HTPF 70/4) only at high radiation doses (12 and 16 J/cm^2). The highest tensile strength was manifested by HT+PFUV 12 (6.37 MPa), which is 1.8 times of

that of HTPF 70/4 (3.49 MPa) and 3.4 times of that of the control (1.89 MPa). HT+PFUV treatments seemed to be more efficient in improving tensile strength of the soy protein film. This may be explained in a similar fashion as in 4.1, in which pre-formed film system is more concentrated with the protein chains exist in closer proximity, and this helps promote the formation of interaction and bonding among protein chains.

Soy protein contains a notable amount of aromatic amino acids such as tyrosine (Tyr). When aromatic amino acids are exposed to UV, the radiation could be absorbed by their aromatic side group that contains conjugated double bond system. Ensuing oxidation produces the amino acid free radical, such as tyrosine radical (Tyr·). With its unpaired electrons, the Tyr· radicals are reactive and upon radical recombination to produce non-radical species, a dityrosine (Tyr-Tyr) is formed, with each monomeric unit linked together via a covalent bond, resulting in cross-linking of proteins (Masutani et al., 2014). Lee et al. (2021) suggested that the photoproducts generated upon UV irradiation could induce the formation of new cross-links and/or oxidized state of aromatic amino acids. This UV-induced protein cross-linking can be experienced in daily life. For example, it is the prime factor responsible for cataract formation by increasing cross-linking of lens crystalline proteins.

This UV-induced cross-linking of protein was reported for gelatin hydrogel by Masutani et al. (2014). In another study, Rhim et al. (1999) reported that irradiating the protein in a solid state can also facilitate cross-linking by amino acid free radical recombination and subsequent polymerization of the polymeric network. The UV-C-induced increase in tensile strength of sesame protein films was reported upon treatment of film-forming solutions and pre-formed films by Fathi et al. (2018). Similar results were reported for whey protein films (Ustunol & Mert, 2004; Schmid et al., 2017), soy protein film (Gennadios et al., 1998), as well as egg albumen, gluten, and zein films (Rhim et al., 1999).

In this study, UV-C induced dityrosine cross-link formation was monitored using fluorescence spectroscopy. Dityrosine, a specific marker of protein oxidation, is particularly associated with radiation-induced oxidation. Dityrosine exhibits a distinct fluorescence peak in the wavelength of 340-500 nm, with its

highest intensity in the 400-420 nm range (Al-Hilaly et al., 2013, 2016). Correia et al. (2012) monitored the formation of dityrosine in insulin that was exposed to UV and reported a dityrosine peak in the wavelength range of 350-550 nm, with the highest intensity around 405 nm. Kerwin & Remmele (2007) noticed dityrosine peak at a wavelength of 420 nm.

Fluorescence emission spectra the film samples are shown in Figure 4.8. Emission peaks at 415 and 475 nm, which are in a typical range of dityrosine, were noticeable for every film sample. Fluorescence intensity of the films was found to increase with increasing UV-C irradiation dose, except HT+PFUV4 which demonstrated lower fluorescence intensity than the untreated control. This increase in fluorescence intensity confirms an increase in dityrosine content.

