

# **Physicochemical Properties and Antioxidant Activity of Enzymatically Modified Soy Protein Isolate Films**

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Doctor of Philosophy

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## **ABSTRACT**

In this study, a sustainable packaging system was developed to provide food safety and security. Soy protein isolate (SPI) was enzymatically modified by transglutaminase under different conditions to ensure desirable and optimized enzyme crosslinking activity before film preparation. Physicochemical properties including viscosity and molecular weight distribution of the modified proteins and films were measured. Results confirmed the enzymatic treatment is an effective way to modify the SPI based biopolymeric film. Modified films with the enzyme had significant increases in tensile strength (TS), percent elongation (%E), initial contact angle, and a reduction in swelling and protein solubility properties compared to the control films. FTIR and XRD spectra revealed that the enzyme treatment modified the structure of SPI film matrix. The optimal film preparation conditions achieved in this part were protein denaturation temperature 80 °C, and enzyme incubation time 2hr.

We attempted to enhance antioxidant activity of enzymatically modified SPI film with the addition of two types of lignin, alkali lignin (AL) and lignosulphonate (LSS), at different concentrations. Results indicated that AL carried higher radical scavenging ability than LSS. Films containing AL showed high absorption in the UV region, and this UV-blocking ability increased with increasing lignin concentration. Deconvoluted FTIR spectra and XRD results suggested that the addition of lignin caused some changes in secondary structure of the protein matrix. The addition of lignin improved TS and thermal stability of films, but reduced %E as a function of lignin concentration. Radical scavenging activity and UV-blocking ability alongside improvement in physicochemical properties of enzymatic modified SPI film with lignin motivated us to apply this bioplastic in two types of oil, soy oil and fish oil. Results revealed that applying enzymatically modified SPI film with AL and LSS in the inner layer of a soy oil packaging system, decreased oxidation rate to around 75%, and pentanal production to about 40% of control. UV-blocking ability of AL caused reduction in oxidation rate for more than 75% compared with the normal packaging system. The effectiveness of this active packaging system in soy oil was greater than fish oil. Thus, the developed biopolymeric materials may have application to food packaging.

**To my parents and my siblings, who have supported me  
during the whole journey of my life**

**To my dearest family and friends.**

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# **CHAPTER 1**

## **GENERAL INTRODUCTION**

Packaging, the third biggest industry in the U.S., has received great attention as businesses and other organizations attempt to create the most efficient environmental “footprint” for their products (Franklin Associates, 2014). According to PIRA International, 70% of packaging is used for food and beverages (Smiths Pira, 2010). The American Chemistry Council (2012) revealed that approximately 30% of thermoplastic resin demand in the U.S. is related to packaging materials, and over twenty billion pounds of synthetic-based plastics was used by the packaging industry. According to the Environmental Protection Agency (EPA), 33 million tons of plastic waste was generated in 2013, whereas, about 14 million tons of the plastic waste was related to packaging and containers, and only 9% of the total plastic waste was recovered for recycling. Therefore, developing technical and practical methodologies to improve sustainability for packaging food product that result in less generation of waste and dependence on petroleum products is of great importance. Principle factors resulting in the need for reduction in usage of traditional petroleum-based polymers are: rising environmental concerns, a need to inhibit greenhouse gas emission, increasing costs of petroleum feedstock, and availability of naturally cheap feedstock (Smith Pira, 2010). Therefore, bio-based materials extracted from biomass may be an attractive alternative to commercial petroleum-based packaging.

According to the European bioplastic organization (2015), bioplastics are defined as plastics based on naturally renewable resources, which are biodegradable or compostable. Since the main function of food packaging systems is to preserve the quality and safety of food products during

storage and transportation, packaging materials should not only be environmentally friendly, but also should extend shelf life. The ideal biopolymeric packaging materials have both excellent mechanical and gas barrier properties and also are biodegradable at the end of their lives.

Biopolymeric materials are derived from agricultural and fishing by-products, waste products of the food processing industry or natural resources. These biopolymeric materials are polysaccharide-based, protein-based, lipid-based or a mixed combination of these components (Wu, et al., 2009; Bertan, et al., 2005; Fabra, et al., 2008; Elizondo, et al., 2009).

Among these biopolymers, soy protein isolate, with higher than 90% protein purity, is derived from soy beans, which is the second most abundant crop in the United States, making it cheap and accessible (Berk, 1992). Soy protein has a globular structure that is created by the combination of strong intermolecular forces such as hydrophobic interactions and hydrogen, ionic and disulfide bonds.

The fractionation of soy protein via different solubility procedures leads to albumin, globulin and glutelin fractions (Jiang et al., 2009). Globulin, which is a water soluble protein and the major component (more than 90%) of soy protein, has two fractions: 7S and 11S. The 7S and 11S fractions contain cysteine residues, which allows sulfhydryl-disulfide interchain reactions that occurs during polymerization upon heating and then cooling to form a consistent covalent film network (Janjarasskul and Krochta, 2010). As mentioned before, these differences in sulfhydryl-disulfide interchange in 7S and 11S fractions cause a variation in protein denaturation temperatures of around 80 °C and 90 °C, respectively (Renkema and Vliet, 2002).

Protein-based biopolymers have good mechanical and gas barrier properties. However, their brittleness and high water vapor permeability are the main restrictions in application of these materials (Andreuccetti *et al.*, 2009 and Guilbert *et al.*, 1996).

Modifying the polymeric network of soy protein isolate-based films through enzymatic and physical treatments can bypass these restrictions and improve the functional properties of the films. Jiang *et al.* (2007) determined the effect of different parameters on the improvement of mechanical properties and surface hydrophobicity of SPI films modified with transglutaminase. Such parameters include the percentage of transglutaminase, pH, and drying temperature. Their findings supported a low concentration of enzyme (4-10 unit/g of protein), an alkaline pH, and a drying temperature of 50 °C.

Active packaging includes a group of technologies giving functions for food preservation other than providing an inert barrier (Brody *et al.*, 2001). Antioxidants in food packaging, especially for those foods sensitive to oxidation, is a type of preservation food system to provide safety and security of food products by inhibiting the initiation or propagation of oxidizing chain reaction (Velioglu *et al.*, 1998). In general, there are two categories of antioxidants: natural and synthetic. Common synthetic antioxidant such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have some restrictions in application in food products due to their potential carcinogenicity (Ito *et al.*, 1983).

Lignin is one of the most abundant organic materials on earth, and large amounts are easily available in bioethanol production. Lignin has an aromatic and highly cross-linked structure and is a reactive macromolecule due to its functional groups such as methoxy groups, aromatic and aliphatic alcohol groups that are possible positions for chemical reactions (Oliviero et al., 2011). Therefore, lignin is able to interact with many polymers and change their wettability, fire resistance, and mechanical properties (Oliviero et al., 2011). Lignin contains phenolic compound that prevent lipid oxidation; lipid oxidation associated with coronary heart disease, atherosclerosis, cancer and the aging process (Fukumoto et al., 2000). According to Pan et al. (2006) some factors that have positive effects on the antioxidant activity of lignin including: more phenolic hydroxyl groups, less aliphatic hydroxyl groups, low molecular weight, and narrow polydispersity. Oliviero et al. (2011) revealed that the addition of lignin to zein protein matrix caused some changes in the secondary structure of zein protein,  $\alpha$ -helix,  $\beta$ -sheet, and  $\beta$ -turn. Therefore, lignin can modify physicochemical properties of the film beside antioxidant activity.

The main objective of this research is to improve cross linking of soy protein using microbial transglutaminase under controlled film preparation conditions, and to increase antioxidant activity of the bioplastic with two types of lignin. The resultant enzymatically modified SPI film with lignin can be applied to food and pharmaceutical products in order to better suit functional requirements. The measurable objectives of this study are listed as follows:

1. To determine the effect of different film preparation conditions, namely: protein denaturation temperatures (80 and 90 °C), enzyme reaction times (1h, 2h, 3h) and enzyme treatments on cross-linking structure of SPI-based biopolymeric solution.
2. To determine the effect of different film preparation conditions, namely: protein denaturation temperatures (80 and 90 °C), enzyme reaction times (1h, 2h, 3h) and enzyme treatments on physicochemical properties of SPI-based biopolymeric film.
3. To investigate the effect of Lignosulphonate (LSS) and Alkali lignin (AL) on physicochemical properties of SPI-based film modified with the enzyme.
4. To determine antioxidant activity of enzymatically modified SPI film with lignin.
5. To investigate the effect of antioxidant activity of modified films with alkali lignin and lignosulfonate on shelf life of soy oil and fish oil.

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## CHAPTER 2

### LITERATURE REVIEW

In this chapter, literature review is divided into three sections: section 2.1 will review the components of soy protein and gelation conditions, section 2.2 will review about transglutaminase, factors and enzyme preparation conditions, and section 2.3 will review about lignin structure and utilization, and its application in active packaging.

#### **2.1. Soy protein isolate (SPI)**

Different types of soy protein, for example, soy flour, soy concentrate, and soy isolate, have different functionalities such as gelling, emulsifying, and foaming capacity (Utsumi et al., 2002). Soy flour contains approximately 50% protein, and applied in a wide range of foods, especially in bakery products and cereals, whereas soy protein concentrates with around 70% protein, applied in greater food qualities due to its improved flavor, color and higher amount of protein. Soy protein isolate, more than 90% protein purity, and particularly interested in this project, prepared commercially with minimum heat treatment (Kinsella, 1979; wolf et al., 1971). Amino acid composition of SPI is shown in Table 2.1.

**Table 2.1.** Amino acid composition of soy protein isolate

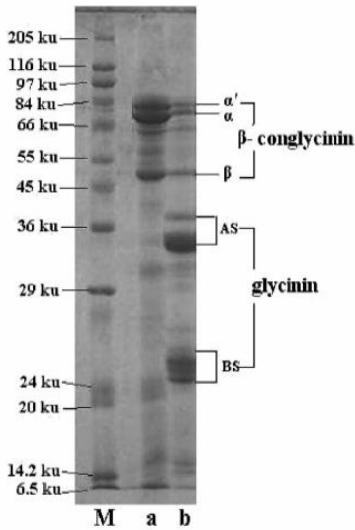
Typical amino acids (g/100g proteins)	
Aspartic acid	11.5
Threonine*	3.8
Serine	5.4
Glutamic acid	19.2
Proline	5.2
Glycine	4.2
Alanine	4.2
Cysteine	1.3
Valine *	4.8
Methionine*	1.3
Isoleucine*	4.8
Leucine*	8.0
Tyrosine	3.8
Phenylalanine*	5.4
Histidine*	2.7
Lysine*	6.4
Arginine	7.8
Tryptophan*	1.4

Source: ADM data sheet, PROFAM 974

\*Essential amino acids

### 2.1.1. Structure of soy protein

The structure of protein is a major determining factor for the usefulness and suitability of protein ingredient in food application (Jiang et al., 2011). According to the solubility of different fractions of soy protein, there are three fractions: albumins (saline solvable), globulin (water solvable) and glutelin (alcohol solvable). Approximately 85-95% of soy protein storage are globulin with isoelectric point (pH 4.2-4.6) (Kinsella, 1975; Wolf and Cowan, 1971). Based on different sedimentation coefficient of soy protein, four major fractions are reported: 2S, 7S, 11S and 15S (Table 2.2). 7S globulin ( $\beta$ -conglycinin) and 11S globulin (glycinin) are considered as the major fractions of SPI composition (Kilara and Sharkasi, 1985). Molecular weight of glycinin and  $\beta$ -conglycinin is shown in SDS-PAGE (Fig.2.1).



**Fig.2.1.** The SDS-PAGE patterns of SPI with high amount of glycinin and  $\beta$ -conglycinin. a:  $\beta$ -conglycinin rich SPI, b: glycinin rich SPI, and M: standard protein markers (Source: Tang et al., 2006b)

**Table 2.2.** Approximate amounts of ultracentrifuge fractions of water extractable soy protein

Fraction	Percent of total	components	Molecular weight
2S	22	Trypsin inhibitors Cytochrome C	8,000-21,500 12,000
7S	37	Hemagglutinins Lipoxygenase $\alpha$ -amylose	110,000 102,000 61,700
11S	31	7S globulin	180,000-210,000
15S	11	11S globulin -	350,000 600,000

Source: Wolf et al. (1971)

### 7S ( $\beta$ -conglycinin) component

$\beta$ -conglycinin is a major component in 7S and 9S forms at 0.5 and 0.1 ionic strength, respectively. 7S fraction is originally a glycoprotein containing carbohydrates as a unit attached to the aspartic acid at the N-terminal of molecule. The carbohydrate portion consists of 12 glucosamine and 38 mannose residue in each protein molecule. The 7S consists of three subunits:  $\alpha$ ,  $\alpha'$  and  $\beta$ . Different subunits have different  $M_w$  in SDS-PAGE gel. Table 2.3 shows

physicochemical properties of different subunits in  $\beta$ -conglycinin. Melting temperature of  $\beta$ -conglycinin is around 80 °C. It has a great emulsifying activity due to the higher molecular flexibility and a large number of hydrophobic groups (Bernard and others 2001).

**Table 2.3.** Physicochemical properties of different subunits of  $\beta$ -conglycinin

	Subunits			
	$\alpha$	$\alpha'$	$\beta$	$\gamma$
<b>Molecular weight</b>				
<b>Electrophoresis</b>				
Urea/acetate (10% acrylamide gel)	68,000	68,000	42,000	-
SDS (10% acrylamide gel)	59,000	58,000	44,000	44,000
Urea/SDS (9% acrylamide gel)	57,000	58,000	46,000	46,000
Gel filtration	57,000	57,000	42,000	-
<b>Isoelectric point</b>	4.9	5.18	5.66-6	-

Source: Kilara and Sharkasi, (1985)

### 11S (glycinin) component

Glycinin (11S) consists of 6 acidic and 6 basic subunits. The molecular weight of glycinin is between 302,000 and 375,000, which are packed to form a hollow cylinder. Physicochemical properties of glycinin is shown in Table 2.4. Melting temperature of glycinin is around 90 °C, and gelling capacity of glycinin is higher than  $\beta$ -conglycinin (Renkema et al., 2001).

**Table 2.4.** Physicochemical properties of glycinin (11S)

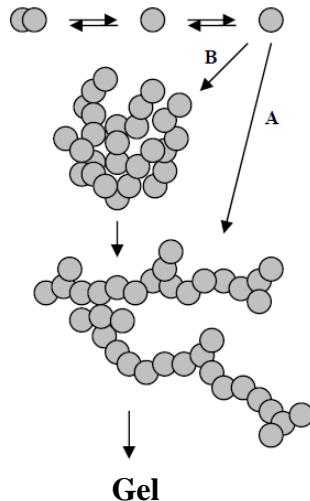
<b>Molecular weight</b>			
Gel filtration		302,000 ± 33,000	
Sedimentation equilibrium		317,000 ± 15,000	
Sedimentation diffusion		322,000 ± 15,000	
From subunit size		326,000 ± 35,000	
Gel electrophoresis		350,000 ± 35,000	
<b>Number of subunits</b>			
<b>Molecular weight of acidic subunits</b>			
A <sub>1</sub> , A <sub>2</sub> , A <sub>4</sub> , A <sub>5</sub>		38,000	
A <sub>3</sub>		45,000	
<b>Molecular weight of basic subunits</b>			
B <sub>1</sub> , B <sub>2</sub> , B <sub>3</sub> , B <sub>4</sub>		21,000	
<b>N-terminal amino acids</b>			
<b>Acidic</b>		<b>Basic</b>	
A <sub>1</sub>	Phe	B <sub>1</sub> -B <sub>4</sub>	Gly
A <sub>2</sub>	Leu		
A <sub>3</sub>	Ile		
A <sub>4</sub>	Ile		
<b>Size</b>			
Electron microscopy		100×100×70 °A	
X-ray scattering		110×110×75 °A	
<b>Isoelectric point</b>			
<b>Acidic</b>		<b>Basic</b>	
A <sub>1</sub> =5.15		B <sub>1</sub> =8.0	
A <sub>2</sub> =5.40		B <sub>2</sub> =8.25	
A <sub>3</sub> =4.75		B <sub>3</sub> =8.50	

Source: Kilara and Sharkasi, (1985)

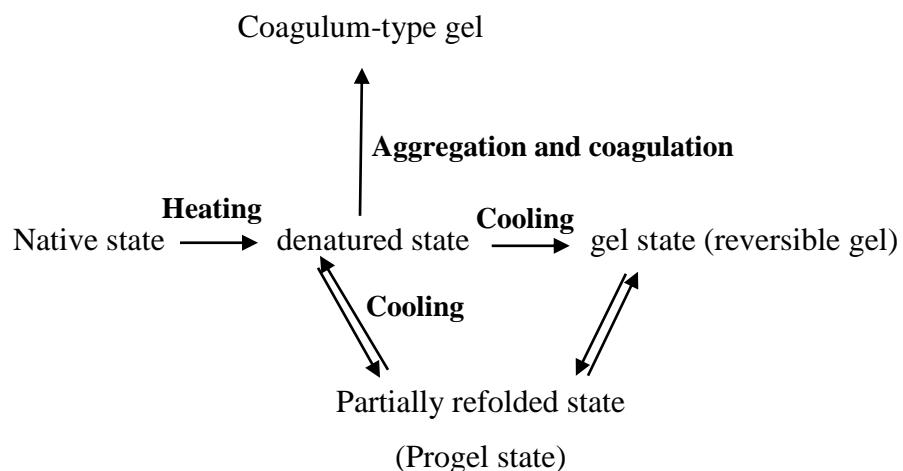
## Gelation

Gelation is a process of producing gel by heating and cooling protein solution (Ferry, 1948). As mentioned before, soy protein has a globular structure and several researches have been done before on gelation of globular protein (Clark et al., 2001; Gosal and Ross-Murphy, 2000). Fig. 2.2 shows the possible aggregation steps in a heat-set globular protein. Fibrils are formed from aggregated monomers by pH adjustment much lower than isoelectric point of protein (A). Pre-

aggregate was formed under other conditions (B). This leads to form more gel formation (Gosal and Ross-Murphy, 2000).



**Fig.2.2.** Formation of heat set gel (Source: Gosal and Ross-Murphy, 2000)



**Fig.2.3.** Mechanism of thermal gelation of globular proteins (Source: Damodaran, 1988)

Another mechanism of thermal gelation of soy protein is shown in Fig.2.3. In this mechanism gelation is a reversible process and occurred after denaturation and cooling of protein.

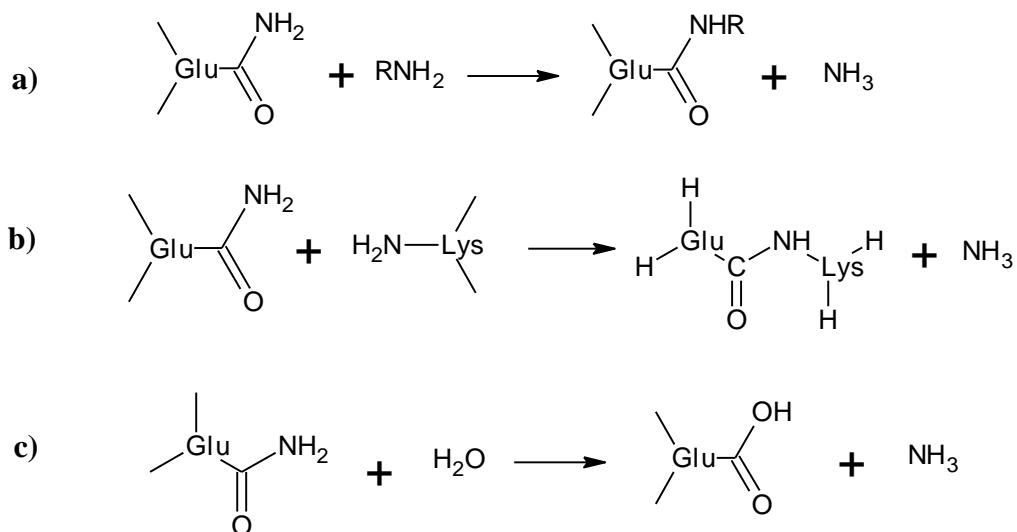
Soy protein isolate showed higher gelling properties at 80 °C than other protein fractions (Babajimopoulos et al., 1983). During heating glycinin and  $\beta$ -conglycinin are dissociated and their subunits subsequently interacted with each other (Utsumi et al., 1983). The ratio of glycinin and  $\beta$ -conglycinin is also important in commercial SPI. Higher proportion of  $\beta$ -conglycinin and basic subunit in water soluble fraction, increased gel forming ability of the protein (Arrese et al., 1991). Raising temperature to above 93 °C increased hardness due to the mix of acid precipitate and  $\beta$ -conglycinin proteins. The elasticity of the gel decreased gradually with increasing the temperature from 80 °C to 100 °C (Kang et al., 1991). Therefore, in current research two temperatures were selected for SPI cooking, 80 and 90 °C.

## **2.2. Microbial transglutaminase**

Transglutaminase is an enzyme that can form crosslinks between proteins. Cross linking has important effects on gelation properties, thermal stability, water holding capacity and physical properties of protein gel. Transglutaminase was first recognized by Mycek et al. (1959). This enzyme had been found in animal tissue, body fluids (Folk, 1980), plants (Icekson and Apelbaum, 1987) and microorganisms (Yan et al., 2005). The only transglutaminase available until late 1980 was guinea pig liver transglutaminase, therefore, the market price was high, and it was applied as a texture enhancer (Motoki and Kumazawa, 2000; Zhu et al., 1995, 1999). Transglutaminase factor XIII in human blood, is one of the most well-known type of

transglutaminase that helps to prevent bleeding by crosslinking of fibrin molecules and stabilizing of fibrin polymers (Kuraishi et al., 2001). Thus, several efforts were made to produce transglutaminase by genetic manipulation of various microorganisms such as *Escherichia coli* (Ikura et al., 1990). However, due to the lack of public acceptability of this enzyme, transglutaminase from *Streptoverticillium* S-8112 was found by Ando et al. (1989), and commercialized as a food enzyme prepared by Ajinomoto Co., Inc.

Transglutaminase improves crosslinking in protein matrix with involving in three types of reactions: acyl-transfer reaction, crosslinking reaction, and deamination. Fig.2.4 shows these reactions.



**Fig.2.4.** General reactions catalyzed by transglutaminase: a) acyl-transfer reaction; b) crosslinking reaction; c) deamination. Source: Kuraishi et al. (2001)

In all of these reactions, glutamine amino acid is involved. In acyl transfer reaction (Fig.4.2. a), transglutaminase catalyzes the reaction between a  $\gamma$ -carboxyamide of peptide and a primary

amine. If transglutaminase catalyzes the reaction between amine group in lysine and amide group in glutamine, crosslinks are formed. (Fig.2.4. b). In the absence or blocking of primary amine, water will be involved as an acceptor (Fig.2.4. c). In the food system crosslinking reaction will happen more often (Kuraishi et al., 2001).

### **Enzyme preparation conditions**

Some important conditions to achieve the optimal activity and stability of enzyme are included pH, temperature and incubation time. The microbial transglutaminase is active over a wide range of temperatures, and stable between pH 5 and 9 (Seguro et al., 1996 and Ando et al., 1989).

The enzyme activity of transglutaminase is determined by the hydroxamate procedure with *N*-carbobenzoxy-L-glutamyl-glycine (Z-Gln-Gly), and the enzyme activity unit is the amount of causing the formation of 1  $\mu$ mole of hydroxamic acid in 1 min at 37 °C (Ando et al., 1989).

Since the goal of the current research is producing a biopolymer to apply in packaging, modifying the polymeric network of soy protein isolate-based films through enzymatic and physical treatments can bypass SPI restrictions. Thus, the addition of the enzyme improves the functional properties of films. Jiang et al. (2007) determined the effect of different parameters on the improvement of mechanical properties and surface hydrophobicity of SPI films modified with transglutaminase. Such parameters include the percentage of transglutaminase, pH, and drying temperature. Their findings supported a low concentration of enzyme (4-10 unit/g of protein), an alkaline pH, and a drying temperature of 50 °C.

In another study reported by Tang et al. (2005) SPI films modified with 60% plasticizer (glycerol, sorbitol and glycerol:sorbitol and transglutaminase (4 U/g of protein) had higher tensile strength and surface hydrophobicity than the control film without the enzyme and plasticizer. Moreover, enzyme treated films had rougher surfaces and more compact cross-sections when compared to the control film.

Gennadios et al. (1993) reported that SPI films prepared between the pH 6 to 11 had significantly ( $P < 0.05$ ) higher TS, higher %E, and lower WVP than films between the pH 1 to 3, and pH 4.6 is the isoelectric point of SPI.

Therefore, in the current research soy protein isolate was unfolded at high temperature (80 and 90 °C) to achieve the highest accessibility of active groups, and then cooled down to 40 °C. Protein solution was incubated with the enzyme under controlling optimal conditions to obtain maximum activity and stability of transglutaminase.

