Porcine Plasma Protein-Based Film: Preparation and Use of Protein Cross-Linking Agents as Film Strengtheners

Pornpot Nuthong

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Thesis Title	Porcine Plasma Protein-Based Film: Preparation and Use of Protein Cross-Linking Agents as Film Strengtheners
Author	Mr. Pornpot Nuthong
Major Program	Food Technology

Major Advisor

……
(Prof. Dr. Soottawat Benjakul)

Co-Advisor

……
(Asst. Prof. Dr. Thummanoon Prodpran)

Examining Committee

……...Chairperson
(Dr. Manee Vittayanont)

…….
(Prof. Dr. Soottawat Benjakul)

…….
(Asst. Prof. Dr. Thummanoon Prodpran)

…….
(Asst. Prof. Dr. Rungsinee Sothornvit)

The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirements for the Master of Science Degree in Food Technology

……..
(Assoc. Prof. Dr. Krerkchai Thongnoo)
Dean of Graduate School

ชื่อวิทยานิพนธ์	ฟิล์มโปรตีนพลาสมาเลือดสุกร: การเตรียมและการใช้สารเชื่อมประสานโปรตีนในการเสริมความแข็งแรงของฟิล์ม
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บทคัดย่อ

จากการศึกษาผลของการปฏิบัติการก่อนและปัจจัยบางประการต่อสมบัติของฟิล์มพลาสมาเลือดสุกร พบว่าทั้งความเข้มข้นของโปรตีน (ร้อยละ 2 และ 3) และกลีเซอรอล (ร้อยละ 50 60 และ 70) มีผลต่อสมบัติของฟิล์ม โดยฟิล์มที่มีความเข้มข้นของโปรตีนร้อยละ 3 และกลีเซอรอลร้อยละ 60 มีค่าการต้านทานแรงดึง (TS) สูงสุด (2.48 เมกกะปาสคาล) ส่วนฟิล์มที่มีความเข้มข้นของโปรตีนร้อยละ 3 และกลีเซอรอลร้อยละ 70 ให้ค่าการยืดตัวเมื่อขาด (EAB) ดีที่สุด (ร้อยละ 18.33) ฟิล์มที่มีความเข้มข้นโปรตีนร้อยละ 3 มีค่าการซึมผ่านไอน้ำ (WVP) สูงกว่าฟิล์มที่มีความเข้มข้นโปรตีนร้อยละ 2 เมื่อระดับความเข้มข้นกลีเซอรอลในฟิล์มเท่ากับร้อยละ 60 หรือ 70 ($p<0.05$) เมื่อความเข้มข้นกลีเซอรอลสูงขึ้น ความใสของฟิล์มเพิ่มขึ้นแต่ฟิล์มมีค่า b* ลดลง จากการศึกษาสมบัติของฟิล์มโปรตีนพลาสมาเลือดสุกรซึ่งเตรียมจากสารละลายฟิล์มที่มีการปรับค่าพีเอชต่างๆ (2-11) และให้ความร้อนที่อุณหภูมิต่างๆ (40 55 และ 70 องศาเซลเซียส) พบว่า การเพิ่มหรือลดค่าพีเอชของสารละลายฟิล์มมีผลให้ค่าการต้านทานแรงดึงและการยืดตัวเมื่อขาดของฟิล์มสูงขึ้น โดยสารละลายฟิล์มที่มีค่าพีเอชเท่ากับ 6 และ 7 ให้ฟิล์มที่มีค่าการต้านทานแรงดึงน้อยที่สุด แต่เมื่อพีเอชของสารละลายฟิล์มสูงหรือต่ำกว่า 7 การซึมผ่านไอน้ำของฟิล์มลดลง สำหรับการให้ความร้อนกับสารละลายฟิล์มซึ่งมีค่าพีเอชเท่ากับ 3 หรือ 10 พบว่า การเพิ่มอุณหภูมิไม่มีผลต่อค่าการต้านทานแรงดึงของฟิล์ม ($p>0.05$) แต่มีผลเพิ่มค่าการยืดตัวเมื่อขาดและการซึมผ่านไอน้ำของฟิล์ม ($p<0.05$)

จากการเปรียบเทียบผลของการใช้ไกลออกซาล ฟอร์มาลดีไฮด์ และ กลูตาราลดีไฮด์ ที่ระดับความเข้มข้นต่างๆ (ร้อยละ 1 2 และ 3) ต่อสมบัติของฟิล์มพลาสมาเลือดสุกรพบว่า การใช้ไกลออกซาล ฟอร์มาลดีไฮด์ และกลูตาราลดีไฮด์ ที่ความเข้มข้นร้อยละ 2 มีผลให้ฟิล์มมีค่าการต้านทานแรงดึงเพิ่มขึ้นร้อยละ 154 90 และ 68 ตามลำดับ และให้ค่าการยืดตัวเมื่อขาดเพิ่มขึ้นร้อยละ 98 73 และ 59 ตามลำดับ สำหรับฟิล์มที่เชื่อมประสานโปรตีนด้วยไกลออกซาลและ กลูตาราลดีไฮด์ มีสีเข้มขึ้นและความใสลดลง เมื่อเปรียบเทียบกับฟิล์มชุดควบคม ($p<0.05$) ส่วนฟอร์มาลดีไฮด์ (ร้อยละ 1-3) ไม่มีผลต่อการเปลี่ยนแปลงการซึมผ่านไอน้ำของฟิล์ม ($p>0.05$)

