EFFECTS OF FERULIC ACID AND UV CURING ON PROPERTIES OF SOY PROTEIN FILM

Mr. Md Shakil

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นายเอ็มดี ชากิล

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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By	Mr. Md Shakil
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Thesis Advisor	Assistant Professor THANACHAN MAHAWANICH, Ph.D.

Accepted by the FACULTY OF SCIENCE, Chulalongkorn University in Partial Fulfillment of the Requirement for the Master of Science

..... Dean of the FACULTY OF SCIENCE
(Professor POLKIT SANGVANICH, Ph.D.)

THESIS COMMITTEE

..... Chairman
(Professor UBONRATANA SIRIPATRAWAN, Ph.D.)

..... Thesis Advisor
(Assistant Professor THANACHAN MAHAWANICH, Ph.D.)

..... Examiner
(Associate Professor KITIPONG ASSATARAKUL, Ph.D.)

..... Examiner
(Assistant Professor SIRIMA PUANGPRAPHANT, Ph.D.)

..... External Examiner
(Associate Professor Warangkana Sompongse, Ph.D.)

เอ็มดี ชากิล : ผลของกรดเฟรูลิกและการบ่มด้วยยูวีต่อสมบัติของฟิล์มโปรตีนถั่วเหลือง. (EFFECTS OF FERULIC ACID AND UV CURING ON PROPERTIES OF SOY PROTEIN FILM) อ.ที่ปรึกษาหลัก : ศ. ดร.ธนจันทร์ มหาวนิช

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาผลของการบ่มด้วยยูวีซีต่อสมบัติของฟิล์มโปรตีนถั่วเหลืองที่เติมกรดเฟรูลิก ตัวอย่างฟิล์มในงานวิจัยนี้เตรียมจากโปรตีนถั่วเหลืองสกัดและเติมกรดเฟรูลิกเข้มข้น 1.5% แปรปริมาณรังสียูวีซีเป็น 4 ระดับ ได้แก่ 0.32, 1.56, 4.00 และ 12.00 จูล/ตารางเซนติเมตร โดยฉายรังสียูวีซีบนแผ่นฟิล์มหรือสารละลายฟิล์ม วิเคราะห์สมบัติเชิงกล สมบัติเชิงเคมีกายภาพ และสมบัติเชิงสัณฐานวิทยาของตัวอย่างฟิล์ม พบว่าการเติมกรดเฟรูลิกและการบ่มด้วยยูวีซีที่ปริมาณรังสี 0.32 จูล/ตารางเซนติเมตร มีผลต่อความหนาของฟิล์มอย่างมีนัยสำคัญ ในขณะที่การเติมกรดเฟรูลิก และ/หรือ การบ่มด้วยยูวีซี มีผลต่อความหนาแน่นของฟิล์มเพียงเล็กน้อย การบ่มด้วยรังสียูวีซีส่งผลให้ฟิล์มที่เติมกรดเฟรูลิกมีความต้านทานแรงดึงขาดและการยืดตัวถึงจุดขาดเพิ่มขึ้น โดยฟิล์มที่ฉายรังสียูวีซีในปริมาณรังสีสูงสุด (12.00 จูล/ตารางเซนติเมตร) มีความต้านทานแรงดึงขาดสูงกว่าตัวอย่างควบคุมประมาณ 1.3 เท่า และมีการยืดตัวถึงจุดขาดสูงกว่าตัวอย่างควบคุมประมาณ 1.7 เท่า การฉายรังสียูวีซีบนแผ่นฟิล์มให้ผลที่ไม่ต่างจากการฉายรังสีบนสารละลายฟิล์ม ในงานวิจัยนี้สามารถยืนยันการเกิดการเชื่อมข้ามของโปรตีนโดยพันธะ C-N และไดไทโรซีนด้วยเทคนิคฟูเรียร์แทรนสฟอร์มอินฟราเรดสเปกโทรสโกปีและฟลูออเรสเซนส์สเปกโทรสโกปี นอกจากสมบัติเชิงกลแล้ว การเติมกรดเฟรูลิกและการบ่มด้วยรังสียูวีซียังมีผลสำคัญต่อสมบัติเชิงแสง ได้แก่ ความโปร่งใส และสีของฟิล์ม การบ่มฟิล์มที่เติมกรดเฟรูลิกด้วยรังสียูวีซีทำให้ฟิล์มมีความโปร่งใสลดลงและความเข้มสีเพิ่มขึ้น โดยฟิล์มที่ได้มีความขุ่นมากขึ้นและมีสีเหลืองเข้มขึ้นเมื่อมองด้วยตาเปล่า เมื่อเปรียบเทียบกับตัวอย่างควบคุม ฟิล์มที่เติมด้วยกรดเฟรูลิกและฉายรังสียูวีซีมีสภาพให้ซึมผ่านได้ของไอน้ำเพิ่มขึ้นเล็กน้อย อย่างไรก็ตามพบว่าปริมาณรังสีที่ต่างกันไม่มีผลต่อสภาพให้ซึมผ่านได้ของไอน้ำของฟิล์มที่เติมกรดเฟรูลิกและฉายรังสียูวีซี นอกจากนี้ยังพบว่าการเติมกรดเฟรูลิก และ/หรือ การบ่มด้วยรังสียูวีซีมีผลเพียงเล็กน้อยต่อความสามารถในการละลายน้ำของฟิล์ม อย่างไรก็ตามในงานวิจัยนี้พบว่าความไม่ชอบน้ำของผิวฟิล์มเพิ่มขึ้นเมื่อปริมาณรังสีเพิ่มขึ้น โดยเฉพาะอย่างยิ่งสำหรับตัวอย่างที่ใช้เทคนิคฉายรังสีบนแผ่นฟิล์ม โดยสรุปพบว่าการฉายรังสียูวีซีเป็นเทคนิคที่มีประสิทธิภาพในการปรับปรุงสมบัติด้านแรงดึงของฟิล์มโปรตีนถั่วเหลืองที่เติมกรดเฟรูลิก อย่างไรก็ตามเทคนิคดังกล่าวจะส่งผลต่อความโปร่งใสและสีของฟิล์มด้วย

สาขาวิชา	วิทยาศาสตร์และเทคโนโลยีทางอาหาร	ลายมือชื่อนิสิต .....
ปีการศึกษา	2564	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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Md Shakil : EFFECTS OF FERULIC ACID AND UV CURING ON PROPERTIES OF SOY PROTEIN FILM. Advisor: Asst. Prof. THANACHAN MAHAWANICH, Ph.D.

The objective of this study was to explore the effect of UV-C curing on the properties of ferulic acid-added soy protein film. The films were fabricated from soy protein isolate and added with 1.5% ferulic acid. UV-C radiation was applied at four different doses (0.32, 1.56, 4.00, 12.00 J/cm^2) to either preformed film or film-forming solution. The mechanical, physicochemical, and morphological properties of the film samples were investigated. Ferulic acid addition and UV-C curing at 0.32 J/cm^2 posed a significant effect on film thickness while film density was slightly affected by ferulic acid addition and/or UV-C treatment. UV-C irradiation of ferulic acid-added film resulted in an increase in tensile strength and elongation at break. The films irradiated at the highest dose (12.00 J/cm^2) exhibited about 1.3-fold increase in tensile strength and a 1.7-fold increase in elongation at break from the control. UV-C treatment on preformed film did not produce any difference in tensile properties from the treatment on the film-forming solution. Protein cross-linking via C-N and dityrosine bonds was confirmed using FTIR and fluorescence spectroscopic techniques. Apart from the mechanical properties, ferulic acid addition and UV-C curing also posed a significant effect on the film's optical properties, including transparency and colour. UV-C irradiation made the ferulic acid-added film become lower in transparency and higher in chroma, as the films appeared more opaque and more intense in yellowness to the naked eye. As compared to the control, UV-C treatment of ferulic acid-added films caused a slight increase in water vapour permeability. However, similar water vapour permeability was observed among the UV-treated ferulic-added films regardless of the UV-C dose used. Ferulic acid addition and/or UV-C irradiation also minimally affected the water solubility of the film samples. In spite of that, an increase in surface hydrophobicity was observed with increasing UV-C dose, especially for the treatments on preformed film. In conclusion, UV-C irradiation was demonstrated as an effective technique for improving the tensile properties of ferulic acid-added soy protein film. It should be noted that, upon utilizing this technique, the transparency and colour of the soy protein film were also affected.

Field of Study:	Food Science and Technology	Student's Signature .....
Academic Year:	2021	Advisor's Signature .....

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Md Shakil

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CHAPTER 1

INTRODUCTION

Packaging is designed to contain the product inside and extend shelf life while maintaining its quality (Zhang & Mittal, 2010). The importance of food packaging materials has increased substantially over the last decades as the world has moved from rural self-sufficiency to a highly-industrialized society (Zink et al., 2016). Globally, more than 280 million tonnes of petrochemical-based plastics are manufactured yearly, with an average annual growth rate of around 5% (B. M. Schmid et al., 2015). Most of the plastic materials are non-biodegradable, and a sizable portion is even non-recyclable. This contributes to the wastage problem and environmental pollution. With this awareness, the packaging and food industries are working together to minimize the packaging volume and develop alternative substitutes to commercial plastics.

Proteins are among the common biopolymers used for preparing biodegradable packaging. Soy protein has been extensively investigated, among others, because of its outstanding film-forming capability. Soy protein film could be made from soy protein isolate, soy protein concentrate, soy flour, and fractionated soy proteins (S. Y. Cho & Rhee, 2004; Guerrero et al., 2011). The amino acid subunits of soy proteins contain both polar and non-polar side groups, facilitating the formation of strong inter- and intra-molecular associations via various chemical interactions, i.e., hydrogen bonding, dipole-dipole interaction, electrostatic interaction, and hydrophobic interaction. This accounts for a strong protein network responsible for a stable film matrix. Segment rotation and molecular mobility are restricted in soy protein film due to charge and polar contacts among the side groups, leading to enhanced stiffness, yield point, and tensile strength (c). Diversity in the property of the amino acid side groups also enables protein film modification using different techniques. In spite of that, soy protein films still have inferior mechanical and vapour barrier properties for practical applications, and these characteristics become extremely impaired in high-humidity conditions (Ou & Kwok, 2004).

It has been reported that inducing cross-linking in protein films by chemical, biochemical, and physical treatments could improve the film properties, particularly

mechanical strength. Aldehydes such as formaldehyde, glutaraldehyde, and glyoxal are efficient protein cross-linkers by reacting mainly with lysine residues of polypeptide chains and inducing inter- and intra-molecular cross-links (Marquié, 2001). However, due to the toxicity of these aldehydes, the safety of aldehyde-added films is questioned, making scientists exploring for safer cross-linking agents or techniques (O'Brien et al., 2005).

Ferulic acid is a phenolic compound widely found in nature with low toxicity. Certain amino acids in proteins, such as tyrosine, lysine, and cysteine, can react with ferulic acid and its oxide, quinoid ferulic acid, to form C-N and C-S bonds (Rawel et al., 2002; Strauss & Gibson, 2004). Ferulic acid-containing films were reported to demonstrate reducing water vapour permeability, water solubility, and increasing tensile strength, elongation at break, and surface hydrophobicity, as compared to the untreated film particularly at high concentration of its oxidized form (Insaward et al., 2015).

Physical methods such as ultraviolet (UV) and gamma irradiation have been reported as an efficient technique to induce cross-linking in proteins. The side groups of aromatic amino acids, like tyrosine and phenylalanine, could absorb the radiation with the production of amino acid-free radicals. Subsequent free radical recombination leads to the formation of a covalent bond between polypeptide chains (Rhim et al., 1999). Gennadios et al. (1998) reported that exposure of soy protein film to UV-C radiation increased tensile strength and yellow coloration with a decrease in elongation at break. However, UV irradiation was found to pose no effect on the water vapour permeability of the film. Besides inducing covalent cross-link, irradiation may also alter protein film properties by instigating molecular degradation (Gennadios et al., 1998).

The objective of this study was, therefore, to investigate the effect of UV-C radiation at different doses as applied to either film-forming solution or preformed film on the properties of ferulic acid-containing soy protein film.

CHAPTER 2

LITERATURE REVIEW

2.1 Proteins and their characteristics

Proteins are macromolecules containing α-amino acid subunits with twenty different amino acids functioning as the building blocks of proteins. The amino acid subunits are assembled through peptide bonds to form a linear polymer called a polypeptide chain. This amino acid sequence is the protein primary structure. Some proteins are made up of multiple polypeptide chains (Dörr, 1980). The other structures (secondary, tertiary, and quaternary) of proteins result from various interactions among the amino acids with varying energy (Schmid & Müller, 2019). Protein structure can be altered using physical (temperature, pressure, and shear) and chemical treatments (pH, organic solvents, organic solutes, detergents, and salts) (Schmid et al., 2014; Zeng et al., 2010). As a polymer base for film fabrication, proteins are popular, as compared to other biopolymers, due to their many distinctive characteristics such as conformational denaturation, electrostatic charges, amphiphilic nature, good film-forming ability, as well as superior gas barrier and mechanical properties of the resulted films (Schmid et al., 2015).