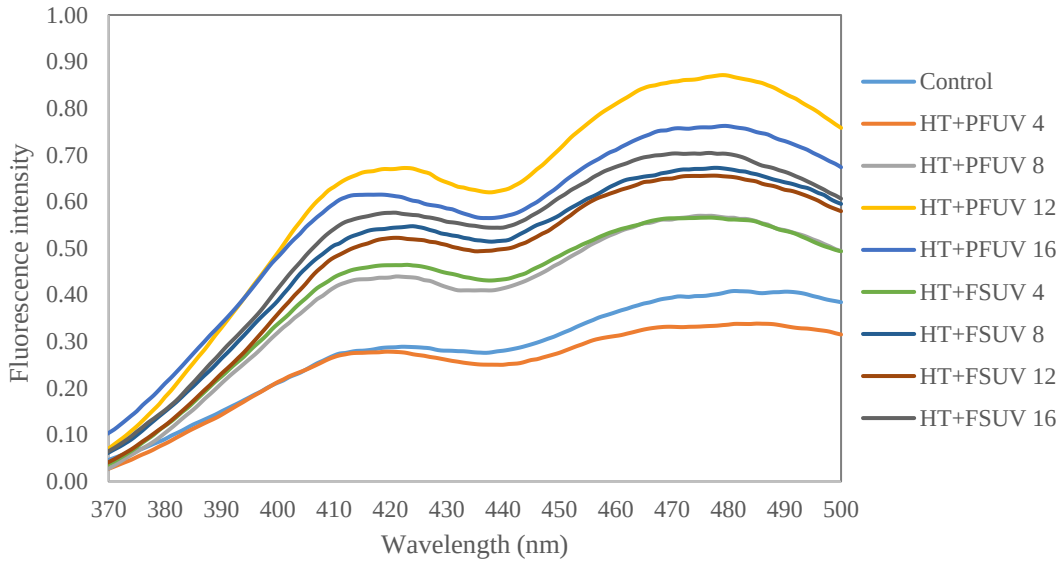

Figure 4. 8 Fluorescence spectra of UV-C-cured heat-treated soy protein films

Regarding elongation at break (Figure 4.9), UV-C treatment on either HT+FSUV or HT+PFUV caused a reduction in elongation at break as compared to the control. However, the differences are not significant ($p>0.05$), except HT+PFUV 4 and HT+PFUV 8 which had significantly lower elongation than the control ($p\leq0.05$). The decrease in elongation at break accompanied by the increase in tensile strength of the UV-C-cured heat-treated films may be attributed to the formation of a

denser film structure as a result of protein cross-linking. This denser structure contributes to stronger but less flexible protein film matrices.

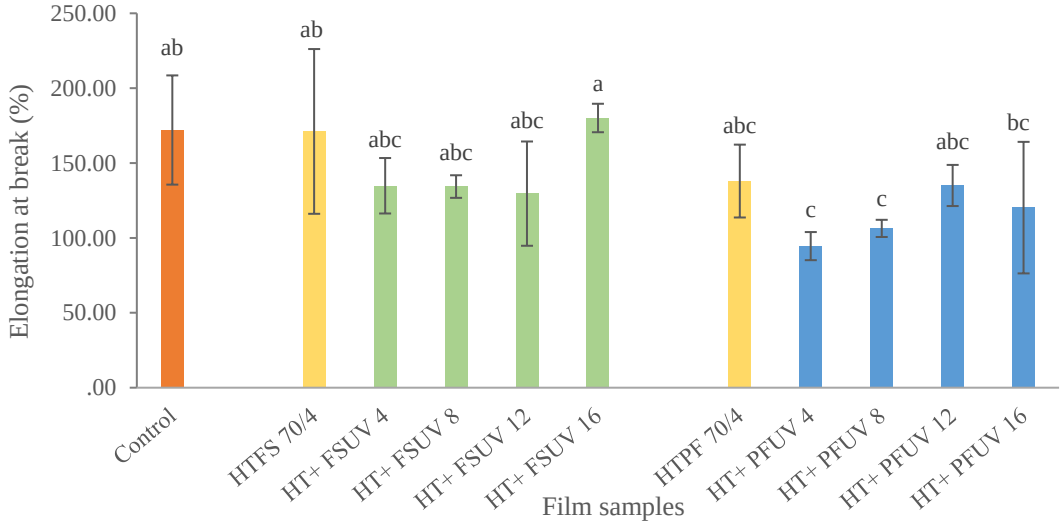

Figure 4. 9 Elongation at break of UV-C-cured heat-treated soy protein films. Means with different letters are significantly different ($p \leq 0.05$).

4.2.3 Color

Table 4.8 displays the CIELAB color parameters UV-cured heat-treated soy protein films. UV-C-curing was shown to help increase lightness of the film samples as all UV-C-cured films exhibited greater L* as compared to the control and their corresponding non-UV-cured heat-treated films. However, different radiation doses produced a film with similar L*. In general, UV-C treatment was found to cause a decrease in a* and an increase in b*. These changes in CIE L*, a*, b* induced an increase in ΔE at lower UV-C doses (4 and 8 J/cm^2) but at higher doses (12 and 16 J/cm^2), ΔE started to decrease again. HT+PFUV films demonstrated the highest ΔE. All film samples had a hue angle of around 90°, conforming to the yellow shade of the films. Chroma of UV-C-cured films was higher than the control and their corresponding non-UV-cured heat-treated films.