จากการศึกษาสมบัติของฟิล์มพลาสมาเลือดสุกรที่เติมกรดแทนนิก กรดคาเฟอิก และกรดเฟอรูลิก ที่ระดับความเข้มข้นต่างๆ (ร้อยละ 1-3) โดยใช้สารละลายฟิล์มที่มีความเข้มข้นโปรตีนร้อยละ 3 (น้ำหนักต่อปริมาตร) และกลีเซอรอลร้อยละ 70 (น้ำหนักต่อน้ำหนักของปริมาณโปรตีน) และผ่านการให้ความร้อนที่ 70 องศาเซลเซียส เป็นเวลา 30 นาที และปรับพีเอชเท่ากับ 10 แล้วเติมสารประกอบฟีนอลก่อนการขึ้นรูปเป็นแผ่นฟิล์ม พบว่า การเติมกรดแทนนิก กรดคาเฟอิก และกรดเฟอรูลิก ที่ระดับความเข้มข้นร้อยละ 3 มีผลให้ฟิล์มมีค่าการต้านทานแรงดึงเพิ่มขึ้นร้อยละ 123.3 194.3 และ 19.5 และค่าการยืดตัวเมื่อขาดเพิ่มขึ้นร้อยละ 71.1 86.3 และ 10.2 ตามลำดับ โดยเปรียบเทียบกับฟิล์มชุดควบคุม การใช้สารประกอบฟีนอลดังกล่าวทำให้ฟิล์มมีค่าการซึมผ่านไอน้ำเพิ่มขึ้นเล็กน้อย ($p<0.05$) นอกจากนี้เมื่อความเข้มข้นของกรดแทนนิก และกรดคาเฟอิกสูงขึ้น ฟิล์มมีสีแดงและเหลืองเพิ่มขึ้น แต่ความใสลดลง การเติมออกซิเจนให้กับสารละลายฟิล์มที่ประกอบด้วยกรดฟีนอลิก ส่งผลให้ฟิล์มมีค่าการต้านทานแรงดึงเพิ่มขึ้นแต่ค่าการยืดตัวเมื่อขาดลดลง ($p<0.05$)

ไกลออกซาล หรือกรดคาเฟอิกในสารละลายฟิล์มให้ผลในการเพิ่มการเชื่อมประสานโปรตีนด้วยพันธะไดซัลไฟด์และพันธะโควาเลนท์ที่ไม่ใช่พันธะไดซัลไฟด์ ฟิล์มที่เติมกรดคาเฟอิกมีความคงตัวต่อความร้อนเพิ่มขึ้น โดยสังเกตจากการเพิ่มจุดหลอมเหลวและอุณหภูมิเริ่มต้นการสลายตัวของฟิล์ม ซึ่งแสดงให้เห็นถึงการเพิ่มขึ้นของการเชื่อมประสานของโมเลกุลโปรตีน จากสเปคตรัมของ FTIR พบว่า เกิดอันตรกิริยาระหว่างโมเลกุลของโปรตีน ซึ่งบ่งชี้จากการเปลี่ยนแปลงของพีคเอไมด์ I และจากการศึกษาโครงสร้างสัณฐานของฟิล์มซึ่งเติมสารประกอบอัลดีไฮด์หรือฟีนอลิก พบว่าฟิล์มมีความสม่ำเสมอ ไม่มีรอยแตกหรือช่องว่าง

จากการศึกษาการเก็บรักษาปลาผงแห้งในถุงซึ่งทำจากฟิล์มโปรตีนพลาสมาเลือดสุกร พบว่าปลาผงแห้งมีปริมาณความชื้นและมีสีเหลืองเพิ่มขึ้นอย่างต่อเนื่องในระหว่างการเก็บรักษานาน 24 วัน แต่การใช้ถุงซึ่งผลิตจากฟิล์มโปรตีนพลาสมาเลือดสุกรที่เติมกรดคาเฟอิกซึ่งผ่านการเติมออกซิเจนสามารถชะลอการเพิ่มขึ้นของค่า TBARS ในปลาผงแห้งตลอดระยะเวลาการเก็บรักษา

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ABSTRACT

Properties of porcine plasma protein-based films as influenced by pretreatment and some factors were studied. Both protein concentrations (2 and 3%) and glycerol contents (50, 60 and 70% of protein) had the impact on film properties. Film prepared at 3% protein and 60% glycerol possessed the highest tensile strength (TS) (2.48 MPa), while that containing 3% protein and 70% glycerol exhibited the greatest elongation at break (EAB) (18.33%). In the presence of 60 or 70% glycerol, water vapor permeability of film containing 3% protein was higher than that with 2% protein ($p<0.05$). Decreases in b*-value but increases in transparency were obtained when films were prepared with higher glycerol content. Pretreatment of film forming solution (FFS) by adjusting pHs (2-11) and temperatures (40, 55 and 70°C) on the properties of resulting films was investigated. TS and EAB became higher with decreasing or increasing pH value of FFS. The lowest TS was observed in film with pretreatment at pHs 6 and 7. WVP of film decreased when the pH values of FFS were above or below pH 7. Heat treatment of FFS with pHs 3 and 10 had no impact on TS of resulting film ($p>0.05$). On the other hand, EAB and WVP increased with increasing temperature of FFS at both pHs 3 and 10 ($p<0.05$).

Porcine plasma protein based film incorporated with glyoxal, formaldehyde and glutaraldehyde at different concentrations (1, 2 and 3%) were characterized. When glyoxal, formaldehyde or glutaraldehyde was incorporated at a level of 2%, TS of resulting film increased by 154, 90 and 68%, respectively, and EAB increased by 98, 73 and 59%, respectively. Films treated with glutaraldehyde and glyoxal were darker and had lower transparency, compared with the control film ($p<0.05$). Films modified with formaldehyde (1-3%) had no changes in WVP ($p>0.05$).