2.2 Fabrication of protein films

The possibility of establishing inter- and intra-molecular bonds and interactions is influenced by protein conformation and film-forming conditions. Fibrous proteins, including collagen and glutenin, possess distinctive characteristics for forming a film with excellent mechanical properties. For globular proteins, like gliadin, glycinin, and casein, it is required that the interactions responsible for higher structures of native proteins must be disrupted in order to unfold the polypeptide chains and thus facilitate the formation of new chemical interactions and bonds necessary for a stable film matrix (Gontard et al., 1994). According to Cuq et al. (1998), three stages of change are required to produce a protein network for film formation: first, the disruption of minimal-energy intermolecular connections that stabilize polypeptide chains in their native state; second, the unfolding and rearrangement of the polypeptide chains; and finally, when the agent that ruptures the intermolecular connections is removed, an

intermolecular network is formed new interactions and bonds stabilize that. Protein films are fabricated using two techniques which are dry and wet processes.

2.2.1 Dry process

Thermoplastic extrusion is employed in the dry process because biopolymers, including proteins, have thermoplastic characteristics. Subsequently, they were plasticized with a small amount of water and heated beyond their glass transition temperature. Because of their cost-effectiveness for larger-scale production, the key technique for commercial processes is compression moulding and extrusion (Belyamani et al., 2014). Thermoplastic biopolymers produce biodegradable packaging or composite packaging components (Galietta et al., 1998; Verbeek & van den Berg, 2010).

2.2.2 Wet process

The process is based on fabricating a film from a protein solution or dispersion (Donhowe, 1994). A protein solution must be prepared under controlled conditions and deposited as a thin layer to form a film. This wet process, or the so-called solvent process, can be classified into the surface film formation and deposition film formation methods (Guilbert et al., 2002; Khwaldia et al., 2004).

2.2.2.1 Surface film formation method

In this method, films are made by heating film-forming solutions over an extended length of time, and the films are periodically collected off the surface of the solution, drained, and then dried (Wittaya, 2012). The high temperature used denatures protein and alters its three-dimensional structure with an exposure of amino acid side groups, such as amino, sulfhydryl, and hydrophobic groups, that are capable of engaging in inter- and intra-molecular interactions (Mallamace et al., 2018). As a result, a protein network is formed, which serves as a matrix for entrapping other film components like plasticizers (Kunte et al., 1997; Schmid et al., 2014).

It is presumed that protein in the film remains in its denatured state when film formation occurs after protein denaturation. Nevertheless, it is believed that the denatured protein will partially be refold during the film formation process, allowing it to regain some of its higher structure. It is indeed possible that the amount

of refolding poses an impact on the number of functional groups accessible for inter- and intra-molecular interaction and subsequent network development and stability (Subirade et al., 1998). Furthermore, interfacial pressures may induce the development of a protein matrix capable of binding water and oil droplets discharged from the surface, therefore promoting protein matrix formation (Farnum et al., 1976).

2.2.2.2 Deposition film formation method

In this technique, the film is formed by pouring a film-forming solution onto a non-stick surface and drying it (Suhag et al., 2020). This method may be used to imitate some industrial procedures, such as dip moulding, for making of a free-standing film. Compared to the surface film formation approach, this deposition method generally produces a more uniform film (Jaynes & Chou, 1975). Film thickness can be readily regulated by controlling the quantity of film-forming solution and its total solid concentration. Several studies have been using this deposition method to make films from various proteins, e.g. wheat gluten, maize zein, casein, whey protein isolate, soy protein isolate, and sesame protein concentrate.

2.3 Plasticizers in protein films

Plasticizers are high-boiling point non-volatile compounds usually contain 14-40 carbon atoms with linear or cyclic structures (Vieira et al., 2011). Upon incorporation, the plasticizer occupies a three-dimensional protein network, boosting the free volume, decreasing protein chain-to-chain contact, as well as promoting film flexibility and chain movement (Vieira et al., 2011). Plasticizers reduce film brittleness by decreasing interactions among protein chains, such as disulfide bonding, hydrogen bonding, hydrophobic interaction, and electrostatic interaction (Sothornvit & Krochta, 2001). Plasticizers also lower polymer deformation stress, hardness, density, viscosity, and electrostatic charge (Vieira et al., 2011).

In polymer science, plasticizers are classified as internal and external. External plasticizers have low volatility, and their molecules interact with polymer chains rather than chemically attached to them by main bonds. Therefore, this type of plasticizer can be readily eliminated through evaporation, migration, and extraction. On the other hand, internal plasticizers are components of the polymer particles that constitute the finished

product and, therefore, can be co-polymerized or reacted with the polymer. Among others, water is the most suitable plasticizer for polar biopolymers as proteins and polysaccharides. However, some plasticizers, such as smaller carbohydrates, polyols, and lipids and their derivatives, are frequently used to plasticize protein film matrix (Yang & Paulson, 2000). The efficacy of plasticizers depends on their molecular weight and ratio to the polymer. Thus, the type and amount of plasticizer used in film-forming solutions pose a significant impact on physical characteristics of protein films. Plasticizers with lower molecular weight are often more efficient in terms of plasticizing activity than those larger ones. The polarity of a plasticizer also affects the film characteristics. Plasticizers with low polarity compete poorly for hydrogen bonding sites and are less efficient in disrupting intermolecular forces in a protein matrix in comparison to those with high polarity. In protein films, glycerol is one of the most efficient and frequently used plasticizers (Sothornvit & Krochta, 2001).

2.4 Common proteins for edible and biodegradable film fabrication

2.4.1 Soy proteins

Soy protein has been extensively investigated because of its outstanding film-forming capabilities. Soy protein films can be made from soy milk, soy flour, soy protein concentrate, soy protein isolate, and fractionated soy proteins (e.g., soy glycinin). Soy protein isolate is a soy protein product with the highest purity containing at least 90% protein on a wet basis (Guerrero et al., 2011). Based on sedimentation coefficients, soy proteins are categorized into four fractions, namely 2S, 7S, 11S, and 15S globulins. Among them, the principal fractions are β-conglycinin (7S globulin) and glycinin (11S globulin), which account for 37 and 31% of total soy globulins, respectively (Cho & Rhee, 2004). It was reported that the film obtained from the 7S fraction was transparent and wrinkled, while that from the 11S fraction was smooth, opaque, and with greater tensile strength than that made from the 7S fraction (Cho & Rhee, 2004). Soy proteins contain both polar and non-polar side groups. As a result, strong inter- and intra-molecular interactions, such as hydrogen bonding, dipole-dipole interaction, ionic interaction, and hydrophobic interaction, could occur between the side groups. Segment rotation and molecular mobility are restricted in soy protein films due to charge and polar contacts between the side groups, leading to enhanced stiffness,

yield point, and tensile strength (Zhang et al., 2001). Soy proteins have an isoelectric point around 4.5. Therefore, soy protein film-forming solutions can be prepared under acidic or alkaline conditions, although the film made from an alkaline solution usually demonstrates superior properties to that made from an acidic solution (Rad et al., 2018; Rayner et al., 2000; Siracusa & Lotti, 2018).

Like most protein films, soy protein film is poor in terms of moisture barrier property due to its hydrophilic nature. This can be improved by adding hydrophobic substances, such as lipids, into the film or altering the protein network by cross-linking the protein chains (Wittaya, 2012).

2.4.2 Corn zein

Zein is the predominant protein, specifically prolamin, in corn. The protein is obtained as a by-product of corn processing. It is present in the endosperm tissue and accounts for 45-50% of corn proteins. Prolamin is insoluble in water but soluble in alcohol (Beck et al., 1996; Shukla & Cheryan, 2001). For edible zein film and coating, an aqueous ethanol medium is mostly used as a solvent (Khwaldia et al., 2004). The presence of non-polar amino acids in zein imparts the hydrophobic nature of the protein, contributing to the excellent water vapour barrier property of zein film (Dickey et al., 2001; Shukla & Cheryan, 2001; Soliman et al., 2009). Physical treatment such as UV- and gamma-irradiation during or after film formation was reported to help improve zein film strength by triggering protein-protein cross-linking within the film structure (Wang & Padua, 2004).

2.4.3 Whey protein

Whey protein film can be made from whey powder, whey protein concentrate, or whey protein isolate (Jauregi & Welderufael, 2010; Kilara & Vaghela, 2004). α-Lactalbumins and β-lactoglobulins are the two major proteins in whey protein which account for 19 and 57% of the total protein, respectively (Dybing & Smith, 1991). The mechanical properties of whey protein film can be improved by treating the film-forming solution or preformed film with high doses of UV radiation. However, the UV treatment was reported to make the film become yellower, greener, and darker than untreated film (Díaz et al., 2016). The properties of whey-based films were reported to

be affected by pH. Alkaline pH assisted denaturation, solubilization, and protein unfolding (Bourtoom et al., 2006). Similar to most protein films, whey protein film demonstrates poor moisture barrier properties but this could be improved by adding lipid components, such as oils (Javanmard & Golestan, 2008), waxes (Soazo et al., 2013), and fatty acids (Fernández et al., 2007), to increase the film hydrophobicity.

2.4.4 Wheat gluten

Gluten is the main storage protein of wheat, comprising of a mixture of proteins, mainly gliadins and glutenins (Rhim et al., 1999; Wieser, 2007). The gliadins and glutenins are prolamins which is insoluble in water but soluble in aqueous ethanol. Gluten films are often prepared by casting a thin layer of film-forming solution and then drying it or boiling protein solution and harvesting the film that forms on top of the solution (Mangavel et al., 2004). The formation of additional disulfide bonds and hydrogen and hydrophobic interactions during film drying is crucial in developing wheat gluten film structure (Gennadios & Weller, 1990).

The most frequent solvent for gluten film-forming solution is aqueous ethanol. Wheat gluten films prepared using an alkaline solution were reported to provide much greater tensile strength than films made in an acidic medium (Zhang & Mittal, 2010). Gluten films have a shiny surface, good oxygen barrier properties but poor resistance to water vapour, and limited mechanical characteristics (Azam et al., 2009). Thermal treatment or chemical cross-linking can improve the mechanical characteristics of gluten films by covalently cross-linking the polypeptide chains.

2.4.5 Sesame protein

Sesame protein film is made from sesame protein isolate or concentrates, which are by-products of the oil extraction process (Achouri et al., 2012; Onsaard et al., 2010). The main components of sesame protein are albumins (8.6%), globulins (67.3%), prolamins (1.4%), and glutelins (6.9%) (Onsaard, 2012). As sesame protein has a high molecular weight with excellent thermal stability, it is a perfect choice for film-forming applications (Lee et al., 2014; Sharma & Singh, 2016). Protein content, pH, temperature, and plasticizer concentration all influence the tensile strength, solubility, and water vapour permeability of the film. Sesame protein film exhibits

superior thermal and moisture barrier properties to those made from other plant proteins, including peanut, soy, lentil, and faba bean proteins. Regarding its optical property, sesame protein film exhibits deeper colour and is less transparent than the films from synthetic polymers (Sharma & Singh, 2016).

2.4.6 Gelatin

Gelatin is a partially hydrolyzed product of collagen, a protein found in animal skin, bone, and connective tissue (Sheng, 2015). It has a uniquely high content of particular amino acids, namely, glycine, proline, and 4-hydroxyproline (Fakirov & Bhattacharyya, 2007). Gelatin has become a key raw material for producing edible films and coatings due to its excellent film-forming ability, availability, reasonable price, biocompatibility, and biodegradability (Jongjareonrak et al., 2006). Gelatin is extremely popular in the pharmaceutical industry because gelatin films and coatings are highly transparent, biocompatible, and have melting temperature close to the body temperature. In addition, gelatin films are oxygen-impervious and thermo-reversible (Hassan et al., 2018).

Gelatin film can be produced by the casting of gelatin aqueous solution. It may be categorized into cold-casted and hot-casted films based on the preparation temperature. The protein in the films prepared with different techniques has distinct conformational states. Gelatin molecule in the cold-casted film has a spiral structure, while that in the hot-casted film has a statistical coil (random coil) shape (Denavi et al., 2009). Films prepared by casting have greater tensile strength, while films made by extrusion have higher extensibility (Andreuccetti et al., 2012). Like most protein films, cross-linking of polymer chains can enhance the function of gelatin films by altering the polymer network (Wittaya, 2012).

2.5 Factors affecting protein film properties

2.5.1 pH of film-forming solution

Among various factors, pH has an impact on the structure and functionality of proteins and their film formation. The isoelectric point of water-soluble proteins like soy protein and whey protein determines their solubility (Zayas, 2012). Proteins have a net negative charge at pH levels higher than their isoelectric point and

a net positive charge at pH levels lower than their isoelectric point. Electrostatic repulsion among protein molecules arises when the pH is adjusted away from the isoelectric point, thus increasing protein solubility (Wittaya, 2012; Zayas, 2012).

Many plant proteins possess an isoelectric point in the pH range of 4-5. Therefore, plant protein film-forming solutions are usually prepared in the alkaline condition in order to enhance protein solubility. Mauri & Añón (2008) reported that soy protein film exhibited the highest tensile strength, elongation, glass transition temperature, and the lowest water vapour permeability when the film-forming solution was prepared at pH 11.0. For mungbean protein film, Wittaya (2009) reported that the film-forming solution at pH 9.5-10.0 resulted in a film with greater tensile strength and elongation at break. Peanut protein film prepared from a pH 9.0 film-forming solution was reported to exhibit the lowest water vapour permeability and oxygen permeability and the greatest tensile strength (Jangchud & Chinnan, 1999). For faba bean protein film, the film prepared using a pH 10.0 film-forming solution demonstrated decreased water vapour permeability and increased puncture strength (Montalvo-Paquini et al., 2013).