Yellowing of proteins upon exposing to UV radiation is widely recognized and can be best observed through the formation of cataracts. In addition to causing cloudiness in the lens of the eyes, UV radiation has also been reported to

cause the lens to turn yellow or brown. Discoloration of the lens has been found to occur more frequently in regions closer to the Equator, where UV intensity is higher compared to regions at higher latitudes. UV radiation is known to trigger photooxidation of proteins and the generation of reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen. This oxidative damage and protein discoloration in the lens are a result of this process (Addepalli et al., 2012).

Table 4. 8 CIELAB color parameters of UV-C-cured heat-treated soy protein films

Film samples[§]	L*	a*	b*	ΔE	Hue angle (°)	Chroma
Control	85.83±0.79^{f}	0.51±0.11^{b}	10.37±1.65^{e}	-	87.05±1.02^{f}	10.38±1.65^{d}
HTFS 70/4	86.69±0.68^{e}	-0.03±0.22^{c}	9.95±2.22^{e}	2.26±1.06^{g}	89.91±1.45^{e}	9.95±2.22^{d}
HT+FSUV 4	90.11±1.02^{cd}	-1.17±0.10^{g}	17.86±3.45^{b}	9.18±2.29^{c}	93.87±0.81^{bc}	17.90±3.44^{b}
HT+FSUV 8	89.83±0.77^{d}	-1.02±0.07^{f}	17.95±1.47^{b}	8.81±0.94^{cd}	93.28±0.43^{c}	17.98±1.47^{b}
HT+FSUV 12	90.54±0.62^{abc}	-1.19±0.08^{g}	16.34±1.86^{c}	7.94±1.12^{de}	94.19±0.39^{ab}	16.38±1.86^{c}
HT+FSUV 16	90.96±0.21^{a}	-1.27±.06^{g}	15.69±0.50^{c}	7.62±0.24^{e}	94.63±0.12^{a}	15.74±0.50^{c}
HTPF 70/4	83.46±0.74^{g}	0.64±0.04^{a}	14.99±1.58^{c}	5.20±1.74^{f}	87.53±0.22^{f}	15.00±1.58^{c}
HT+PFUV 4	90.81±0.69^{ab}	-0.61±0.12^{d}	19.91±1.11^{a}	10.88±0.65^{b}	91.78±0.42^{d}	19.92±1.10^{a}
HT+PFUV 8	90.25±0.81^{bcd}	-0.63±0.18^{d}	21.42±1.37^{a}	12.02±1.00^{a}	91.72±0.55^{d}	21.43±1.36^{a}
HT+PFUV 12	90.19±0.46^{bcd}	-0.79±0.05^{e}	21.29±0.73^{a}	11.85±0.55^{ab}	92.11±0.10^{d}	21.30±0.73^{a}
HT+PFUV 16	90.31±0.51^{abcd}	-0.70±0.23^{de}	21.39±0.87^{a}	11.98±0.60^{a}	91.90±0.65^{d}	21.40±0.87^{a}

[§] Control: soy protein film without heat curing and UV-C curing

HTFS 70/4: film sample obtained by heat-curing of film-forming solution at 70°C for 4 h, and without UV-C curing

HT+FSUV *i*: film sample obtained from film-forming solution heat-treated at 70°C for 4 h and later UV-C-cured at a radiation dose of *i* J/cm^2

HTPF 70/4: film sample obtained by heat-curing of pre-formed film at 70°C for 4 h, and without UV-C curing

HT+PFUV *i*: film sample obtained from pre-formed film heat-treated at 70°C for 4 h and later UV-C-cured at a radiation dose of *i* J/cm^2

Mean±SD of three replicates

Means with different superscript letters in a column are significantly different ($p \leq 0.05$).

Similar results were reported by previous studies. An increase in yellowness was reported in UV-cured films from soy protein, wheat gluten, egg albumen, sodium caseinate, and whey protein (Gennadios et al., 1998; Rhim et al., 1999; Díaz et al., 2016). Masutani et al. (2014) also noticed the presence of a yellowish color in UV-treated gelatin/glucose film. They proposed that the color

change was caused by a by-product resulting from the cross-linking reaction, potentially involving protein glycation with glucose. Díaz et al. (2016) and Schmid et al. (2015) observed a linear increase in b* with increasing UV-C doses.

4.2.4 Transparency

Film transparency, expressed as %transmittance, of the UV-C-cured heat-treated films is illustrated in Figure 4.10. UV-C-cured heat-treated films showed a slightly decreasing trend in %transmittance as compared to their corresponding sample undergoing heat treatment alone. A significant reduction in %transmittance was observed in those films cured at the highest UV-C dose (HT+FSUV16 and HT+PFUV16) ($p \leq 0.05$).