Additionally, properties of porcine plasma protein-based film incorporated with tannic acid, caffeic acid and ferulic acid at different concentrations (1-3%) were determined. Film-forming solution (FFS) containing 3% protein (w/v) and 70% glycerol (w/w of protein content) was preheated at 70ºC for 30 min and adjusted pH to 10 followed by the addition of phenols and film casting. Tensile strength (TS) of resulting film increased by 123.3, 194.3 and 19.5% and elongation at break (EAB) increased by 71.1, 86.3 and 10.2%, respectively, compared with the control film, when tannic acid, caffeic acid and ferulic acid at a level of 3% was added. The use of all phenolic compounds slightly increased water vapor permeability (WVP) of resulting films ($p<0.05$). The increases in a*- and b*-values of films were observed as the higher concentrations of tannic acid and caffeic acid were used. This was associated with the lowered transparency of resulting films. FFS containing phenolic acid with prior oxygenation yielded the film with increased TS but lowered EAB ($p<0.05$).

The incorporation of glyoxal or caffeic acid resulted in the increase in protein cross-links stabilized by both disulfide and non-disulfide covalent bonds. Greater thermal stability with an increase in the melting point was observed in film incorporated with caffeic acid, indicating a greater degree of cross-linking. The coincidental increase in initial temperature of film degradation was noticeable. FTIR spectra revealed the intermolecular interaction between protein molecules as indicated by the shift of amide I peak. Morphology study revealed that film incorporated with aldehyde or phenolic compounds possessed the continuous matrix with less pores and cracks.

When the dried fish powder was packaged in bags produced from porcine plasma protein-based films, the moisture content and the formation of yellowish color increased continuously during 24 days of storage ($p<0.05$). However, the increase in TBARS value was retarded when kept in bag from film treated with caffeic acid with prior oxygenation throughout the storage time ($p<0.05$).

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Pornpot Nuthong

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CHAPTER 1

INTRODUCTION

Chemically synthesized polymeric films are widely used for packaging in food industry but they are not biodegradable, leading to environmental pollution and serious ecological problems (Tharanathan, 2003). Recently, the recycling or substitution of non-biodegradable synthetic materials has been paid increasing attention. Biodegradable packaging including film has become important issue. Edible and/or biodegradable films can be defined as a thin layer of natural biopolymers, which can be formed on a food as coating or performed as a film that can be placed between food components, used as a food wrap, or formed into a pouch to contain foods (Hernandez-Izquierdo & Krochta, 2008).

Protein-based films are biodegradable and act as the effective lipid, oxygen and aroma barriers at low to intermediate relative humidity, however they exhibit poor water vapor barrier property (McHugh & Krochta, 1994). The chemical natures of the polymeric matrix and additives affect the film characteristics (Kester & Fennema, 1986). Properties of protein-based films depend on various factors such as source of protein, pH of protein solution, plasticizers, preparation conditions and substances incorporated into the film-forming solutions (FFS) (Park, Weller, Vergano & Testin, 1993; Park & Chinnan, 1995).

Plasticizers are necessarily used in protein films because they reduce brittleness by lowering the intermolecular interactions between adjacent chains of the biopolymers (Cuq, Aymard, Cuq & Guilbert, 1995; Vanin, Sobral, Menegalli, Carvalho & Habitane, 2005). Generally, plasticizers such as glycerol and sorbitol are

used to modify the functional and physical properties of the film (Bergo & Sobral, 2007). Type and concentration of plasticizer determine the properties of protein-based films (Sobral, dos Santos & Garcia, 2005; Vanin, Sobral, Menegalli, Carvalho & Habitane, 2005).

The solubilization of protein is the important process for film formation. The pH of the solution affects the nature and the distribution of the protein's net charge. The proteins are more soluble in low (acid) or high (alkaline) pH values because of the repulsive force among the molecules and, therefore, contributing to its solubility (Pelegrine & Gasparetto, 2005). The muscle proteins-based films prepared at acid (pH 1-4) and alkaline (pH 10-12) conditions had better mechanical properties than those prepared at neutral pHs (Hamaguchi, Yin & Tanaka, 2007).

Temperature and time used to solubilize proteins directly affect the mechanical properties of protein-based films. Increases in heating time and temperature resulted in the increases in tensile strength, Young's modulus and elongation at break of protein-based films (Perez-Gago & Krochta, 2001; Choi & Han, 2002; Shaw, Monahan, O'Riordan & O'Sullivan, 2002).

Protein chains possess reactive side groups which can be potentially modified via chemical, physical or enzymatic cross-linking to enhance the properties of films (Hernandez-Munoz, Villalobos, & Chiralt, 2004). Aldehydes such as glutaraldehyde, glyoxal or formaldehyde enhanced mechanical and barrier properties of biodegradable films from sunflower protein isolate (Orliac, Rouilly, Silvestre, & Rigal, 2002), soy protein (Park, Bae, & Rhee, 2000; Rhim, Gennadios, & Weller, 1998), collagen (Lieberman & Gilbert, 1973) and whey proteins (Galietta, di Gioia, Guilbert, & Cuq, 1998). Apart from aldehydes, polyphenols are known to react under oxidizing conditions with side chain amino groups of peptides, leading to the formation of

protein cross-links (Strauss & Gibson, 2004). Ferulic acid can cross-link with protein and polysaccharides by producing a resonance-stabilized free radical intermediate (Oudgenoeg, Hilhorst, Piersma, Boeriu, Gruppen, Hessing, Voragen, & Laane, 2001). Oxidized ferulic acid can react with amino and thiol groups in protein (Figueroa-Espinosa, Morel, Surget, Asther, Moukha, Sigoillot, & Rouau, 1999). Additionally, free radical formed from ferulic acid can react with tyrosine and with itself to form diferulic acid (Vansteekiste, Babot, Rouau, & Micard, 2004), which act as a bridge between protein molecules. Tannic acid also shows the ability to bind proteins (Henson, Niemeyer, Ansong, Forkner, Makkar, & Hagerman, 2004).