2.5.2 Heat treatment and drying temperature

The temperature has an impact on the interaction forces in proteins. Heat is generally considered an influential denaturing factor for proteins, and their amino acid content influences their thermal stability and structure. During heating of film-forming solution, the degree of protein unfolding determines the type and number of covalent bonds (disulfide bond) and noncovalent interactions (hydrophobic interaction, ionic interaction, and hydrogen bond) among protein chains. When proteins denature, chains can connect more firmly and rapidly, particularly through a disulfide bond (Denavi et al., 2009). Moreover, protein configuration undergoes changes during drying process as water gradually evaporates and this affects properties of the resulted film (Denavi et al., 2009; Sun, 2005).

Heating and drying temperature were reported to have an effect on the physical and barrier properties of films(Bourtoom, 2008; Fernández-Pan et al., 2010). At pH around 9.50 and heating temperature at 75°C for 20 min, mung bean protein film

exhibited high tensile strength and elongation at break and low water vapour permeability as well as water solubility as compared to the control (Bourtoom, 2008). In the case of peanut protein film, water solubility, water vapour permeability, and oxygen permeability were found to decrease, while tensile strength and elongation at break were found to increase with increasing heating temperature of the film-forming solution (Liu et al., 2004). In another study, Jangchud & Chinnan (1999) reported that peanut film had the lowest water activity, moisture content, water vapour permeability, and oxygen permeability but the highest tensile strength at pH 9 and heat treated at 90°C. Moreover, Perez-Gago & Krochta (2000) reported that drying temperature significantly lower water vapour permeability of lipid-whey protein emulsion film. The researchers also noticed that the film dried at 80°C exhibited lower water vapour permeability compared to that dried at room temperature and at 40°C. However, in this study, the film mechanical properties were found to be unaffected by drying conditions (Perez-Gago & Krochta, 2000).

2.5.3 Protein concentration

Protein-protein interactions affect protein mobility and film formation capabilities. The concentration of film-forming solution influences the self-adhesion of polymers, protein matrix development, and speed of matrix formation (Kaewprachu et al., 2016; Wittaya, 2012). Compared to optimum concentration, either lower or higher protein concentration probably leads to a lower degree of protein-protein interaction and inferior properties of the film obtained. An intermediate viscosity may be attained at the optimum concentration of film solution, resulting in maximum cohesive strength (Barman et al., 2018; Wittaya, 2012).

It was reported that the development of disulfide bridges in whey protein film required a comparatively high protein content (>8%) of the film-forming solution (Nandane & Jain, 2015; Sothornvit & Krochta, 2001). The protein concentration of the film-forming solution also affects the film's physical, mechanical, and barrier properties (Chen et al., 2019). A higher concentration of whey protein isolate may result in a thicker film, eventually affecting water vapour and oxygen permeability (Gounga et al., 2007). For fish myofibrillar protein film, an increase in protein concentration of the film-forming solution was reported to cause an increase in thickness, tensile strength,

elongation at break, and water vapour permeability. In contrast, film solubility was found to decrease (Kaewprachu et al., 2016).

2.5.4 Relative humidity

The moisture content of the film may induce modification of its physical property. Water vapour adsorption by dry materials is thought to be linked to the attachment of water molecules to certain hydrophilic sites, such as carboxyl, amino, and hydroxyl groups (Barman et al., 2018; Wittaya, 2012). Swelling and molecular structure alteration cause multimolecular adsorption at high relative humidity. Characteristics of protein-based films may undergo changes with storage time due to the intrinsic instability of their basic ingredients, including moisture (Wittaya, 2012). Anker et al., 2002 stated that polymer rearrangement could result in changes that induce physical instability of protein films owing to the movement of low molecular weight components, like plasticizers and water, in film formulation (Anker et al., 2002). Cuq et al. (1997) investigated the effect of relative humidity on mechanical and barrier properties of myofibrillar protein-based films and reported that an increase in relative humidity induced a decrease in elastic modulus and water vapour barrier properties and an increase in elongation at break. Pochat-Bohatier et al. (2006) reported that the gas permeability of wheat protein film increased as the relative humidity reached 96%. This was due to that the polymer matrix expanded when exposed to moisture, enabling chemical reactions between amino acids and gases. Chinma et al. (2015) described that an increase in relative humidity caused an increase in tensile strength, elastic modulus, as well as water vapour permeability, and a decrease in elongation at break of soy protein film. Gennadios et al. (1993) described a reduction in tensile strength of corn zein film and wheat gluten film with increasing relative humidity.

2.6 Modification of protein films through cross-linking

2.6.1 Physical modification

Heat curing and irradiation are two physical techniques that are widely used to modify protein film properties through the formation of protein cross-linking.

2.6.1.1 Heat curing

According to Soroka (1999), heat curing involves exposing a substrate to one or more heating cycles to modify the molecular structure and reorder the polymers. For protein films, this could be done by heating either the film-forming solution or the preformed film. Jensen (1959) proposed that heating a protein could facilitate a thiol-disulfide exchange reaction which results in a disulfide cross-link (Figure 2.1).

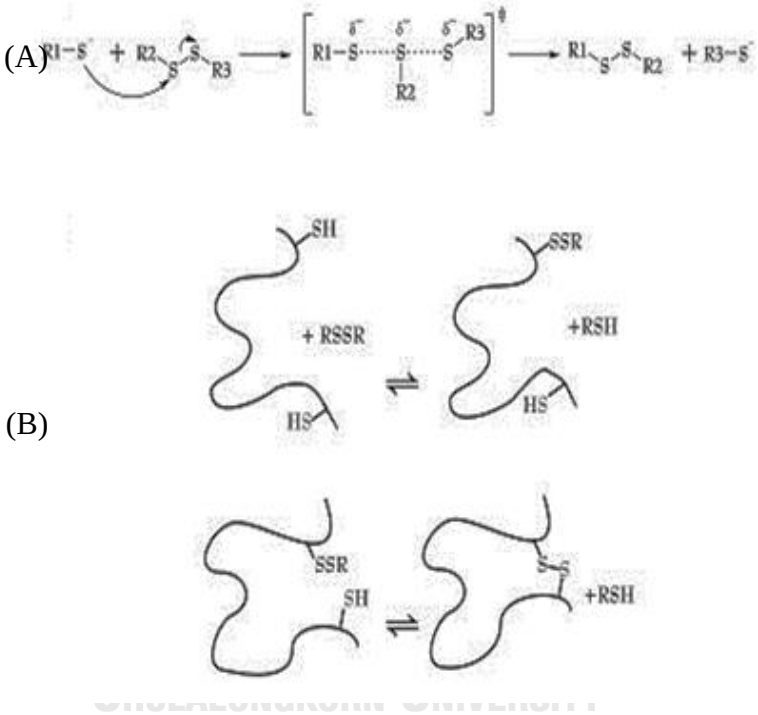


Figure 2.1 (A) Thiol-disulfide exchange reaction and (B) formation of disulfide cross-link in polypeptide

Source: Ágoston et al. (2005)

Heat curing was reported to improve the tensile strength of whey protein, soy protein, and gluten-based films (Zubeldía et al., 2015). Stuchell & Krochta (1994) reported that heat curing enhanced the elongation of soy protein film and gluten film. Perez-gago & Krochta (2001) demonstrated that heat curing resulted in a whey protein film with decreasing oxygen permeability.

2.6.1.2 Irradiation

Irradiation is another technique that could be used to induce cross-linking in protein. The radiation could be classified into two types, ionizing and non-ionizing radiation (Reisz et al., 2014). The ionizing radiations that have been used in protein film modification are gamma radiation and electron beam (or beta radiation). This type of radiation is of higher energy and can induce the displacement of electrons from atoms or molecules. The non-ionizing radiation frequently used in protein film modification is UV radiation. Being of lower energy, this type of radiation does not remove electrons but rather transfers its energy to the atoms or molecules.

Ionizing irradiation can result in irreversible protein conformation changes, amino acid oxidation, covalent bond breaking, protein-free radical generation, and recombination and polymerization processes. During gamma irradiation, water forms hydroxyl radicals. Aromatic amino acids in protein like phenylalanine and tyrosine react with those hydroxyl radicals (Sabato et al., 2001). Inter-protein cross-linking, hydrophobic and electrostatic interactions and the formation of disulfide bonds can convert proteins to higher molecular weight aggregates (Davies & Delsignore, 1987).

Regarding UV, it is an electromagnetic radiation with a wavelength of 100-400 nm and a frequency of 10^{15}-10^{18} Hz. UV radiation can be classified as UV-A, UV-B, and UV-C. UV-A or long wave UVR or black light has a wavelength of 315-400 nm and a photon energy of 3.10-3.94 eV, which is the lowest energy among the three types of UV radiation. UV-B or middle UVR or sunburn radiation has a wavelength of 280-315 nm and a photon energy of 3.94-4.43 eV. Lastly, UV-C or short wave UVR or germicidal radiation has a wavelength of 100-280 nm and the highest photon energy of 4.43-12.4 eV. Most studies involving UV curing of protein films utilized the UV-C at 253.7 nm. UV radiation could be absorbed by the side group aromatic of amino acids, mainly tyrosine, followed by oxidation of the amino acid and production of its free radical. Upon recombination of the free radicals, dityrosine cross-link is produced (Figure 2.2).

However, it should be noted that irradiation can directly affect proteins and indirectly pose an effect on its surroundings, which can alter the properties of protein film either by covalent cross-linking or molecular degradation (Gennadios et al., 1998; Puchala & Schuessler, 1995; Ressouany et al., 1998). The effect of irradiation on protein structure is influenced by several factors, including protein concentration, oxygen availability, and the quaternary structure of the protein (Cho et al., 1999).

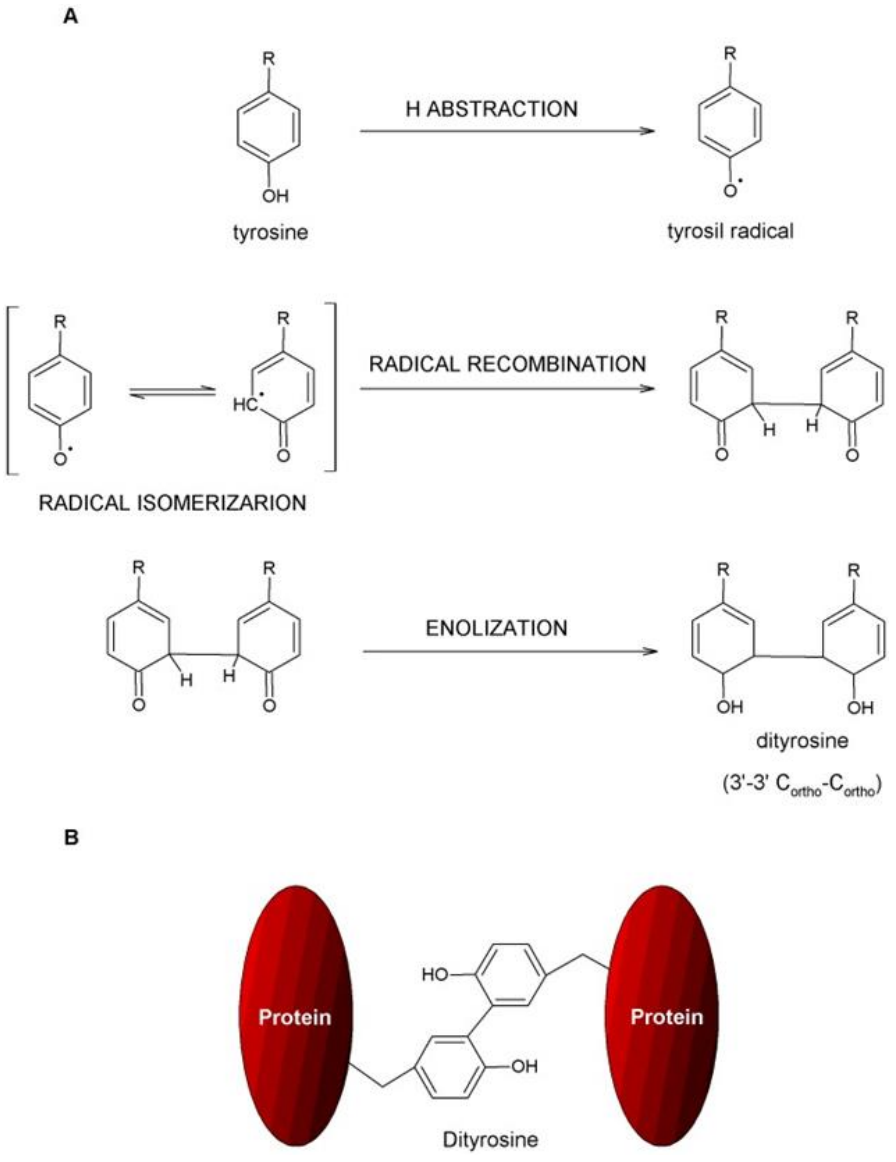


Figure 2.2 Mechanism of dityrosine cross-link formation showing the reactions involving dityrosine formation (A) and dityrosine cross-link in protein (B)

Source: Correia et al. (2012)

Gennadios et al. (1998) investigated the impact of UV-C irradiation on the properties of soy protein film and reported that increasing UV-C dose up to 103.7 J/m^2 resulted in an increase in tensile strength and a decrease in elongation at break. The authors explained that this was due to the UV-induced protein cross-linking. Additionally, it was found that UV-C treatment posed no effect on water vapour permeability while it intensified the yellow colouration of the film. Using a much lower UV-C dose (4.5 J/m^2), Vaz et al. (2003) found that UV-C treatment of film-forming solution and preformed film caused a slight increase in the film's mechanical properties.