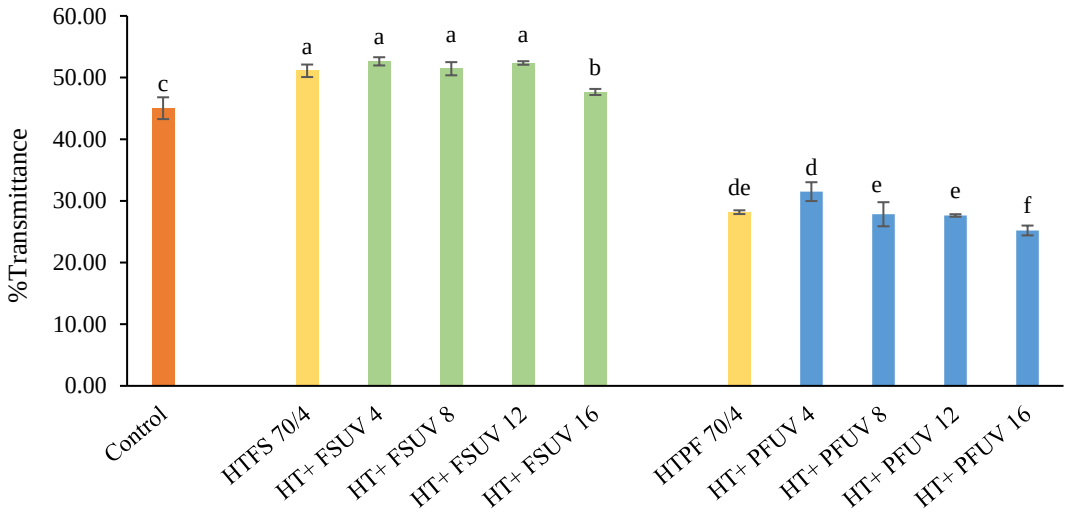

Figure 4. 10 Elongation at break of UV-C-cured heat-treated soy protein films. Means with different letters are significantly different ($p \leq 0.05$).

Schmid et al. (2015) previously reported a decrease in light transmission with increasing UV-C doses in whey protein film. It is widely recognized that UV radiation can induce protein crosslinking by facilitating the recombination of aromatic amino acid-free radicals, particularly Tyr·. UV radiation has been known as one of the major contributors to the development of cataracts, or the clouding of the ocular lens due to protein aggregation (Cetinel et al., 2017). The reduction in transparency of protein films at higher UV-C dose may be attributed to a similar mechanism.

4.2.5 Water Solubility

Figure 4.11 depicts water solubility, as %total soluble matter, of the UV-C-cured heat-treated soy protein films. Heat-curing alone was shown to decrease the film solubility, as discussed earlier in 4.1.5. Subsequent UV-C treatment to either film-forming solution or pre-formed films, further decreased the water solubility. In general, water solubility exhibited a decreasing trend with increasing UV-C doses. HT+PFUV 16 demonstrated the lowest %total soluble matter of 27.87, a 2-fold decrease from that of the control. This may be attributed to the formation of covalent dityrosine likages and a concomitant decrease in free tyrosine, a polar amino acid, resulting in a reduction in water solubility of the films subjected to UV treatment.