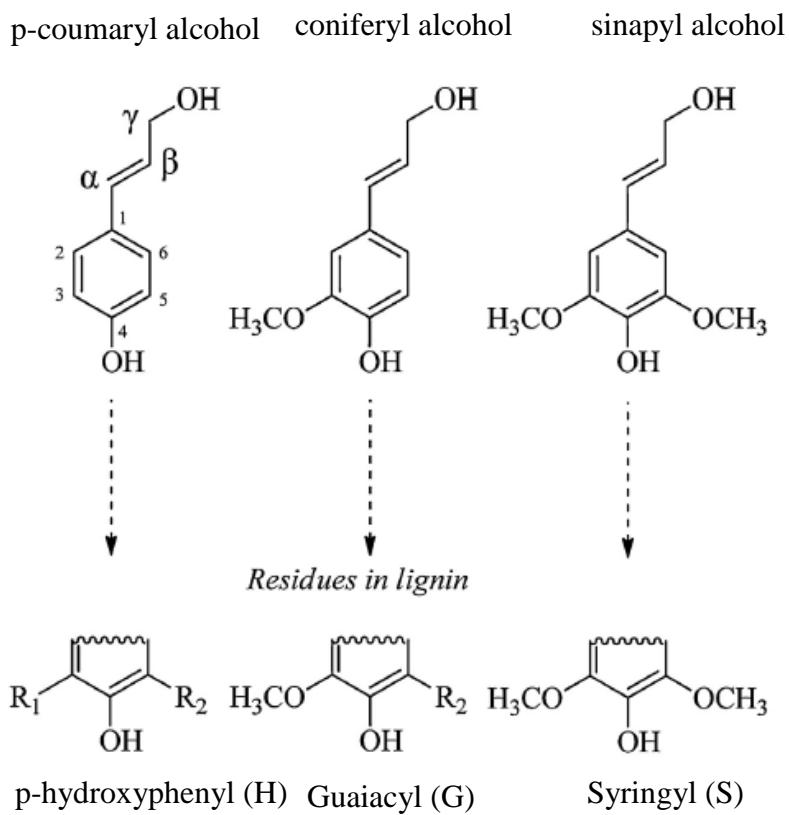
### **2.3. Lignin**

Lignin is the second most abundant organic materials on earth after cellulose, and large amounts are easily available in paper and bioethanol production companies. Lignin was first discovered through 1795-1871 by a French chemist. However, the chemical structure of lignin remained a mystery for a long time. The aromatic structure of lignin was recognized by Benedikt and

Bamberger in 1890. Freudenberg et al. (1959) stated that lignin is an amorphous and unordered material, with a kind of structural order consisting phenylpropane units on connected blocks.

### **Lignin chemical structure**

Lignin has aromatic and highly cross-linked structure with a greatly reactive macromolecular compound due to its functional groups such as aromatic ring, methoxy groups, and aromatic and aliphatic alcohol groups that are possible positions for chemical reaction (Oliviero et al., 2011). Therefore, lignin is able to interact with many polymers and change their wettability, fire resistance, and mechanical properties (Oliviero et al., 2011). Chemical structure of lignin consists of three types of aromatic alcohols, namely: p-coumaryl, coniferyl and sinapyl alcohols (Dorrestijn et al., 2000). Fig.2.5 shows the structure of these components.



**Fig.2.5.** The three main precursors of lignin and their corresponding structure in lignin polymers  
 $R_1, R_2 = H$  or lignin  
 Capital letters are the abbreviations of each monolignol  
 Source: Laurichesse and Avérous, (2014)

Lignin composition is affected by the species and the environment. In hardwood lignin, G and S units are more than H units, and in softwood lignin high level of G units with low level of H units was observed (Sarkanen et al., 1971a). Due to the different possible chemical sites, lignin could play a central role as a new chemical feedstock.

### Physical properties of lignin

Lignin is an amorphous polymer with a wide range of glass transition temperature ( $T_g$ ), which is depending on method of isolation, sorbed water, molecular weight, and thermal history.

Therefore, glass transition temperature ( $T_g$ ) and decomposition temperature ( $T_d$ ) are important in lignin properties (Sarkanen et al., 1971b). It was reported that  $T_g$  of lignin is varied between 90 to 150 °C depending on plant species and extraction methods (Irvine et al., 1984).

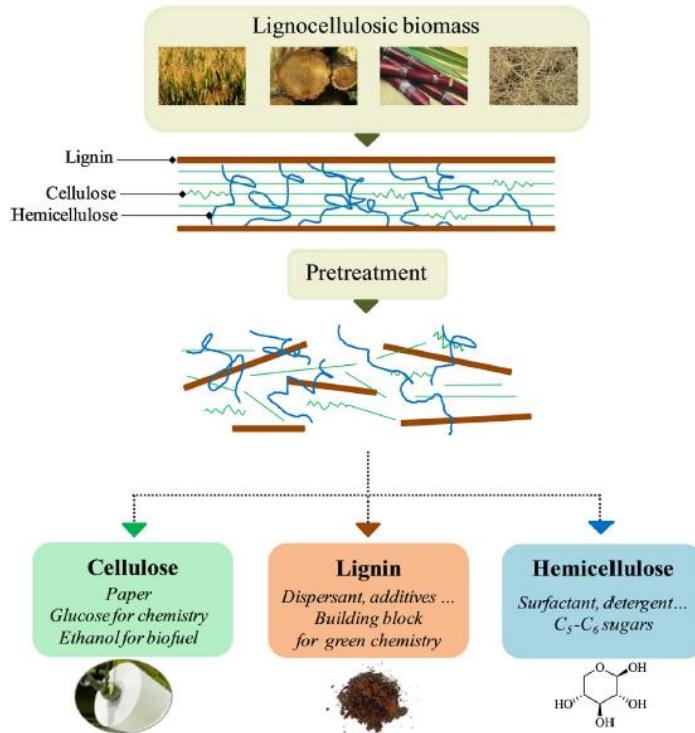
Lignin is hydrophobic in planta and its hydroxyl sites involve in hydrogen bonds with water molecules. Chemical modification of hydroxyl groups in lignin by esterification or alkylation caused some changes in inter and intra-molecular interactions, resulted in glass transitions that are more easily detected (Glasser, 2000).

Thermal degradation of lignin is over a wide range of temperatures due to the various oxygen-based functional groups. The decomposition temperature of lignin starts from 150-275 °C due to the dehydration from the hydroxyl groups located on benzyl groups (Brebu & Vasile, 2010). The breakage of  $\alpha$  and  $\beta$ -aryl-alkyl-ether linkages happened between 150 and 300 °C, and carbon-carbon cleavage occurs at 370-400 °C.

### **Lignin extraction**

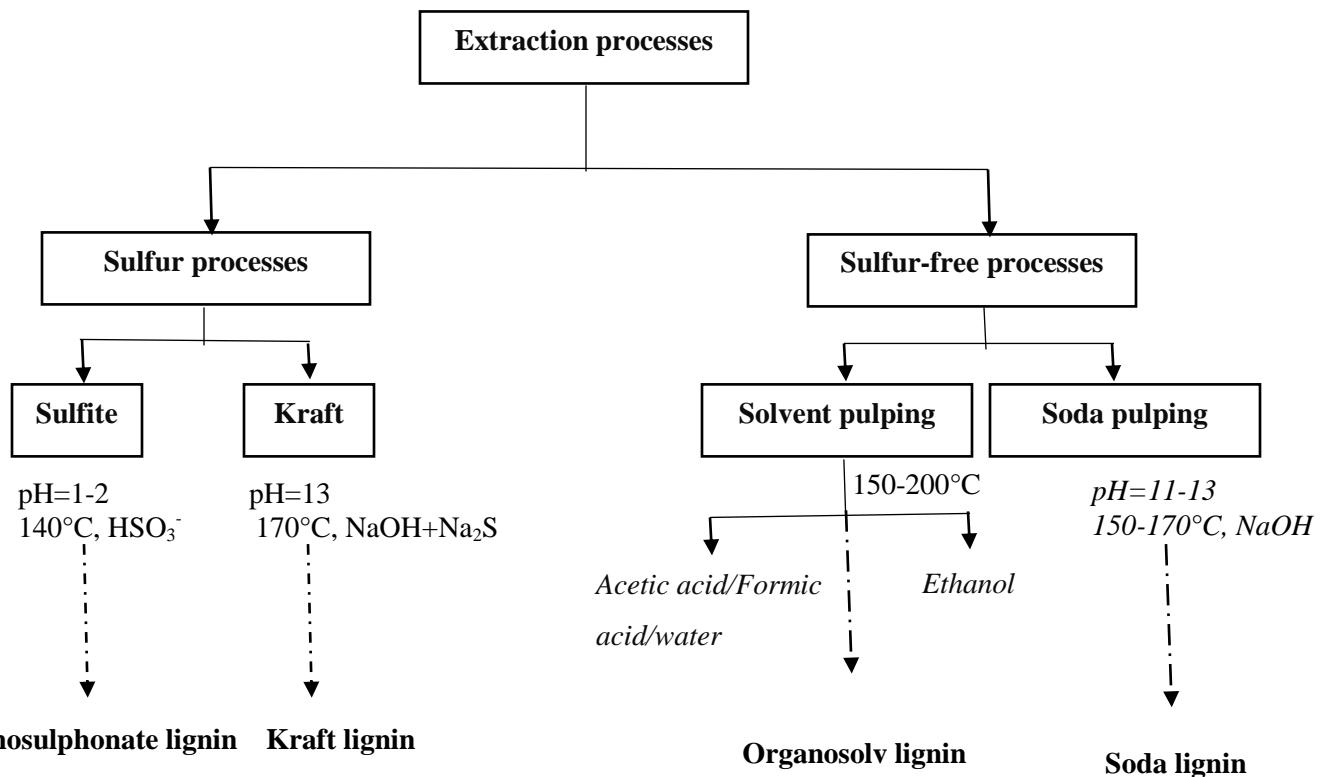
Lignin is extracted from other lignocellulosic parts of wood by physical, chemical and biochemical treatments. The purity and final structure of lignin is highly dependent on botanical source, pulping process and extraction procedure (Vázquez et al., 1997). Schematic concept of lignin extraction is shown in Fig.2.6. Fully integrated biorefinery can be considered as

conversion of pulp mills into (i) cellulose to make paper, (ii) high value co-products such as lignin, and (iii) hemicellulose.



**Fig.2.6.** Schematic concept of biorefinery based on lignocellulosic biomass (Source: Laurichesse and Avérous, 2014)

There are two main lignin extraction procedures: sulfur process and sulfur-free process (Fig.2.7). chemical structure of lignin is affected by extraction procedure (Table 2.5).



**Fig.2.7.** Different extraction processes to separate lignin from lignocellulosic biomass and the corresponding production of technical lignins (Source: Laurichesse and Avérous, 2014)

**Table 2.5.** Properties of Kraft lignin and lignosulphonate

Lignin type	Sulfur-lignin	
	Kraft	Lignosulphonate
Raw materials	Softwood, hardwood	Softwood, hardwood
Solubility	Alkali	Water
Average molecular mass ( $M_n\text{-gmol}^{-1}$ )	1,000-3,000	15,000-50,000
Polydispersity	2.5-3.5	6-8
$T_g$ (°C)	140-150	130

(Source: Laurichesse and Avérous, 2014)

## **Application of lignin in active packaging**

Lignin contains phenolic compound that prevent lipid oxidation; lipid oxidation associated with coronary heart disease, atherosclerosis, cancer and the aging process (Fukumoto & Mazza, 2000). According to Pan et al. (2006) some factors that have positive effects on the antioxidant activity of lignin include: more phenolic hydroxyl groups, less aliphatic hydroxyl groups, low molecular weight, and narrow polydispersity. Several researches have been done before on applying different types of lignin in biopolymer film (Table 2.6). In composite film with starch lignin is applied as a plasticizing agent (Wu et al., 2009). In corn starch biopolymer modified with acidified starch microparticles, the addition of lignin improved tensile strength and reduced percent elongation (Spiridon et al., 2011). Lignin also stabilize composite material against photo-oxidation and reduce water absorbency and transparency (Kosikova et al., 1993; Ban et al., 2007).

Oliviero et al. (2011) revealed that the addition of lignin to zein protein matrix caused some changes in the secondary structure of zein protein,  $\alpha$ -helix,  $\beta$ -sheet, and  $\beta$ -turn. Therefore, lignin can modify physicochemical properties of the film beside antioxidant activity.

**Table 2.6.** Effect of different types of lignin on physicochemical properties of different biopolymers

Film	Condition	Type of lignin	Important points	References
Zein protein+lignin+ PEG	Melt mixing method	AL, LSS	<ul style="list-style-type: none"> <li>• AL was more effective,</li> <li>• Both improve mechanical properties</li> <li>• 1% AL increased 150% max strength and decreased 33% water uptake,</li> </ul>	Oliviero et al., 2011, <i>J. Agr.Food chemistry</i>
Fish gelatin+LSS (85:15)+sorbitol	Solution casting	LSS	<ul style="list-style-type: none"> <li>• Antioxidant activity</li> <li>• Reduce mechanical properties</li> <li>• Less transparency</li> <li>• Light barrier properties</li> <li>• No anti-microbial capacity</li> </ul>	Nunez-Flores et al., 2013, <i>J.Food hydrocloid</i>
SPI:AL+Gly	Compression molding	AL, LSS	<ul style="list-style-type: none"> <li>• 50 parts AL increased TS</li> <li>• Reduce water absorption</li> <li>• Reduce water sensitivity</li> </ul>	Huang et al., 2002, <i>J.App.Pol. Sci.(a,b)</i>
SPI+2% HL+Gly	Compression molding	HL	<ul style="list-style-type: none"> <li>• Increased TS 2.3 times than SPI sheet</li> <li>• Decreased %E</li> </ul>	Wei et al., 2006, <i>J.Macromol.Mater.Eng</i>
Starch+lignin	Casting	ASL	<ul style="list-style-type: none"> <li>• Reduced TS and EM</li> <li>• Increased %E</li> <li>• High resistanse to thermal degradation</li> <li>• Antioxidant activity of the film</li> </ul>	Acosta, et al., 2015

Abbreviations: SPI: soy protein isolate, AL: alkali lignin, LSS: lignosulphonate, HL: hydroxypropyl lignin, Gly: glycerol, PEG: poly ethylene glycol, ASL: alcohol-soluble lignin

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## **CHAPTER 3**

### **Evaluation of Enzymatically Modified Soy Protein Isolate Film Forming Solution and Film at Different Manufacturing Conditions**

#### **Abstract**

Effect of transglutaminase on Soy Protein Isolate film forming solution and films was investigated by tracking rheological behavior and physicochemical properties based on different manufacturing conditions, namely: enzyme treatments (with the enzyme, without the enzyme, with deactivated enzyme), enzyme incubation times (1hr, 2hr, and 3hr), and protein denaturation temperatures (80, and 90 °C). Enzymatic crosslinking reaction and change in molecular weight distribution was confirmed by viscosity measurement and SDS-PAGE, respectively comparing to both non-enzyme treated and deactivated enzyme treated controls. Films treated with both the enzyme and deactivated enzyme showed significant increase in TS, %E and initial contact angle of the films comparing to the non-enzyme control film. This implies that the bulk stabilizers of commercial enzyme may mask the effect of treatment on above properties. Contact angle of films showed two-sidedness due to the influence of orientation of molecules during drying and material of the plate. Statistically, enzyme incubation time and film preparation temperature showed significant effect ( $p<0.05$ ) on film properties. Accordingly, the treatment at 2 hr enzyme incubation time and protein denaturation temperature 80 °C showed the highest tensile strength and surface hydrophobicity. Water absorption property and protein solubility in water was significantly reduced by enzyme treatment comparing to both controls. FTIR and XRD spectra were in good agreement with other results, while enzyme treatment modified the structure of SPI film matrix. Based on above observations, the enzymatic treatment to protein based polymer can

be a useful way to control the physical properties of protein based biopolymeric film and this treatment can be applied to the wide spectrum of scientific areas such as packaging, food, pharmaceutical, and agricultural industries.

### **3.1. Introduction**

Biopolymer-based materials such as proteins, polysaccharides, lipids or a mixed combination of these materials are useful alternatives to synthetic polymers due to their safety, fast biocompatibility and biodegradability rates (Di Pierro et al., 2006). Among these biopolymers, soy protein isolate, higher than 90% protein purity, is derived from soy beans, which is the second most abundant crop in the United States, making it cheap and accessible (Berk, 1992). Soy protein has a globular structure that is created by the combinations of strong intermolecular forces such as hydrophobic interactions and hydrogen, ionic and disulfide bonds.

The fractionation of soy protein via different solubility procedures leads to albumin, globulin and glutelin fractions (Jiang et al., 2009). Globulin, which is a water soluble protein and the major component of more than 90% of soy protein content, has two fractions: 7S and 11S. The 7S and 11S fractions consist of cysteine residue; which causes a sulfhydryl disulfide interchain reaction that occurs during polymerization upon heating and then cooling to form a consistent covalent film network (Janjarasskul and Krochta, 2010). As mentioned before, these differences in sulfhydryl disulfide interchain in 7S and 11S fractions cause a variation in protein denaturation temperatures of around 80 °C and 90 °C, respectively (Renkema and Vliet, 2002).

In recent years, researchers have noticed the benefits of consuming plant-based protein as an alternative to animal-based proteins due to social-cultural and hygienic reasons (Pranoto et al., 2007). Moreover, soy protein isolate brings many benefits including its ability to maintain its nutritional value and support human health. However, low elasticity, high water vapor permeability and low surface hydrophobicity are some restrictions of soy protein-based films.

Modifying the polymeric network of soy protein isolate-based films through enzymatic and physical treatments can bypass these restrictions and thus improve the functional properties of the films. Jiang et al. (2007) determined the effect of different parameters on the improvement of mechanical properties and surface hydrophobicity of SPI films modified with transglutaminase. Such parameters include the percentage of transglutaminase, pH, and drying temperature. Their findings supported a low concentration of enzyme (4-10 unit/g of protein), an alkaline pH, and a drying temperature of 50 °C.

In another study reported by Tang et al. (2005), SPI films modified with 60% plasticizer (glycerol, sorbitol and glycerol:sorbitol) and transglutaminase (4 U/g of protein) had higher tensile strength and surface hydrophobicity than the control film without enzyme and plasticizer. Moreover, enzyme treated films had rougher surfaces and more compact cross-sections when compared to the control film.

The sequence of amino acids, with an abundance of lysine and glutamine (6.4% and 19% respectively) in soy protein allows it to readily react with transglutaminase. Transglutaminase crosslinking between  $\epsilon$ - amine group in lysine and  $\delta$ -carboxyamide group on protein-bound glutamine residues, leading to the improvement of covalent crosslinking of the proteins (Kuraishi et al., 2001).

Other crucial factors affecting the quality of film network include the denaturation of the protein by heat, type of solvent, and pH. The ionization state of amino acids is altered by a change in pH

(Berg et al., 2002). Gennadios et al. (1993) reported that SPI films prepared between the pH 6 to 11 had significantly ( $P < 0.05$ ) higher TS, higher %E, and lower WVP than films between the pH 1 to 3, and pH 4.6 is the isoelectric point of SPI.

Accordingly, film preparation conditions such as pH, the amount of enzyme, protein denaturation temperature, enzyme reaction time and temperature had significant effects on the properties of SPI-based films (Tang et al., 2005; Jiang et al., 2007; Mariniello et al., 2003). However, optimized conditions of SPI-based film fabrication using the enzymatic reaction have not yet been fully investigated.

Preliminary results showed that the modified films with the enzyme prepared at pH 7 and 10 were not significantly different in mechanical and surface hydrophobicity properties. This result is in agreement with the finding of Gennadios et al. (1993), who modified the structure of SPI-based films with different pH values, but without the enzyme. Moreover, the highest activity and stability of the enzyme is at pH 7 (Ho et al., 2000).

Therefore, in this experiment, pH 7 was used during enzyme incubation time to verify the enzymatic crosslinking activity, also the viscosity of film forming solutions were compared and the effects of different enzyme treatments (with the enzyme, without the enzyme and with deactivated enzyme), protein denaturation temperatures (80 and 90 °C), and enzyme incubation times (1h, 2h and 3h) on the physicochemical and surface hydrophobicity of the films were determined.

### **3.2.Materials and method**

#### **3.2.1. Materials**

Soy protein isolate (PROFAM 974) with 90% protein on a dry basis was supplied by ADM, Protein Specialties Division, Netherlands. SPI had 5% moisture, 4% fat and 5% ash. Transglutaminase Activa RM, was donated from Ajinomoto, Japan. According to the data sheet provided by the company, Activa RM contains 99.5% sodium caseinate and maltodextrin and 0.5% the enzyme. Glycerol was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Reagents for enzyme activity measurements such as Tris-HCl, Tris-acetate buffer, CaCl<sub>2</sub>, glutathione, hydroxylamine, CBZ-glutamylglycine, L-glutamic acid  $\gamma$ -monohydroxamate, FeCl<sub>3</sub>-trichloroacetic were purchased from Sigma Aldrich. Coomassie brilliant blue was purchased from Bio-Rad. Citric acid, ethanol and phosphoric acid were obtained from Fisher Scientific.

#### **3.2.2. Treatment of transglutaminase and its activity determination**

The activity of enzyme was determined by a method explained by Tang et al. (2005). One gram of enzyme was completely dissolved in 0.05 mol/L Tris-HCl and adjusted pH to 6, and placed at 4 °C for a week at constant stirring. After that the solution centrifuged at 1000×g for 15 min at 4 °C to remove precipitate composed of impure proteins. The enzymatic activity of transglutaminase was measured by colorimetric procedure describing by Folk and Cole (1965). The measured activity of the enzyme was 47.8 unit per gram of enzyme.

### **3.2.3. Film preparation**

Modified soy protein isolate-based film was produced by dissolving 5 g SPI in 100 g distilled water with glycerol 30% (w/w of protein) according to the preliminary experiment. The pH of the protein solution was adjusted to 7 using HCl 0.1 N. This was followed by heating the solution to 80 or 90 °C for 30 min with hot plate equipped with temperature controller to achieve the denaturation of SPI sample. Transglutaminase solution was prepared separately by dissolving 4 unit per gram of protein in a part of distilled water and stirring at room temperature for 30 min to achieve a constant solution (according to the preliminary study). The enzymatic solution was deactivated by heating at 70 °C for 30 min. SPI film forming solution was incubated with transglutaminase at 40 °C and for different reaction times (1h, 2h and 3h). One hundred gram of solution was weighted and cast on Teflon-coated glass plates ( $20\times20\text{ cm}^2$ ) to achieve the consistency of thickness (roughly 0.1 mm) in all samples and dried at room temperature for 24 hr and then carefully peeled off manually. The films were then preconditioned at 25°C and 50% RH for 2-3 days before analysis according to ASTM D618-61 (1995).

### **3.2.4. Rheology of film forming solution**

The effect of transglutaminase on the rheological properties of SPI film forming solution with 7.5% solid content were determined by using a rotational rheometer (AR G2, TA instrument Ltd, New Castle, DE, USA) with a conical concentric cylinder (30.2 mm diameter and 78.4 mm length and a geometry with 28.1 mm diameter and 80.8 mm length). This is according to the method described by Gauche et al. (2008). The measurements were replicated three times for each sample and the data were evaluated using the Origin software, version 9.0 (Microcal Software Inc., Northampton, MA, USA). The temperature of rheometer was controlled by

Brookfield temperature controller with nitrogen gas to set in 25 °C. The sample was preconditioned at 25 °C for 3 min to ensure stability. The shear rate increased linearly from 10 to 1000 s<sup>-1</sup> with 8 points per decade in a steady state flow rate. Viscosity of the SPI solution was expressed by Pa.s. The flow behavior of SPI solution with the enzyme was measured according to the Power Law model (1). Consistency index was applied to verify the viscosity alteration of SPI solution followed by enzymatic reaction:

$$\sigma = K(\dot{Y})^n \quad \text{Equation 1.}$$

Where  $\sigma$  is shear stress, K is consistency index,  $\dot{Y}$  is shear rate and n is flow behavior.

### **3.2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

The protein solution samples were prepared by dissolving 0.1 ml of soy protein isolate solutions (5% w/w) modified with the enzyme, without the enzyme and with the deactivated enzyme, with 0.4 ml distilled water and 0.5 ml Leamml solution. The samples were heated for 5 min with dithiothreitol (DTT) in boiling water to separate S-S bonds before electrophoresis. The electrophoresis was applied on a discontinued buffer system according to Leammi (1970) with 12% separating gel and 4% stacking gel. A low range (14.4-97.4 kD) molecular weight protein marker was purchased by BIO-RAD. SDS-PAGE standards to estimate molecular weights of the protein solution samples. Standard curve is the linear regression coefficient of log MW and R<sub>f</sub> (distance migrated by protein/distance migrated by dye). Staining solution contains 0.25% Coomassie brilliant blue (R-250) in 50% methanol and 7.5% acetic acid. The gel was destained overnight at 7.5% acetic acid solution.

### **3.2.6. Fourier transforms infrared spectroscopy (FTIR)**

Infrared spectroscopy (FTIR) was performed with an 8700 Nicolet Thermo Electron, with transmission mode and a resolution of  $4\text{ cm}^{-1}$ , in the range of  $4000\text{-}500\text{ cm}^{-1}$ . The samples were preconditioned in a dissector containing  $\text{P}_2\text{O}_5$  for a week to achieve the lowest moisture content.