Schmid (2015) reported that UV-C dose up to 31.4 J/cm^2 led to no significant changes in elongation at break, water vapour permeability, and oxygen permeability of whey protein film. However, the radiation caused an increase in tensile strength and yellowness of the film. Fathi et al., (2018) studied the effect of different UV radiation (UV-A, UV-B, and UV-C) on the properties of sesame protein film. The treatments were done on both film-forming solution and preformed film. It was reported that UV exposure caused a decrease in moisture content, water solubility, and water vapour permeability while increasing film density and hydrophobicity. UV-C treatment on film-forming solution produced a film with the greatest tensile strength and Young's modulus. Compared to UV treatment of the preformed film, UV irradiation of film-forming solution provided a superior outcome.

Rhim et al., (1999b) revealed that UV-C treatment (51.8 J/m^2) increased the tensile strength of gluten, corn zein, and egg albumen films but had no effect on that of sodium caseinate film. Gluten, egg albumen, and caseinate films exhibited increasing yellowness upon UV-C irradiation, while this posed no effect on the colour of corn zein film. Díaz & Candia (2017) studied the effect of UV-C irradiation on the properties of soy protein film. The radiation was applied to film-forming solutions of pH 9 and 11, and an increase in protein aggregation was observed at both pH values. It was also found that UV-C treatment of pH 9 film-forming solution produced a film with higher solubility, tensile strength, elastic modulus, puncture deformation and lower elongation at break as compared to the untreated control. In contrast, treatment of pH 11 film-forming solution resulted in a film with higher solubility and elastic modulus but lower puncture deformation and elongation at break.

Micard et al. (2000) reported that UV-C treatment on wheat gluten film enhanced its tensile strength but reduced its elongation at break. The treatment was found to have no effect on water vapour permeability. Ustunol & Mert (2004) applied UV-C radiation at a dose of 324 J/cm^2 to a whey protein film-forming solution and found that the resulted film demonstrated increasing tensile strength and decreasing elongation.

2.6.2 Chemical modification

Chemical modification of protein films by cross-linking is the formation of covalent bonding either inter- or intra-molecularly. These chemicals are known as cross-linking reagents (Arora et al., 2017). Chemical cross-linking of protein film can potentially modify its properties, for example, mechanical, optical, and barrier properties. Protein side groups play a significant role in such a mechanism. In the past, low molecular weight aldehydes, such as formaldehyde, glutaraldehyde, and glyoxal, were very popular because of their exceptional cross-linking ability (Guilbert et al., 1995; Marquie et al., 1995). However, due to the toxicity of these aldehydes, scientists have been looking for alternative and safer cross-linking agents such as genipin, thiol oxidant, and phenolic compounds (Anker et al., 2002; Bondeson & Oksman, 2007).

Ferulic acid is a low-toxicity phenolic compound that has protein cross-linking ability. Side groups of certain amino acids in polypeptide chains, such as tyrosine, lysine, and cysteine, can react with ferulic acid and its oxide, quinoid ferulic acid, to form C-N and C-S bonds (Rawel et al., 2002; Strauss & Gibson, 2004). As a result, it can be used as a cross-linking agent to enhance the properties of protein-based films (Ou & Kwok, 2004). Insaward et al. (2015) reported that soy protein film fortified with ferulic acid demonstrated decreasing water vapour permeability and water solubility and increasing tensile strength, elongation at break, and surface hydrophobicity, compared to the untreated film, particularly at high concentrations of oxidized ferulic acid.

Strauss & Gibson (2004) explained the cross-linking reaction between phenolic acid and chemical groups of amino acid subunits of polypeptide chains (Figure 2.3). A phenolic acid **(1)** can be oxidized and turned into its quinone. The resulting quinone could undergo a side reaction, with a production of a phenolic acid dimer **(2)**,

which, in this case, only larger phenolic molecule is obtained, without cross-linking between phenolic compound and polypeptide chain. In terms of phenolic-protein cross-linking reaction, the quinone could react with the amino or sulfhydryl group of the polypeptide, producing a covalent C-N or C-S bond with the regeneration of hydroquinone. This hydroquinone, again, can undergo oxidation and react with the amino or sulfhydryl group of another polypeptide chain, resulting in a covalent cross-link between two polypeptide chains **(3)**. Alternatively, two polypeptides with quinone attaching to each chain can dimerize to form a covalent cross-link **(4)**.

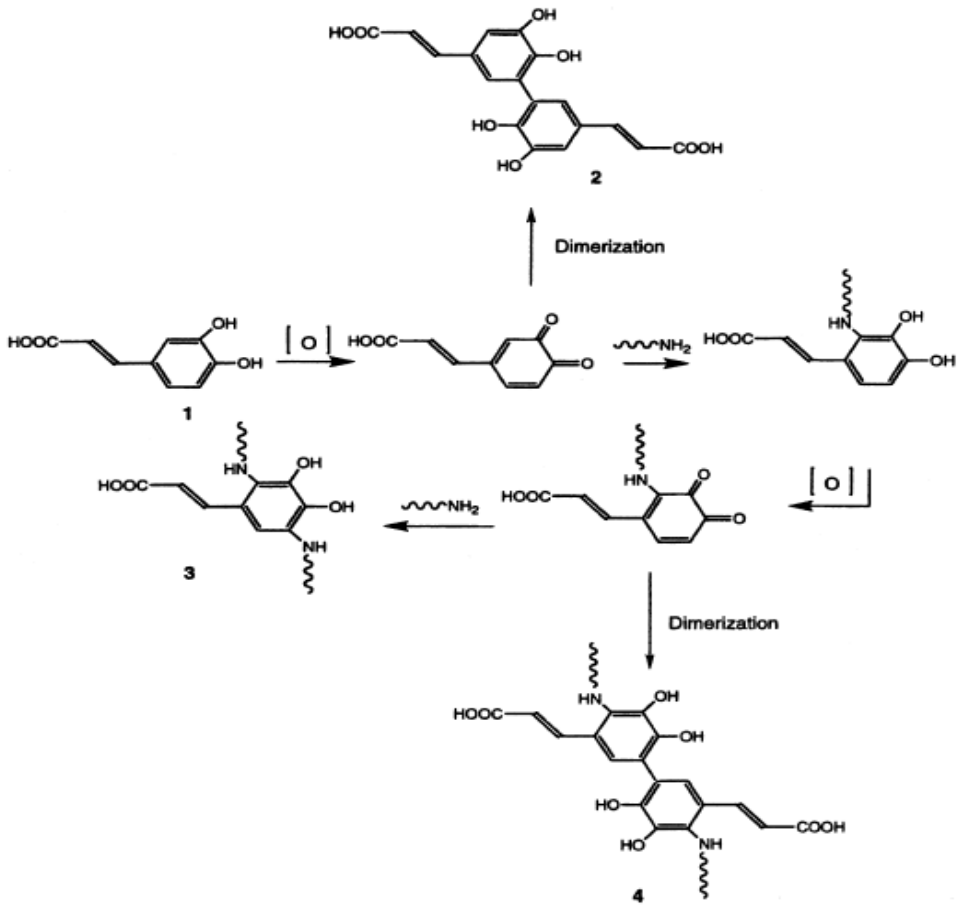


Figure 2.3 Reaction of phenolic acid and amino side group producing covalent cross-link of polypeptide chains

Source: Strauss & Gibson (2004)

Ustunol & Mert. (2004) investigated the properties of whey protein film modified using glutaraldehyde, formaldehyde, dialdehyde starch, and carbonyldiimidazole as cross-linkers. It was reported that each cross-linker was able to

improve tensile strength but had no effect on elongation at the break of the film. Water solubility was also significantly lowered upon cross-linking, in which the films treated with formaldehyde exhibited the lowest water solubility. The authors also observed that chemical cross-linking increased water vapour permeability while lowering oxygen permeability compared to the control. The film treated with glutaraldehyde had the greatest water vapour permeability, followed by those treated with carbonyldiimidazole, formaldehyde, and dialdehyde starch, in descending order. It was explained that the different polar groups generated by the cross-linkers may have contributed to this difference in barrier property. The lowered oxygen permeability of the cross-linked films might be due to the that non-polar oxygen molecule was not able to permeate through the polar structure of the cross-linked films. Liu et al. (2004) reported that formaldehyde and glutaraldehyde significantly increased tensile strength and decreased water vapour permeability and oxygen permeability of peanut protein film.

Besides the direct addition of aldehyde to the film-forming solution, treating a preformed film with aldehyde vapour was also reported as an effective way to cross-link the protein. Micard et al. (2000) used formaldehyde vapour from ethanolic solution of formaldehyde (10% v/v) to treat preformed wheat gluten films. The treatment was carried out for 24 h in an airtight container. It was reported that the films exposed to formaldehyde vapour demonstrated significantly higher tensile strength and lower elongation at break as compared to the untreated film. Formaldehyde vapour treatment also reduced the film water solubility and water vapour permeability (Micard et al., 2000).

Cao et al. (2007) studied the effect of phenolic acids, ferulic and tannic acid, on properties of gelatin film. It was found that both phenolic acids had cross-linking ability on gelatin film. Different amounts of ferulic and tannic acid were added to the film-forming solutions of different pH, ranging from 6 to 10. With increasing phenolic concentrations, tensile strength was found to increase while elongation at break was found to decrease. Ferulic acid was more efficient in terms of improving tensile strength. Addition of tannic acid, especially at high concentrations, resulted in a film which was more intense in yellowness. Maximum mechanical strength of gelatin

film was achieved when the pH of the film-forming solution was 7 for ferulic acid and 9 for tannic acid. Both phenolic cross-linkers were shown to reduce the film swelling ratio but had no noticeable effect on water vapour permeability.

Insaward et al. (2015) investigated the effect of phenolic acids, namely ferulic, caffeic, and gallic acid, as well as their oxidized derivatives, on properties of soy protein film. It was found that the film added with gallic acid exhibited highest tensile strength and elongation at break, followed by those added with caffeic and ferulic acid. Tensile strength and elongation at break of the films added with oxidized phenolic acids were greater than those added with unoxidized ones. Regarding colour, the films containing phenolic acids appeared darker than the control. Phenolic-containing film samples exhibited lowered water vapour permeability and water solubility, and greater surface hydrophobicity as compared to the control, particularly at high concentrations of oxidized phenolic acid.

Ou et al. (2005) demonstrated the cross-linking ability of ferulic acid, which resulted in enhanced mechanical strength of soy protein film. The ferulic cross-linked soy protein showed higher tensile strength and elongation at break, with lower oxygen permeability than the control. Additionally, ferulic acid in its oxidized state further enhanced mechanical properties and decreased water vapour permeability. The authors suggested that ferulic acid is a suitable cross-linking agent for protein films.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Soy protein isolate, 90.20% protein, wet basis, food grade (Krungthepchemi, Bangkok, Thailand)

Ferulic acid, food grade (Chanjao Longevity, Bangkok, Thailand)

Glycerol, food grade (Krungthepchemi, Bangkok, Thailand)

3.2 Equipment

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

Contact angle measuring instrument, model OCA15EC (Data Physics Instrument, Filderstadt, Germany)

Fourier transform infrared spectrometer (FTIR), model Spectrum One (Perkin Elmer, Waltham, MA, USA)

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

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

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

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

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

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

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

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

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

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

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

Visible spectrophotometer (model GENESYS20, Thermo Scientific, Waltham, MA, USA)

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

3.3 Methodology

For this study, the effect of UV-C radiation on the properties of ferulic acid-containing soy protein film was investigated. The ferulic acid concentration was fixed at 1.5% by the weight of soy protein isolate. UV-C at four different doses (0.32, 1.56, 4.00, and 12.00 J/cm^2) was applied to either film-forming solution or preformed film. Soy protein film without ferulic acid addition and without UV-C treatment was used as a control. In addition, non-UV-treated ferulic-added film (FE) and non-ferulic-added film treated with UV-C at 0.32 J/cm^2 (UV0.32) were also used as references.

3.3.1 Film preparation

3.3.1.1 Control film

Non-UV-treated soy protein film without ferulic acid addition was used as a control. The film was prepared according to the method described by Insaward et al. (2015) with some modifications. To prepare the film-forming solution, 5 g of soy protein isolate were dissolved in 100 g of phosphate buffer (pH 7.4), which contained 2.75 g of glycerol (55% by weight of soy protein isolate) as a plasticizer. After being homogenized using Ystral homogenizer (model X10/25, Ystral, Ballrechten-Dottingen, Germany) for 2 min at 22,000 rpm, the protein solution was then heated in a water bath (model SW23, Julabolabortechnik, Seelbach, Germany) at 70°C for 30 min to partially denature the protein and then cooled to ambient temperature. The ultrasonic degassing technique was done in an ultrasonic bath (model 136H, Fisher Scientific, Schwerte, Germany) to remove air bubbles from the film-forming solution. Film casting was done by transferring 45 mL of the film-forming solution onto a 150 mm × 150 mm acrylic mould. The film was then dried at 40°C for 24 h. The film sample was equilibrated at 50% RH and 25°C for 48 h before being subjected to further analyses.