In slaughtering process, blood is produced and requires the treatment before disposal. Some blood has been used as coagulated blood for local consumption. Plasma protein from blood, such as beef plasma (Foegeding, Dayton & Allen, 1986), chicken plasma (Rawdkuen, Benjakul, Visessanguan & Lanier, 2004; 2005), bovine plasma (Lu & Chen, 1999) and porcine plasma (Benjakul, Visessanguan & Chantarasuwan, 2004), can be used to improve or modify the textural properties of surimi gel. Porcine plasma protein was used for the preparation of Maillard reaction product (MRPs) with antioxidative activity (Lertittikul, Benjakul & Tanaka, 2007). The use of porcine plasma protein as a biomaterial for film preparation should be another approach for maximization the use of this protein. However, no information regarding porcine plasma protein-based film and some factors affecting the properties of the film has been reported. Therefore, the objectives of this study were to investigate the effect of protein and plasticizer contents as well as pretreatment of film forming solution by adjusting pH or temperature prior to film formation on the properties of porcine plasma protein-based film. Then improve the properties of

porcine plasma protein-based film using the different phenolic compounds and aldehyde compounds.

Literatures reviews

1. Blood and plasma

1.1 Composition of the blood

Blood is a complex tissue that consists of blood cells and cell-like components suspended in a clear, straw-colored liquid called plasma (Table 1) (Brum et al., 1994). The porcine can yield 10-12 liters of blood (Wismer-Pederson, 1979), about 55% of which is plasma. Blood transports dissolved nutrients, gases, hormones, and water through the body. Approximately two-thirds of the weight of blood is a plasma composed of 90% water and 7% protein (Chan et al., 2001). Porcine blood consists of 79.2% water, 18.5% protein, and 0.1% fat (FAO, 1996). Blood constituents vary depending on animal species (Table 2) (Lenzell, 1974).

Table 1 Blood components and their functions

Component	Percent	Functions
Plasma	55	Suspends blood cells so they flow. Contains substances that stabilize pH and osmotic pressure, promote clotting, and resist foreign invasion. Transports nutrients, wastes, gases, and other substances
White blood cells	<0.1	Allow phagocytosis of foreign cells and debris. Acts as mediators of immune response.
Platelets	<0.01	Seal leak in blood vessels.
Red blood cells	45	Transport oxygen and carbon dioxide.

Source: Brum et al. (1994)

Table 2 Concentrations (mg/ml) of some blood constituents in the pig, goat and cow

	Pig	Goat	Cow
Blood			
Glucose	560	460	500
Acetase	20	90	90
Lactate	130	70	80
Plasma			
3-Hydroxybutyrate	10	60	50
Acetoacetate	6	3	6
Triacylglycerols	320	220	90
Free fatty acids	4	90	80
Phospholipids	550	1600	800
Cholesterol	170	370	230
Cholesterol esters	240	1000	1830
Free glycerol	Not determined	3.4	Not determined
Methionine	8	3	3
Phenylalanine	21	7	7
Luecine	43	21	22
Threonine	27	10	10
Lysine	42	21	12
Arginine	41	25	13
Isoleucine	30	18	17
Histidine	24	10	10
Valine	49	28	31
Glutamate	62	19	9
Tyrosine	26	10	7
Asparagine	6	9	4
Proline	45	26	8
Ornithine	19	11	9
Aspartate	4	3	2
Alanine	37	17	16
Glutamine	64	37	26
Glycine	51	69	18
Citruline	Not determined	19	12
Serine	17	14	9
Total amino acids	630	380	250

Source: Lenzell (1974)

1.2 Blood plasma

Blood plasma is a straw-colored liquid made up of water and dissolved substances. These soluble constituents can be grouped into three categories (Alters, 1996):

1. Proteins. Three majors plasma proteins are albumins, which help maintain the blood's osmotic pressure; globulins, which transport nutrients and play a

role in the immune system; and fibrinogen, which is important in blood clotting (Audesirk et al., 2002; Donnelly and Delaney, 1977). Plasma is an important source of protein (Table 3) (Howell and Lawrie, 1983).

2. Nutrients, hormones, respiratory gases, and wastes. These substances move from one place to another in the body and are used or produced by the metabolism of cells. These substances include glucose, lipoproteins (a soluble form of lipids), amino acid, vitamins, hormones, and the respiratory gases.

3. Salts and ions. Plasma is a dilute salt solution. In water, salts dissociate into their component ions. The main plasma ions are sodium (Na^+), chloride (Cl^-), and bicarbonate (HCO_3^-). In addition, there are trace amounts of other ions, such as calcium (Ca^{2+}), magnesium (Mg^{2+}), copper (Cu^{2+}), potassium (K^+), and zinc (Zn^{2+}). In living systems, these ions are called electrolytes, which serve general functions in the body.

Table 3 Compositional analysis of porcine plasma protein sample

Parameter	Source of plasma	
	Porcine plasma	Freeze dried porcine plasma
Protein % (w/w) (Nx6.25)	6.8 (±0.1)	68.0 (±0.1)
Moisture % (w/w)	91.0 (±0.1)	9.2 (±0.1)
Ash % (w/w)	1.1 (±0.1)	11.5 (±0.1)
Sodium % (w/w)	0.5 (±0.1)	5.2 (±0.1)
Citrate % (w/w)	0.44 (±0.1)	4.6 (±0.1)
Lipid % (w/w)	0.15 (±0.1)	2.0 (±0.1)
pH of 6% (w/w) protein solution	8.1 (±0.1)	8.1 (±0.1)
Total bacterial count at 37°C	390/ml to $4x10^3$/ml	$3.8 x10^3$/g

Source: Howell and Lawrie (1983)

1.3 Blood processing

Blood is the first by-product obtained in slaughtering process. Approximately 4% of the animal live weight (Chan et al., 2001) or 50% of the total animal's blood

volume can be collected during bleeding in the slaughter operation and the remaining portion is retained in the capillary system throughout the carcass (Ranken, 1980; Wismer-Pederson, 1988; Ockerman and Hansen, 2000). In general, blood is collected hygienically and then an anticoagulant, usually trisodium citrate (0.2% with or without water), is added. Processing of blood includes centrifugation to separate light plasma (52 to 70%) from heavy erythrocytes and then chilling to 2°C, if not previously done, to minimize bacterial growth (Ockerman and Hansen, 2000).