### **3.2.7. X-ray diffraction**

X-ray diffraction was performed by XRD (Bruker D8 Discover, Karlsruhe, Germany) from  $2\Theta$  values of  $5$  to  $50^\circ$  at the rate of  $0.1\text{ sec/step}$ , and equipped with  $\text{CuK}\alpha$  radiation ( $\lambda=0.1542\text{ nm}$ ).

### **3.2.8. Scanning electron microscopy (SEM)**

The samples were dried in a desiccator containing pentoxide phosphate for a week, and then conducted with a 60:40 gold-palladium mixture deposited by Denton Sputter Coater, Desk II. The samples were tested with JEOL Neoscope electron microscopy model JCM 5000.

### **3.2.9. Mechanical properties**

For determination of tensile strength (TS) and percent elongation (%E), the ASTM (1997) method was used and evaluated by TA.XTPLUS texture analyzer (Stable Micro Systems, Surrey, UK) with 50 Kg loading capacity. The films were cut in rectangles  $2.5 \times 8\text{ cm}^2$  and put in the environmental chamber (50% RH and  $23^\circ\text{C}$ ) chamber for two days before testing. The samples were fixed between the grips with initial grip separation 5 cm and pulled apart at cross head speed of 50 mm/min. Tensile strength was determined by dividing peak load (N) to cross section

area ( $\text{m}^2$ ) and the results expressed by MPa. Percent elongation was calculated by dividing the extended length by initial length multiply by 100.

### **3.2.10. Initial Surface hydrophobicity**

The initial surface hydrophobicity of SPI films was assessed by measuring contact angle (FTA 200; Dynamic Contact Angle Analyzer). A drop of 1  $\mu\text{l}$  distilled water at the rate of 0.3  $\mu\text{l/sec}$  was placed on the surface of the film with an automatic piston syringe (100  $\mu\text{l}$ ) and photographed. Initial contact angle was measured after 0.3 sec to minimize the noise in the picture. The method is based on image processing and curve fitting for contact angel according to measuring the contact angel between the baseline of the drop and the tangent of drop boundary. The contact angles were measured on both sides of the drop and the average recorded.

### **3.2.11. Water absorption property**

Water absorption property of the SPI films at pH 7 was determined according to ASTM D570 method. The films were cut in  $25\times25$  mm and preconditioned at environmental chamber for two days, and dried in oven 50 °C for 24 hours before the analysis. Dry samples were weighted ( $w_1$ ) by AG104 Mettler Toledo with 0.1 mg precision. Then they immersed in sodium phosphate dibasic solution 0.1 M and adjust the pH with NaOH 50% w/w to 7 at 25 °C for 2 hours. The wet samples then wiped with filter paper to remove extra water and weighted ( $w_2$ ). Swelling (%) calculated by the following equation:

$$\text{Swelling (\%)} = 100 \times (w_2 - w_1) / w_1 \quad \text{Equation.2}$$

### **3.2.12. Protein solubility**

The protein solubility of films in deionized water during water absorption test was determined by using Bradford (1976) method. The film samples were cut in 25×25 mm and preconditioned at environmental chamber for 2 days, then dried at oven 50 °C for 24 hours. The samples were immersed in 10 ml deionized water for 2 min and 2 hours and the amount of soluble protein in water was detected by UV-spectroscopy at 595 nm absorbance. Coomassie Brilliant Blue G-250 100 mg was dissolved in 50 ml 95% (w/v) ethanol and 100 ml 85% (w/v) phosphoric acid and this solution was diluted to 1 liter. Standard curve was plotted as a function of concentration (0%, 20%, 40%, 60%, 80% and 100% v/v) of 10 mg/ml bovine serum albumin (BSA). To prepare standard curve, 0.1 ml of the protein standard added with 5 ml of Coomassie blue solution to the glass tubes, and flipped several times. Protein solubility results were reported as the percentage of total protein detected in the solution.

### **3.2.13. Factorial design and statistical analysis**

Soy protein isolate was modified with three different enzymatic solutions: with the enzyme, with the deactivated enzyme, without the enzyme at different processing conditions: protein denaturation temperatures (80 and 90 °C) and enzyme incubation times (1hr, 2hr and 3hr). The experiment was designed by fully factorial design with 18 treatments. Each type of film was prepared 3 times and each film replicated 5 times for mechanical test and 3 times for surface hydrophobicity and swelling properties. Therefore, totally 15 replicates for mechanical test and 9 replicates for the others were done.

All data were analyzed by one-way analysis of variance (ANOVA) to determine the difference between samples, and general linear model (GLM) to determine the best interaction of enzyme solution type, protein denaturation temperatures (80 and 90 °C) and enzyme incubation times (1h, 2h, 3h). Minitab Software model 14.12.0 (Minitab Inc., State Collage, Penn., USA) was used. Tukey test was used to carry out the difference of means between pairs with 95% confidence interval.

### **3.3. Results and discussion**

#### **3.3.1. Viscosity of the film solution**

The viscosity of the SPI film forming solution with and without transglutaminase is shown in Fig. 3.1. As displayed in the Fig. 3.1 (e), the behavior of protein solution without the enzyme was Newtonian. All samples prepared at different incubation times (1h, 2h and 3h) had similar viscosities, which was about 0.035 Pa.s. After enzymatic treatment, the behavior of protein solution is non-Newtonian and pseudoplastic. The changing behavior from Newtonian to non-Newtonian confirms that the crosslinking improved with increasing enzyme incubation time. However, in both 80 and 90 °C protein denaturation temperatures, a significant effect ( $p<0.05$ ) of different enzymatic incubation times on the viscosity of the SPI film forming solution was observed (Fig.3.1.a & c).

Gauche et al. (2008) measured the viscosity of milk whey protein solution and they found that the addition of transglutaminase causes the transition of Newtonian to non-Newtonian behavior. Malhotra and Coupland (2004) concluded that SPI solution (5% w/w) prepared at pH 6 had the same trend at different shear rates between  $10\text{-}2500\text{ s}^{-1}$ , and they believed that the viscosity

measured at a high shear rate was more reproducible than those at a lower shear rate (standard error decreased). However, the viscosity of the SPI solution measured in our research was constant until the shear rate of about  $500\text{ s}^{-1}$ . These differences between their findings and ours may be due to the differences in the pH values of the solution or the conditioning of the viscosity measurements, but the values of the viscosity were similar.

According to Fig.3.1.e, the viscosity of protein solution with the deactivated enzyme is significantly lower than those with the enzyme, higher than the solution without the enzyme, and the behavior of the solution is non-Newtonian.

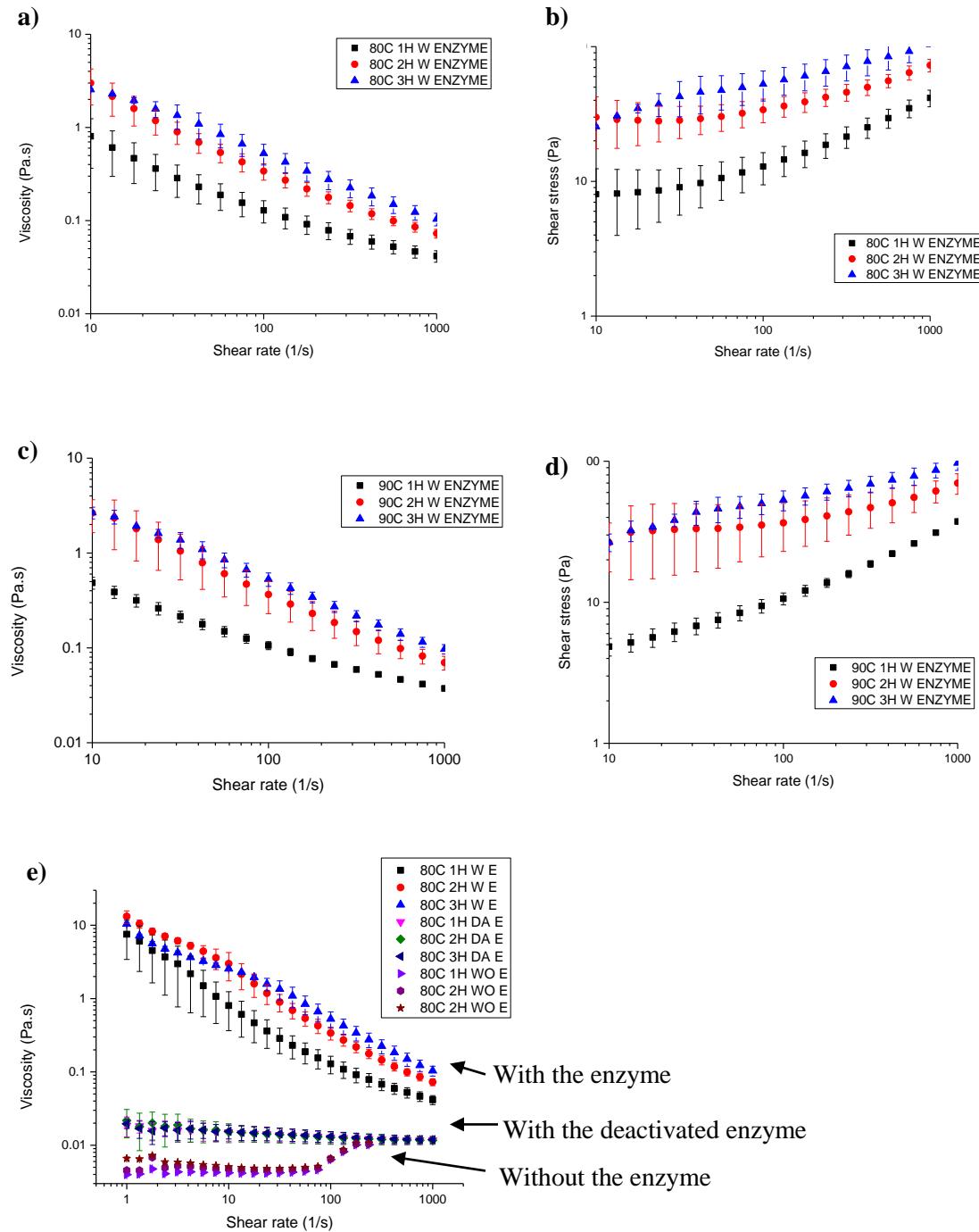
Power law is an equation applied for non-Newtonian fluids. The values of consistency index ( $k$ ) and flow behavior ( $n$ ) are determined as the intercept and slope of the log-log plot of shear rate versus shear stress respectively (Shukla and Rizvi, 1995) (see Fig.3.1 b & d). Consistency index of SPI solution determined the effectiveness of enzymatic incubation time and efficiency of transglutaminase on the inter- and intra-molecular crosslinking of the protein solution (Ando et al., 1989). Table 3.1 shows the consistency and flow indices of different enzymatic modified films at different protein denaturation temperatures and different enzyme incubation times. At 7.5% solid content, increasing enzyme incubation time from 1 hour to 2 hours considerably increased the K-values of the solution prepared at both denaturation temperatures (80 and 90 °C). However, there is not a significant change in the K-value of the solutions prepared at 2 and 3 hr incubation times ( $p>0.05$ ). The consistency index of the solution prepared at 90 °C and 2 or 3 hr incubation time was higher than the same condition prepared at 80 °C. Thus, increasing enzyme incubation time more than 2 hours does not change the consistency index of the SPI solution.

The flow behavior index confirmed non-Newtonian behavior of enzymatic modified film forming solutions due to the number of this value, which is not 1, whereas in Newtonian fluids it is near 1 (Wilcox and Swaisgood, 2002). Consequently, rheological properties of the solution do not depend on the enzyme incubation time over 2 hours.

**Table 3.1.** Consistency and flow indexes of different enzymatic SPI solution at different protein denaturation temperatures and different enzyme incubation times

Film	Consistency index (k) (Pa.s <sup>n</sup> ) <sup>a</sup>	Flow index (n)
90 °C, 1h incubation	6.171±0.445 <sup>a</sup>	0.033±0.001 <sup>a</sup>
90 °C, 2h incubation	31.540±0.800 <sup>b</sup>	0.041±0.002 <sup>b</sup>
90 °C, 3h incubation	33.002±0.703 <sup>c</sup>	0.081 ±0.009 <sup>c</sup>
80 °C, 1h incubation	8.779±0.355 <sup>d</sup>	0.035±0.001 <sup>d</sup>
80 °C, 2h incubation	28.986±0.582 <sup>e</sup>	0.0468±0.001 <sup>e</sup>
80 °C, 3h incubation	29.167±0.193 <sup>e</sup>	0.109±0.013 <sup>f</sup>

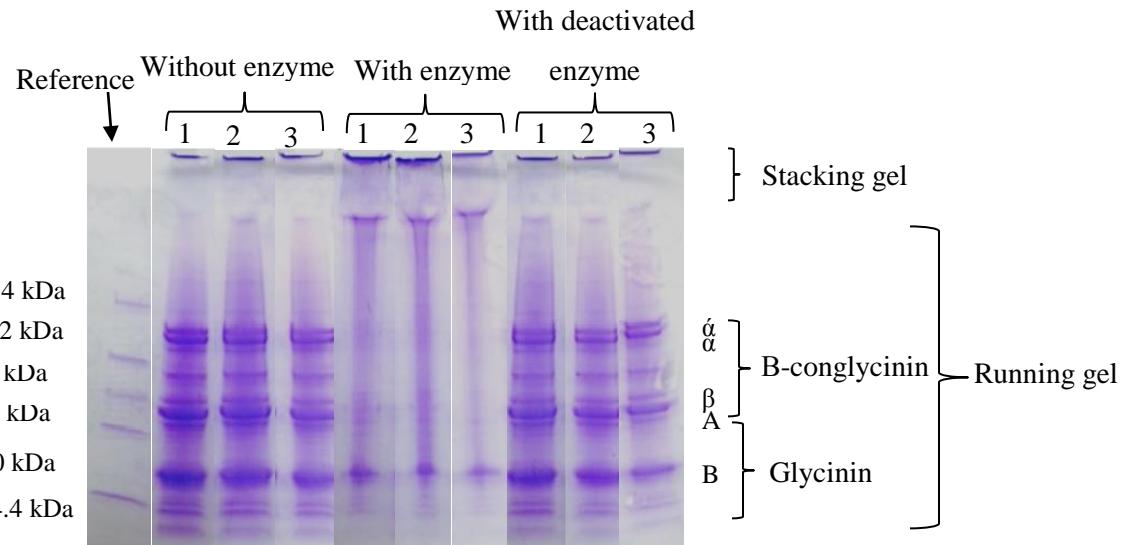
Values were at <sup>a-f</sup> Means ± standard deviation. Different alphabets within a column indicate significant difference at p< 0.05.



**Fig.3.1.** Effect of different protein denaturation temperatures and enzyme reaction times on the viscosity of SPI-solution treated with transglutaminase: a) Viscosity and shear rate of SPI with enzyme at 80°C, b) Viscosity and shear rate of SPI with the enzyme at 90°C, c) effect of different enzyme treatments on viscosity of SPI solution (error bars are Mean  $\pm$  Standard Error of Mean at 95% CI)

### **3.3.2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)**

The effect of enzyme activity on the molecular weight distribution of SPI solution was determined by SDS-PAGE. According to Fig.3.2, SPI solutions without the enzyme and with the deactivated enzyme have similar patterns,  $\beta$ -conglycinin and glycinin globulins at the same concentrations. However, modified solution with the enzyme had high molecular mass (>97000 Da) that could not go into the gel. High molecular weight subunits of  $\beta$ -conglycinin,  $\alpha$ ,  $\alpha$  and  $\beta$ , and the AB subunit of glycinin were observed in the patterns without the enzyme and with the deactivated enzyme. Different enzyme incubation times 1, 2 and 3 hours did not have a significant effect on the molecular weight of the protein solution. Molecular weight estimation of SPI-based protein solutions modified with the enzyme, without the enzyme and with the deactivated enzyme were determined based on the regression equation achieved by standard plot. Enzyme incubation time in less than 1 hour showed more bonds in SDS-PAGE gel (Jiang et al., 2007). The pattern of molecular weight distribution of protein solutions modified with the enzyme, without the enzyme and with the deactivated enzyme in SDS-PAGE is a great evidence of enzymatic reaction and well matched with rheological properties.

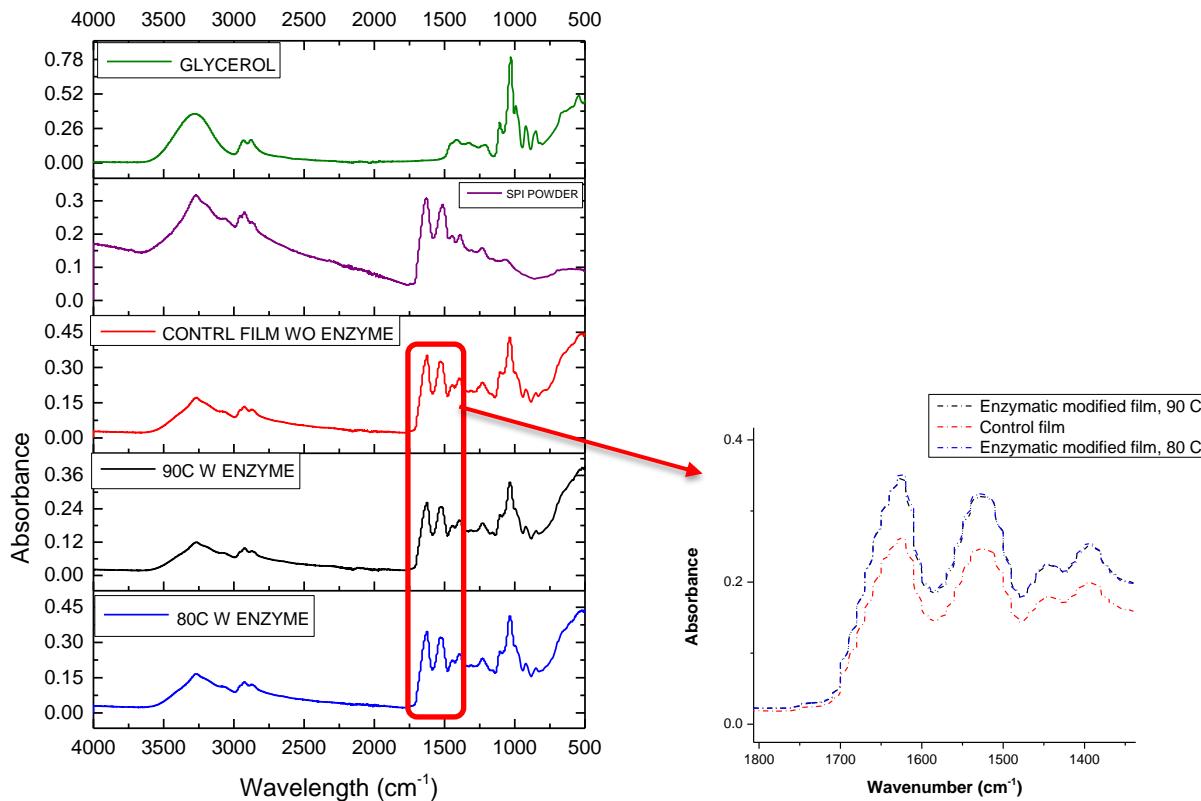


**Fig. 3.2.** SDS-PAGE analysis of formed precipitates in the film-forming solutions of SPI. Numbers are enzyme incubation times.

### 3.3.3. Fourier Transform Infrared (FTIR) spectra

Functional groups and structural changes of films followed by modification with the enzyme can be determined by FTIR spectra (Zadeh et al., 2014). As it is shown in Fig.3.3, in all SPI-based films both modified with and without the enzyme, the significant broad absorption bands are seen at 3267, 2921, 1624 and 1527  $\text{cm}^{-1}$  which are related to O-H stretching, C-H stretching, C=O stretching and N-H stretching, respectively. Protein crosslinked by the enzyme made some changes in amide I and II region. Amide I and amide II bands are two major bands of the protein infrared spectrum. Amide I band ( $1600\text{-}1700 \text{ cm}^{-1}$ ) is the closest absorption band of the polypeptide, and C=O has a predominant role in amide I. Amide II results from the N-H bending vibration and from the C-N stretching vibration. This band is conformationally sensitive (Schmidt, et al., 2004). SPI can participate in crosslinking reactions easily due to possessing many reactive side groups such as N-H, O-H and S-H groups (Su et al., 2010). Modified SPI films with the enzyme at both temperature conditions showed higher intensity in amide I and amide II bands compare to the control film. This difference in the peak intensity would be a good

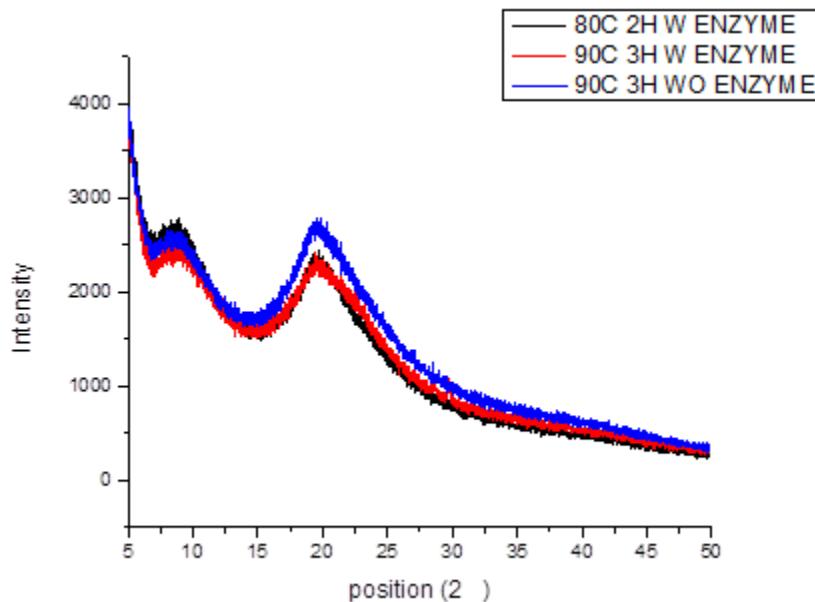
evidence to prove that the enzyme modified the structure of protein matrix. It results from the formation of new amide linkages between SI and the enzyme. It was in accordance with the results observed by González et al. (2011), who modified the structure of SPI with genipin as a cross-linker. The secondary structure of protein can be detected by amide I band, whereas in the current research this band is at  $1624\text{ cm}^{-1}$  in all SPI-based films; therefore, it has  $\beta$ -sheet structure and this  $\beta$ -sheet structure increased surface hydrophobicity of the film (Wang et al., 2011). The spectra of SPI films show an absorption peak at  $1034\text{ cm}^{-1}$ , which is attributed to the vibration of O-H groups (from glycerol) combined with N-H groups of the amino acid residues of SPI protein (Zadeh et al., 2014). Therefore, it is indicated that glycerol reacts with the protein through covalent linkages.