3.3.1.2 Ferulic acid-added films

Ferulic acid at a concentration of 1.5% by weight of soy protein isolate was chosen based on the result of the previous report (Insaward et al., 2015). To prepare the film-forming solution, 5 g of soy protein isolate were dissolved in 70 g of phosphate buffer (pH 7.4), which contained 2.75 g of glycerol (55% by weight of soy protein isolate) as a plasticizer. After being homogenized using Ystral homogenizer

(model X10/25, Ystral, Ballrechten-Dottingen, Germany) for 2 min at 22,000 rpm, the protein solution was then heated in a water bath (model SW23, Julabolabortechnik, Seelbach, Germany) at 70°C for 30 min to partially denature the protein and then cooled to ambient temperature. Separately, 0.075 g of ferulic acid (1.5% by weight of soy protein isolate) was dissolved in 30 g of phosphate buffer (pH 7.4). The ferulic solution was then added to the protein solution and homogenized for 2 min at 22,000 rpm. The ultrasonic degassing technique was done in an ultrasonic bath (model 136H, Fisher Scientific, Schwerte, Germany) to remove air bubbles from the film-forming solution. Film casting was done by transferring 45 mL of the film-forming solution onto a 150 mm × 150 mm acrylic mould. The film was then dried at 40°C for 24 h. The film sample was equilibrated at 50% RH and 25°C for 48 h before being subjected to further analyses. In this study, non-UV-treated ferulic acid-added film (FE) was used as a reference.

3.3.2 UV-C irradiation

UV-C radiation at a wavelength of 253.7 nm was applied to either film-forming solution (denoted by SUV) or preformed film (denoted by FUV) at four different doses (0.32, 1.56, 4.00, and 12.00 J/cm^2). Irradiation treatment was carried out in a UV-C cabinet (model PIS-88C, P Inter Supply, Bangkok, Thailand) with a radiation intensity of 2500 μW/cm^2. All UV-treated films were prepared similar to the protocol described in 3.3.1, except that for UV-C treatment on the film-forming solution, UV-C radiation was applied to the film-forming solution before the drying step. In the case of UV-C treatment on preformed films, UV-C radiation was applied to the film after being dried at 40°C for 24 h and cooled to room temperature. All film samples were equilibrated at 50% RH and 25°C for 48 h before being subjected to further analyses. In this study, a preformed film without ferulic acid addition and UV-treated at 0.32 J/cm^2 (FUV0.32) were also prepared and used as a reference. Table 3.1 summarizes all film samples prepared in this study.

Table 3.1 Film samples with the applied UV-C doses and irradiation time

Film samples	Treatments		UV-C dose (J/cm^2)	Irradiation time
	Ferulic acid*	UV-C**		
Control			-	-
FE	✓		-	-
FUV0.32		✓	0.32	2 min 8 s
UV-treatment on preformed film				
FE+FUV0.32	✓	✓	0.32	2 min 8 s
FE+FUV1.56	✓	✓	1.56	10 min 24 s
FE+FUV4.00	✓	✓	4.00	26 min
FE+FUV12.00	✓	✓	12.00	80 min
UV-treatment on film-forming solution				
FE+SUV0.32	✓	✓	0.32	2 min 8 s
FE+SUV1.56	✓	✓	1.56	10 min 24 s
FE+SUV4.00	✓	✓	4.00	26 min
FE+SUV12.00	✓	✓	12.00	80 min

* Ferulic acid was added at 1.5% by weight of soy protein isolate

** UV-C radiation intensity was 2500 μW/cm^2.

3.3.3 Determination of film properties

3.3.3.1 Thickness

The film sample was cut into a 100 mm × 30 mm strip. The thickness was measured using a thickness gauge (model 7301, Mitutoyo, Tokyo, Japan).

3.3.3.2 Mechanical properties

Tensile test on the film samples was performed with uniaxial tension according to the ASTM D882 standard method (ASTM, 2009) using a Texture Analyzer (model TA.XTplus, Stable Micro System, Godalming, UK) equipped with tensile grips (A/TG) probe and 1 kg load cell. A strip of film sample (100 mm × 30 mm) was held by both grips with an initial grip separation of 50 mm. The film sample was stretched at a constant speed of 8.33 mm/s until failure. Tensile strength and elongation at break were calculated using 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 moved by the top grip (mm); and L_i is the initial grip separation (mm).

3.3.3.3 Colour

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

$$\text{Hue angle (Quadrant II)} = 180 + \arctan(b^*/a^*) \quad \ldots(3.3)$$

$$\text{Chroma} = (a^{*2} + b^{*2})^{1/2} \quad \ldots(3.4)$$

3.3.3.4 Water vapour permeability

Water vapour permeability of the film samples was determined following the ASTM E96 method (ASTM, 2022). A film sample without leaks and scratches was cut into a 60 mm × 60 mm piece. Twenty g of dried silica gel was placed into a glass permeation cup. Silicone grease was applied to the rim of the cup. After being mounted on the cup, the film piece was tightened with a rubber ring and parafilm. The 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 the water vapour permeability using Equation (3.5):

$$\text{Water vapour permeability} = \frac{W\,d}{A\,t\,(P_2\text{-}P_1)} \quad \ldots(3.5)$$

where W is weight gain of the permeation cup (g); d is the film thickness (m); A is the 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 vapour across both sides of the film (Pa).

3.3.3.5 Water solubility

The water solubility of the film samples, expressed in terms of total soluble matter, was determined according to the method outlined by Insaward et al. (2015). A 20 mm × 20 mm piece of film sample and Whatman grade 4 filter paper were dried in a hot air oven (model 5200, Kubota, Fujioka, Japan) at 70°C for 24 h. The dried film and filter paper was weighed to obtain their initial dry weight. The film sample was transferred to a 50-mL test tube containing 20 mL of distilled, and the test tube was then continuously shaken using an Innova® laboratory shaker (model 2050, New Brunswick Scientific, Edison, NJ, USA) at room temperature (25°C) for 24 h. The sample was filtered through the dried filter paper. The filter paper, together with the retentate, was then dried at 70°C for 24 h and later weighed to obtain the final dry weight. Water solubility was calculated using Equation (3.6):

$$\text{Water solubility (\%)} = \frac{W_i - W_f}{W_i} \times 100 \quad \ldots(3.6)$$

where W_i is the initial dry weight of the film sample (g); and W_f is the final dry weight of the film sample (g).

3.3.3.6 Surface hydrophobicity

Film surface hydrophobicity was determined in terms of a contact angle between the film surface and a water droplet using a contact angle measuring instrument (model OCA15EC, Data Physics Instruments, Filderstadt, Germany). A droplet (4 μL) of distilled water was deposited on the film surface. The contact angle of the water droplet and the film surface (θ) was measured, with θ<90° and θ>90° characterizing hydrophilic and hydrophobic surfaces, respectively.

3.3.3.7 Film density

Film density was calculated from dry mass and dimensions of a film piece using Equation (3.7) (Fathi et al., 2018):

Film density = $\frac{m}{A\,d}$ …(3.7)

where m is the dry mass of the film piece (g); A is the film area (cm^2); and d is the film thickness (cm).

3.3.3.8 Transparency

Transparency of a film sample was measured in terms of %transmittance according to the ASTM D1746 method (ASTM, 2015). A film sample was cut into a precise dimension (10 mm × 40 mm) and mounted onto the inside of a glass cuvette. %Transmittance was measured at 500 nm using a visible spectrophotometer (model GENESYS20, Thermo Scientific, Waltham, MA, USA).

3.3.3.8 Film microstructure

The cross-sectional microstructure of a film sample was investigated using a scanning electron microscope (model JSM-IT300, JEOL, Tokyo, Japan). The film sample was first to cut into a 5 mm-wide strip and stored in a desiccator with silica gel to dry for seven days. The film sample was then cut using a sharp razor blade to expose a clean cross-sectional area. The sample was mounted on a sample stub and sputter coated with gold. The microstructure of the film matrix was observed at 800× magnification.

3.3.3.9 Fourier transform infrared (FTIR) spectroscopy

The formation of the C-N bond was monitored using an FTIR spectrometer (model Spectrum One, Perkin Elmer, Waltham, MA, USA) with an attenuated total reflectance (ATR) accessory. Transmittance in a wavenumber range of 4000-500 cm^{-1} was measured using a scanning resolution of 4.00 cm^{-1} and 64 scans per sample.

3.3.3.10 Fluorescence spectroscopy

To monitor dityrosine cross-link formation, fluorescence intensity was measured using a spectrofluorometer (model FP-6200, Jasco, Tokyo, Japan) using an excitation wavelength of 320 nm and emission wavelength of 400-420 nm (Correia, 2016). The scanning speed was set at 125 nm/min.

3.3.4 Experimental design and statistical analysis

All experiments were done in three replicates. A completely randomized design was used. Data were analysed using Analysis of Variance. Duncan's new multiple range tests 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

In the current study, ferulic acid-containing soy protein film was prepared by adding 1.5% ferulic acid by weight of soy protein isolate. UV-C at four different doses (0.32, 1.56, 4.00, and 12.00 J/cm^2) was applied to either preformed film (for which the samples are designated as FE+FUV0.32, FE+FUV1.56, FE+FUV4.00, and FE+FUV12.00, respectively) or film-forming solution (for which the samples are designated as FE+SUV0.32, FE+SUV1.56, FE+SUV4.00, and FE+SUV12.00,

respectively). The control was soy protein film without ferulic acid addition and without UV-C treatment. Non-UV-treated ferulic-added film (FE) and non-ferulic-added preformed film treated with UV-C at 0.32 J/cm^2 (FUV0.32) were also used as references. The results of this study are as follows:

4.1 Film thickness

The thickness of the film samples, shown in Table 4.1, was in the range of 0.165-0.193 mm. Film thickness was found to be significantly affected ($p \leq 0.05$) by ferulic acid addition and/or UV-C treatment, as evidenced by the lower thickness of the control film. However, all ferulic acid-added and/or UV-treated films were not different in thickness ($p > 0.05$). Nuthong et al. (2009) investigated the effect of tannic, caffeic, and ferulic addition at 1, 2, and 3% on the thickness of porcine plasma protein-based film and reported that 3% tannic acid addition resulted in a film with a significantly greater thickness ($p \leq 0.05$) than the control. However, all other samples were of similar thickness to the control ($p > 0.05$). In terms of UV irradiation, Díaz et al. (2016) studied the effect of UV irradiation on the properties of whey protein concentrate film and reported that the films treated with UV-C at 0.12, 4.00, and 12.00 J/cm^2 were of higher thickness than the control. This increase in thickness could possibly be due to the induced protein aggregation that may influence the structure of the film (Díaz et al., 2016).

Table 4.1 Thickness of ferulic acid-added soy protein films treated with UV-C at 0.32, 1.56, 4.00 and 12.00 J/cm^2

Film samples	Thickness (mm)
Control	0.165±0.133^{c}
FE	0.185±0.179^{ab}
FUV0.32	0.188±0.127^{ab}
UV-treatment on preformed films	
FE+FUV0.32	0.186±0.152^{ab}
FE+FUV1.56	0.181±0.132^{ab}
FE+FUV4.00	0.193±0.107^{a}
FE+FUV12.00	0.177±0.143^{ab}
UV-treatment on film-forming solutions	
FE+SUV0.32	0.184±0.141^{ab}
FE+SUV1.56	0.177±0.130^{ab}
FE+SUV4.00	0.189±0.102^{ab}
FE+SUV12.00	0.178±0.079^{ab}

Mean±SD of three replicates.
Sample means within a column that do not share a common superscript letter differ significantly at p=0.05.

4.2 Mechanical properties

Mechanical properties of the film samples are reported in terms of tensile strength and elongation at break, as shown in Table 4.2. Increasing tensile strength and elongation at break were observed upon ferulic acid addition and/or UV-C curing. However, the difference was statistically insignificant (p>0.05), except at higher doses of UV-C treatment on ferulic acid-added films.

As to phenolic cross-linking of protein films, Insaward et al. (2015) reported that the addition of either oxidized or unoxidized ferulic acid at 1.5% of soy protein isolate caused an increase in tensile strength and elongation at the break of the film. Ou et al. (2005) also reported that ferulic acid addition at 50-200 mg/100 g of soy protein isolate was shown to improve tensile strength and elongation at the break of the film. The development of a cross-linked structure owing to reactions between protein and ferulic acid might be responsible for the increase in the tensile properties (Insaward et

al., 2015). In the contrary, Arcan & Yemenicioğlu, (2011) reported that the addition of ferulic acid resulted in a zein film with decreasing tensile strength but increasing elongation at break.