1.4 Utilization of blood and plasma

Animal blood produced during slaughter is a valuable protein source that has many possibilities for use in the food, laboratory, medical, industrial, and fertilizer areas (Ockerman and Hansen, 1988). Animal blood is utilized mainly in the food and feed industries. Blood proteins are added as emulsifiers, stabilizers, clarifiers, or nutritional components to enhance the properties of foods, and as lysine supplements, vitamin stabilizers, milk substitutes, or nutritional components in feedstuffs. When used in products not for human consumption, blood is simply dried and processed into blood meal or mixed and dried with meat and bone meal (Hyun and Shin, 1998).

Blood or plasma has been used as a nutritional supplement in the bakery products such as cake (Myhara and Kruker, 1998). Bovine plasma, a by-product of animal slaughtering, has received attention as an egg substitute in cake (Lee et al., 1991) bread and biscuit (Johnson et al., 1979). The use of blood protein in meat products is becoming increasingly popular as an important dietary iron source, as well as protein source, and as a meat substitute due to its lower cost compared to meat (Chan et al., 2001).

Blood is used in many countries in the manufacture of traditional products, such as blood sausage and meat loaf. It can be added at low concentrations (0.5-2.0%)

in order to improve the color of meat products (FAO, 1996). The utilization in large proportions is limited by its effect on the sensory properties of the products, particularly the color and strong metallic flavor (Howell and Lawrie, 1983). This problem can be overcome by using plasma instead of whole blood. Generally, plasma and red blood cells can be separated by centrifuging the whole blood at 1000xg for 30 min at 4°C (Benjakul et al., 2001).

Blood plasma consists of various proteins, mainly albumin and globulins, which act as efficient emulsifiers in the sausages (Cofrades et al., 2000; Chan et al., 2001). Animal plasma proteins have a good emulsifier property (Saito and Taira, 1987) and have been used as fat replacement in bologna sausage (Cofrades et al., 2000), associated with the improvement of the quality of ham pate with increased the moisture and protein content after the fat replacement (Viana et al., 2005). Gordon (1971) and Tybor et al. (1973) reported that plasma protein isolates were excellent emulsifiers and binders in cook meat products. However, losses of their emulsifying properties were found after thermal process. In general, 60% of the emulsifying properties of plasma proteins was lost after heat treatment at 80°C for 30 min. (Saito et al., 1988). So, the utilization of plasma protein has been limited.

Furthermore, additives are commonly used to improve or modify the textural properties. Protein additives have also been widely used in surimi such as beef plasma protein, egg white and potato extract (Morrissey et al., 1993). Kang and Lanier (1999) found that the addition of 1% bovine plasma protein to Pacific whiting surimi enhanced strength of gels. Porcine plasma protein containing both proteinase inhibitor and transglutaminase also effectively increased surimi gel strength (Benjakul et al., 2000; Benjaku et al., 2001; Jiang and Lee, 1992). Addition of chicken plasma protein in combination with $CaCl_2$ induced the cross-linking of surimi proteins with the

increase in breaking force and deformation and the formation of ordered microstructure (Rawdkuen et al., 2005). Blood plasma proteins have been extensively used in the elaboration of cooked meat products due to its useful functional properties, especially for gel formation upon heating (Cofrades et al., 2000; Pietrasilk et al., 2007). Foegeding et al. (1986) reported that the fibrinogen-rich fraction of beef plasma increased the strength of heat-induced myosin gels.

2. Protein based-films and coating

An edible film can be defined as a thin layer of edible material, which can be formed on a food as a coating or performed as a film that can be placed between food components, used as a food wrap, or formed into a pouch to contain foods. It purpose is to inhibit migration of moisture, oxygen, carbon dioxide, aromas, and lipids; carry food ingredients (for example, antioxidants, antimicrobials, and flavors); and, or improve mechanical integrity or handling characteristics of the food (Krochta and De Mulder-Johnston, 1997). The utility of edible films lies in their capacity to act as an adjunct of edible films lies in quality, extending shelf life and possibly improving economic efficiency of packaging materials (Kester and Fennema, 1986).

Materials used to produce edible films can be divided into 4 categories: biopolymer hydrocolloids, lipids, resins, and composites. Biopolymer hydrocolloids include proteins such as gelatin, keratin, collagen, casein, soy protein, whey protein, myofibrillar proteins, wheat gluten, and corn zein; and polysaccharides such as starch, starch derivatives, cellulose derivatives, and plant gums. Suitable lipids include waxes, acylglycerols, and fatty acids. Resins include shellac and wood rosin. Composites generally contain both lipid and hydrocolloid components in the form of a bilayer or an emulsion (Krochta et al., 1994; Perez-Gago and Krochta, 2005).

Among these materials, proteins have been extensively used for the development of edible films because of their relative abundance, good film-forming ability and nutritional qualities (Shiku *et al*., 2003). The mechanical and barrier properties of protein-based films are generally better than those of polysaccharide-based films. This is due to the fact that, contrary to polysaccharides which are homopolymers, protein has a specific structure (based on 20 different monomers) which confers a wider range of potential functional properties, especially high intermolecular bonding potential (Cuq *et al*., 1995).