**Fig. 3.3.** FTIR spectra of modified SPI films with transglutaminase in 3 hr incubation and pure SPI film.

### 3.3.4. X-ray diffraction

Fig.3.4 shows the X-ray patterns of the SPI-based films with and without the enzyme. In all SPI-based films there are two peaks at  $2\Theta$  values of around 9 and  $19^\circ$  which are in agreement with the previous studies (Wang et al., 2014; Su et al., 2007). Enzymatically modified SPI films decreased the intensity of the peaks comparing to the controls. The modified films have broader and flatter peaks, which shows good compatibility of the enzyme and SPI. This behavior was also observed in XRD peaks of SPI-based films modified with konjac glucomannan prepared by Wang et al. (2014). Su et al. (2007) reported that the addition of glycerol reduced the crystallinity of SPI-based films modified with poly vinyl alcohol (PVA).In our study, all films were plasticized by glycerol and the crystallinity of the films might be reduced accordingly as shown in Fig. 3.4



**Fig. 3.4.** X-ray position of SPI-based film with and without the enzyme

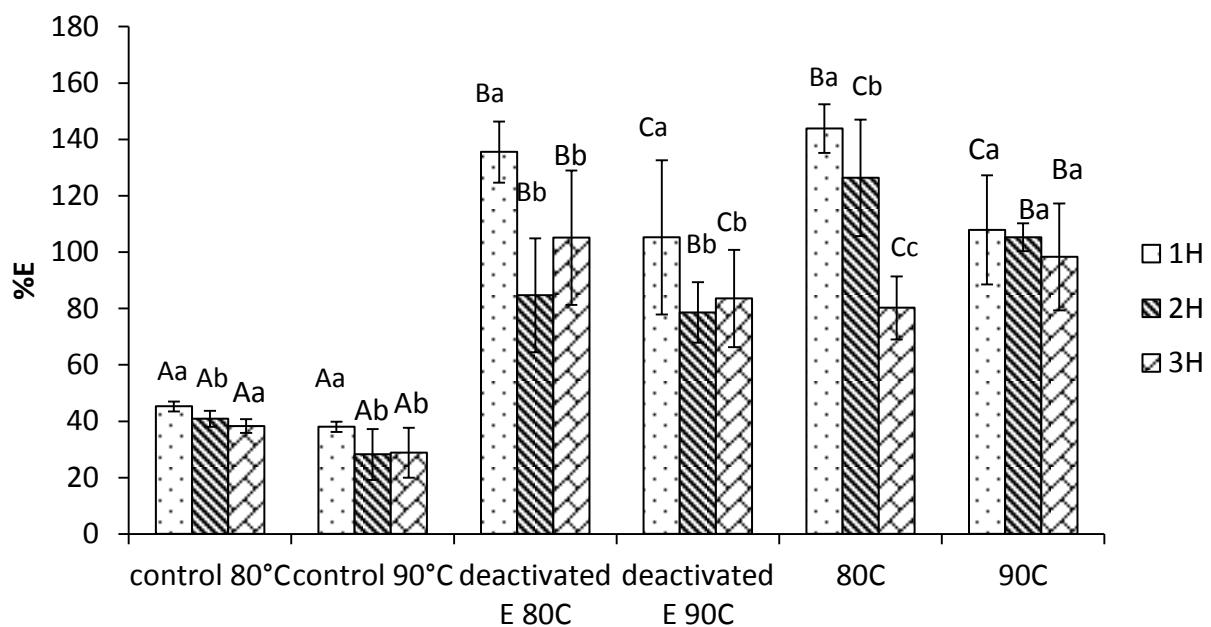
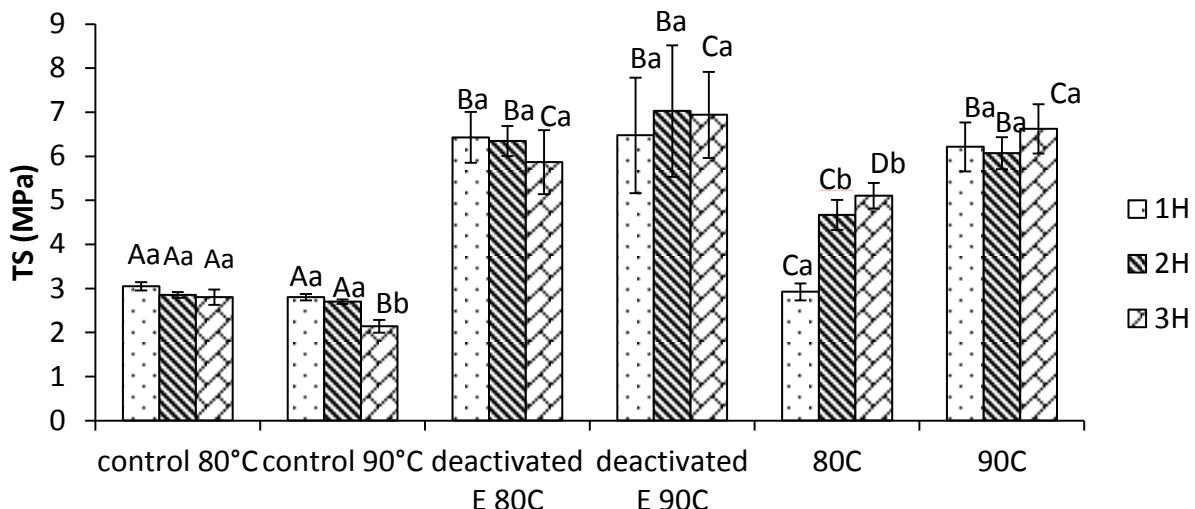
### **3.3.5. Mechanical properties**

Mechanical properties of SPI-based film at different protein denaturation temperatures and different enzyme reaction times are shown in Fig. 3.5. Different enzyme incubation times did not have a significant effect on the tensile strength (TS) of films prepared at 90°C ( $p>0.05$ ). However, TS of the films prepared at 80 °C grew gradually as the enzymatic incubation time increased ( $p<0.05$ ) and both of them were considerably higher than the control film. Moreover, protein incubation time (1, 2 and 3h) and protein denaturation temperature (80 °C and 90 °C) did not show a significant effect on the TS of the control films ( $p>0.05$ ). Tang et al. (2005) reported that increasing the protein denaturation temperature to 90 °C caused higher amounts of free active groups as a result of unfolding protein, therefore, coagulation occurred more while cooling the solution. Thus, the physicochemical properties of SPI films treated with the enzyme will be dependent on the denaturation temperature.

Jiang et al. (2007) reported that pre-incubation time with microbial transglutaminase for 30-60 min slightly increased TS of SPI films. However, longer incubation time (120 min) significantly decreased TS compared to the control film. They believed that more additional enzyme incubation time could cause the higher protein aggregation and result in decreasing TS. Mariniello et al. (2003) reported that enzymatic modification of pectin-soy flour film with transglutaminase (20 U/g of protein) improved TS two times more than that of the control film without the enzyme. High amounts of enzyme caused aggregation. In their experiment, although the amount of enzyme was high, the presence of pectin delayed the aggregation of soy protein.

Modified films with the deactivated enzyme and prepared at 80 °C had higher TS than the others with the enzyme at the same preparation condition. However, there is no significant difference in TS of modified films prepared at 90 °C with the enzyme and with the deactivated enzyme. Activa RM prepared by Ajinomoto contains 99.5% sodium caseinate and maltodextrin. Therefore, intermolecular hydrogen bonds between soy protein and maltodextrin can form a compact structure to increase TS. Statistical analysis showed that enzyme treatment and temperature and their interactions had significant effects on the TS of modified films (p-value<0.05).

The percentage of elasticity (%E) of the films prepared at 80 °C was higher than those prepared at 90 °C (Fig.3.5). In the films prepared at 80 °C increasing enzyme incubation time had a negative effect on %E, which is opposite of the findings in TS. Larre et al. (2000) indicated that the presence of transglutaminase increased simultaneously TS and %E of gluten films and they explained that the formation of crosslinking by the enzyme gained flexibility to the film structure. At a higher temperature (90 °C) more protein denatured and unfolded, resulting in a compact structure of films. This caused fewer opportunities for glycerol molecules to interact between protein chains. Therefore, the chain mobility reduced; the films prepared at 90 °C have higher TS and lower %E than the others prepared at 80 °C (Guerrero and Caba, 2010). In addition, increasing enzymatic incubation times showed a significant effect (p<0.05) on %E of the films prepared at 80 °C. Percent elongation of modified films with the enzyme was not notably different with the ones with the deactivated enzyme (p-value>0.05). According to Table.3.2, enzyme treatment, protein denaturation temperature, enzyme incubation time and their interactions had significant effects (p<0.05) on %E.



**Fig.3.5.** Effect of different enzyme treatments, protein denaturation temperature and enzyme reaction time on the mechanical properties of SPI-film treated with transglutaminase (error bars are Mean  $\pm$  Standard deviation at 95% CI). Small letters show significant differences in each group. Capital letters shows significant differences within groups.

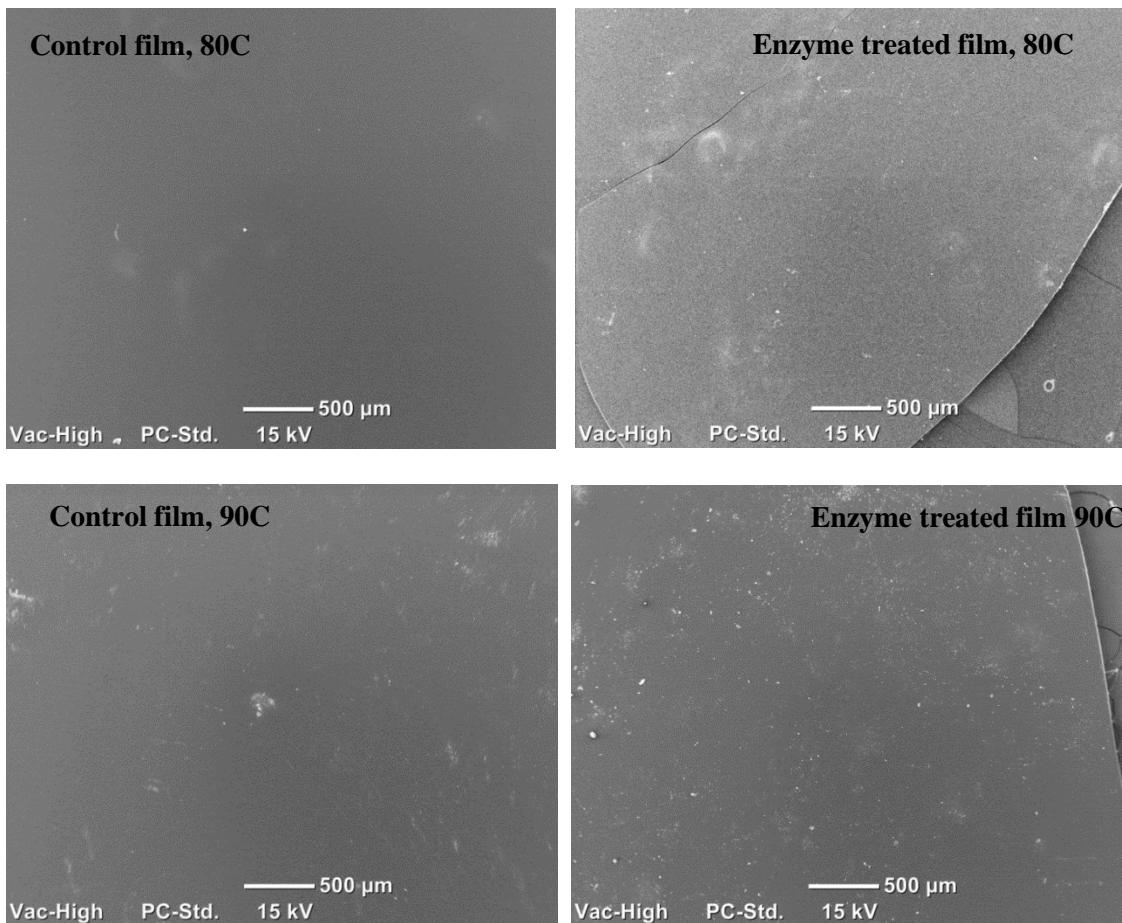
**Table 3.2.** The effectiveness of enzyme treatment, protein denaturation temperature, enzyme incubation time and their interactions on physico-chemical properties of SPI-based edible film treated with transglutaminase by GLM method and confidence interval 95%.

Analyze	Condition	F-ratio	P-value	R <sup>2</sup>	R <sup>2</sup> -adj
TS	Enzyme treatment	426.33	0.000		
	Temperature	57.34	0.000		
	Reaction time	2.86	0.065		
	Enzyme*temp	43.81	0.000		
	Enzyme* time	8.76	0.000		
	Temp*time	1.31	0.274		
	Enzyme*time*temp	4.76	0.001		
%E	Total			88.04	86.63
	Enzyme treatment	383.67	0.000		
	Temperature	19.38	0.000		
	Reaction time	45.86	0.000		
	Enzyme*temp	6.34	0.002		
	Enzyme*time	9.18	0.000		
	Temp*time	7.51	0.001		
Contact angle (airside)	Enzyme*temp*time	5.25	0.001		
	Total			87	85.47
	Enzyme treatment	596.99	0.000		
	Temperature	5.19	0.024		
	Reaction time	1.84	0.162		
	Enzyme*temp	77.00	0.000		
	Enzyme*time	8.34	0.000		
Contact angle (plateside)	Temp*time	2.08	0.129		
	Enzyme*temp*time	12.92	0.000		
	Total			90.94	89.87
	Enzyme treatment	390.33	0.000		
	Temperature	0.34	0.558		
	Reaction time	4.78	0.010		
	Enzyme*temp	13.48	0.000		
	Enzyme*time	4.15	0.003		
	Temp*time	1.50	0.227		
	Enzyme*temp*time	5.60	0.000		
	Total			85.65	83.96

Note: Enzyme treatments (with enzyme, without enzyme and with deactivated enzyme); temperature (80 and 90 °C); time (enzyme incubation time 1, 2 and 3 hr).

### 3.3.6. Scanning electron microscopy

The airside surface morphology of both control and modified films with transglutaminase at different temperatures is shown in Fig.3.6. Modified films with the enzyme have higher roughness on the surface than those of control films and this roughness induced effect on the surface hydrophobicity of the enzyme-treated films. The same result was also observed by Tang et al. (2005), and they believed that this roughness can be accounted for the aggregation of hydrophobic groups of SPI made by transglutaminase.



**Fig.3.6.** SEM micrographs (at 30 $\times$  magnification) of top surfaces of control and the enzyme-treated films of SPI for 3hr enzyme incubation.

### **3.3.7. Surface hydrophobicity**

Contact angle is defined as the angle between the solid surface and a tangent drawn on the drop surface, which passes through the triple point solid-liquid-vapor (Karbowiak et al., 2006). It is varied between 0-180° at the high hydrophilic surfaces to absolutely no wetting surfaces. A surface with a water contact angle below 60° is called hydrophilic and a surface above 60° is called hydrophobic (Vogler, 1998).

Surface hydrophobicity is a very important factor in packaging materials because it has effects on the water affinity, water vapor permeability, and adsorption/desorption of the film; thus the type of application will be influenced.

As it is shown in Fig.3.7, modified films with the enzyme and the deactivated enzyme had higher surface hydrophobicity than the control; however, no significant difference was observed between enzyme treated film and enzyme deactivated films at different incubation times.

The films prepared at 80 °C and 1hr enzyme reaction time showed the highest contact angle at the airside of the film, which was about 97°, while control shows 60°.

It is attributed by the influence of hydrophobic groups of SPI which are initially hidden in the inside of the molecules after enzyme treatment (Tang et al., 2005). It is well known that the contact angle of the biopolymeric film is affected by the type, concentration, and combination of plasticizers (Tang et al., 2005). The contact angles in our study, whereas glycerol was used as a single plasticizer, are showing slightly lower than other literatures due to the type of plasticizer

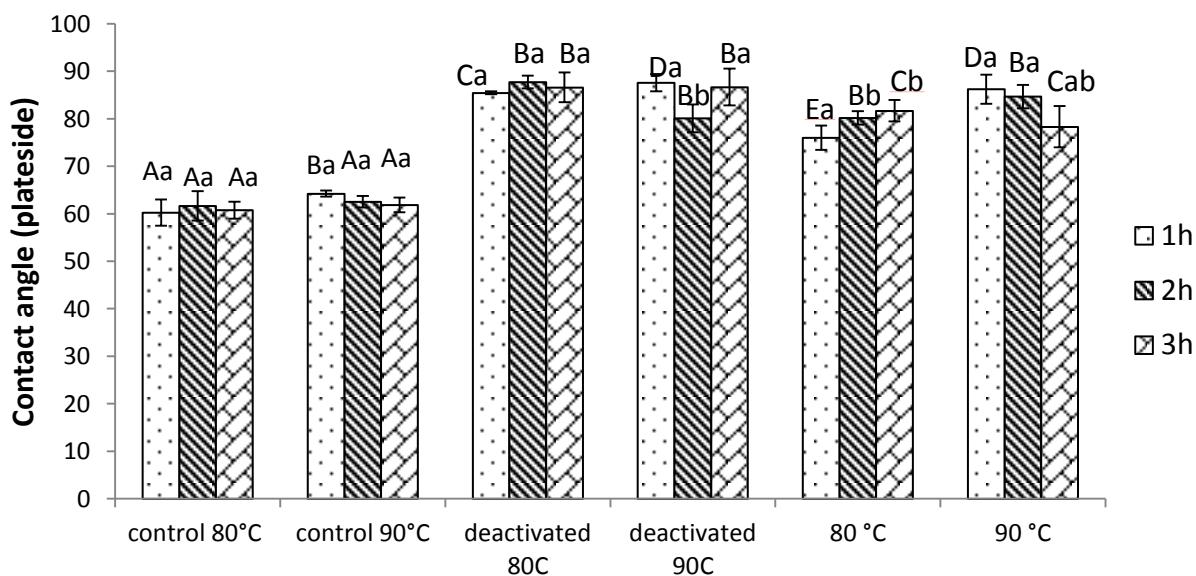
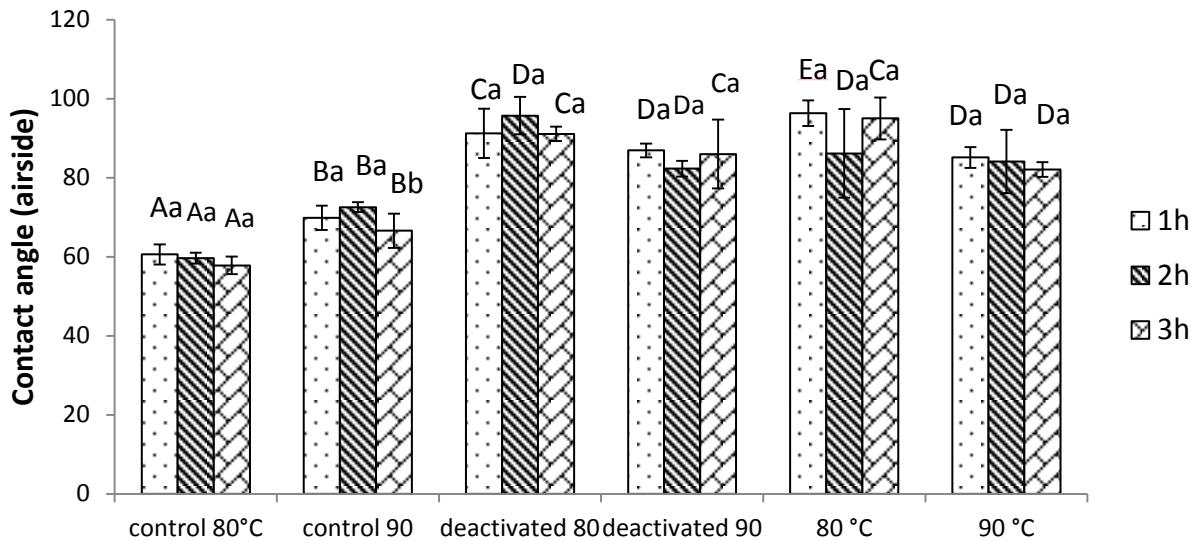
and concentration (Jiang et al., 2007).. The contact angle of films was also affected by casting methods, preparation temperature, enzymatic treatment and its incubation time processes

As shown in Table 3. 2, different enzyme incubation times and protein denaturation temperatures did not show a significant effect ( $p>0.05$ ) on the surface hydrophobicity of plateside of the films. However, the hydrophobicity of the treated films with the enzyme and deactivated enzyme was significantly higher than the control film. Treated films had lower contact angle at the plateside of the films compare to the airside. Similar phenomena is observed at many other biopolymeric products such as two-sideness in paper (Samyn, 2013). The contact angles in two different sides is attributed to the reorientation of the molecules during drying and their affinity to the surface of the plate (Karbowski et al., 2006).

According to the GLM table (Table 3.2), the interaction of enzyme treatment and temperature had a significant effect ( $p\leq 0.05$ ) on the contact angle of airside of the film, and the interaction of enzyme treatment and time had a significant effect ( $p\leq 0.05$ ) on the plateside contact angle of the film. Modified films prepared at 90 °C had more roughness on the airside surface compare to films prepared at 80 °C, therefore, temperature had significant effect ( $p<0.05$ ) on the airside contact angle of films. Moreover, different enzyme treatments (with the enzyme, without the enzyme, and with deactivated enzyme) had significant effect ( $p<0.05$ ) on the contact angle of both sides of the films. It implies that the enzymatic treatment and incubation time is crucial to modify the surface hydrophobicity of bipolymeric film

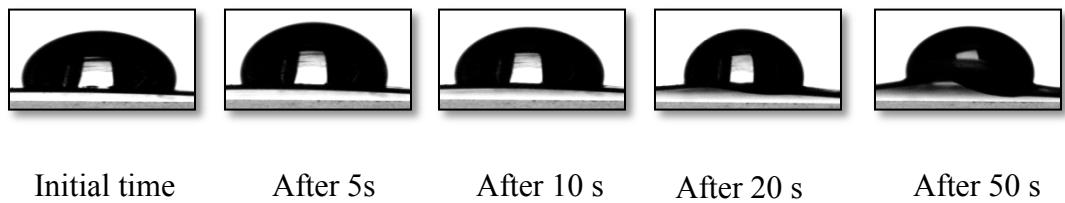
As shown in Fig.3.8, contact angle of both sides of films have the same trend during the first 25 sec, after that some changes were observed on the surface of the film due to the water absorption and swelling. This is not completely in agreement with Karbowiak et al. (2006) who observed swelling at the airside and absorption at the plateside of the films prepared with iota-carrageenan modified with Grindsted barrier system (GBS) as a kind of fat and glycerol monoestearate (GMS) as an emulsifier. This difference was due to the addition of fat to increase surface hydrophobicity of films.

Based on our observation in contact angle, the hydrophobicity of enzyme treated sample was improved comparing to control to approximately 40%.

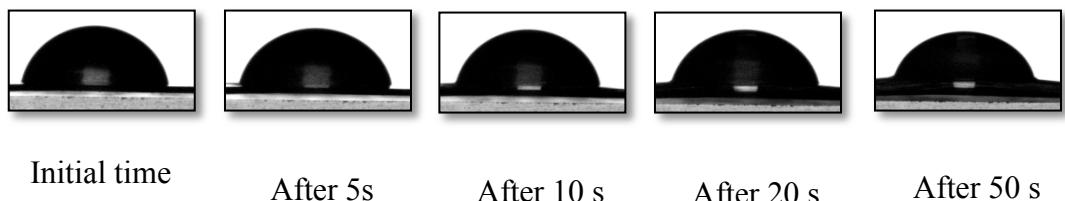


**Fig.3.7.** Effect of different enzyme treatments, protein denaturation temperature and enzyme reaction times on the contact angle of SPI-film treated with transglutaminase (error bars are Mean  $\pm$  Standard deviation at 95% CI). Small letter shows significant difference in each group by Tukey test. Capital letter shows significant difference within groups.

Contact angle films in 80 °C, 1h: airside



Contact angle films in 80 °C, 1h: plateside



Initial time      After 5s      After 10 s      After 20 s      After 50 s

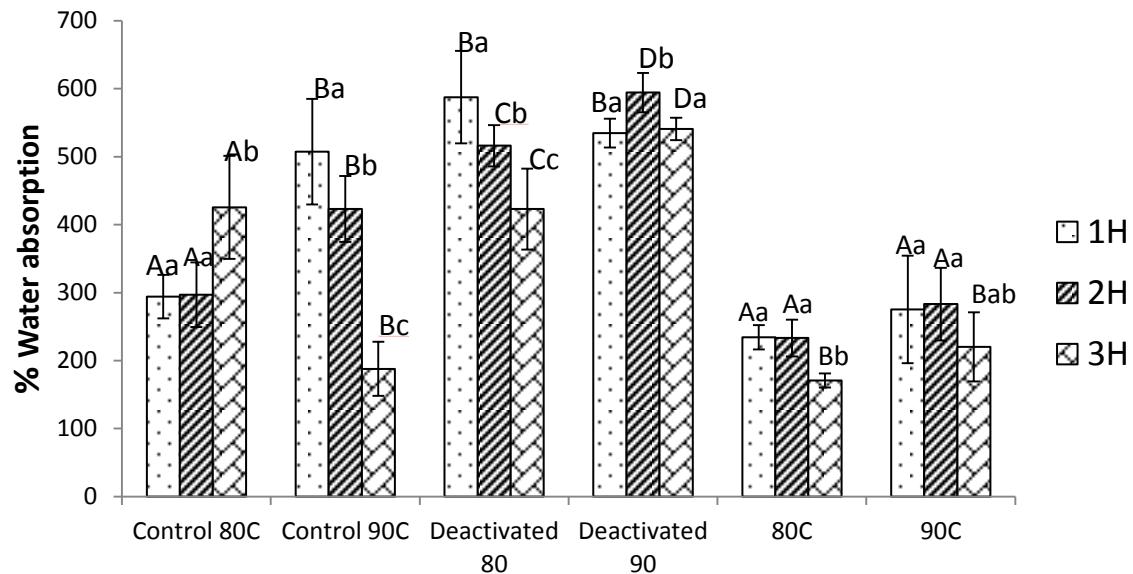
**Fig.3.8.** Different kinetics observed for water droplets on the SPI-based films treated with transglutaminase on both sides of the film during 4 minutes.