Table 4.2 Mechanical properties of ferulic acid-added soy protein films treated with UV-C at 0.32, 1.56, 4.00 and 12.00 J/cm^2

Film samples	**Tensile strength (MPa)**	**Elongation at break (%)**
Control	2.48±0.10^{b}	100.25±12.62^{c}
FE	2.61±0.14^{b}	117.19±18.90^{bc}
FUV0.32	2.49±0.10^{b}	122.50±17.05^{bc}
UV-treatment on preformed films		
FE+FUV0.32	2.56±0.16^{b}	107.75±16.02^{c}
FE+FUV1.56	2.72±0.05^{b}	124.11±17.02^{bc}
FE+FUV4.00	2.74±0.08^{b}	140.64±26.95^{ab}
FE+FUV12.00	3.12±0.16^{a}	166.67±27.65^{a}
UV-treatment on film-forming solutions		
FE+SUV0.32	2.61±0.07^{b}	110.81±30.25^{bc}
FE+SUV1.56	2.64±0.07^{b}	118.98±22.71^{bc}
FE+SUV4.00	2.74±0.39^{b}	150.95±26.92^{ab}
FE+SUV12.00	3.29±0.13^{a}	165.45±21.15^{a}

Mean±SD of three replicates.
Sample means within a column that do not share a common superscript letter differ significantly at p=0.05.

UV-C treatment on preformed film and film-forming solution induced an increasing trend in tensile strength with increasing UV-C dose (Table 4.2). The improvement in tensile strength was evident in those samples treated at the highest UV-C dose (12.00 J/cm^2). UV-C treatment at the same radiation dose seemed to pose a similar effect on both preformed film and film-forming solution. Fathi et al. (2018) also found a similar trend in sesame protein isolate film. Díaz et al. (2016) reported that UV treatment on whey protein film-forming solution was more effective in terms of improving tensile strength as compared to the same treatment on preformed film. In the current study, FE+SUV12.00 possessed greater tensile strength than FE+FUV12.00, but this difference was not statistically significant (p>0.05). Both film samples

exhibited about a 1.3-fold increase in tensile strength from the control. A similar trend was previously reported by Gennadios et al. (1998) for UV-treated soy protein film. Diaz et al. (2016) also observed an increase in tensile strength at a high dose (12.00 J/cm^2) of UV-C for whey protein concentrate film.

With respect to elongation, UV-C treatment tended to increase elongation at the break of the ferulic acid-added film (Table 4.2). However, elongation at break of the films treated at lower UV-C doses (0.32 and 1.56 J/cm^2) was not significantly different from that of the control and reference films (FE and FUV0.32) (p>0.05). In contrast, higher UV-C doses (4.00 and 12.00 J/cm^2) were shown to induce an increase in elongation at break. As compared to the control, the FE+FUV4.00 and FE+SUV4.00 exhibited about a 1.5-fold increase in elongation at the break, while the FE+FUV12.00 and FE+SUV12.00 were about 1.7 times higher in elongation at break than the control.

The improvement in mechanical properties of ferulic acid-added soy protein film upon exposure to high doses of UV-C could be due to the induced protein cross-linking. UV radiation could be absorbed by a side group of aromatic amino acids, mainly tyrosine, with a production of the amino acid-free radical (Tyr•). Recombination of these free radicals leads to the formation of dityrosine cross-link both inter- and intra-molecularly (Correia et al., 2012; Masutani et al., 2014). Apart from dityrosine, isodityrosine and trityrosine could also form. However, these products are usually produced at a much lower concentration than the dityrosine (Corriea et al., 2012). Rhim et al. (2000) reported the development of covalent bonds other than disulfide bonds in the structure of UV-treated soy protein film.

The development of cross-linked structure in the UV-treated ferulic-added samples increases the film integrity, and this could be the possible explanation for the increase in tensile strength along with elongation at break.

4.3 Film density

The density of the film samples is summarized in Table 4.3. The film density seemed to be unaffected (p>0.05) by ferulic acid addition (FE) or 0.32 J/cm^2 UV-C curing (FUV0.32) of soy protein film. The density of the control, FE, and UV0.32 films were approximately 1 g/cm^3. Likewise, the density of ferulic acid-added soy protein

film was also minimally affected by UV-C treatment. This finding is in contrast to that reported earlier by Fathi et al. (2018). The authors irradiated sesame protein film with UV-A, UV-B, and UV-C at a dose of 32.6 J/cm^2 and found that all UV radiations induced a significant increase in the density of the protein film. The indifference found in this study may be due to the lower radiation doses used as compared to Fathi et al. (2018).

Table 4.3 Density of ferulic acid-added soy protein films treated with UV-C at 0.32, 1.56, 4.00 and 12.00 J/cm^2

Film samples	**Density (g/cm^3)**
Control	0.983±0.042^{bcd}
FE	0.923±0.033^{d}
FUV0.32	1.020±0.040^{abc}
UV-treatment on preformed films	
FE+FUV0.32	0.969±0.051^{cd}
FE+FUV1.56	1.000±0.034^{bc}
FE+FUV4.00	1.019±0.015^{abc}
FE+FUV12.00	1.039±0.013^{ab}
UV-treatment on film-forming solutions	
FE+SUV0.32	1.073±0.020^{a}
FE+SUV1.56	1.017±0.026^{abc}
FE+SUV4.00	0.966±0.064^{cd}
FE+SUV12.00	0.955±0.042^{cd}

Mean±SD of three replicates.

Sample means within a column that do not share a common superscript letter differ significantly at p=0.05.

4.4 Transparency

Transparency, expressed in %transmittance, of the film samples was shown in Table 4.4. It was found that film transparency was significantly affected by both ferulic acid addition and UV-C curing (p≤0.05). The FE and FUV0.32 samples demonstrated transmittance of 61.90 and 62.50%, respectively, which were significantly lower than the control (70.21%). The decrease in film transparency upon the addition of ferulic acid is possibly due to protein aggregation by ferulic-induced cross-linking and the development of the colored product of phenolic-protein reaction (Pierpoint, 1969; Tang

et al., 2005; Yi et al., 2006). This is in good agreement with that reported by Insaward et al. (2015) for soy protein film incorporated with ferulic, caffeic, and gallic acids. However, this finding contrasts with Y. Wang & Xiong. (2021) who observed that the addition of oxidized ferulic acid at 2.5 and 5.0% did not pose any significant changes in the transparency of whey protein isolate film.

In terms of UV treatment, UV radiations are known to induce protein cross-linking through recombination of aromatic amino acid-free radicals (particularly Tyr•), and it has been reported that the radiations are among the major factors facilitating cataract formation or opacification of the ocular lens, which is caused by protein aggregation (Cetinel et al., 2017). The decrease in protein film transparency caused by UV-C radiation may be explained using the same mechanism.

Table 4.4 Transparency (expressed as %transmittance) of ferulic acid-added soy protein films treated with UV-C at 0.32, 1.56, 4.00 and 12.00 J/cm^2.

Film samples	**%Transmittance**
Control	70.21±1.53^{a}
FE	61.90±1.72^{b}
FUV0.32	62.50±2.64^{b}
UV-treatment on preformed films	
FE+FUV0.32	61.38±0.62^{b}
FE+FUV1.56	56.83±2.47^{c}
FE+FUV4.00	54.91±0.69^{cd}
FE+FUV12.00	51.73±0.76^{e}
UV-treatment on film-forming solutions	
FE+SUV0.32	62.40±0.32^{b}
FE+SUV1.56	62.73±1.60^{b}
FE+SUV4.00	52.41±1.33^{de}
FE+SUV12.00	52.36±0.85^{de}

Mean±SD of three replicates.
Sample means within a column that do not share a common superscript letter differ significantly at p=0.05.

Pertaining to UV-C irradiation of ferulic acid-added films, it was found that the treatment caused a decrease in film transparency with increasing UV-C doses. The

pronounced effect was detected at higher UV-C doses (4.00 and 12.00 J/cm2) in which the film samples exhibited transmittance of around 50%. The UV-C treatment on preformed film and film-forming solution similarly affected transparency of the resulted films. The reduction in transparency of UV-irradiated ferulic-added films is most probably due to the effect of both ferulic acid and UV-C as discussed above. Similar result was reported by Schmid et al., (2015) for whey protein isolate film.

4.5 Water vapour permeability

The water vapour permeability of the film samples is presented in Table 4.5. Water vapour permeability of the soy protein film seemed to be unaffected by ferulic acid addition nor UV-C treatment. The control, FE, and FUV0.32 were of similar water vapour permeability (p>0.05). As compared to the control, UV-C treatment of ferulic acid-added films generally tended to slightly increase the water vapour permeability. However, all the UV-treated ferulic-added film samples demonstrated similar water vapour permeability, which fell within a range of 5.40×10^{-7} to 6.43×10^{-7} g m/m^2 h P. The overpowering of the hydrophilic nature of protein film to the increase in the degree of cross-linking could be the most probable reason for this phenomenon (Schmid et al., 2017).

Table 4.5 Water vapor permeability of ferulic acid-added soy protein films treated with UV-C at 0.32, 1.56, 4.00 and 12.00 J/cm^2

Film samples	Water vapour permeability ($\times 10^{-7}$ g m/m^2 h P)
Control	5.52±0.14^{cd}
FE	5.68±0.33^{bc}
FUV0.32	5.40±0.57^{cd}
UV-treatment on preformed films	
FE+FUV0.32	6.22±0.64^{ab}
FE+FUV1.56	5.99±0.50^{bc}
FE+FUV4.00	6.43±0.56^{a}
FE+FUV12.00	6.28±0.53^{ab}
UV-treatment on film-forming solutions	
FE+SUV0.32	5.95±0.29^{bc}
FE+SUV1.56	5.80±0.68^{bc}
FE+SUV4.00	6.24±0.45^{ab}
FE+SUV12.00	5.76±0.18^{bc}

Mean±SD of three replicates.
Sample means within a column that do not share a common superscript letter differ significantly at p=0.05.

As to previous studies dealing with phenolic cross-linking of protein films, there was a controversy that cross-linking may cause an increase, a decrease, or have no effect at all on water vapour permeability. (González et al., 2011) proposed that phenolic compounds could interact with proteins via various interactions, such as hydrogen bond and covalent bond, yielding a protein matrix with decreasing free volume. This, in turn, allows the water vapour to permeate through the film matrix at a reduced rate. In contrast, Strauss & Gibson (2004) suggested that too high phenolic concentration may result in polymerization of the phenolics instead of reacting and cross-linking with proteins. This may give rise to an increase in water vapour permeability of the phenolic-added film. The minimal effect of phenolic addition on water vapour permeability was also reported by Insaward et al. (2015) for soy protein film and Wang (2021) for whey protein film. Ou et al. (2005) reported that ferulic addition to soy protein film at a concentration lower than 100 mg/100 g protein had no effect on its water vapour

permeability, while at 100 mg/100 g protein, water vapour permeability of the film was found to increase.

In terms of UV-C irradiation, many of the previous studies reported that UV-C treatment posed no effect on the water vapour permeability of protein films. For example, Schmid et al. (2017) investigated UV-C curing of whey protein film and found that the radiation doses used (1.2-42 J/cm^2) did not induce any differences in water vapour permeability, in which all the film samples had water vapour permeability in a range of 5.95×10^{-7} to 6.15×10^{-7} g m/m^2 h P. This is also in agreement with those reported by Ustunol & Mert (2004) and Díaz et al., (2017) for UV-C curing of whey protein films and Gennadios et al. (1998) for UV-C curing of soy protein film. On the other hand, Fathi et al. (2018) declared that UV-C treatment at 32 J/cm^2 on sesame protein isolate film significantly reduced its water vapour permeability. The authors also reported that UV-C treatment on the film-forming solution and preformed film showed a similar result.

4.6 Water solubility

The water solubility of the film samples is tabulated in Table 4.6. Either ferulic acid addition (FE) or UV-C treatment at a lower dose (FUV0.32) on soy protein film had no significant effect on water solubility (p>0.05). The water solubility of the control, FE, and FUV0.32 film was found to be around 40%. The insignificant effect of ferulic addition on water solubility is similar to that reported by (Arabestani et al., 2016). Contrarily, Insaward et al. (2015) reported that ferulic acid addition to soy protein film caused a reduction in its water solubility. In terms of UV-C curing, Wongoun (2020) investigated the effect of different UV-C doses on the water solubility of soy protein film reinforced with cellulose nanocrystals and reported that, at lower UV-C doses (0.06-0.45 J/cm^2), the radiation posed no effect on the water solubility of the film. Meanwhile, higher UV-C doses (0.65-1.56 J/cm^2) instigated a significant decrease in the film solubility.

For ferulic acid-added soy protein films, the application of UV-C on preformed film brought about a gradual decrease in water solubility with increasing radiation dose. At the highest UV-C dose, the FE+FUV12.00 exhibited 37.49% solubility. On the other

hand, UV-C treatment at 0.32-4.00 J/cm^2 on film-forming solution caused a slight but insignificant (p>0.05), increase in water solubility from the control. The FE+SUV12.00 demonstrated 40.23% solubility, similar to the 41.86% solubility of the control. It should be noted that all samples in this experiment were not statistically different in terms of water solubility from the control (p>0.05).