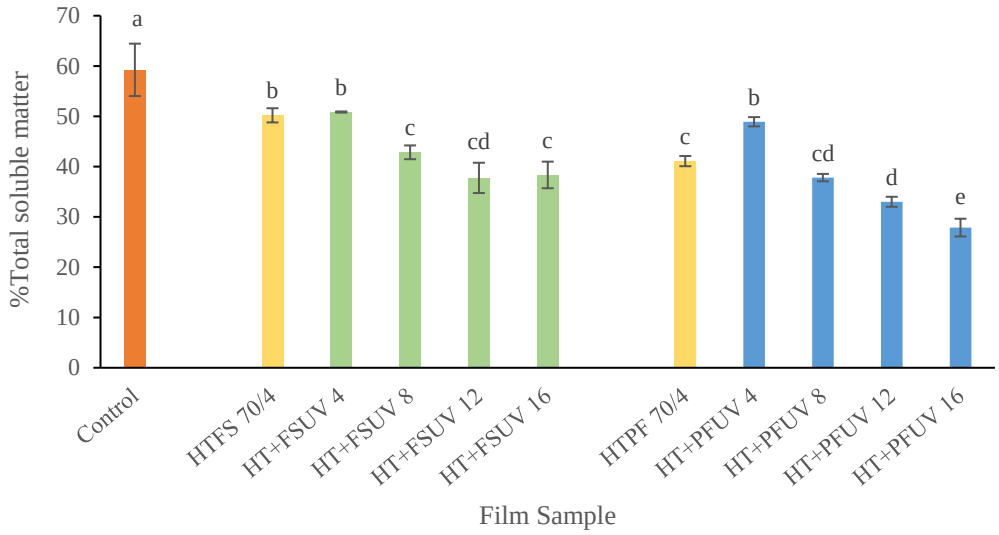

Figure 4. 11 Water solubility, expressed as %total soluble matter, of UV-C-cured heat-treated soy protein films. Means with different letters are significantly different ($p \leq 0.05$).

Our findings are in accordance with Fathi et al. (2018) who reported a decrease in water solubility of sesame protein films upon exposure to UV-C radiation. Diaz et al. (2016) also observed reducing water solubility of whey protein film upon UV-C treatment of the film-forming solution or pre-formed film. Similar findings were reported for sodium caseinate film (Rhim et al., 1999), soy protein film (Rhim et al., 2000), peanut protein films (Liu et al., 2004), and whey protein film (Schmid et al., 2015). Schmid et al. (2015) ascertained that UV radiation- induced covalent bond formation between aromatic amino acids might play a crucial role in reducing protein solubility.

4.2.6 Water vapor permeability

Water vapor permeability of UV-C-cured heat-treated soy protein films is summarized in Table 4.9. UV-curing of heat-treated film-forming solution (HT+FSUV) posed no effect on water vapor permeability of the films. All HT+FSUV samples had similar water vapor permeability to the film undergoing heat treatment alone (HTFS 70/4) ($p>0.05$). In contrast, HT+PFUV possessed a significantly higher water vapor permeability than HTPF 70/4 ($p\leq0.05$).

Table 4. 9 Water vapor permeability the UV-C-cured heat-treated soy protein films

Film samples[§]	Water vapor permeability (g m/m^2 h Pa)
Control	0.72±0.014^b
HTFS 70/4	0.81±0.007^a
HT+FSUV 4	0.85±0.021^a
HT+FSUV 8	0.83±0.014^a
HT+FSUV 12	0.82±0.021^a
HT+FSUV 16	0.84±0.021^a
HTPF 70/4	0.72±0.007^b
HT+PFUV 4	0.82±0.012^a
HT+PFUV 8	0.82±0.028^a
HT+PFUV 12	0.80±0.014^a
HT+PFUV 16	0.80±0.025^a

[§] Control: soy protein film without heat curing and UV-C curing

HTFS 70/4: film sample obtained by heat-curing of film-forming solution at 70°C for 4 h, and without UV-C curing

HT+FSUV *i*: film sample obtained from film-forming solution heat-treated at 70°C for 4 h and later UV-C-cured at a radiation dose of *i* J/cm^2

HTPF 70/4: film sample obtained by heat-curing of pre-formed film at 70°C for 4 h, and without UV-C curing

HT+PFUV *i*: film sample obtained from pre-formed film heat-treated at 70°C for 4 h and later UV-C-cured at a radiation dose of *i* J/cm^2

Mean±SD of three replicates

Means with different superscript letters are significantly different ($p \leq 0.05$).

Crack and pinhole formation in the matrix of UV-C-cured films as observed by SEM (discussed later in 4.2.8) may facilitate water diffusion, resulting in increasing water vapor permeability as compared to non-UV-treated films. Several studies reported that there was no difference in water vapor permeability of films treated using different UV-C doses (Fathi et al., 2018; Díaz et al., 2016; Liu et al., 2004; Ustunol & Mert, 2004; Micard et al., 2000; Gennadios et al., 1998; Rhim et al., 1999). Many of these authors attributed the absence of changes to the need for more extensive covalent cross-linking that could induce a noticeable decrease in the water vapor permeability.

4.2.7 Surface hydrophobicity

Surface hydrophobicity of the UV-C-cured heat-treated soy protein films, expressed as the contact angle between a water droplet and the film surface, is

shown in Figure 4.12. As compared to the corresponding non-UV-cured films, UV-C-curing at lower dosed tended to produce a film with lower contact angle, or in other words, a film with more hydrophilic surface. However, at higher UV-C doses, contact angle became increasing again, with HT+FSUV 12, HT+FSUV 16, and HT+PFUV 16 showing greater contact angle than their corresponding non-UV-cured films.