### 3.3.8. Water absorption properties

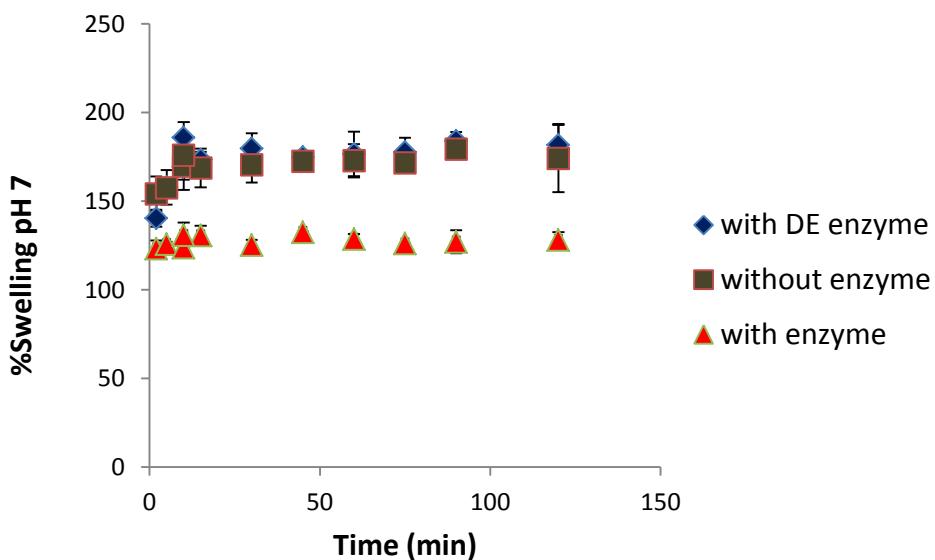
Treated soy protein isolate film with the enzyme is soluble in water and a few minutes of storing in water is sufficient to induce weight gain. The preliminary experiment showed that more than 2 minutes immersing the films in water caused partial dissolution. As it is shown in Fig.3.9, modified films with the enzyme had lower capacity to gain weight in water compared to both the control film and modified films with the deactivated enzyme at both protein denaturation temperatures. However, control film prepared at 90 °C and 3 hr incubation time showed similar water absorption properties as low as those of enzymatically modified films, which might be due to the high number of aggregations that caused compact structure to decrease the ability of water absorption. Moreover, increasing enzyme incubation time reduced the degree of water absorption

capacity of the films. It is in good agreement with the previous literature published by Bigi, et al. (2001) who showed that water absorption of the gelatin films in physiological solution decreased after modification with permanent chemical crosslinker: glutaraldehyde. This indicates that increasing enzyme incubation time reduces free space for water absorption by increased crosslinking

Fig.3.10 shows water absorption behaviors of the films prepared at 80°C and 3 hr incubation time and pH 7 conditions. Films were stable in the physiological solution in the period of 2 hours; however, they were not stable in distilled water, which might be due to the ionic solution of buffer causing protein not to dissolve fast. Moreover, the enzyme treated SPI film showed significant reduction of the water absorption compared to both control and deactivated enzyme treated films. This clearly shows that the SPI molecules were crosslinked by the enzyme and reduced the water holding capacity due to the reduction of free volume and functional moiety which is interacting with water molecules.



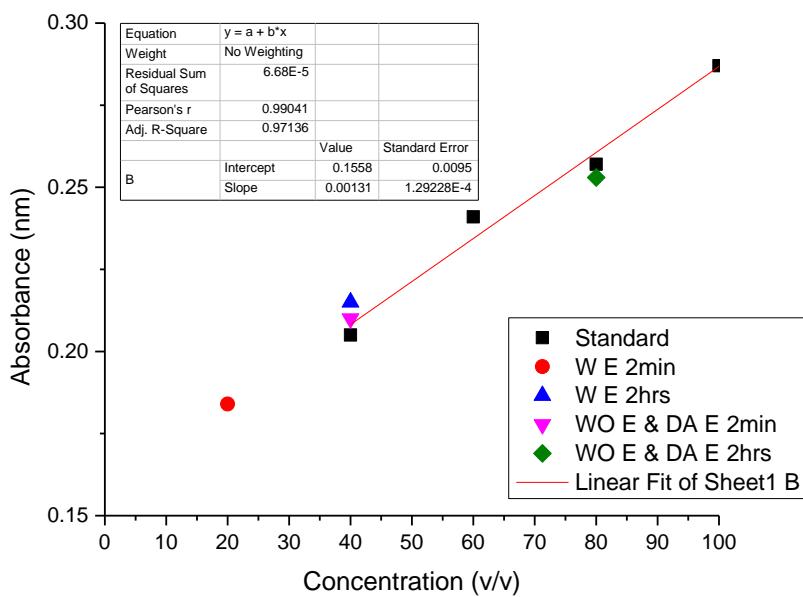
**Fig.3.9.** Water absorption properties of SPI-treated films with transglutaminase immersed in deionized water in 2 min (error bars are Mean  $\pm$  Standard Error of Mean at 95% CI).



**Fig.3.10.** Effect of enzyme treatment on the water absorption property of SPI-film treated with transglutaminase at different pH buffer solutions in 2 hours (error bars are Mean  $\pm$  Standard Error of Mean at 95% CI).

### 3.3.9. Protein solubility

Protein solubility of modified SPI-film with the enzyme, without the enzyme and with deactivated enzyme, which were prepared at 80 °C and 3 hr incubation, is shown in Fig.3.11. SPI film modified with the enzyme showed the lowest protein solubility in 2 min, which was about 20%. However, the solubility of those control and the deactivated enzyme treated films was not significantly different from enzymatic modified films immersing in 2 hours in distilled water. This implies the crosslinking by enzyme doesn't change the ultimate water solubility of protein based film in the view of long term stability. Instead, it may be useful to control the instant solubility in potential application. This result is well-matched to water absorption of the films; whereas enzymatic modified films had lower water absorption and protein solubility compared to the films without the enzyme and with deactivated enzyme.



**Fig. 3.11.** Standard curve of the protein concentration versus the wavelength absorbance (nm)

### **3.4. Conclusion**

Enzymatic treatment of soy protein isolate with transglutaminase improved crosslinking of protein network; SDS-PAGE and rheological data are excellent evidences for this. The behavior of enzymatic treated protein solution with 7.5% solid contents was non-Newtonian and pseudoplastic, and the viscosity increased with increasing enzyme incubation time. Mechanical and surface hydrophobicity properties of the films prepared at 80 °C were significantly affected by different enzymatic incubation times ( $p<0.05$ ). SPI-based films modified with transglutaminase showed significant improvement in mechanical strengths, surface hydrophobicity and water absorption properties compared to the control film due to the crosslinked structure in the molecule. SPI films treated with the deactivated enzyme exhibited similar mechanical and surface hydrophobicity to the films with the enzyme. However, viscosity of the solution and water absorption properties of film treated with deactivated enzyme was not changed because of lack of crosslinking by enzyme. Based on the above observations, the effect of enzymatic treatment on biopolymeric product can be easily determined by both viscosity and SDS-PAGE evaluation and it shows the enzymatic treatment at protein based polymer can be a useful way to control the physical properties of protein based biopolymeric film, and this treatment can be applied to a wide spectrum of business areas such as packaging, food, pharmaceutical, and agricultural industries.

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## **CHAPTER 4**

### **Improving Physicochemical and Functional Properties of Enzymatic Modified SPI Film with Lignin**

#### **Abstract**

The effect of lignin on enzymatically modified soy protein isolate (SPI) biopolymeric films was investigated in regards to functionality and physicochemical properties. Two types of lignin, alkali lignin (AL) and lignosulphonate (LSS), potentially carrying antioxidant activity, were added to the protein solution at different concentrations (2%, 4%, 6%, and 10% w/w of SPI). Films incorporating lignins were characterized by physicochemical and morphological properties, along with radical scavenging activity and UV-blocking ability. The radical scavenging test (DPPH assay) showed that AL material carries higher radical scavenging ability than that of LSS. However, films containing LSS showed slightly higher radical scavenging activity. It is attributed to the higher solubility and compatibility of LSS in the SPI film matrix. The UV-spectroscopy test revealed that films containing AL showed high absorption range in UV region, and this UV-blocking ability increased with increasing the level of lignin. By deconvoluted FTIR spectra, it was confirmed that the addition of lignin made some changes in the secondary structure of the protein matrix, which was well matched to XRD results. Thermal and mechanical analysis tests showed that the addition of lignin to the films improved tensile strength and thermal stability of films while it reduced the %E as a function of lignin concentration. The addition of lignin reduced viscosity of film forming solution. Higher

reduction in viscosity was observed when AL was added comparing to LSS due to more interruption of protein matrix by AL. Scanning electron microscopy pictures showed that films with LSS had smoother surfaces compared to those with AL due to the higher water solubility and compatibility with SPI matrix. It resulted in lower differences in the contact angles between plateside and airside of the films. Lignin acts as a filler and reduces water vapor permeability of films compared to the control film. Water absorption properties of films containing AL showed 50% higher than both control and films containing LSS. Based on our observation of the incorporation of lignin, which was relatively cheap, nontoxic, and naturally abundant, into biopolymeric film, will bring additional functionality and modified physicochemical properties for food packaging systems. It will also increase the potential of active food packaging structures.

#### **4.1. Introduction**

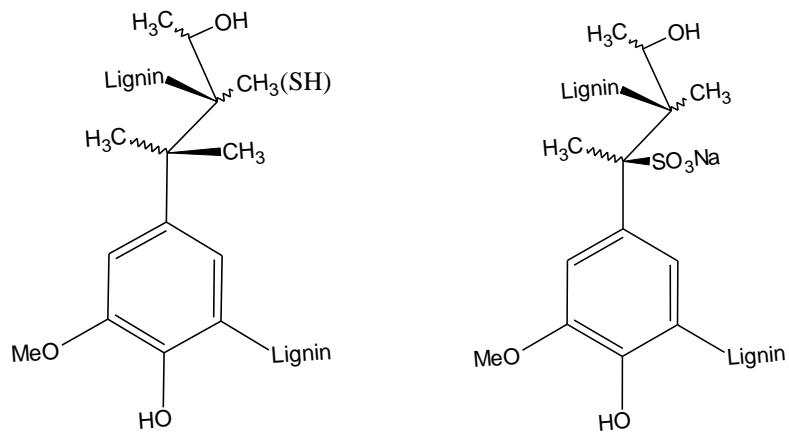
Active packaging is a group of technologies giving some functions for food preservation other than providing an inert barrier (Brody et al., 2001). Due to the customer demand and market trends, the area of active packaging is becoming increasingly important. Antioxidants in food packaging, especially for those sensitive to oxidation, is a kind of preservation food system to provide safety and security of food products by inhibiting the initiation or propagation of oxidizing chain reaction (Velioglu et al., 1998). Lipid oxidation is associated with coronary heart disease, atherosclerosis, cancer, and the aging process (Fukumoto & Mazza, 2000). Antioxidants prevent oxidation by three mechanisms: UV-blocking, radical scavenging, and chelation (Núñez-Flores et al. 2013; Zheng and Wang, 2001; Schreiber et al., 2013). In general, there are two categories of antioxidants: natural and synthetic. Common synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) have some restrictions in application in food products due to their carcinogenicity (Ito et al., 1983).

Lignin, as a natural antioxidant, contains phenolic compounds that prevent lipid oxidation in a similar way to BHT or BHA. According to Pan et al. (2006) some factors that have positive effects on the antioxidant activity of lignin include: more phenolic hydroxyl groups, less aliphatic hydroxyl groups, low molecular weight, and narrow polydispersity. Lignin is one of the most abundant organic materials on earth, and large quantity of volume has been produced as byproducts in many agricultural business sectors. Lignin has an aromatic and highly cross-linked structure with a greatly reactive macromolecular compound due to its functional groups. These groups include aromatic ring, methoxy groups, and aromatic and aliphatic alcohol groups that make chemical reactions possible (Oliviero et al., 2011). Therefore, lignin is able to interact with

many polymers and change their wettability, fire resistance, and mechanical properties (Oliviero et al., 2011). Oliviero et al. (2011) revealed that the addition of lignin to zein protein matrix caused some changes in the secondary structure of the zein protein,  $\alpha$ -helix,  $\beta$ -sheet, and  $\beta$ -turn. Therefore, lignin can modify physicochemical properties, other than antioxidant activity, in the film.

According to the source of lignin and its extraction procedure, lignin structure, purity, and corresponding properties were influenced and changed (Laurichesse and Avérous, 2014). Alkali lignin (AL) and lignosulphonate (LSS) are derived from sulfur processes. Lignosulphonates are water soluble, and possess higher molecular weight than AL. Alkali lignins are soluble at high pH, and both are commercially available (Laurichesse and Avérous, 2014).

Several authors investigated the effect of different types of lignin on the functional and physicochemical properties of natural polymers at different film preparation conditions (Oliviero et al., 2011; Nunez-Flores et al., 2013; Huang et al., 2003a,b; Wei et al., 2006). The characteristics of biopolymeric products incorporating lignin were highly dependent upon functional groups of both lignin and natural polymers. However, the interaction between soy protein isolate (SPI) and lignin under enzyme treatment, which may potentially cross-link them, has not been explored yet. The ultimate objective of this study is to investigate the effect of enzyme treatment on physicochemical properties of biopolymeric films fabricated with SPI and lignin as functions of types and concentrations of lignin, as well as other processing conditions.



**Fig. 4.1.** Chemical structure of alkali lignin (left), and lignosulfonate (right)

## 4.2. Materials and methods

### 4.2.1. Materials

Soy protein isolate (PROFAM 974) with 90% protein on a dry basis was supplied by Archer Daniels Midland company (ADM) Protein Specialties Division, Netherlands. SPI has 5% of moisture, 4% of fat and 5% of ash. Transglutaminase (Activa RM) was donated by Ajinomoto, Japan. According to the data sheet provided by the company, Activa RM contains 99.5% sodium caseinate and maltodextrin and 0.5% the enzyme. Based on our preliminary study, measured activity of the enzyme was 47.8 unit per gram of enzyme. Glycerol and alkali lignin (code 370959) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Calcium lignosulphonate was donated from Green Agrochem, grade one, Jinan, China. Reagents for enzyme activity measurements such as Tris-HCl, Tris-acetate buffer, CaCl<sub>2</sub>, glutathione, hydroxylamine, CBZ-glutaminylglycine, L-glutamic acid  $\gamma$ -monohydroxamate, FeCl<sub>3</sub>-trichloroacetic were purchased from Sigma Aldrich. Citric acid, ethanol, methanol, BHT, and

phosphoric acid were bought from Fisher Scientific. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) was obtained from Sigma Chemical Co.

#### **4.2.2. Antioxidant activity of lignin material**

Antioxidant activity of different lignins (AL and LSS) was determined based on DPPH (1, 1-diphenyl-2-picrylhydrazyl) method, and according to radical scavenging ability of lignin material at neutral pH. The assay was carried out as described by Brand-Williams et al. (1995) with some modifications. Six different concentrations of lignin solution in methanol ranging from 0 to 800 µg/ml were prepared and DPPH solution 0.06 mM in methanol was added to each antioxidant solution and the value of absorbance at 517 nm was measured by Genesys 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA). Butylatedhydroxytoluene (BHT) was used as a reference. Radical scavenging activity of each antioxidant was expressed from the graph by linear regression of absorbance versus concentration.

#### **4.2.3. Film preparation**

Enzymatically modified soy protein isolate-based film was prepared by dissolving 5 g SPI in 100 ml distilled water with glycerol 30% (w/w of SPI) according to the preliminary study. Lignin solution (2%, 4%, 6%, and 10% w/w of SPI) and enzyme solution (4 U/g of protein) were prepared separately and added to the final solution under a specific pH condition. Maximum solubility of alkali lignin was at pH 10-11. The pH of water soluble lignosulphonate was adjusted to pH 7 with sodium hydroxide 50% w/w. After heating SPI solution at 80 °C in 30 min, the solution was cooled down to 40 °C and incubate with the lignin for 1 hour. Transglutaminase was added to the solution and incubated at the same temperature condition for another 2 hours. One hundred gram of solution was weighted and cast on Teflon-coated glass plates ( $20\times20\text{ cm}^2$ )

to produce the consistency of thickness in all samples and dried at room temperature for 24 hr and then carefully peeled off manually. The films were then preconditioned at 25°C and 50% RH for 2-3 days before analysis according to ASTM D618-61 (1995).

#### **4.2.4. DPPH free radical scavenging test of films**

Radical scavenging activity of film samples with and without lignin was determined according to the method described by Yen et al. (1995) with a slight modification. A rectangular piece of soy protein-based film ( $1 \text{ cm}^2$ ) with and without lignin was soaked into 5 ml of DPPH 0.05 mM in methanol. Film samples are insoluble in methanol-based solution. The solution was shaked at room temperature in the dark for 2 hours. The absorbance of the solution was determined in 517 nm by Genesys 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA). Radical scavenging activity of films was calculated according to the following equation:

$$\text{Radical Scavenging activity (\%)} = (1 - \left( \frac{A_{\text{sample}}}{A_{\text{control}}} \right)) \times 100$$

Whereas,  $A_{\text{sample}}$  is the absorbance of sample and  $A_{\text{control}}$  is the absorbance of control without any sample.

#### **4.2.5. Color and opacity of films**

The color of film surface was measured using a Konica Minolta (CR-200; Minolta Co. Ltd, Osaka, Japan). Prior to analysis, the colorimeter was calibrated to standard black and white tiles. Five different parts of film samples were measured, and the mean and standard deviation were recorded. The color scale was included L-value, from 0-100, a-value and b-value, from negative to positive scales.

Opacity of films was measured by Genesys 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific, USA), and according to a method described by Núñez-Flores et al. (2013). The films were cut into a rectangle piece and directly placed into a cuvette. An empty cuvette was used as the reference. The opacity of films was calculated by the equation  $O = \text{Abs}600/X$ , whereas Abs600 is the value of absorbance at 600 nm and X is the thickness of the film. Results were obtained in triplicate.

#### **4.2.6. Film UV-blocking measurement**

UV-absorbance of the films was measured by the UV/Vis spectrometer (Shimadzu 2550 UV-Vis spectrometer spectrum, Kyoto, Japan) with wavelength range of 200-800 nm.

#### **4.2.7. Rheology of film forming solutions**

The effect of lignin on the rheological properties of enzymatically modified SPI film forming solution with 7.5% solid content were determined by using a rotational rheometer (AR G2, TA instrument Ltd, New Castle, DE, USA) with a conical concentric cylinder (30.2 mm diameter and 78.4 mm length and a geometry with 28.1 mm diameter and 80.8 mm length). This is according to the method described by Gauche et al. (2008). The measurements were replicated three times for each sample and the data were evaluated using the Origin software, version 9.0 (Microcal Software Inc., Northampton, MA, USA). The temperature of rheometer was controlled by Brookfield temperature controller with nitrogen gas to set in 25 °C. The sample was preconditioned at 25 °C for 2 min to ensure stability. The shear rate increased linearly from 1 to 1000 s<sup>-1</sup> with 8 points per decade in a steady state flow rate. Viscosity of the SPI solution was expressed by Pa.s.

#### **4.2.8. Fourier transforms infrared spectroscopy (FTIR)**

Infrared spectroscopy (FTIR) was performed with an 8700 Nicolet Thermo Electron, with transmission mode and a resolution of  $4\text{ cm}^{-1}$ , in the range of  $4000\text{-}500\text{ cm}^{-1}$ . Number of scans for background was 32 and for the sample was 96. The samples were preconditioned in a dissector containing  $\text{P}_2\text{O}_5$  for a week to achieve the lowest moisture content. To see the effect of lignin on secondary structure of protein matrix, the absorbance region between  $1800\text{-}1400\text{ cm}^{-1}$  was deconvoluted by OriginPro, version 9.0 (Microcal Software Inc., Northampton, MA, USA) by using the best fit by  $R^2 > 99\%$  to specify the position of the peaks correspond to the different secondary conformations of protein matrix (Oliviero et al., 2011).

#### **4.2.9. X-ray diffraction**

In order to analyze the crystalline structure of enzymatically modified SPI-based film with and without the lignin X-ray diffraction was performed by XRD, Bruker D8 Discover, from  $2\Theta$  values of 5 to  $50^\circ$  at the rate of 0.1 sec/step and equipped with  $\text{CuK}\alpha$  radiation ( $\lambda=0.1542\text{ nm}$ ).

#### **4.2.10. Environmental scanning electron microscopy (ESEM)**

The cross section of film samples was frozen in liquid nitrogen and snapped immediately, then coated with gold 60:40 gold-palladium mixture deposited by Cressington 208HR coater. The samples were tested with FEI Quanta 600 FEG, Oregon, USA.

#### **4.2.11. Mechanical properties**

For determination of tensile strength (TS) and percent elongation (%E), ASTM (1997) method was used and evaluated by TA.XTPLUS texture analyzer (Stable Micro Systems, Surrey, UK) with 50 Kg loading capacity. The films were cut in rectangles  $2.5 \times 8 \text{ cm}^2$  and put in the environmental chamber (50% RH and 23 °C) chamber for two days before the analysis. The samples were fixed between the grips with initial grip separation 5 cm and pulled apart at cross head speed of 50 mm/min. Tensile strength was determined by dividing peak load (N) to cross section area ( $\text{m}^2$ ) and the results expressed by MPa. Percent elongation was calculated by dividing the extended length by initial length multiply by 100.

#### **4.2.12. Surface hydrophobicity**

The surface hydrophobicity of enzymatically modified films activated with different levels of lignin was assessed by measuring contact angle (FTA 200; Dynamic Contact Angle Analyzer). A drop of 1  $\mu\text{l}$  distilled water at the rate of 0.3  $\mu\text{l/sec}$  was placed on the surface of the film with an automatic piston syringe (100  $\mu\text{l}$ ) and photographed. Initial contact angle was measured after 0.3 sec to minimize the noise in the picture. The method is based on image processing and curve fitting for contact angel according to measuring the contact angel between the baseline of the drop and the tangent of drop boundary. The contact angles were measured on both sides of the drop and the average recorded.

#### **4.2.13. Water absorption property**

Water absorption property of the modified films at pH 7 was determined according to ASTM D570 method. The films were cut in  $25 \times 25 \text{ mm}$  and preconditioned at environmental chamber

for two days, and dried in oven 50 °C for 24 hours before the analysis. Dry samples were weighted ( $w_1$ ) by AG104 Mettler Toledo with 0.1 mg precision. Then they immersed in sodium phosphate dibasic solution 0.1 M and adjust the pH with NaOH 50% w/w to 7 at 25 °C for 2 hours. The wet samples then wiped with filter paper to remove extra water and weighted ( $w_2$ ). Swelling (%) calculated by the following equation:

$$\text{Swelling (\%)} = 100 \times (w_2 - w_1) / w_1 \quad \text{Equation.1}$$

#### **4.2.14. Thermogravimetric analysis (TGA)**

Thermal decomposition of modified SPI-based films with lignin was measured using a TA Q500 thermogravimetric analyzer (TGA, TA instrument Inc., New Castle, DE) at a heating rate of 10 °C/min from 25 to 800 °C under a nitrogen gas. Film samples were preconditioned in desiccator containing P<sub>2</sub>O<sub>5</sub> for a week to achieve the lowest moisture content. A palladium pan was used for the experiment.

#### **4.2.15. Water vapor permeability (WVP)**

Water vapor permeability coefficients (WVP, g · cm / cm<sup>2</sup> s · pa) of the films were measured with i-Hydro 7500 Water Vapor Transmission Rate Testing System (Labthink Instruments Co., Ltd., China) according to an internal standard ISO 15106-3 with the electrolytic detection sensor method (ASTM 1997). The thicknesses of the three parts of each film were measured by the micrometer then averaged thickness of each film. The WVP coefficient of each film was calculated using as an equation: WVP coefficient (g · cm / cm<sup>2</sup> s · pa) = WVTR × x / Δp, where WVTR is water vapor transmission rate (g / m<sup>2</sup> · 24 hr) through the film and x indicates the mean thickness of each films (μm). Δp (Pa) is the difference in water vapor partial pressure between

the two sides (a dry chamber and controlled humidity chamber) of the film. Each film ( $7\text{ cm} \times 7\text{ cm}$ ) was attached between two sides of the punched aluminum plates. The diameter of the punched area on the plates was 5.08 cm. The actual test area of WVP was  $3.5\text{ cm} \times 3.5\text{ cm}$  based on the exposed punched area of the plates. The aluminum plate containing the film was inserted in the cell chamber and closed it tight. The flow rate was adjusted to 100 ml/min, and the test relative humanity and temperature was controlled at  $50 \pm 2\%$  and at  $23.5^\circ\text{C}$  receptively. The standard testing mode was programmed with four cycles testing mode for each cell chamber. Each test time interval per a cycle was 30 minutes. Before four cycles of each test was started, the zero purging for 60 minutes was conducted.

#### **4.2.16. Factorial design and statistical analysis**

Enzymatically modified soy protein isolate was activated with lignin in different concentrations. Two factors: lignin type (AL and LSS), and lignin concentration (0%, 2%, 4%, 6% and 10%) were determined. Therefore, there were 10 treatments. Each type of film was prepared 3 times and each film replicated 5 times for mechanical test and 3 times for surface hydrophobicity and swelling properties. Therefore, totally 15 replicates for mechanical test and 9 replicates for the others were done.

All data were analyzed by one-way analysis of variance (ANOVA) to determine the difference between samples. Minitab Software model 14.12.0 (Minitab Inc., State Collage, PA., USA) was applied. Tukey test was used to carry out the difference of means between pairs with 95% confidence interval.