Table 4.6 Water solubility of ferulic acid-added soy protein films treated with UV-C at 0.32, 1.56, 4.00 and 12.00 J/cm^2

Film samples	Water solubility (%)
Control	41.86±1.88^{ab}
FE	40.47±1.11^{ab}
FUV0.32	41.36±2.39^{ab}
UV-treatment on preformed films	
FE+FUV0.32	40.53±3.44^{ab}
FE+FUV1.56	38.91±2.70^{b}
FE+FUV4.00	38.52±2.79^{b}
FE+FUV12.00	37.49±0.75^{b}
UV-treatment on film-forming solutions	
FE+SUV0.32	45.94±5.32^{a}
FE+SUV1.56	45.57±4.99^{a}
FE+SUV4.00	43.45±3.24^{ab}
FE+SUV12.00	40.23±2.66^{ab}

Mean±SD of three replicates.
Sample means within a column that do not share a common superscript letter differ significantly at p=0.05.

This insignificant difference in water solubility may also be due to the highly hydrophilic nature of soy protein film. A similar result has been previously reported by Díaz et al. (2016) for whey protein film. Oppositely, Fathi et al. (2018) found that sesame protein isolate film displayed a significant decrease in water solubility upon being exposed to UV-C at 32.6 J/m^2. The authors also reported that UV-C treatment on film forming solution and preformed film yielded a similar result.

4.7 Surface hydrophobicity

The contact angle of a water droplet with a film surface is an indicator of surface hydrophobicity. The contact angle of the film samples is presented in Table 4.7. It was found that the control, FE, and FUV0.32 had a similar contact angle (p>0.05) of a value around 50° which implies that the film samples possess a hydrophilic surface, typical of most protein films.

The contact angle of the ferulic acid-added film was not significantly affected by lower doses of UV-C (0.32 J/ cm^2 for preformed film and 0.32 and 1.56 J/cm^2 for film-forming solution). For UV-C treatment on ferulic acid-added preformed film, the contact angle of the irradiated film samples was significantly greater than that of the control at the UV-C dose of ≥1.56 J/cm^2 and a noticeable increase in contact angle was observed with increasing UV-C dose, with the FE+FUV12.00 possesses the greatest contact angle of 60.40° indicating that the film surface became more hydrophobic. However, being less than 90° in contact angle, this is still considered a hydrophilic surface.

Table 4.7 Contact angle of a water droplet with the surface of ferulic acid-added soy protein films treated with UV-C at 0.32, 1.56, 4.00 and 12.00 J/cm^2

Film samples	Contact angle (°)
Control	47.77±11.56^{d}
FE	47.17±1.98^{d}
FUV0.32	48.77±2.02^{d}
UV-treatment on preformed films	
FE+FUV0.32	46.57±2.25^{d}
FE+FUV1.56	53.17±2.70^{c}
FE+FUV4.00	56.47±2.02^{b}
FE+FUV12.00	60.40±3.64^{a}
UV-treatment on film-forming solutions	
FE+SUV0.32	46.80±3.01^{d}
FE+SUV1.56	47.90±2.99^{d}
FE+SUV4.00	52.53±3.44^{c}
FE+SUV12.00	56.23±3.23^{b}

Mean±SD of three replicates.

Sample means within a column that do not share a common superscript letter differ significantly at p=0.05.

For UV-C treatment of film-forming solution, this posed less effect on the film surface hydrophobicity. The contact angle of the film samples also increased with increasing UV-C dose, but to a lesser extent as compared to the treatment of the preformed film.

Fathi et al. (2018) proposed that the increase in protein film surface hydrophobicity upon UV-C irradiation is probably due to the development of cross-linking and the decrease in free hydrophilic groups in the polypeptide chains. Moreover, this may also be due to UV-induced protein conformational changes, which, in turn, result in the exposure of hydrophobic regions that, in a native state, are buried inside the protein structure (Kristo et al., 2012).

The higher effectiveness of UV-C treatment on preformed films than the treatment on film-forming solutions could be due to the higher protein concentration of preformed (i.e., dried) films resulting in the close proximity of the polypeptide chains and thus facilitating the cross-linking of proteins at the surface. A similar result was reported by Fathi et al. (2018) for sesame protein isolate film.

4.8 Colour

The CIELAB colour parameters of the film samples are given in Table 4.8. It was found that all the colour parameters, namely, L^*, a^*, b^*, hue angle, and chroma, of soy protein film were not affected by either ferulic acid addition (FE) and UV-C treatment at 0.32 J/cm^2 (FUV0.32) (p>0.05). The control, FE, and FUV0.32 demonstrated a hue angle of around 96° which is close to the yellow hue angle (90°). In general, phenolic addition to protein film tended to increase the yellowness due to the coloured product of a reaction between phenolic compound and protein (Rawel et al., 2002). The type and concentration of phenolic compounds were found to have an impact on the colour of the reaction product of phenolic and protein (Pierpoint, 1969). Insaward et al. (2015) reported an increase in yellowness upon adding ferulic, caffeic, or gallic acid to soy protein film and this discolouration got even pronounced upon oxidation of the phenolic compound to its corresponding quinone. (Prodpran et al., 2012) reported a similar finding for ferulic acid-added fish myofibrillar protein film.

However, a noticeable increase in yellowness upon ferulic acid addition was not found in our study.

Table 4.8 Colour parameters of ferulic acid-added soy protein films treated with UV-C at 0.32, 1.56, 4.00 and 12.00 J/cm^2

Film samples	*L**	*a** ^{ns}	*b**	Hue angle (°)	Chroma
Control	90.44±0.87^{ab}	-1.87±0.13	17.91±2.01^{d}	95.96±0.54^{ab}	18.01±2.10^{d}
FE	90.01±0.77^{bc}	-2.01±0.16	18.65±2.29^{cd}	96.15±0.75^{ab}	18.76±2.28^{cd}
FUV0.32	90.47±0.92^{ab}	-1.88±0.13	17.94±1.78^{d}	95.99±0.32^{ab}	18.04±1.18^{d}
UV-treatment on preformed films					
FE+FUV0.32	90.28±0.66^{ab}	-2.11±0.16	18.88±1.89^{cd}	96.39±0.19^{ab}	18.99±1.89^{cd}
FE+FUV1.56	90.79±0.69^{ab}	-2.18±0.24	19.53±1.95^{cd}	96.38±0.54^{ab}	19.66±1.96^{cd}
FE+FUV4.00	91.13±0.76^{a}	-1.87±0.13	19.53±2.29^{cd}	95.50±0.34^{b}	19.62±2.38^{cd}
FE+FUV12.00	90.57±1.06^{ab}	-1.66±0.15	21.35±2.70^{bc}	94.48±0.39^{d}	21.42±2.71^{bc}
UV-treatment on film-forming solutions					
FE+SUV0.32	90.32±0.81^{ab}	-2.16±0.20	18.76±2.40^{cd}	96.61±0.34^{a}	18.88±2.41^{cd}
FE+SUV1.56	90.49±1.11^{ab}	-2.09±0.24	19.44±306^{cd}	96.18±0.40^{ab}	19.56±3.06^{cd}
FE+SUV4.00	90.12±0.74^{bc}	-2.13±0.27	22.45±3.07^{ab}	95.42±0.16^{b}	22.54±3.09^{ab}
FE+SUV12.00	89.36±1.25^{c}	-1.96±0.20	24.89±4.80^{a}	94.58±0.49^{c}	24.97±4.80^{a}

Mean±SD of three replicates.

Sample means within a column that do not share a common superscript letter differ significantly at p=0.05.

^{ns} Sample means within the column do not differ significantly at p=0.05.

UV-C treatment at 0.32-4.00 J/cm^2 on preformed ferulic acid-added soy protein (FE+FUV0.32, FE+FUV1.56, FE+FUV4.00) and treatment at 0.32-1.56 J/cm^2 on soy protein film-forming solution (FE+SUV0.32, FE+SUV1.56) did not pose a significant effect on colour parameters of the films (p>0.05). However, UV-C treatment at 12.00 J/cm^2 on preformed ferulic acid-added soy protein (FE+FUV12.00) and treatment at 4.00 and 12.00 J/cm^2 on soy protein film-forming solution (FE+SUV4.00, FE+SUV12.00) caused a significant change in +*b**. This, in turn, resulted in a shift of hue angle towards a lower value and an increase in chroma, implying that the samples became more intense in yellowness as they appeared to the naked eye.

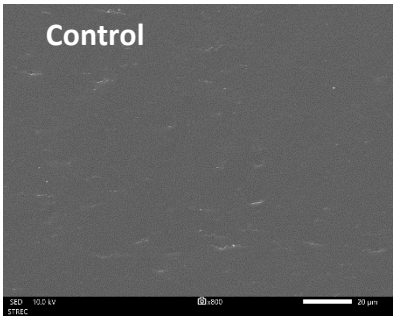
The more intense yellowness of UV-C cured film-forming solution as compared to the treatment on preformed film was also observed by Díaz et al. (2016) for whey protein film. This may be due to the fact that the greater molecular mobility in film-forming solution facilitates photochemical reactions induced by UV.

Yellowing of protein upon being exposed to UV radiations is widely recognized and is best exemplified by cataract formation, which, apart from the clouding of the lens of the eyes, yellow to the brown colouration of the lens has also been reported. Lens discolouration has been found with a higher incidence in regions near the Equator where UV intensity is greater compared to those regions at higher latitudes. UV radiations are known to induce photooxidation of proteins and photochemical generation of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, resulting in oxidative damage and discolouration of proteins in the lens (Addepalli et al., 2012). In the same manner, this photodamage could also be responsible for the discolouration of UV-treated protein films.

UV treatment has been reported to cause an increase in yellowness in many protein films. Similar to our study, Wongoun, (2020) treated cellulose nanocrystal-reinforced soy protein film with UV-C at 0.06-1.56 J/cm^2 and found that at higher UV-C does (0.45-1.56 J/cm^2), hue angle became decreasing, approaching the yellow hue angle of 90°, while +b* and chroma became increasing. Schmid et al. (2015) investigated the effect of UV-C radiation (2.3- 31.4 J/cm^2) on the colour of whey protein film and reported an increasing +b* value with increasing radiation dose, while L* and -a* remained relatively constant. UV-C was also reported to induce yellowness in soy protein film without phenolic addition (Gennadios et al., 1998; Rhim et al., 2000). However, Rhim & Gennadios. (1999) declared a contrary finding in which UV-C was found to induce a decrease in +b* of zein film. The authors proposed that UV-C may prompt the degradation of pigment, contributing to the yellow colour in zein protein. Micard et al. (2000) reported that there was no significant impact of UV-C on the colour of wheat gluten films. For our study, apart from the direct effect of UV-C on the protein film itself, we have postulated that UV-C may accelerate the oxidation of ferulic acid, turning it into the corresponding quinone, leading to greater chroma of the film samples.

4.9 Film microstructure

The cross-sectional structure of selected film samples is depicted in Figure 4.1. The control, FE, FUV0.32, FE+FUV0.32, FE+FUV12.00, FE+SUV0.32, and FE+SUV12.00, were selected for investigation.



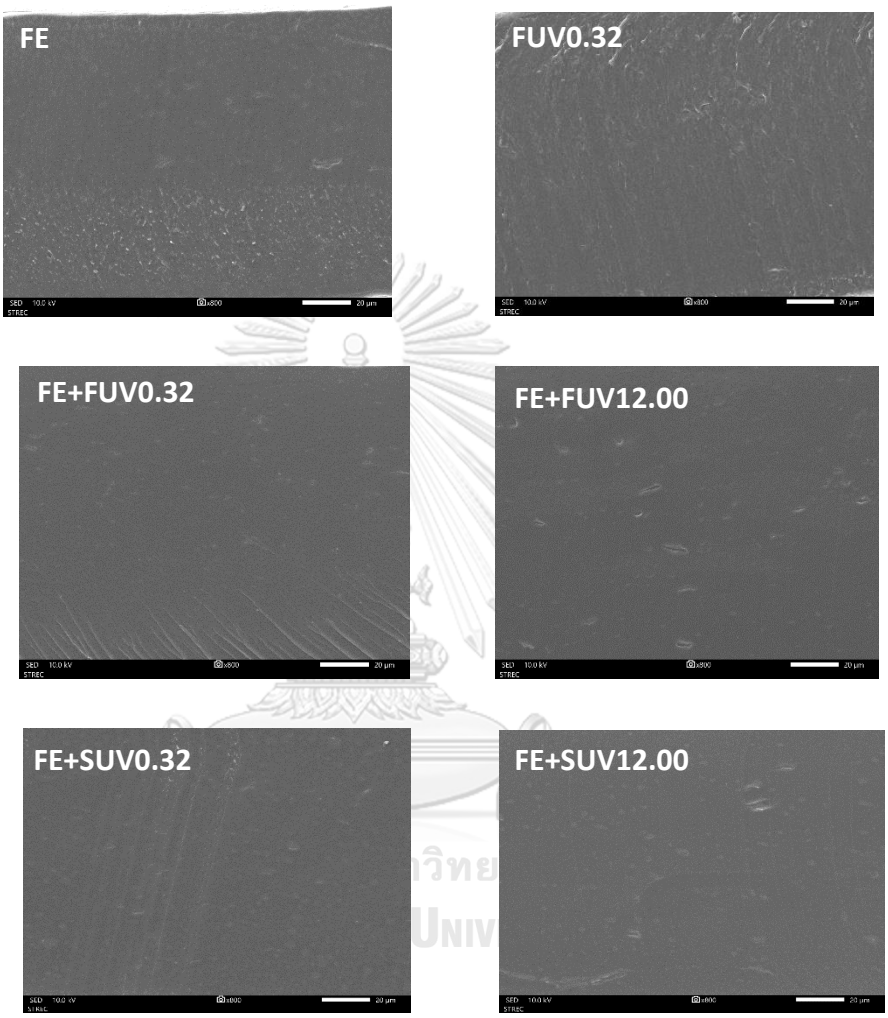


Figure 4.1 Cross-sectional scanning electron micrographs of ferulic acid-added and/or UV-treated soy protein films obtained at 800× magnification

All the film samples appeared quite homogeneous in terms of their cross-sectional structure. The UV-treated ferulic-added films seemed to have a slightly more homogeneous microstructure as compared to the control, FE, and FUV0.32.(C. Zhang et al., 2014) reported that the addition of 5% ferulic acid to soy protein/chitosan composite film resulted in a much uniform and compact structure as compared to the control film that showed some inhomogeneous zones and small pores. In another study, (Arabestani et al., 2016) found that the addition of ferulic acid at 50 mg/100 g to bitter vetch (Vicia ervilia) protein film produced a film with more compactness and continuous zones, while the control sample presented discontinuous zones and some small pores.