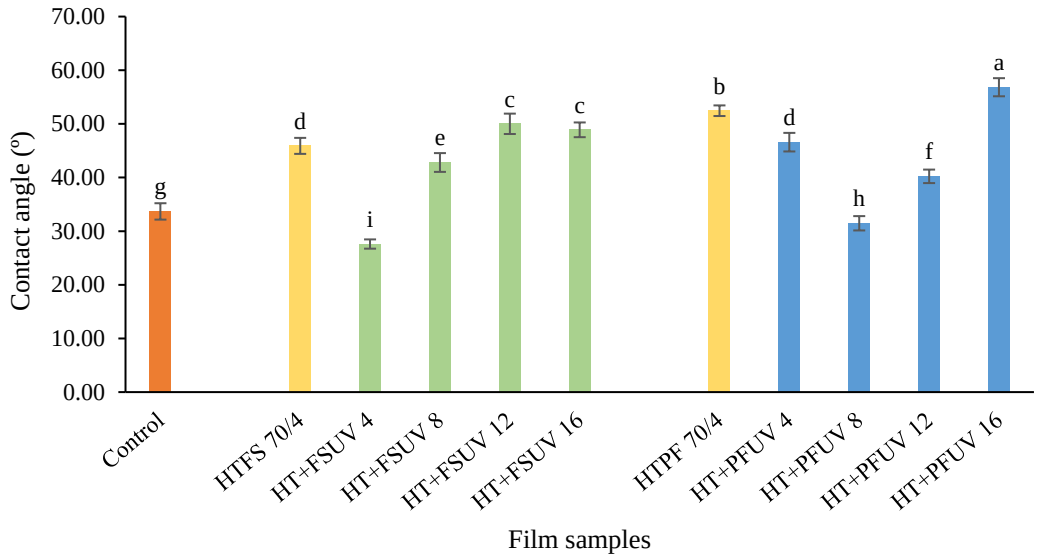

Figure 4. 12 Surface hydrophobicity, expressed as contact angle between water droplet and the film surface, of UV-C-cured heat-treated soy protein films. Means with different letters are significantly different ($p \leq 0.05$).

The increase in contact angle at higher UV-C doses may be due to that UV could also induce protein conformational changes that expose hydrophobic regions which are buried in the native protein structure (Kristo et al., 2012). Fathi et al. (2018) proposed that the increase in surface hydrophobicity of sesame protein film upon UV-C irradiation was probably due to the development of cross-linking and the decrease in free hydrophilic groups in the polypeptide chains.

4.2.8 Cross-sectional microstructure

SEM micrographs of the UV-C-cured heat-treated soy protein films are shown in Figures 4.13 and 4.14. UV-C treatment on either heat-treated film-forming solutions or heat-treated pre-formed films reveals a great impact on the film microstructure. The control which is the film without heat treatment and UV-C treatment exhibited a dense and homogeneous structure without pinholes and cracks.

UV-C curing of the film-forming solution (HT+FSUV) revealed a less homogeneous film matrix, with noticeable pinholes and cracks (Figure 4.13).

In contrast HT+PFUV, UV-C curing to pre-formed film produced a film with less cracks, but more pinholes (Figure 4.14). Fathi et al. (2018) examined the effect of UV-C on sesame protein film and reported that the holes and cracks were increased upon exposure of the films to UV-C radiation. This formation of pinholes and cracks may be due to the increased degree of cross-linking in the protein matrix, which, in turn, may induce protein aggregation, resulting in the film with less homogeneous microstructure.