Proteins are renewable, biodegradable/edible resource with great potential in improve the quality and stability of a large range of food products by using a number of processing techniques (Pommet et a., 2005). Protein-based films are lack of mechanical strength and poor water vapor barrier property because of their hydrophilic nature. Protein chains posses reactive side groups which can be potentially modified via chemical, physical or enzymatic cross-linking to enhance the functional properties of films (Hernandez-Munoz *et al*., 2004).

3. Mechanism of protein film formation

Protein films are generally formed from solutions or dispersions of the protein as the solvent/carrier evaporates. The solvent/carrier is generally limited to water, ethanol or ethanol-water mixture (Krochta, 1997). Generally, globular protein must be denatured by heat, acid, base and/or solvent in order to form the more extended structures that are required for film formation. Once extended, protein chains can associate through hydrogen, ionic, hydrophobic and covalent bondings. The chain to chain interaction that produces a cohesive film is affected by the degree of chain extension (protein structure) and the nature and sequence of amino acid residues

during drying film (Krochta, 1997). Protein-based film can be formed in three steps (Marquie and Guilbert, 2002).

3.1 Denaturation of protein

Proteins must be denatured by heat or adjusting pH to transition from the native form to the denatured state. This is to break inter-and intramolecular bondingd that stabilize protein molecules in their native forms, resulting in mobile chains. However, protein films posses different properties depending upon the structure of protein. Sano *et al*. (1994) reported that natural actomyosin (NAM) exhibited the higher denaturation when heating at 30°C and higher protein aggregation and rigidity with increasing temperature above 30 °C.

3.2 Aggregation of protein

Aggregation of mobile protein chains is very important for network formation of the film matrix. This aggregation involves arrangement and orientation of mobile chains in such desired way that film network are formed (in the film step) through various molecular interactions such as hydrophobic interaction, hydrogen bond, disulfide bond etc. Factors affect protein aggregation such as protein type and concentration and pH of the film forming solution (Xiong and Brekke, 1989).

3.3 Film formation

Drying the cast film-forming solutions eliminated solvents and allowed the proteins to form three-dimensional networks through new inter/and intra molecular linkage. Mechanism of film formation is illustrated in Figure 1.

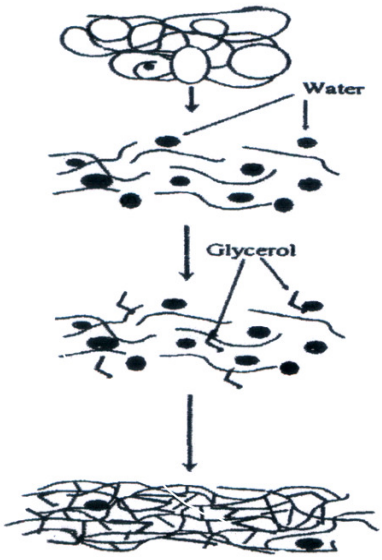


Figure 1 Mechanism of film formation

Source: Adapted from Marquie and Guilbert (2002)

4. The types of crosslinks found in food

Protein crosslinking refers to the formation of covalent bonds between polypeptide chain within a protein (intramolecular crosslinks) or between proteins (intermolecular crosslinks) (Feeney and Whitaker, 1988). Food processing often involves high temperatures, extremes in pH, particularly alkaline, and exposure to oxidizing conditions and uncontrolled enzyme chemistry, can result in the introduction of protein crosslinks, producing substantial changes in the structure of proteins, and the functional and nutritional properties of the final product (Singh, 1991).

4.1 Disulfide crosslinks

Disulfide bonds are the most common and well-characterized types of covalent crosslink in proteins in biology. They are formed by the oxidative coupling of two cysteine residues that are adjacent within a food protein matrix. A suitable oxidant accepts the hydrogen atoms from the thiol groups of the cysteine residues, producing disulfide crosslinks. The ability of proteins to form intermolecular disulfide

bonds during heat treatment is considered to be vital for the gelling of some food protein, including milk proteins, surimi, soybeans, eggs, meat and some vegetable proteins (Zayas, 1997).

4.2 Crosslinks derived from dehydroprotein

The alkaline conditions combines with thermal processing induced racemization of amino acid residues and the formation of covalent crosslinks, such as dehydroalanine, lysinoalanine and lanthionine (Friedman, 1999). Dehydroprotein is very reactive with various nucleophilic groups including the ε-amino group of lysine residues and the sulfhydryl group of cysteine.

4.3 Crosslinks derived from tyrosine

Various crosslinks formed between two or three tyrosine residues have been found in native proteins and glycoprotiens, for example in plant cell walls (Singh, 1991). Polyphenol oxidase can also lead indirectly to protein crosslinking, due to reaction of cysteine, tyrosine, or lysine with reactive benzoquinone (Feeney and Whitaker, 1988).

4.4 Crosslinks derived from the Maillard reaction

The Maillard reaction is a complex of chemical reactions between an amine with a carbonyl group and reducing sugar or fat breakdown product. Glycosylation or glycation is an important reaction, which induces the covalent attachment of sugar to α- or ε-NH_2 groups of amino acids and protein to form glycated proteins (Friedman, 1996). The Maillard reaction produces a variety of intermediate products, such as α-dicarbonyl compounds and deoxysones, and finally brown pigments (melanoidins) are formed (Van Boekel, 1998).

4.5 Crosslinks formed via transglutaminase catalysis

Transglutaminase catalyses the acyl-transfer reaction between the γ-carboxyamide group of peptide bound glutamine residues and various primary amines. The ε-amino groups of lysine residues in proteins can act as the primary amines, yielding inter- and intramolecular ε-*N*-(γ-glutamyl)lysine crosslinks (Motoki and Seguro, 1998).

5. Cross-linking method of protein-based films

Crosslinking provides an opportunity to create gel structures from protein solutions, dispersions, colloidal systems, protein coated emulsion droplets or protein-coated gas bubbles and create new types of food (Dickinson, 1997). In addition, judicious choice of starting proteins for crosslinking can produce through crosslinking of different proteins containing complementary amino acids (Zhu et al., 1995).