## **4.3. Results and discussion**

### **4.3.1. Antioxidant activity of lignin material**

Antioxidant activity of lignin was measured by the ability of material to absorb or scavenge free radicals in the food system. Free radicals accelerate lipid oxidation, and lipid oxidation decreases shelf life of food products. As shown in Table 4.1, the radical scavenging activity of alkali lignin at pH 7, was approximately 55%. It was higher than lignosulphonate and some other natural antioxidants extracted from 1 mg/ml of different berries (Nakajima et al., 2004). However, the antioxidant activity of alkali lignin was lower than that of the synthetic antioxidant, BHT. The antioxidant activity of lignin is affected by some factors, namely: structural features, presence of functional groups such as phenolic hydroxyl groups, and molecular weight (Pan et al., 2006; Ugartondo et al., 2008). Therefore, the higher antioxidant ability of alkali lignin resulted from the structural and chemical composition differences, or possibly the lower molecular weight (Laurichesse and Averous, 2014). This conclusion is based on the literature review and higher hydrogen donation possibility in the structure of AL (-SH group), compared to lignosulphonate (Fig. 4.1).

**Table 4.1.** Radical scavenging activity of two types of lignin and BHT material

	AL material (%)	LSS material (%)	BHT material (%)
pH 7	54.9 ± 2.31	12.78 ± 1.23	98.9 ± 0.52
pH 11	71.45 ± 2.15	21 ± 0.62	ND

Results are mean ± SD in triplicate.

Abbreviations: AL: alkali lignin, LSS: lignosulphonate, BHT: butylated hydroxytoluene, ND: not detected

#### **4.3.2. DPPH free radical scavenging test of films**

Radical scavenging activity of SPI films with and without lignin and control containing BHT is shown in Table 4.2. SPI films modified with the enzyme didn't show antioxidant activity (2.25%). However, after addition of lignin to SPI films, radical scavenging activity of films in the DPPH methanol solution increased dramatically. Films incorporating LSS showed a slightly higher radical scavenging ability compared with that of AL-SPI film. This increase could be attributed to high solubility of LSS in methanol solution. This is an opposite result compared to the radical scavenging activity of each material. SPI films with AL and LSS in this study showed higher radical scavenging activity than the composite film made of starch-lignin (90:10) prepared by Acosta et al. (2015), who applied wheat straw alcohol-soluble lignin.

**Table 4.2.** Radical scavenging activity of two types of lignin and BHT film

	SPI film (control) (%)	AL film (%)	LSS film (%)	BHT film (%)
Radical scavenging activity	2.15 ± 0.08	24.46 ± 0.12	30.93 ± 0.09	89.74 ± 0.65

Results are mean ± SD in triplicate.

Abbreviations: AL: alkali lignin, LSS: lignosulphonate, BHT: butylated hydroxytoluene, SPI: soy protein isolate

#### **4.3.3. Color, opacity and UV-blocking of films**

The color of packaging film is important in terms of the type of application, general appearance, and consumer acceptance. The enzymatically modified soy protein isolate film was transparent, unmarked, and had a homogenous texture. After the addition of lignosulphonate, films were still transparent and the visual appearance was well-matched to the L-value measured by Hunter Lab color meter. However, both the greenness and yellowness of these films increased as a function of concentration. This increase showed the same trend with opacity (Fig. 4.2 and Table 4.3). On

the other hand, films incorporating AL were dark, and the darkness increased with the increase of concentration (Fig. 4.2). It was also well-matched to opacity measurement. Modified films with AL were more red and yellow compared with those containing LSS. The reddish color was due to the chromophoric nature of lignin (Espinoza Acosta et al., 2015). Unsaturated functional groups, such as carbonyl moieties, aromatic rings, and carbon-carbon double bond, in lignin absorbed UV light, and when they conjugated with other unsaturated structures, the absorption peaks may shift to visible regions (Espinoza Acosta et al., 2015). Similar results have been reported by Espinoza Acosta et al. (2015) who prepared a composite film from starch and lignin. Consequently, the observation in Fig. 4.2 was well-matched to opacity and color meter. It indicated that lignin is homogeneously distributed in the SPI matrix.



**Fig.4.2.** Soy protein isolate based film modified with different concentrations of lignosulphonate (left) and alkali lignin (right)

**Table 4.3.** Effect of different concentrations of lignosulphonate (LSS) and alkali lignin (AL) on color parameters (L-value, a-value, and b-value) and opacity of enzymatic modified SPI film.

Film	Opacity	L-value	a-value	b-value
SPI	0.90 ± 0.06 <sup>Aa</sup>	89.18 ± 0.10 <sup>Aa</sup>	0.84 ± 0.21 <sup>Aa</sup>	2.95 ± 0.84 <sup>A</sup>
LSS 2%	0.88 ± 0.04 <sup>B</sup>	87.88 ± 0.41 <sup>A</sup>	0.26 ± 0.12 <sup>B</sup>	7.06 ± 0.51 <sup>B</sup>
LSS 4%	0.93 ± 0.07 <sup>A</sup>	86.64 ± 0.19 <sup>A</sup>	-0.47 ± 0.06 <sup>C</sup>	12.19 ± 0.27 <sup>C</sup>
LSS 6%	1.22 ± 0.08 <sup>C</sup>	83.5 ± 0.25 <sup>A</sup>	-0.46 ± 0.07 <sup>C</sup>	20.09 ± 0.34 <sup>D</sup>
LSS 10%	1.05 ± 0.04 <sup>D</sup>	81.33 ± 0.40 <sup>A</sup>	-0.32 ± 0.12 <sup>C</sup>	24.85 ± 0.62 <sup>E</sup>
AL 2%	1.73 ± 0.21 <sup>b</sup>	76.67 ± 0.64 <sup>b</sup>	2.34 ± 0.24 <sup>b</sup>	24.39 ± 1.28 <sup>b</sup>
AL 4%	2.24 ± 0.22 <sup>c</sup>	67.93 ± 1.91 <sup>c</sup>	5.88 ± 1.00 <sup>c</sup>	32.48 ± 1.07 <sup>c</sup>
AL 6%	2.67 ± 0.1 <sup>d</sup>	54.62 ± 1.03 <sup>d</sup>	12.99 ± 0.47 <sup>d</sup>	36.76 ± 0.14 <sup>d</sup>
AL 10%	4.01 ± 0.19 <sup>e</sup>	42.96 ± 1.83 <sup>e</sup>	17.71 ± 0.40 <sup>e</sup>	29.57 ± 2.08 <sup>c</sup>

Results are mean ± SD in triplicate.

Abbreviations: AL: alkali lignin, LSS: lignosulphonate, BHT: butylated hydroxytoluene, SPI: soy protein isolate.

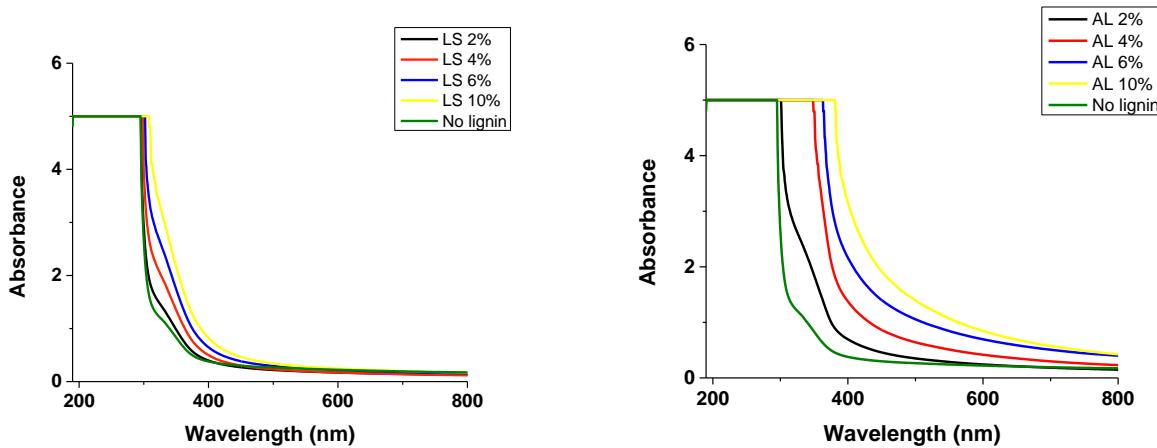
\*Small letters shows significant differences in different AL samples, and capital letters shows significant differences in LS samples.

#### 4.3.4. Film UV-blocking properties

Antioxidant activity of lignin in polymeric matrix results from both radical scavenging activity and UV-blocking ability due to its ring structure, proton donation ability and opacity in the film.

Films containing AL are reddish, which is due to the chromophoric nature of lignin, and this characteristic is known to block UV radiation (Ban et al., 2007; Pereira et al., 2007). As shown in Fig. 4.3, modified films with AL had a broader range of absorption within UV region (under 400nm) compared to others. The UV absorption region increased as a function of concentration in the films modified with both lignins. UV spectroscopic scanning of modified films were well-matched to other results such as visual observation (Figure 4.2), colorimeter, and opacity (Table 4.3). Modified films with AL 10% had UV absorption region similar to modified fish gelatin films prepared by Núñez-Flores et al. (2013) with 15% lignin, although their lignin type was

different with this current experiment. It could be a beneficial property in food packaging if the product is sensitive to UV induced oxidation process.



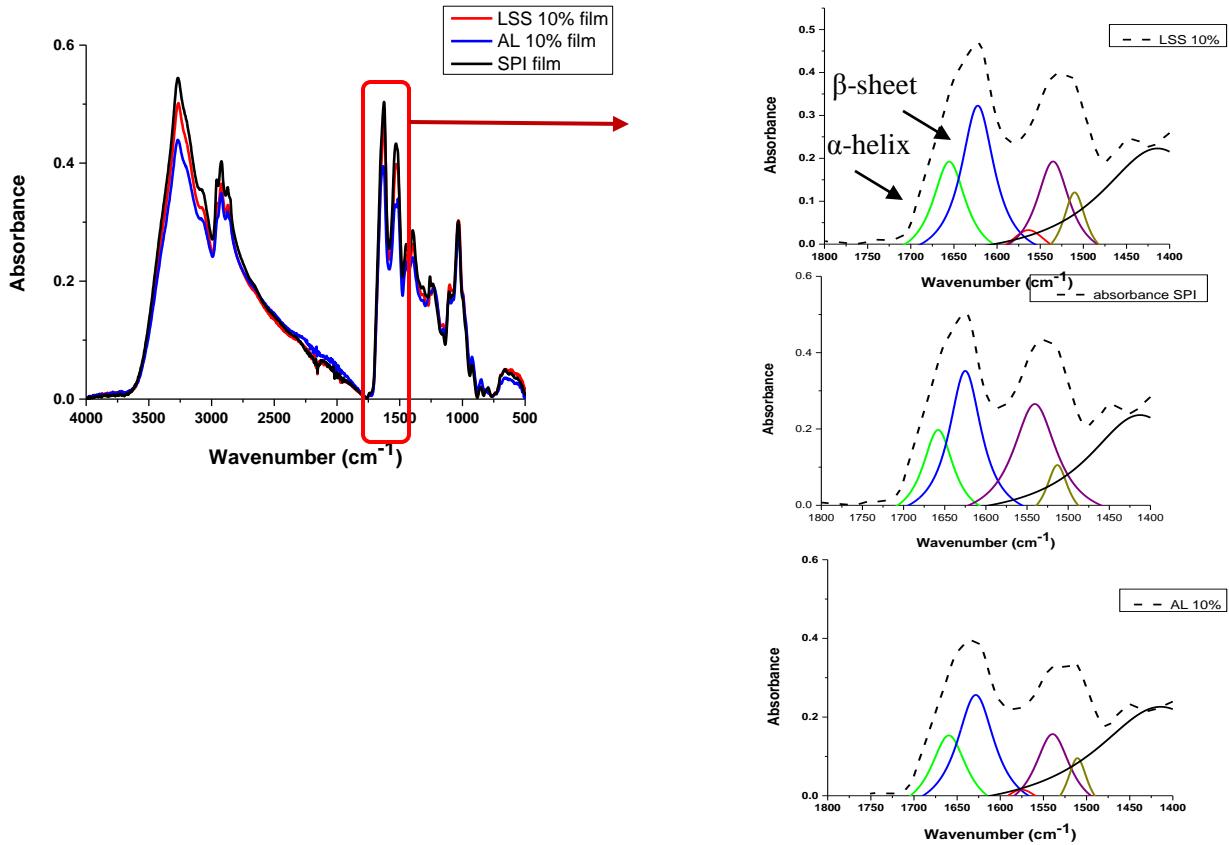
**Fig.4.3.** Effect of lignosulphonate (LSS) and alkali lignin (AL) on light absorbance at wavelength 200 to 800 nm.

#### 4.3.5. FTIR

To assess the effect of lignin on the secondary structure of enzymatically SPI film, FTIR analysis can be used (Athamneh et al., 2008). As shown in Fig.4.4, FTIR spectra in control film and films contain 10% AL and 10% LSS were not significantly different. However, it was expected that the addition of lignin changes secondary structure ( $\alpha$ -helix and  $\beta$ -sheets) of enzymatically modified SPI matrix (Oliviero et al., 2011). Therefore, to evaluate possible changes in the secondary structure of SPI matrix, amide I and amide II region ( $1800\text{-}1400\text{ cm}^{-1}$ ), in FTIR spectra were deconvoluted. Amide I created from C=O stretching vibration, and a sensitive indicator of changes in the secondary structure of protein such as  $\alpha$ -helix,  $\beta$ -sheets and  $\beta$ -turn, where their stretching vibrations are at  $1651$ ,  $1623$  and  $1676\text{ cm}^{-1}$ , respectively (Oliviero et al., 2010; Yao et al., 2009). The deconvolutions of the analyzed spectral region of the control film

and the modified films with 10% AL and 10% LSS are showed in Fig. 4.4. The addition of AL showed shifts of  $\alpha$ -helix and  $\beta$ -sheet peaks to the higher wavenumber or left side, while LSS based film showed shifts in the other side. In general, shifting amide I to lower values of wavenumber indicates more ordering in secondary structure (Athamneh et al., 2008). This is good evidence that shows AL caused more disordering on the secondary structure of protein matrix compared to LSS, which was in agreement with Oliviero et al. (2011) finding in zein protein matrix. Low concentration of lignin (1% and 3%) to zein protein shifted amide I spectra to lower frequencies due to the formation of hydrogen bonding between amino acids of zein and functional groups of lignin (Oliviero et al., 2011).

Amide II band created from C-N and N-H stretching deformation vibration (Pelton & McLean, 2000). AL based film showed shifts of amide II band to higher frequencies, while LSS films showed shifts to lower frequencies compared to the control film.



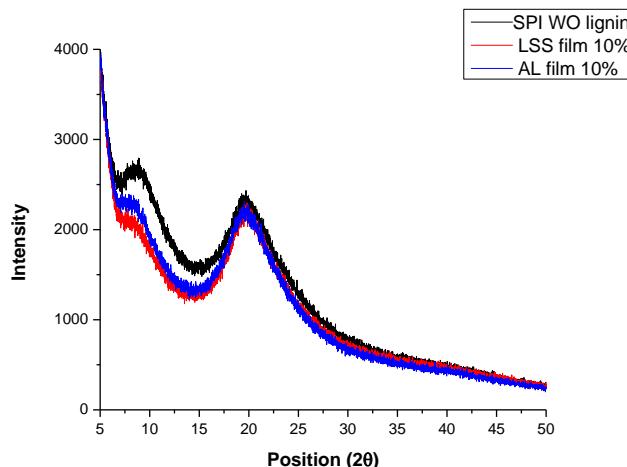
**Fig.4.4.** FTIR spectra of enzymatic modified SPI film with lignosulphonate (LSS) and alkali lignin (AL) (left), and deconvoluted spectra in the amide I and II region (wavenumber 1800-1400 cm<sup>-1</sup>).

Abbreviations: AL: alkali lignin, LSS: lignosulphonate, SPI: soy protein isolate

#### 4.3.6. XRD

The XRD patterns of enzymatically modified SPI film with 10% AL and LSS was reported in Fig. 4.5. XRD spectra of films showed a typical pattern of protein. Two peaks at  $2\theta=9.5^\circ$  and  $20^\circ$  positions are related to two d-spacing around  $4.6^\circ\text{A}$  ( $d_0$ ) and  $8.96^\circ\text{A}$  ( $d_i$ ). Olivero et al. (2012) suggested that these two peaks are attributed to the average backbone distance within  $\alpha$ -helix structure of the protein and d-spacing of  $\alpha$ -inter-helix packing, respectively, and they observed these two peaks in thermoplastic zein foam nanocomposite modified with 10% AL and LSS. Lower intensity of modified films with lignin compared with the control film showed the

hierarchical changes in SPI matrix due to the addition of lignin. This reduction may be attributed to the disruption of  $\alpha$ -inter helix packing (Olivero et al., 2012). XRD spectra was well-matched to FTIR spectra. Besides, the addition of lignin did not change the pattern of XRD spectra significantly. It implies that there is a high compatibility between lignin and enzymatically modified SPI. Huang et al. (2003a) reported that modifying the structure of soy protein with LSS improved the order of the protein structure due to the physical cross linking of lignin with the protein matrix, and it increased the crystallinity of the matrix. This ordered structure relates to high strength and elongation of the film, which was prepared by melt extrusion method.

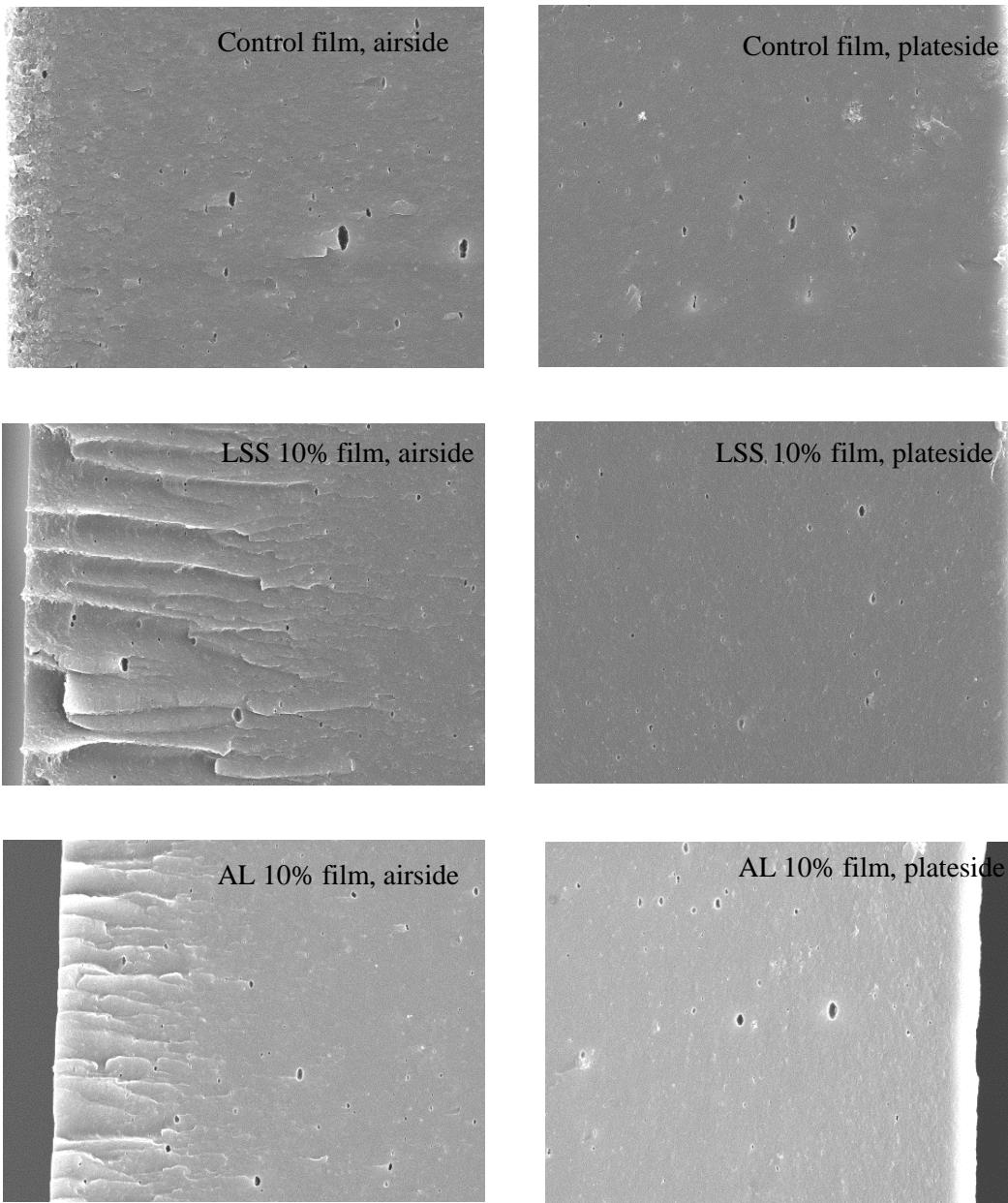


**Fig.4.5.** X-ray pattern of enzymatic modified SPI films with lignosulphonate (LSS) and alkali lignin (AL)  
Abbreviations: AL: alkali lignin, LSS: lignosulphonate, SPI: soy protein isolate

#### 4.3.7. Environmental scanning electron microscopy (ESEM)

SEM images of freeze-fractured cross section of films were observed in Fig. 4.6. Two sides of these films have different morphological structures. It resulted from the high thickness (approximately 0.1 mm) of films. The control film showed smooth surfaces in both sides, airside and plateside. However, the roughness at the airside of films increased after modifying the film

with lignin, and these differences in roughness resulted in an effect on the contact angle of these two sides of the film. This roughness was only observed to the airside of the film, which has been subjected to the water evaporation during the drying process (Núñez-Flores et al., 2013). The same pattern was also observed by Huang et al. (2002) who produced a soy protein modified lignin nano-composite by extrusion method, that the addition of lignin resulted in a fluctuant fracture surface. Zhang et al. (2001) believed that this kind of morphology enhanced toughness of the blends.

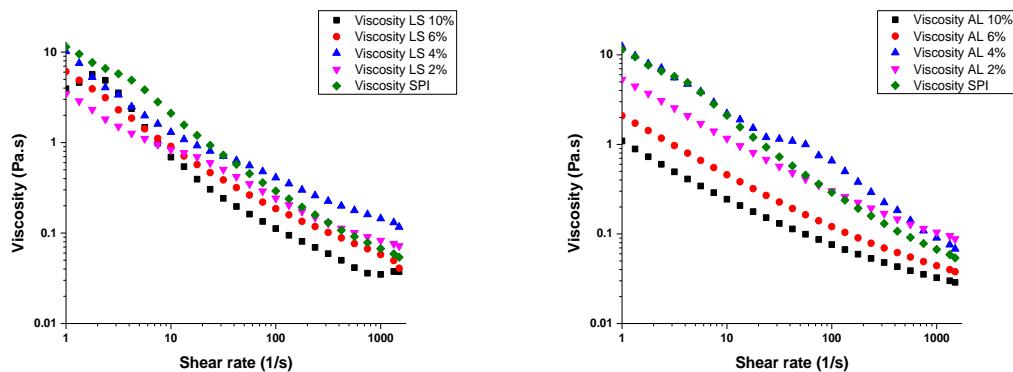


**Fig.4.6.** SEM micrographs (at 10 $\times$  magnification) of airside and plateside surfaces of control and the modified enzyme-treated films with lignosulphonate (LSS) and alkali lignin (AL).

#### 4.3.8. Rheology of film forming solution

Originally, it was expected that the enzyme can catalyze crosslinking of protein and lignin, which increased viscosity of the solution. However, as it was observed in Fig. 4.7, lignin acted

like a lubricant and reduced viscosity of the protein solution. When the concentration of AL increased, viscosity dropped to 10 times compared to the control. High concentrations of AL (6 and 10%) showed significant reduction in viscosity of the solution compared to the protein solution with LSS. At lower shear rates (less than 10 1/s), the protein solution with 4% AL showed similar viscosity to the control protein solution. This observation is well matched to what we observed in FTIR and XRD spectra. The addition of AL produced more interference in protein structure compared to LSS. Núñez-Flores et al. (2013) investigated that the addition of lignin to gelatin solution strongly reduced gelling capacity of gelatin, which was due to a greater degree of interference in the triple helical structure of gelatin.



**Fig. 4.7.** Effect of different concentrations of lignosulphonate (LSS), left, and alkali lignin (AL), right, on viscosity of enzymatic modified SPI film.