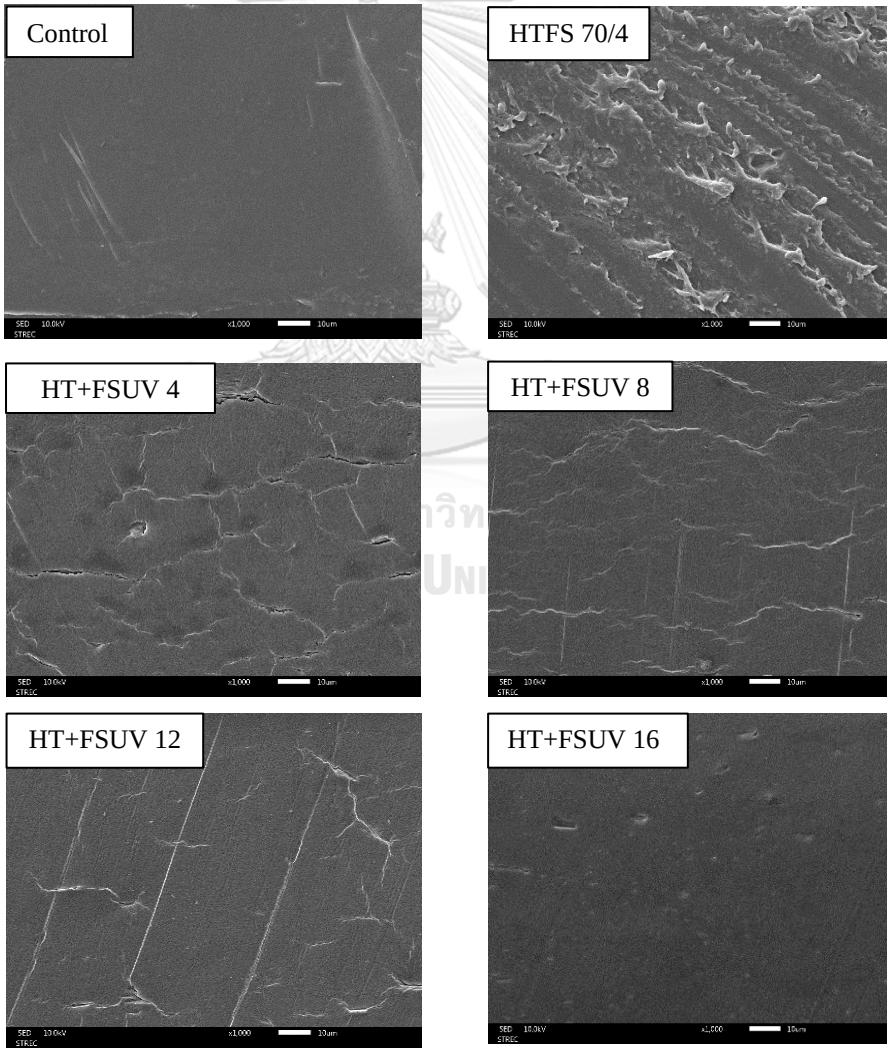

Figure 4. 13 Cross-sectional SEM micrographs of soy protein films prepared from UV-C-cured heat-treated film-forming solution, taken at 1000× magnification