5.1 Chemical method

Of the chemical cross-linking agents, the aldehydes bond very quickly to proteins (Donohue et al., 1983). Aldehydes such as glutaraldehyde, glyoxal or formaldehyde enhanced mechanical and barrier properties of biodegradable films from sunflower protein isolate (Orliac et al., 2002), soy protein (Park et al., 2000; Rhim et al., 1998), collagen (Lieberman and Gilbert, 1973) and whey proteins (Galietta et al., 1998). These cross-linking agents can enhance the mechanical and barrier properties of biodegradable film for food packaging applications.

Formaldehyde, the simplest of cross-linking agents, has the broadest reaction specificity. In addition to amine group of lysine, it reacts with the side chains of cysteine, tyrosine, histidine, tryptophan, and arginine (Hernandez-Munoz et al., 2004). Most glutaraldehyde induced protein cross-linking reactions are carried out at highly alkaline pH, with α,β- unsaturated molecules predominating. The aldehyde functions of these molecules react with primary amines to form imines (Schiff bases)

stabilized by resonance and resistant to acid hydrolysis (Monsan et al., 1975). Protein cross-linking by glyoxal involves a key reaction with arginine guanidyl groups. This reagent is preferentially active at alkaline pH. The first step of the reaction resembles Schiff base formation and then gives rise to different products. Secondary reactions can also occur with primary amines and thiol groups (Marquie, 2001).

Aldehydes which bond very quickly to proteins are usually used to cross-linking, but aldehydes have toxicity which may not be tolerable in many fields. Thus, the use of natural cross-linking could be a better alternative for use in food (Galietta et al., 1998). Over the past few years, the use of phenolic acid and flavonoids as cross-linking agents to produce stabilized food protein for use as novel ingredients for the modification of food systems has been recognized (Strauss et al., 2004). Recently, phenolic compounds as cross-linking agents have more interest for film preparation. Though some phenolic compound such as ferulic acid, caffeic acid, and tannic acid have been investigated as cross-linking agents for soy protein isolate films (Guo and Ou, 2002; Ou et al., 2004; Ou et al., 2005), gelatin based films (Cao et al., 2007), starch-chitosan blend films (Mathew and Abraham, 2008).

5.2 Enzymatic methods

Enzymes have high specificity to modify the functional properties of foods required the catalyst under the mild conditions and they are less likely to produce toxic products (Singh, 1991). Thus enzymes are commonplace in many industries for improving the functional properties of food proteins (Poutanen, 1997). In recent years, the enzyme transglutaminase have been recognized due to its ability to induce the gelation of protein solutions. The use of this enzyme has been investigated (Motoki and Seguro, 1998; Kuraishi et al., 2001; Saguer et al., 2007). Transglutaminase (TGase) can catalyze the cross-linking reaction between proteins,

catalyses an acyl-transfer reaction between the γ-carboxyamide group of peptide bound glutamine residues (acyl donors) and a variety of primary amines (acyl acceptors), including the ε-amino group of lysine residues in certain proteins, and thus modify the several selective properties of protein films (Motoki and Seguro, 1998). The effects of TGase treatment on the film properties have been studied for many proteins, such as soy protein isolate films (Tang et al., 2005), casein-based films (Chambi and Grosso, 2006), gelatin-based films (de Carvalho and Grosso, 2004), pectin-soy flour films (Mariniello et al., 2003), whey protein films (Yildirim and Hettiarachchy, 1998).

6. Film properties

6.1 Mechanical properties

Stress/strain curves that result from the tensile testing provide valuable information on film flexibility, toughness, and elongation**.** These results are use in prediction of the package performance during handling and storage. Cunningham et al. (2000) reported elongation at break of soy protein films increased from 1.5 to 106%, and the tensile strength of the films decreased from 15.8 to 1.6 MPa, when glycerol content increased from 20% to 40%. As glycerol content increased from 40% to 50% , the percent elongation of whey protein films increased from 85% to 94% and decreased tensile strength from 8 to 4 MPa (Sothornvit et al., 2007). Liu et al. (2006) found that addition of corn oil increased the tensile strength and elastic modulus, while reducing the percent elongation of gelatin/sodium alginate films. Film with 2.5% corn oil had lower tear resistance than films without oil. Rojas-Grau et al. (2007) studied the tensile properties of alginate-apple puree edible films incorporated with plant essential oils. The percent elongation of control film (without essential oils) was 51.06% and increased in all films containing essential oils, reaching a maximum

value of 58.33% with carvacrol, while decreasing the tensile strength of films. Sabato et al. (2007) found that increasing radiation dose from 25 to 200 kGy in muscle protein films from Nile Tilapia slightly increased tensile strength and percent elongation.

6.2 Solubility

Solubility in water is an important property of edible films. The solubility of protein depends upon protein-solvent interactions having a lower free energy than the sum of the protein-protein and solvent-protein interactions (Mangino, 1984). Perez-Gago and Krochta (2001) reported that protein solubility of whey protein-based film in water decreased by increasing heat denaturation time and temperature. Film pieces formed using low denaturation temperatures and short times for film-forming solutions dissolved rapidly after being in contact with water. Protein solubility (PS) of film from soy protein isolate and soy protein fraction, fractionated with an ultrafiltration (100 and 300 kDa cutoff) was reported by Cho and Rhee (2004). The molecular weight distribution of proteins had little influence on the PS values of fractionated soy protein films due to the fractionated soy protein film networks were formed by hydrogen and disulfide bonding. Liu et al. (2004) studied the effect of physical treatments (heat denaturation, ultraviolet radiation and ultrasound) on the solubility of peanut protein film. The water solubility of films produced from heated film-forming solution decreased from 49.99% to 42.74% relates with an increase in hydrophobic and disulfide binding. However, ultrasound treatment increased the water solubility of the film from 49.99% to 53.12%. It was possibly due to the quaternary structure, releasing smaller peptides and facilitating could be solubilize in water. The washing process can affect the properties of protein-based film from the meat of round scad (*Decapterus maruadsi*). Artharn et al. (2007)

found that film prepared from unwashed mince had greater protein solubility than film from washed mince due to the ease of sarcoplasmic proteins being leached from the film.