#### 4.3.9. Mechanical properties

Tensile strength (TS) and percent elongation (%E) of enzymatically modified film with two types of lignin are shown in Table 4.4. The addition of lignin, typically 6% and 10% AL, increased TS of SPI modified films to around two times higher than the control film, while elongation decreased as a function of concentration. Percent elongation in films containing 6% and 10% AL dropped significantly compared to the control film. It implies that AL had more

disruption in the protein matrix than LSS. Increasing the concentration of LSS to over 2% improved TS of SPI films. The addition of 2% LSS did not change TS and %E of the modified films compared to the control film, which might be attributed to the high compatibility of lignin in the protein matrix. According to the technical data sheet from the producer, glass transition temperature ( $T_g$ ) of AL is higher than LSS ( $T_g$  of AL is 160 °C, and LSS is 140 °C). It may result in higher TS and lower %E in AL based film than LSS based film. Huang et al. (2003a), who modified the structure of soy protein with different ratios of LSS by melt extrusion method, showed similar patterns to our observation. Núñez-Flores et al. (2013) reported that the addition of a low concentration of lignin (0.6% w/v in the solution) dropped TS and improved %E of fish gelatin based films plasticized with 15% glycerol and 15% sorbitol. They reported that lignin produced plasticizing effects on fish gelatin film and it increased %E and reduced TS. The same pattern was also achieved by Acosta et al. (2015), who modified wheat starch structures with lignin, and the addition of lignin significantly reduced TS, but increased %E. Accordingly, it implies lignin acts as a reinforcing agent in enzymatically modified SPI matrix.

**Table 4.4.** Effect of different concentrations of lignosulphonate (LSS) and alkali lignin (AL) on mechanical and permeability properties of enzymatic modified SPI film

Film	TS (MPa)*	%E	WVP ( $10^{-13}$ g.cm/cm <sup>2</sup> .s.Pa)**
Control	4.74 ± 0.34 <sup>Aa</sup>	126.33 ± 17.9 <sup>Aa</sup>	7.16 ± 0.83 <sup>Aa</sup>
LSS 2%	4.11 ± 0.33 <sup>A</sup>	146.17 ± 14.88 <sup>A</sup>	3.28 ± 0.24 <sup>B</sup>
LSS 4%	7.22 ± 0.41 <sup>B</sup>	63.99 ± 10.89 <sup>B</sup>	4.6 ± 0.42 <sup>C</sup>
LSS 6%	7.73 ± 0.13 <sup>B</sup>	86.93 ± 6.95 <sup>B</sup>	4.78 ± 0.14 <sup>C</sup>
LSS 10%	8.01 ± 0.89 <sup>B</sup>	79.95 ± 5.32 <sup>B</sup>	4.13 ± 0.07 <sup>C</sup>
AL 2%	7.07 ± 0.35 <sup>b</sup>	57.5 ± 11.8 <sup>b</sup>	4.33 ± 1.03 <sup>b</sup>
AL 4%	6.49 ± 0.41 <sup>a</sup>	51.61 ± 6.15 <sup>b</sup>	4.43 ± 0.4 <sup>b</sup>
AL 6%	10.13 ± 0.73 <sup>c</sup>	14.7 ± 3.7 <sup>c</sup>	3.52 ± 0.6 <sup>b</sup>
AL 10%	10.98 ± 1.02 <sup>c</sup>	7.45 ± 1.24 <sup>c</sup>	4.23 ± 0.3 <sup>b</sup>

Abbreviations: TS: tensile strength, %E: percent elongation, WVP: water vapor permeability

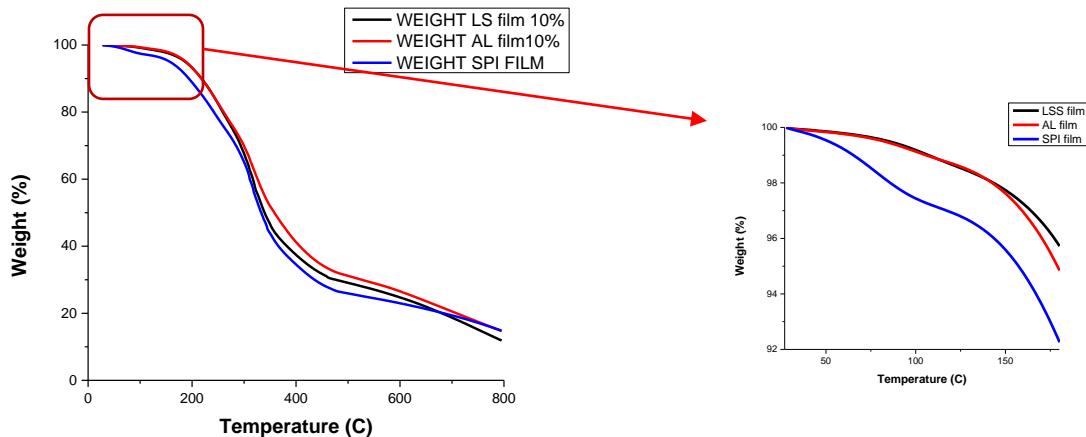
\*Results are mean ± SD after five times replication

\*\* Results are mean ± SD after three times replication

\*\*\*Small letters shows significant differences in different AL samples, and capital letters shows significant differences in LSS samples.

#### 4.3.10. Thermogravimetric analysis (TGA) of films

Thermal analysis techniques such as TGA provides information on the thermal stability of biopolymer films. As shown in Fig. 4.8, modified films with lignin, both AL and LSS, showed higher thermal stability compared to the control film based on onset temperatures at around 150 °C and 50 °C, respectively. The first weight loss was attributed to the evaporation of water from the films. Both AL and LSS showed similar trends in the TGA graph. The second weight loss occurred around 300 °C in both films containing lignin and around 275 °C in the control film, which is responsible for decomposition. Opposite results were observed by Acosta et al. (2015), who modified the structure of durum wheat starch with lignin that the weight loss of films modified with lignin was about 275 °C. On the other hand, the weight loss of their control film was around 300 °C. They believed that this reduction might be due to the discontinuity of the starch matrix after the addition of lignin. Therefore, based on the TGA graph, it is concluded that the addition of lignin not only improved thermal stability, but also increased the homogenous dispersion of lignin in the film matrix.



**Fig. 4.8.** Effect of lignosulphonate (LSS) and alkali lignin (AL) on thermal stability of enzymatic modified SPI film

#### **4.3.11. Contact angle of the film**

Contact angle is a helpful measurement to determine wettability and surface hydrophobicity of biopolymer films. The contact angle of both sides, airside and plateside, of films modified with two types of lignin is shown in Table 4.5. The addition of lignin did not have a significant effect on the airside contact angle of enzymatically modified films with lignin compared to the control film ( $p>0.05$ ). On the other hand, plateside contact angle reduced after the addition of lignin and this reduction was higher in the films modified with LSS compared to those with AL, which was attributed to the surface roughness of the airside of films. SEM images, Fig. 4.6, confirmed that the surface roughness on the airside was higher than the plateside. Different concentrations of lignin did not have a significant effect on the airside contact angle of films ( $p>0.05$ ). Thus, it was concluded that lignin reduced surface hydrophobicity of the enzymatically modified films. The contact angle results achieved in this experiment were in agreement with polypropylene (PP) film modified with lignin proposed by Kosikova et al. (1995), where the addition of lignin reduced surface hydrophobicity of PP film, and increasing the concentration of lignin, reduced contact angle of modified PP film. Additionally, increasing the concentration of lignin to more than 5 *wt%*, reduced surface contact angle of biopolymer films based on agar, due to the higher hydrophilic nature of lignin compared to agar (Shankar et al., 2015). Therefore, the addition of lignin resulted in the increase of surface hydrophilicity of films.

**Table 4.5.** Effect of lignosulphonate (LSS) and alkali lignin (AL) on initial contact angle and water absorption of enzymatic modified SPI film

Film*	Airside contact angle	Plateside contact angle	Water absorption (%)
Control	86.14 ± 5.60 <sup>Aa</sup>	80.21 ± 1.2 <sup>Aa</sup>	128.73 ± 5.25 <sup>Aa</sup>
LSS 2%	86.65 ± 2.87 <sup>A</sup>	62.77 ± 0.75 <sup>B</sup>	145.58 ± 8.05 <sup>B</sup>
LSS 4%	81.87 ± 3.01 <sup>A</sup>	62.56 ± 1.35 <sup>B</sup>	120.94 ± 27.3 <sup>A</sup>
LSS 6%	82.63 ± 2.65 <sup>A</sup>	66.46 ± 4.87 <sup>B</sup>	132.45 ± 1.76 <sup>A</sup>
LSS 10%	83.15 ± 2.12 <sup>A</sup>	66.90 ± 3.21 <sup>B</sup>	119.05 ± 9.79 <sup>A</sup>
AL 2%	77.98 ± 4.25 <sup>a</sup>	68.79 ± 2.27 <sup>b</sup>	220.77 ± 65.51 <sup>b</sup>
AL 4%	79.64 ± 0.52 <sup>a</sup>	67.26 ± 6.23 <sup>b</sup>	204.73 ± 4.68 <sup>b</sup>
AL 6%	87.07 ± 3.02 <sup>a</sup>	74.27 ± 4.62 <sup>a</sup>	235.88 ± 9.08 <sup>b</sup>
AL 10%	68.23 ± 0.67 <sup>b</sup>	81.63 ± 3.21 <sup>a</sup>	235.17 ± 4.46 <sup>b</sup>

\*Results are mean ± SD after three times replication

\*\*Small letters shows significant differences in different AL samples, and capital letters shows significant differences in LSS samples.

### 4.3.12. Water absorption properties

Water sensitivity is one of the main limitations of bioplastics. The effect of different types of lignin on the water absorption property of enzymatically modified SPI film is shown in Table 4.5. Films modified with AL had approximately 100% higher water absorption compared to the films modified with LSS. Water absorption results are well matched to what we observed previously in FTIR, XRD and viscosity graph (Fig. 4.5, 4.6 and 4.7), that AL increased more interference in SPI matrix compared to LSS. The addition of LSS at different concentrations did not have a significant effect ( $p>0.05$ ) on the water absorption of modified films after 2 hours swelling in buffer solution at pH 7. Opposite results were observed by Oliviero et al. (2011), who modified the structure of zein protein with AL and LSS by melt extrusion method, and they investigated that modifying zein protein structure with LSS did not have a significant effect on the water uptake capacity of films, however, AL reduced water absorption of the modified film; which was due to the decrease in the concentration of hydrophilic amino acids regarding the interaction of OH and SH groups of AL with amino acids. Huang et al. (2003b) investigated that

the addition of LSS to SPI matrix dramatically reduced water absorption to around 80% in films containing 40 parts LSS, although the films were prepared by compression molding. They believed that sulfonic acid groups of LSS formed cross linked network in SPI matrix. Thus, in the following experiment LSS showed a good miscibility in enzymatically modified SPI film whereas, it did not have a significant effect on water absorption, contact angle, and mechanical properties (specially at concentration of 2% and 4%), which was well-matched to what we observed in FTIR spectra.

#### **4.3.13. Water vapor permeability**

The effect of type and concentration of lignin on water vapor permeability of modified SPI films are shown in Table 4.4. The addition of lignin to enzymatically modified SPI film improved water vapor barrier property up to approximately 50% compared to the control film. But, there was not a significant difference between WVP of modified films with AL and LSS ( $p>0.05$ ). The composite film of agar/lignin revealed reducing WVP with increasing lignin concentration to 10 wt% (Shankar et al., 2015). Consequently, it was concluded that lignin act as a filler that can provide high barrier property to the film. Opposite results were observed by Núñez-Flores et al. (2013), who modified the structure of fish gelatin based film with two types of lignin, that the mixture of gelatin to lignin at the ratio of 85:15, increased water vapor permeability to nearby 50%, which was due to the either water-induced plasticization effect of lignin or poor compatibility of lignin with gelatin. Moreover, fish gelatin based films modified with LSS had lower WVP than those bovine gelatin films modified with the same lignin, which might be due to the greater aggregation in the protein matrix (Núñez-Flores et al., 2012).

#### **4.4. Conclusion**

Alkali lignin (AL) and lignosulphonate (LSS) materials themselves showed strong radical scavenging activity. They carry strong potentials in use for active packaging structure. Films containing AL showed strong UV-blocking ability. FTIR and XRD spectra revealed that lignin modified secondary structure of SPI matrix. The addition of lignin increased TS and thermal stability and reduced %E of modified films. Radical scavenging activity and UV-blocking ability beside improvement in physicochemical properties of enzymatic modified SPI film with lignin motivated us to apply this bioplastic in further food application.

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## **CHAPTER 5**

### **Effect of Antioxidant Active Films Containing Lignin on Shelf Life of Soy Oil and Fish Oil**

#### **Abstract**

Antioxidant activity of enzymatic modified soy protein film with lignin was investigated using two stimulated food systems, direct contact and indirect contact on soy oil and fish oil. Autoxidation of oil samples, direct contact system, was determined by peroxide value, color, headspace oxygen and volatile compound, while photoxidation, indirect contact system, was determined by peroxide value and color of samples compared to the control, oil sample without active packaging. In the direct contact system in soy oil, peroxide value reduced to approximately 75%, and lignin did not have an effect on the color of the oil. Films tested in this study did not have a significant effect on headspace oxygen of oil samples, however, it reduced pentanal concentration in soy oil samples. Based on our observation, SPI films with lignin showed significant impact in soy oil than fish oil due to the intrinsic characteristics of two different oils. It implies that our testing methods might be highly effective for certain type of products. In conclusion, SPI films with lignin is an alternative active packaging materials for highly sensitive to oxidation by radical and UV light.

## **5.1. Introduction**

Lipid oxidation is initiated by the formation of free radicals, which is produced by a high activation energy. This energy may be supplied by heat, UV-light, singlet oxygen or other sources. The following mechanisms show three stages involving in lipid oxidation: initiation, propagation, and termination (Kamal-Eldin and Pokorny, 2005).

During lipid oxidation, oxygen reacts with lipid in a series of free radical chain reactions; this leads to change chemical composition, and quality deterioration. Lipid oxidation caused different health problems such as coronary heart disease, atherosclerosis, cancer, and aging process (Jadhav et al., 1996). Antioxidants are direct or indirect food ingredients in packaging and food that can postpone or inhibit oxidation by involving in one or more stages in lipid oxidation (Fukumoto and Mazza, 2000). It enhances quality and safety of food products to increase shelf life. Common antioxidants applying commercially in food products are selected spices namely rosemary, sage, thyme, oregano, ginger, and turmeric (Loliger, et al., 1996). The release of natural antioxidants from package to the food is highly desirable due to the reduction of lipid oxidation and growth of food nutritional value (Barbosa-Pereira et al., 2013).

This study is focusing on exploration of effect of alkali lignin and lignosulphonate on highly oil based products: soy oil and fish oil, using two simulated systems: indirect contact and direct contact, which are responsible for effects on photooxidation and autoxidation, respectively.

Soy oil, derived from soy bean, consists of almost 58% polyunsaturated fatty acids, namely: 7-10% alpha-linolenic acid (C-18:3), 51% linoleic acid (C-18:2), and 23% monounsaturated oleic

acid (C-18:1) (Ivanov et al., 2010). Peroxide value of fresh soy bean oil is very low, around 0.75 miliequivalent peroxide/1000 g sample. Therefore, PO value will be a good indicator for antioxidation activity triggered by SPI films with lignins.

Fish oil is a rich source of omega-3 fatty acids, namely: eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), that are known to prevent heart attacks or strokes. Chemical structure of EPA is C-20:5, and DHA is C-22:6. Therefore, they are sensitive to oxidation due to the vast number of double bonds within the fatty acid chain (Albert et al., 2015).

To determine oxidation rate in oil products, peroxide value (PV), oxygen headspace and volatile compounds were measured. Peroxide value reflects the primary oxidation products, while volatile compounds reflects secondary oxidation products (Albert et al., 2015).

Applying active packaging to prevent oxidation in fats and oils have been done, previously. Jongjareonrak et al. (2008) covered lard with modified fish gelatin film with BHT or  $\alpha$ -tocopherol, and found the prevention effect of this film on oxidation of lard. The addition of lignin not only improves antioxidant properties of food products, but also in some cases has antimicrobial properties (Arancibia et al., 2014). Modified fish gelatin film with lignin prevented lipid oxidation and formation of carbonyl groups in salmon muscle stored at 5°C (Ojagh et al., 2011). Blue shark muscles were packaged in low density polyethylene (LDPE) containing barley husks to reduce oxidation rate during storage (de Abreu et al., 2011). However, there was not enough information regarding application of enzymatic modified SPI film with lignin on oil products.

Thus, the goals of the current experiment were to investigate the effect of two types of lignin, alkali lignin (AL) and lignosulphonate (LSS), on radical scavenging activity and UV-blocking ability of soy oil and fish oil based on peroxide value, color, headspace oxygen and volatile compound of the oil samples, and UV-oxidation was determined by peroxide value and color of samples.

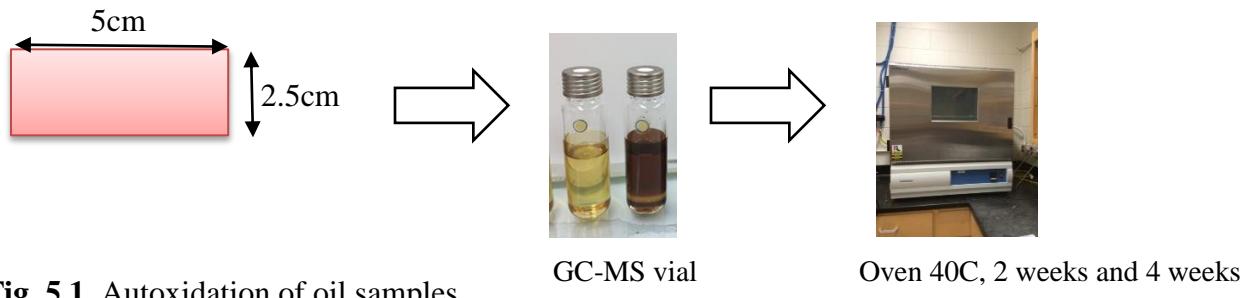
## **5.2. Materials and method**

### **5.2.1. Materials**

Soy oil was purchased from local grocery store. Fish oil bought from OmegaPure, Virginia, USA. Potassium iodide, sodium thiosulfate 0.1 N, acetic acid glacial, and chloroform were purchased from Fisher Scientific.

### **5.2.2. Sample preparation for autoxidation**

Enzymatically modified soy protein films with two types of lignin, AL and LSS, were prepared according to the methodology explained in chapter 4. Then, samples were cut in  $2.5 \times 5 \text{ cm}^2$ , and placed in 20 ml GC-MS vial. An OxyDot (purchased from OxySense 5250I, Dallas, TX) was placed inside each vial to measure oxygen headspace of samples. Ten gram of soy oil or fish oil added to the vial, and placed in the oven 40 °C for 2 weeks or 4 weeks to accelerate oxidation (Fig. 5.1). Oil characterization were carried during storage for 2 or 4 weeks. The quality of oil samples was measured by peroxide value, oxygen headspace, volatile compound and color of oil samples.



**Fig. 5.1.** Autoxidation of oil samples

### 5.2.3. Sample preparation for UV oxidation

Enzymatically modified soy protein films with two types of lignin, AL and LSS, were prepared according to the methodology explained in chapter 4. Film samples were cut in  $12.5 \times 8 \text{ cm}^2$  and covered a box containing 10 vials filled with soy oil or fish oil (Fig. 5.2). Then, samples were placed under fluorescence light for 2 weeks and 4 weeks. Fluorescent light bulbs were produced by Sylvania (Designer cool white 30W, F30T12/DCW/RS, Ontario, Canada). The light intensities and light spectrum were measured by Upertek MK350S (1B) (Miaoli County, Taiwan). The average UV-intensity was 4711 LUX. Peroxide value and color of oil samples were carried out after that period of time.



**Fig. 5.2.** UV-oxidation of oil samples

#### **5.2.4. Sample characterization**

The peroxide value (PV) of soy oil or fish oil was measured according to American Oil Chemical Society (AOCS). Approximately 5 g of oil was weighted in an Erlenmeyer 250 ml, then 30 ml acetic acid glacial-chloroform (3:2) was added to the oil sample. The content was stirred to dissolve oil in the solution completely. 0.5 ml saturated potassium iodide was added to the oil solution for 1 min. Immediately after that 30 ml distilled water was added to the solution. Two ml of starch indicator solution 1% was added to the Erlenmeyer, and the solution was titrated with 0.02 N sodium thiosulfate to achieve a white cloudy solution, which was the end point of titration. The PV was calculated as follows:

$$PV \text{ (meqO}_2 \text{ kg oil}^{-1}) = (S-B) \times N \times 1000 / \text{sample weight}$$

Where S is the volume consumed during titration (ml), B is the volume of the blank (ml), N is normality of sodium thiosulfate, and sample weight is on gram.

The effect of autoxidation and UV-oxidation on the color of soy oil or fish oil was determined by a Konica Minolta (CR-200; Minolta Co. Ltd, Osaka, Japan). The color parameters were brightness (L-value), redness (a-value), and yellowness (b-value), and each point was the mean of three replications.

The headspace volatile compound was analyzed by GC-MS (Shimadzu, QP2010, Kyoto, Japan) with an integrated headspace autosampler (AOC-5000 Plus, Shimadzu, Tokyo, Japan). Split injection was conducted with a ratio of 20. The type of fiber was a 65um Gray SPME (PDMS/DVB/Carboxen). Samples were pre incubated at 45 °C and volatile binding took place at 45 °C for 30 minutes. Carrier gas was helium at 0.5 mL/min purge flow. The capillary column

of GC system was DB-5 (30m × 0.25mm× 0.25um). The oven temperature program 40 °C, and injection temperature 240 °C for a sampling time of 1 min.

Oxygen headspace was measured by OxySense 5250I, Dallas, TX. The system was calibrated before the analysis, and headspace oxygen was measured during 4 weeks' incubation of oil samples in an oven 40 °C. Three replications were done for each sample.

### **5.2.5. Statistical analysis**

All data were analyzed by one-way analysis of variance (ANOVA) to determine the difference between samples. Minitab Software model 14.12.0 (Minitab Inc., State Collage, Penn., USA) was applied. Tukey test was used to carry out the difference of means between pairs with 95% confidence interval.

## **5.3. Results and discussion**

### **5.3.1. Autoxidation of soy oil samples**

#### **Peroxide value of soy oil**

Peroxide value (PV) was applied to monitor the rate of oxidation in soy oil samples. Peroxide value of soy oil samples during autoxidation in the oven 40 °C for 2 weeks and 4 weeks is shown in Table 5.1. Soy oil packed with both AL and LSS based SPI film reduced oxidation rate up to approximately 75% comparing to the control. It resulted from the radical scavenging activity of lignin in the packaging system.. The rate of oxidation in oil samples was low within the first 2 weeks, then increased rapidly in the next 2 weeks (Table 5.1), while control showed the opposite results wherein oxidation rate in the first 2 weeks was very sharp, and then low rate of oxidation

happened in the other next 2 weeks. This might be due to the rate of decomposition of peroxide to secondary products (Boselli et al., 2005).

**Table 5.1.** Effect of autoxidation on peroxide value (PV) of soy oil packaged in enzymatic modified SPI film with lignin

Soy oil condition	Day zero	PV after 2 weeks*	PV after 4 weeks
Soy oil without film	0.79 ± 0.01	2.59 ± 0.25 <sup>a</sup>	2.98 ± 0.3 <sup>a</sup>
Soy oil with LSS film	0.79 ± 0.01	1.19 ± 0.0 <sup>b</sup>	1.78 ± 0.27 <sup>b</sup>
Soy oil with AL film	0.79 ± 0.01	1.0 ± 0.27 <sup>b</sup>	1.6 ± 0.0 <sup>b</sup>

Abbreviations: LSS: lignosulphonate, AL: alkali lignin

\*Miliequivalent peroxide/1000 g sample

Results are mean ± SD in triplicate

### Color of soy oil

Oxidation resulted in significant changes on the brightness, redness and yellowness of soy oil samples (Table 5.2) as compared to the fresh soy oil. The brightness (L-value) of soy oil increased after 2 weeks' incubation in the oven, then reduced in the next 2 weeks' storage in the oven. This might be attributed to color alterations due to oxidation of tocopherol in soy bean oil, which was in colorless form (Komoda et al., 1966). According to negative value of a-value, soy oil samples are green, and autoxidation increased greenness of soy oil samples. The blueness of oil sample increased during autoxidation. it was reported that oxidation may lead to discoloration of oil due to oxidative deterioration and increased conjugation (Anwar et al., 2007; Sherwin, 1978). Colored compound such as chlorophylls may affect with the analytical reactions, especially at the beginning of oxidation, and change the color of oxidized products. The reaction of the reagent with a precursor of a carbonyl product increased color of oxidized product

(Kamal-Eldin and Pokorny, 2005). In conclusion, SPI films with lignin did not show a significant effect ( $p>0.05$ ) on the color of soy oil samples.