As compared to UV-C treatment on the film-forming solution at 12.00 J/cm^2 (FE+SUV12.00), the preformed film treated at the same UV-C dose (FE+FUV12.00) appeared to have more pinholes in the film structure. Fathi et al. (2018) reported that UV-C irradiation of film-forming sesame protein solution exhibited less irregularities and was more compact and denser. In the same study, UV-C treatment on preformed film resulted in a film with more pinholes and minor cracks.

4.10 Fourier transform infrared (FTIR) spectroscopy

The FTIR spectra of the control, FE, UV0.32, FE+FUV12.00, and FE+SUV12.00 are shown in Figure 4.2. FTIR analysis was carried out to monitor the development of the C-N bond induced by ferulic acid cross-linking of protein. A strong absorption peak was observed at 1040 cm$^{-1,}$ which corresponds to the C-N stretching vibration (Ikhmal et al., 2018). The ferulic acid-added film samples exhibited lower %transmittance, signifying the presence of a greater number of C-N bonds than the control. The lower %transmittance of FE+FUV12.00 and FE+SUV12.00 (i.e., more C-N bonds) might be due to the that UV-C promoted the oxidation of ferulic acid to its corresponding quinone, which is more efficient in cross-linking protein than the unoxidized form.

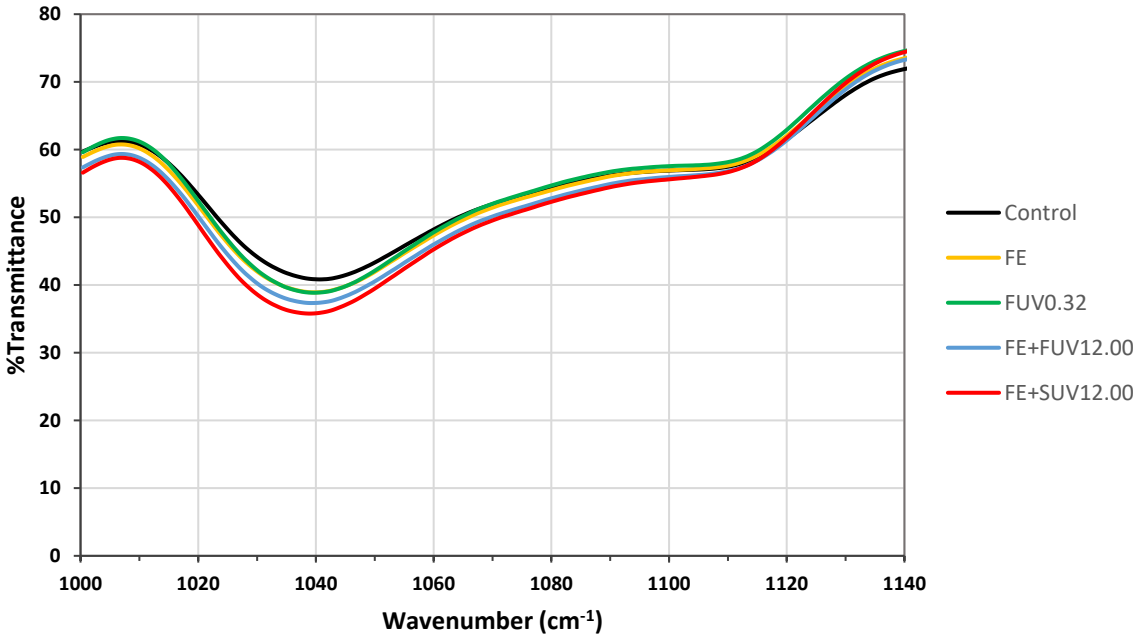


Figure 4.2 FTIR spectra of ferulic acid-added and/or UV-treated soy protein films

4.11 Fluorescence spectroscopy

Dityrosine is formed in many proteins as a result of UV-irradiation (Malencik & Anderson, 2003; Sionkowska et al., 2006) and gamma-irradiation (Terryn et al., 2006). In these cases, dityrosine cross-linking can be either intra- or inter-molecular (Malencik & Anderson, 2003) and could result in protein aggregation (Balasubramanian & Kanwar, 2002). Dityrosine is one of the specific markers of protein oxidation, especially for that induced by radiation.

In this study, dityrosine cross-linking of the film samples was monitored using fluorescence spectroscopy. (Al-Hilaly et al., 2013, 2016) reported that dityrosine produces a specific fluorescence peak in the wavelength of 340-500 nm, with the highest intensity in the wavelength of 400-420 nm. Correia et al. (2012) monitored dityrosine formation in insulin that was exposed to UV and detected dityrosine peak in the wavelength of 350-550 nm, with the greatest intensity around 405 nm. Fluorescence emission spectra of the film samples are shown in Figure 4.3.

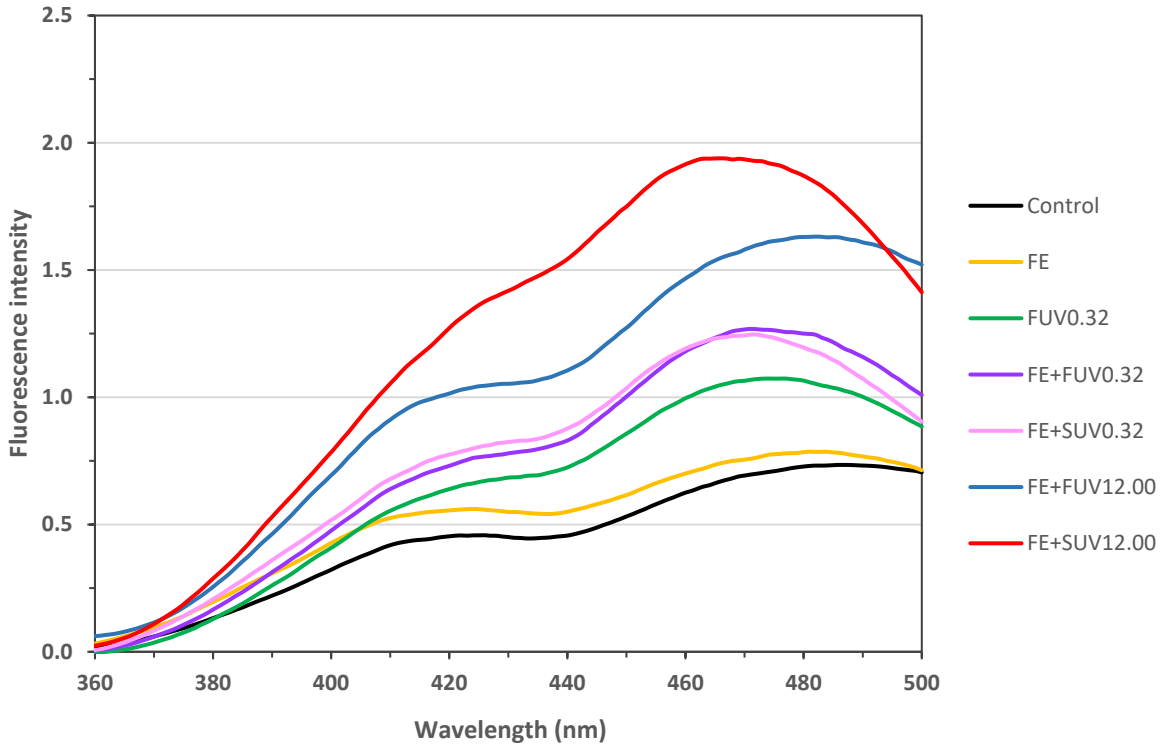


Figure 4.3 Fluorescence emission spectra of ferulic acid-added and/or UV-treated soy protein films

In this study, a fluorescence peak confirming dityrosine formation was detected in the wavelength of 360-500 nm, with the highest intensity around the wavelength of 470 nm. It was evident that the UV-C treated samples possessed greater fluorescence intensity than the unirradiated samples (control and FE). Fluorescence intensity was found to increase with increasing UV-C dose. In this experiment, FE+SUV12.00 exhibited higher fluorescence intensity than FE+FUV12.00. In spite of the difference in fluorescence intensity between FE+SUV12.00 and FE+FUV12.00, it should be noted that the samples were generally similar in other properties.

CHAPTER 5

CONCLUSION

Ferulic acid addition and UV-C curing of either preformed film or film-forming solution were found to modify certain properties of soy protein film. As compared to the control, the film thickness was found to significantly increase upon adding ferulic acid and/or irradiating with UV-C. Film density was slightly affected by ferulic acid addition and/or UV-C treatment, but the value was found to be in a narrow range of about 1 g/cm^3.

UV-C treatment to preformed film and film-forming solution induced an increase in tensile strength and elongation at break, with a significant difference from the control manifested at higher UV-C doses (12.00 J/cm^2 for tensile strength, 4.00 and 12.00 cm^2 for elongation at break). However, the tensile properties of UV-C treatment on preformed film were not significantly different from the treatment on the film-forming solution. This improvement in tensile properties was proposed to be due to the induced protein cross-linking, specifically the C-N bond via ferulic-protein reaction and dityrosine bond via UV-induced free radical formation and recombination. In this study, C-N bond formation was confirmed by the decrease in FTIR transmittance detected at a wavenumber of 1040 cm^{-1} which corresponds to C-N stretching vibration. Dityrosine cross-link was also substantiated by an increase in the intensity of the fluorescence peak observed in the wavelength of 360-500 nm.

Regarding the optical properties, feluric acid addition and/or UV-C curing posed a significant effect on both transparency and colour. All modified films exhibited significantly lower transparency than the control, and the transparency became decreasing with increasing UV-C dose. In terms of colour, UV-C treatment of ferulic acid-added film brought about a significant increase in $+b^*$. This, in turn, resulted in a decrease in hue angle, changing towards a value of 90°, along with an increase in chroma, signifying that the films became more intense in yellowness. The changes in optical properties were proposed to be due to protein aggregation, coloured products of the ferulic-protein reaction, and products of photooxidation of ferulic acid and protein.

UV-C treatment of ferulic acid-added films tended to make a slight increase in water vapour permeability as compared to the control. However, regardless of the UV-C dose, all the UV-treated ferulic-added film samples demonstrated similar water vapour permeability. Likewise, the water solubility of the film seemed to be minimally affected by ferulic acid addition and/or UV-C irradiation. The indifference may be due to the fact that changes in these properties upon ferulic acid addition or UV-C treatment might become overpowered by the highly hydrophilic nature of the protein film. Surface hydrophobicity, on the other hand, was found to be influenced by radiation dose. An increase in surface hydrophobicity (an increase in contact angle) was evident with increasing UV-C dose, particularly in those samples treated on preformed film. It should be noted that all the samples in this study demonstrated a contact angle of lower than 90°, implying that they still had a hydrophilic surface.

To summarize, UV-C irradiation was proven as an effective tool for modifying tensile strength and elongation at the break of ferulic acid-added soy protein film by facilitating the formation of covalent cross-linking in protein. In addition, the transparency and colour of the film were also affected by the treatments, owing to the cross-linking and other reactions induced by ferulic acid and UV-C.

Suggestions for future study

Addition research might be required, for instance, the application of UV-C at higher doses than those used in this study, to obtain a film with greater tensile strength and elongation at break. Changes in other properties might also be detectable if higher doses of UV-C are applied.

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VITA

NAME	Md Shakil
DATE OF BIRTH	10 October 1994
PLACE OF BIRTH	Bangladesh
INSTITUTIONS ATTENDED	Hajee Mohammad Danesh Science and Technology University, Dinajpur, Bangladesh Chulalongkorn University, Bangkok, Thailand
HOME ADDRESS	Village: Baradushia, P.O: Baradushia, P.S: Brahmanpara, Dist: Cumilla, Country: Bangladesh
PUBLICATION	Shakil, M. D., & Mahawanich, T. (2022). Effect of UV-C curing on properties of ferulic acid-added soy protein film. Proceedings of the 24th Food Innovation Asia Conference 2022 (FIAC2022), virtual conference http://www.fiac-thailand.com, June 16-17.