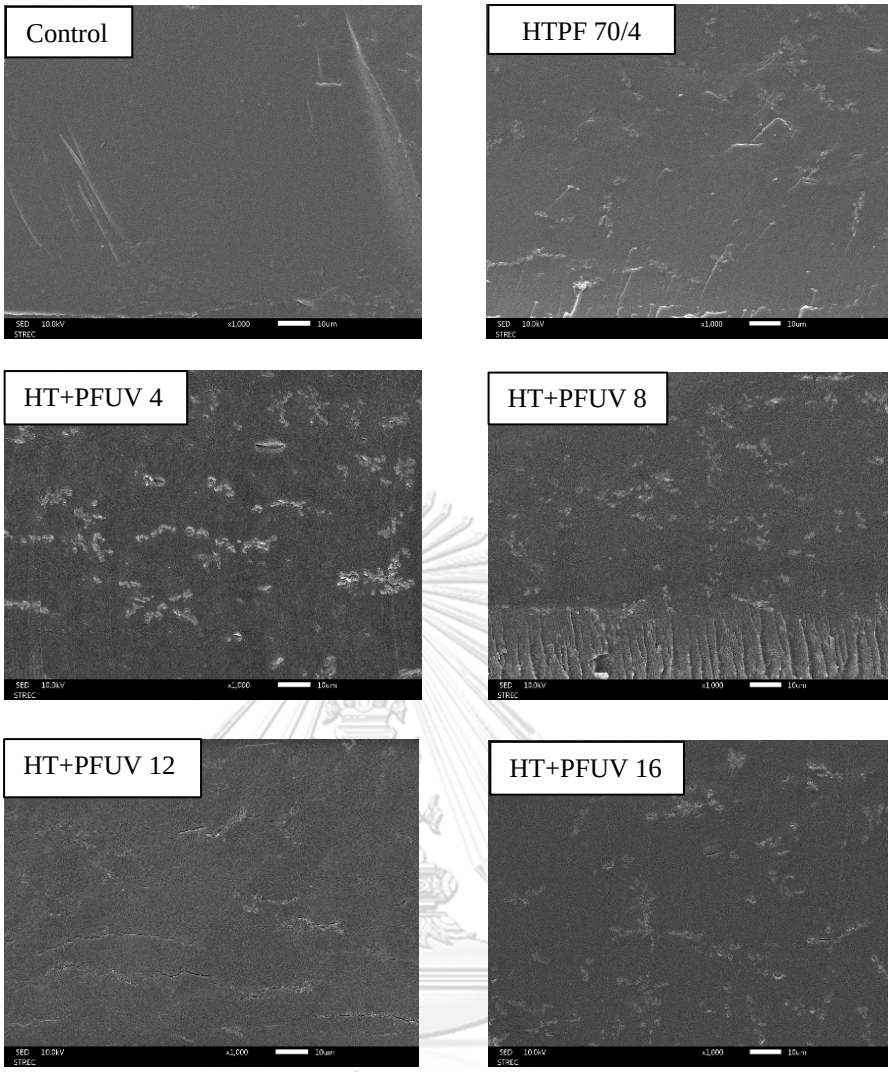

Figure 4. 14 Cross-sectional SEM micrographs of UV-C-cured heat-treated pre-formed soy protein films, taken at 1000× magnification

CHAPTER 5
CONCLUSION

In this study, the effects of heat curing and UV-C curing on properties of soy protein film were investigated. Both the heat and UV-C treatments were applied to either film-forming solution or pre-formed films.

Regarding heat curing, the treatment was found to have no effect on film thickness (p>0.05) but it did improve tensile strength of the films undergoing heat-curing as film-forming solution or pre-formed film. PF 70/4 exhibited the greatest tensile strength (3.49 MPa), a 1.8-fold increase from that of the control (1.89 MPa). An increase in tensile strength upon heat curing was proven to be due to the heat-induced sulfhydryl-disulfide exchange which produces disulfide cross-links, as revealed by the increase in intensity peak in the S-S stretching region of the Raman spectra. This increasing degree of cross-linking may also be responsible for the lower elongation at break of the heat-cured films. An increase in yellowness intensity was shown to be induced by the heat treatment. In terms of transparency, heat curing on FS significantly increased %transmittance of the resulted film while PF samples exhibited lower %transmittance as compared to the control. Heated films demonstrated a decrease in water solubility. As to water vapor permeability, heat-cured FS films exhibited significantly higher water vapor permeability than the control (p≤0.05), but different treatments on FS resulted in a film with similar water vapor permeability (p>0.05). However, all heat-cured PF samples had similar water vapor permeability to the control (p>0.05). Heat curing of either FS or PF was found to induce an increase in contact angle, implying that the film possesses a surface with increasing hydrophobicity upon heat treatment. In spite of that, all film samples still had a hydrophilic surface owing to the contact angle of lower than 90º. For the microstructure, that of heat-cured films appeared less homogenous as compared to the control.

To study the effect of UV-C curing, the film samples heat-treated at 70ºC for 4 h were selected as the base films for further investigation. UV-curing also posed no effect on film thickness while causing a significant increase in tensile strength. Formation of UV-induced dityrosine cross-links was substantiated by the increase in

fluorescence intensity at 415 and 475 nm, which fall in a typical range of dityrosine fluorescence emission. On the other hand, UV-C treatment posed a minimal effect on elongation at break. An increase in yellowness intensity was observed for the UV-cured films. Meanwhile, a slight decrease in transparency was demonstrated upon UV-C curing. The UV treatment resulted in a film with lower water solubility. UV-curing of heat-treated film-forming solution posed no effect on water vapor permeability of the films but did cause a significant increase in water vapor permeability of the pre-formed films. This may be due to the formation of pinholes and cracks in the film matrices, as revealed by the SEM micrographs. In terms of surface hydrophobicity, UV-C-curing at lower doses tended to produce a film with lower surface hydrophobicity. However, at higher UV-C doses, contact angle became increasing again, signifying an increase in surface hydrophobicity.

To summarize, heat curing and combined heat/UV-C curing were proven as an effective technique for improving tensile strength of soy protein film by facilitating the formation of covalent cross-links among protein chains. Additionally, the treatments also affected optical and moisture barrier properties of the film.

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