**Table 5.2.** Effect of autoxidation on color of soy oil packaged in enzymatic modified SPI film with lignin

Sample	2 weeks incubation			4 weeks incubation		
	L-value	a-value	b-value	L-value	a-value	b-value
Day zero	35.68±0.26	-1.17±0.1	5.62±0.04	35.7±0.1	-1.37±0.14	4.52±0.1
No film	38.29±0.16	-2.14±0.08	8.39±0.10	36.64±0.12	-2.58±0.24	8.32±0.05
LSS	37.83±0.64	-2.48±0.12	8.30±0.08	36.79±0.06	-2.48±0.10	8.35±0.03
AL	37.98±0.79	-2.25±0.02	8.72±0.05	36.50±0.18	-2.65±0.08	8.44±0.08

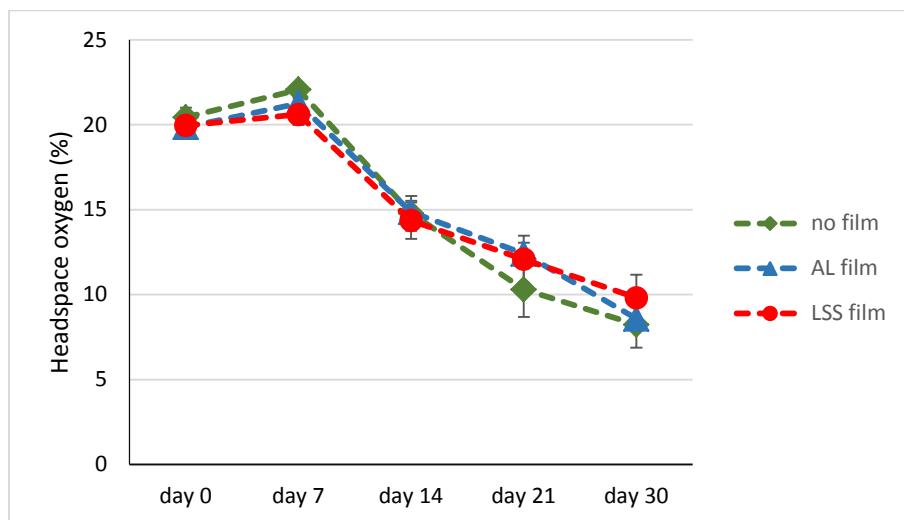
Abbreviations: LSS: lignosulphonate, AL: alkali lignin

Results are mean ± SD in triplicate

### Headspace oxygen

Oxygen is an essential substance for the degradation of lipids. Three stages are involved in lipid oxidation, namely: initiation, propagation and termination. Free radicals attached to oxygen in propagation step, and accelerate oxidation rate due to the formation of peroxy radicals and hydroperoxides to produce volatile compounds (Johnson and Decker, 2015; Jung et al., 1989). Therefore, measuring oxygen headspace is a precise method to determine oxidation in the package. As shown in Fig. 5.3, oxygen headspace increased after a week to approximately 22%, which was due to release of dissolved oxygen to the headspace. Temperature is an important factor influencing on oxygen solubility in food, which by increasing the temperature, more oxygen is moved to headspace from the dissolved state (Chen et al., 2011). The food's composition effects on the level of dissolved oxygen, whereas, oxygen is approximately 5-10

times more soluble in oil than pure water at 20 °C (Aho & Wahlroos 1967, Montgomery et al. 1964). Headspace oxygen decreased significantly to approximately 15% after a week later and around 10% after a month autoxidation in the oven 40 °C. There was not a significant difference ( $p>0.05$ ) in the headspace oxygen of soy oil samples stored in active packaging with the control. Unfortunately, it implies measuring headspace oxygen may not be effective to monitor the antioxidation progress by SPI films with lignin and the oxidation progress is still existing in food.



**Fig. 5.3.** Effect of autoxidation on oxygen headspace of soy oil packaged in enzymatic modified SPI film with alkali lignin (AL) and lignosulphonate (LSS)

### Volatile compound

During oxidation oxygen attached to double bond and volatile carbonyl compounds are formed. The main volatile compounds in oxidized soy oil are pentanal, hexanal, heptanal, octanal and nonanal. The effect of SPI film with lignins on the profile of volatile compound of soy oil oxidized in the oven 40°C in one month is shown in Table 5.3. Day zero soy oil (fresh soy oil)

showed low amount of volatile compound formation. The tested films showed a positive effect on pentanal concentration in soy oil samples while it did not show a significant effect on the concentration of hexanal in the soy oil sample, and they were considerably higher than day zero sample. Heptanal, octanal and nonanal were not detected in day zero soy oil. SPI film with AL showed lower concentration of heptanal in oil samples. Same pattern was also observed in nonanal concentration in samples packaged with AL and LSS film. In conclusion, packaging soy oil with SPI-based film with both AL and LSS reduced the formation of volatile compound, off-flavor during autoxidation.

**Table 5.3.** Effect of autoxidation on volatile compound of soy oil packaged in enzymatic modified SPI film with alkali lignin (AL) and lignosulphonate (LSS)

Sample	Pentanal	Hexanal	Heptanal	Octanal	Nonanal
Day zero	$5.5 \times 10^6 \pm 158054.6^a$	$0.24 \times 10^6 \pm 5470.9^a$	ND	ND	ND
No film	$8.7 \times 10^6 \pm 2139915^b$	$7.1 \times 10^6 \pm 8567.5^b$	$0.1 \times 10^6 \pm 4521.2^a$	ND	$0.2 \times 10^6 \pm 12541^a$
LSS film	$4.1 \times 10^6 \pm 481294.4^c$	$7.9 \times 10^6 \pm 4756.3^c$	$0.1 \times 10^6 \pm 3562.4^b$	$4.2 \times 10^6 \pm 65412.3$	$0.1 \times 10^6 \pm 1425^b$
AL film	$5.8 \times 10^6 \pm 561376.3^a$	$8.6 \times 10^6 \pm 5239.1^d$	$0.09 \times 10^6 \pm 1254.2^c$	ND	$0.1 \times 10^6 \pm 14149^b$

Abbreviations: AL: alkali lignin, LSS: lignosulphonate, ND: not detected  
Small alphabets show significant effect in each column with confidence interval=0.95

### **5.3.2. UV-oxidation of soy oil samples**

#### **Peroxide value of soy oil**

Soy oil samples were covered by AL, LSS, Al-foil and those without cover, and placed under florescence light at room temperature. This system is designed for indirect contact to see the effect of UV triggered oxidation. As shown on Table 5.4, the rate of oxidation in soy oil covered by AL was approximately 75% lower than those covered with LSS and the control samples without any cover during storage under UV for 2 and 4 weeks. Films containing AL showed some dark brown reddish color leading to a reduction of lightness in films, and this chromophoric nature of AL is highly capable of protecting against UV-light. There was not a significant difference in oxidation rate of samples covered by LSS and those without cover. It implies LSS is not appropriate to UV blocking packaging system. The rate of oxidation at the first 2 weeks were significantly higher in all samples comparing to the next 2 weeks, which was attributed to the consumption of headspace oxygen in the first 2 weeks. UV-blocking is a very important factor in food packaging, because UV light breaks conjugated bonds in poly unsaturated fatty acid oils and accelerates free radicals in food system, therefore, UV-absorption is proportional to peroxide value of oil (Kamal-Eldin and Pokorny, 2005). Consequently, UV-blocking ability of films containing AL was found to be very effective in food packaging.

**Table 5.4.** Effect of UV-oxidation on peroxide value (PV) of soy oil packaged in enzymatic modified SPI film with lignin

Soy oil condition	Day zero	PO value after 2 weeks*	PO value after 4 weeks
<b>Covered with Al foil</b>	0.79 ± 0.01	1.25 ± 0.57 <sup>a</sup>	2.36 ± 1.3 <sup>a</sup>
<b>Uncovered samples</b>	0.79 ± 0.01	9.73 ± 0.89 <sup>b</sup>	16.54 ± 2.02 <sup>b</sup>
<b>Covered with LSS</b>	0.79 ± 0.01	9.57 ± 0.58 <sup>b</sup>	15.69 ± 1.57 <sup>b</sup>
<b>Covered with AL</b>	0.79 ± 0.01	3.57 ± 0.08 <sup>c</sup>	6.76 ± 0.53 <sup>c</sup>

Abbreviations: LSS: lignosulphonate, AL: alkali lignin

\*Miliequivalent peroxide/1000 g sample

Results are mean ± SD in triplicate

Small alphabets show significant effect in each column with confidence interval=0.95

### Color of soy oil

The color of oils changed during storage under UV-light. It was due to the oxidation discoloration. As shown in Table 5.5, brightness (L-value), greenness (a-value) and yellowness (b-value) of soy oil samples covered by Al-foil and alkali lignin in 4 weeks were not significantly different ( $p>0.05$ ). Al-foil was applied as a second control for absolute UV-blocking material (Page et al., 1992) besides to the control which is not covered with any film. On the other hand, soy oil samples covered by LSS and uncovered, were not significantly different ( $p>0.05$ ) in terms of brightness, greenness and yellowness of preservation under UV-light in 4 weeks. This is in agreement with the results in previous chapter measured by UV-spectroscopy, where films containing LSS did not have any UV-blocking ability. Pristouri et al. (2010) reported that color of olive oil was unaffected by packaging material during 12-month storage if they store in the dark, therefore, light-blocking is one of the main factors to extend shelf life of oil products.

**Table 5.5.** Effect of UV-oxidation on color of soy oil packaged in enzymatic modified SPI film with lignin

Sample	2 weeks incubation			4 weeks incubation		
	L-value	a-value	b-value	L-value	a-value	b-value
Day zero	35.65±0.26 <sup>a</sup>	-1.21±0.04 <sup>a</sup>	5.70±0.31 <sup>a</sup>	35.7±0.1 <sup>a</sup>	-1.38±0.10 <sup>a</sup>	4.52±0.07 <sup>a</sup>
Al-foil	36.91±0.04 <sup>b</sup>	-1.40±0.05 <sup>b</sup>	4.83±0.07 <sup>b</sup>	35.70±0.02 <sup>a</sup>	-1.71±0.11 <sup>b</sup>	5.67±0.13 <sup>b</sup>
No film	36.25±0.31 <sup>c</sup>	-1.14±0.13 <sup>a</sup>	2.8±0.16 <sup>c</sup>	36.15±0.06 <sup>b</sup>	-1.05±0.02 <sup>c</sup>	2.53±0.11 <sup>c</sup>
LSS	36.34±0.27 <sup>c</sup>	-1.04±0.19 <sup>a</sup>	4.00±0.33 <sup>d</sup>	36.15±0.23 <sup>b</sup>	-0.73±0.07 <sup>d</sup>	2.73±0.06 <sup>d</sup>
AL	36.35±0.18 <sup>c</sup>	-1.49±0.03 <sup>c</sup>	4.30±0.14 <sup>d</sup>	35.44±0.52 <sup>a</sup>	-1.82±0.14 <sup>b</sup>	5.92±0.22 <sup>b</sup>

Abbreviations: LSS: lignosulphonate, AL: alkali lignin

Results are mean ± SD in triplicate

Small alphabets show significant effect in each column with confidence interval=0.95

### 5.3.3. Autoxidation of fish oil samples

#### Peroxide value of fish oil

Autoxidation of fish oil was determined by quantification of either first or secondary products of oxidation reactions, and peroxides are primary reaction products of lipid oxidation (de Abreu et al., 2011). As shown in Table 5.6, fish oil samples packaged with enzymatic modified SPI film treated with AL and LSS had slightly lower oxidation than the control in 2 weeks and 4 weeks storage in the oven. Day zero (fresh) fish oil showed high initial peroxide value (around 12.82 meq/1000g sample).

Rancidity occurred based on this reaction: fresh oil  $\xrightarrow{A}$  peroxide  $\xrightarrow{B}$  rancid oil. If the temperature of storage is high, the rate of reaction B increased, therefore,

peroxide value reduced in samples stored in the oven for 2 weeks. Thus, peroxide numbers become less reliable in measuring of rancidity at high temperature (Stansby, 1941). Yu et al. (2013) investigated fish oil stored in active packaging incorporated with caffeic acid for 10 days

had around 2 times lower peroxide value than the control sample without active packaging, which was due to high radical scavenging activity of caffeic acid compared to lignin.

**Table 5.6.** Effect of autoxidation on peroxide value (PV) of fish oil packaged in enzymatic modified SPI film with lignin

Fish oil condition	Day zero	After 2 weeks*	After 4 weeks
<b>Fish oil without film</b>	12.82 ± 1.3	13.18 ± 0.05 <sup>a</sup>	18.72 ± 0.1 <sup>b</sup>
<b>Fish oil with LS film</b>	12.82 ± 1.3	11.74 ± 0.03 <sup>b</sup>	17.2 ± 0.4 <sup>c</sup>
<b>Fish oil with AL film</b>	12.82 ± 1.3	11.97 ± 0.01 <sup>c</sup>	17.18 ± 0.1 <sup>c</sup>

Abbreviations: LSS: lignosulphonate, AL: alkali lignin

\*Miliequivalent peroxide/1000 g sample

Results are mean ± SD in triplicate

Small alphabets show significant effect in each column with confidence interval=0.9

### Color of fish oil

Autoxidation of fish oil changed brightness after 2 weeks and 4 weeks storage in the oven, as compared to day zero (fresh) fish oil. However, greenness and yellowness were not significantly changed during oxidation ( $p>0.05$ ). This might be due to high peroxide value of day zero fish oil, that changes in color after oxidation was not significant (Table 5.7).

**Table 5.7.** Effect of autoxidation on color of fish oil packaged in enzymatic modified SPI film with lignin

Sample	2 weeks incubation			4 weeks incubation		
	L-value	a-value	b-value	L-value	a-value	b-value
Day zero	33.14±0.18 <sup>a</sup>	-2.25±0.11 <sup>a</sup>	10.44±0.13 <sup>a</sup>	33.67±0.27 <sup>a</sup>	-2.26±0.10 <sup>a</sup>	13.44±0.12 <sup>a</sup>
No film	34.12±0.19 <sup>b</sup>	-2.06±0.17 <sup>a</sup>	10.30±0.19 <sup>a</sup>	34.11±0.20 <sup>ab</sup>	-2.11±0.42 <sup>a</sup>	9.91±0.14 <sup>b</sup>
LSS	34.17±0.19 <sup>b</sup>	-2.19±0.15 <sup>a</sup>	11.33±0.12 <sup>b</sup>	34.69±0.28 <sup>c</sup>	-2.26±0.15 <sup>a</sup>	13.27±0.13 <sup>ac</sup>
AL	34.01 ± 0.25 <sup>b</sup>	-2.13±0.26 <sup>a</sup>	11.31±0.09 <sup>b</sup>	34.1±0.96 <sup>a</sup>	-1.45±0.55 <sup>b</sup>	13.15±0.15 <sup>c</sup>

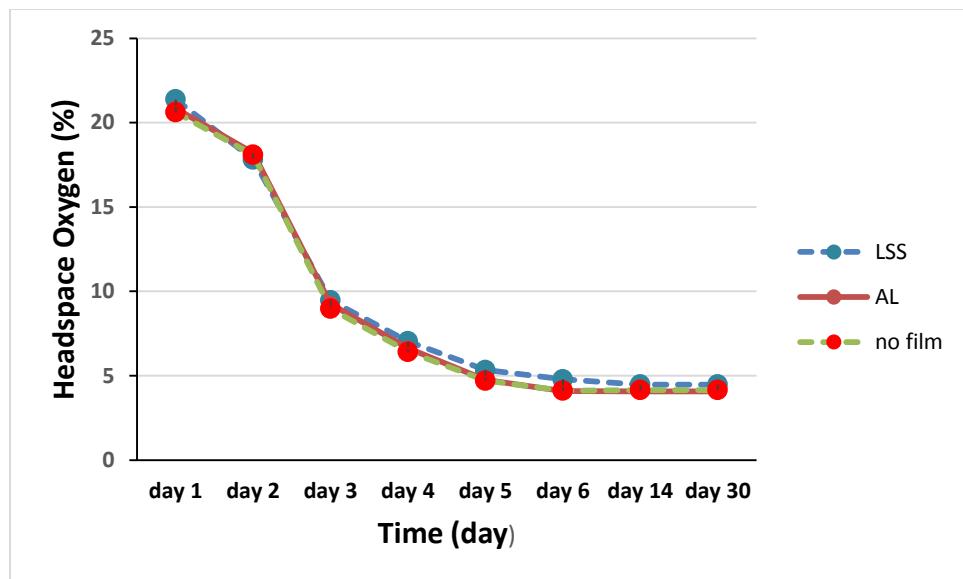
Abbreviations: LSS: lignosulphonate, AL: alkali lignin

Results are mean ± SD in triplicate

Small alphabets show significant effect in each column with confidence interval=0.95

### Headspace oxygen of fish oil samples

Figure 5.4 shows the percentage of headspace oxygen in fish oil packaging with and without lignin. The amount of oxygen after 3 days incubation in the oven dramatically dropped up to 9%, and after 5 days incubation it remained stable to around 5%. There was not a significant difference ( $p>0.05$ ) in headspace oxygen among tested samples. As compared with results in soy bean oil, headspace oxygen in soy oil increased after a week, then dropped gradually to 10% after a month incubation in the oven, which was different with the results achieved in fish oil. This difference was attributed to the chemical structure of lipid, whereas, fish oil had high initial peroxide value and higher degree of unsaturated double bond (Johnson and Decher, 2015), which by increasing the temperature, rancidity increased.



**Fig. 5.4.** Effect of autoxidation on oxygen headspace of fish oil packaged in enzymatic modified SPI film with alkali lignin (AL) and lignosulphonate (LSS)

### Volatile compound of fish oil

The effect of active packaging on volatile compound of fish oil is shown in Table 5.8. Volatile compounds are the carbonyl products of unsaturated fatty acid oxidation. As major fatty acid in fish oil are DHA (C22:6) and EPA (C20:5), hexanal, heptanal, octanal and nonanal are major volatile compounds, off flavors, in fish oil package. SPI films with lignins showed positive effect on formation of hexanal, heptanal, and octanal of fish oil comparing to the control. It is a good evidence of antioxidant activity of SPI films with lignins

**Table 5.8.** Effect of autoxidation on volatile compound of fish oil packaged in enzymatic modified SPI film with alkali lignin (AL) and lignosulphonate (LSS)

Sample	2-pentenal	Hexanal	Heptanal	Octanal	Nonanal
Day zero	$1.1 \times 10^6$ ± 7975.5 <sup>a</sup>	ND	$0.6 \times 10^6$ ± 58421 <sup>a</sup>	ND	$0.05 \times 10^6$ ± 4123 <sup>a</sup>
No film	$8.7 \times 10^6$ ± 73526 <sup>b</sup>	$25.3 \times 10^6$ ± 66935 <sup>a</sup>	$16.8 \times 10^6$ ± 86529 <sup>b</sup>	$15.4 \times 10^6$ ± 12506 <sup>a</sup>	$8.8 \times 10^6$ ± 35957 <sup>b</sup>
LSS film	$13.5 \times 10^6$ ± 120666 <sup>c</sup>	$20.0 \times 10^6$ ± 141715 <sup>b</sup>	$11.2 \times 10^6$ ± 115024 <sup>c</sup>	ND	$7.1 \times 10^6$ ± 65281 <sup>c</sup>
AL film	$10.4 \times 10^6$ ± 191125 <sup>d</sup>	$18.9 \times 10^6$ ± 110675 <sup>c</sup>	$11.2 \times 10^6$ ± 872719 <sup>c</sup>	$12.1 \times 10^6$ ± 35778.9 <sup>b</sup>	$6.6 \times 10^6$ ± 5692.3 <sup>d</sup>

Abbreviations: AL: alkali lignin, LSS: lignosulphonate, ND: not detected

Results are mean ± SD in triplicate

### 5.3.4. UV oxidation of fish oil

#### Peroxide value

Peroxide value of fish oil samples covered by SPI film with AL, LSS, Al-foil, and the control, without any cover, is shown in Table 5.9. Fish oil samples without any cover had the highest peroxide value compared to those covered by AL, LSS and Al-foil. It might be due to increasing rancidity in oil samples by raising temperature, which reduced peroxide number (Stansby, 1941).

**Table 5.9.** Effect of lignin in UV-blocking ability of enzymatic modified SPI film

Fish oil sample	Day zero	After 2 weeks	After 4 weeks
No covered oil	12.82 ± 1.30	19.65 ± 0.51 <sup>a</sup>	21.41 ± 0.42 <sup>a</sup>
LSS-covered oil	12.82 ± 1.30	18.75 ± 0.23 <sup>b</sup>	20.95 ± 0.70 <sup>a</sup>
AL-covered oil	12.82 ± 1.30	15.26 ± 0.52 <sup>c</sup>	17.88 ± 0.11 <sup>b</sup>
Al-foil covered oil	12.82 ± 1.30	15.75 ± 0.16 <sup>c</sup>	16.62 ± 0.15 <sup>c</sup>

Abbreviations: LSS: lignosulphonate, AL: alkali lignin

\*Miliequivalent peroxide/1000 g sample

Results are mean ± SD in triplicate

Small alphabets show significant effect in each column with confidence interval=0.95

### Color of fish oil

Table 5.10 shows UV-blocking effect of different packaging materials on the color of fish oil after a month incubation under UV-light. As compared with day-zero fish oil covered by enzymatic modified SPI films with lignins, there was not a significant difference in their colors in terms of brightness, greenness and yellowness. On the other hand, fish oil samples covered by modified film containing LSS did not show a significant difference comparing to the control ( $p>0.05$ ). It is well matched to the previous report by Stansby, (1941). Fish oil with increasing temperature the rate of rancidity is higher than rate of peroxide formation, therefore, peroxide value is less applicable in our simulated testing system.

**Table 5.10.** Effect of different types of packaging on brightness (L-value), greenness (a-value), and yellowness (b-value) of fish oil incubated under UV-light for a month

Fish oil sample	L-value	a-value	b-value
Day-zero oil	34.91 ± 0.01 <sup>a</sup>	-2.89 ± 0.09 <sup>a</sup>	10.81 ± 0.02 <sup>a</sup>
No covered oil	35.75 ± 0.48 <sup>b</sup>	-1.67 ± 0.06 <sup>b</sup>	7.34 ± 0.08 <sup>b</sup>
LSS-covered oil	35.04 ± 0.13 <sup>ac</sup>	-1.77 ± 0.05 <sup>c</sup>	7.01 ± 0.13 <sup>c</sup>
AL-covered oil	35.52 ± 0.3 <sup>d</sup>	-2.26 ± 0.08 <sup>d</sup>	9 ± 0.07 <sup>d</sup>
Al-foil-covered oil	35.07 ± 0.01 <sup>ac</sup>	-2.69 ± 0.05 <sup>e</sup>	10.57 ± 0.01 <sup>e</sup>

Abbreviations: LSS: lignosulphonate, AL: alkali lignin, Al-foil: aluminum foil

Results are mean ± SD in triplicate

Small alphabets show significant effect in each column with confidence interval=0.95

#### 5.4. Conclusion

Direct contact between SPI films with lignins and oils significantly decreased the oxidation rate to around 75% and the formation of pentanal to about 40%. UV-blocking of AL resulted in significant reduction in oxidation rate for higher than 75% compared with the control. Lignin itself did not show a negative effect on the color of oil samples. In both soy oil and fish oil, active packaging developed with lignin reduced oxidation rate during autoxidation and photoxidation; however, this active packaging system is more effective in soy oil than in fish oil. Films containing AL had UV-blocking ability, and reduced oxidation rate of oil samples.

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## **CHAPTER 6**

### **CONCLUSION AND FUTURE WORK**

#### **6.1. Summary**

In the current research, soy protein isolate was enzymatically modified by transglutaminase to improve physicochemical properties of the film under controlling film preparation conditions. Then, antioxidant activity of the modified SPI film was developed with two types of lignin: alkali lignin (AL) and lignosulphonate (LSS). The modified film was tested in real food application. The summary of achievements is listed below:

1. Enzymatic treatment of soy protein isolate with transglutaminase improved crosslinking of protein network, SDS-PAGE and rheological data are excellent evidences for this reason.
2. Mechanical and surface hydrophobicity properties of the films prepared at 80 °C were significantly affected by different enzymatic incubation times ( $p<0.05$ ).
3. SPI-based films modified with transglutaminase showed improvement in not only tensile strength, but also surface hydrophobicity and water absorption properties compared to the control film.
4. Modified SPI films with the deactivated enzyme had mechanical and surface hydrophobicity similar to the modified films with the enzyme; however, viscosity of the solution and water absorption properties was somewhat similar to the control film without the enzyme.
5. The contact angle of the airside of the film was higher than the plateside.

6. Alkali lignin (AL) and lignosulphonate (LSS) material had radical scavenging activity
7. Films contain AL had UV-blocking ability
8. FTIR and XRD spectra revealed that lignin modified secondary structure of SPI matrix.
9. The addition of lignin increased TS and thermal stability and reduced %E of modified films.
10. Based on direct contact to oil, SPI films with AL and LSS increased antioxidant activity up to around 75% and decreased pentanal concentration to about 40%.
11. UV-blocking of AL caused reduction in oxidation rate for more than 75% compared to normal packaging systems.
12. Lignin did not have a negative effect on the color of oil samples
13. In both soy oil and fish oil, our active packaging with lignin reduced oxidation rate during storage; however, our testing method may be more appropriate for less unsaturated fatty acids like soy oil.

## **6.2. Future work**

1. Enzymatic modified soy protein isolate film with lignin can be applied in inner layer of both flexible and rigid food packaging systems.
2. The current biopolymeric material can be applied in pharmaceutical products, such as capsule production.
3. We can apply lignin to other types of protein to have different properties, such as zein protein, which is not water soluble and has different properties with soy protein.