6.3 Permeability

Sobral et al. (2001) studied the effect of plasticizer content on water vapor barrier properties of gelatin based edible films plasticized with sorbitol. The water vapor permeability (WVP) of the films of pigskin and bovine hide gelatin increased from 1.8 to 3.2 x 10^{-8} g mm h^{-1} cm^{-2} Pa^{-1}, and from 1.7 to 3.8 x 10^{-8} g mm h^{-1} cm^{-2} Pa^{-1}, respectively, between 15 and 65 g sorbitol/100 g gelatin. Bertan et al. (2005) found that the incorporation of Brazilian elemi, as hydrophobic substances, into gelatin films using a blend of stearic and palmitic acids to dissolve the elemi were efficient in reducing WVP and increasing oxygen permeability (OP). To improve the barrier properties of peanut protein films, Liu et al. (2004) increased in the heating treatment temperature from 60°C to 80°C caused a decrease in WVP and OP while heating at 90°C increased the WVP and OP of the film. Maria Martelli et al. (2006) also reported a continuous increase in water vapor permeability (WVP) with increasing glycerol concentration in chicken feather keratin films. WVP values of some protein films plasticized with glycerol are also differenced. Feather keratin films plasticized with glycerol presented WVP values in the same range as edible methylcellulose films and lowers than peanut proteins films, meat myofibrillar proteins and fish water-soluble proteins. A direct relation between WVP with plasticizer content and film thickness were reported by Bertuzzi et al. (2007) in edible starch based films. WVP increased linearly as the concentration of glycerol increased in the range of 10 g to 60 g/100g starch. The film provided an increased resistance to mass transfer across it with increased film thickness and led to reducing WVP values.

6.4 Thermal properties

The thermal transition of the films was used to predict protein-based package performance under different preparation conditions. DSC, TGA, and DMTA have been the most common thermal analysis techniques used in determining the transition temperature of various protein-based films (Hernandez-Munoz et al., 2004; Bertan et al., 2005; Schmidt and Soldi, 2006; Sabato et al., 2007; Carvalho et al., 2008). Cuq et al. (1997) used both DSC and DMTA to study the thermal transitions of fish myofibrillar protein-based films as affected by moisture content. The storage modulus, loss modulus and tan δ of films were decreased when increasing water content from 5 g to 68 g of water per 100 g of dry matter. When the water content is increased, the temperature noted at endothermic heat drop flow by DSC is lowered. Berreto et al. (2003) studied the thermal degradation of edible films prepared from sodium casemate, whey protein concentrate and gelatin in the presence and absence of sorbitol. When sorbitol was present, TGA indicated that the initial temperature of degradation decreased from 300 to 280°C due to during heating the initially ordered structure of films is destroyed. Lodha and Netravali (2005) compared the effect of stearic acid and glycerol on the thermal properties of the soy protein isolate (SPI) resin sheet with DSC and TGA. The lowered melting temperatures and reduced enthalpies for steric acid modified-SPI resin with glycerol as compared to the stearic acid modified-SPI resin (without glycerol) indicates that the presence of glycerol also leads to formation of less stable and smaller crystal structure as well as reduced crystallinity. As the TGA thermogram indicates the glycerol plasticized-SPI resin and stearic acid modified-SPI resin started to degrade at 250 and 275°C, respectively. Vanin et al. (2005) found that increasing all plasticizers (glycerol, ethylene glycol, diethylene glycol and propylene glycol) content from 10 g to 30 g plasticizer/100 g

gelatin in gelatin-based films decreased a glass transition temperature. Similar result was observed by Sobral et al. (2001) studied the thermal properties of gelatin based edible films plasticized with different concentration of sorbital.

6.5 Microstructural properties

The microstructure characteristics of protein-based films are a function of the formulations and processing conditions used to manufacture the films. Prodpran et al. (2007) used scanning electron microscopy (SEM) to study the surface texture of muscle protein-based films as affected by palm oil and chitosan incorporation. The addition of chitosan, the surface of film became rougher with increasing chitosan content. For films added with palm oil, the surface of film had the irregular surface with the distribution of oil droplets. Maria Martelli et al. (2006) studied the microstructure of chicken feather keratin (CFK) films carried out without and with different plasticizer concentrations. The films made with glycerol and sorbitol appear to be more flexible compared with the CFK film made with PEG 4000. The plasticizers have a great influence on the microstructure of CFK films. With the use of sorbitol, the film surfaces showed a more uniform aspect. The SEM micrographs of the fracture surfaces of the soy protein isolate (SPI) resin containing different concentration of glycerol or stearic acid. SPI resin with 30% glycerol showed lower roughness at the fracture surface than SPI resin containing 25% stearic acid. The height and contour of the asperities at the fractured surface of the resin containing stearic acid indicate that the presence of stearic acid resulted in a more ductile failure (Lodha and Netravali, 2005).

7. Protein-based films from different sources

Proteins are a renewable, biodegradable/edible resource with great potential to improve the quality and stability of a large range of food product by using a number

of processing techniques (Hernandez-Izquierdo and Krochta, 2008). Proteins have been used to produce edible materials. Proteins are heteropolymers with α-amino acids being their monomer units. Proteins differ from polysaccharides where only a few monomers are involved in the range of polysaccharides that exist. During heating processing, proteins disaggregate, denature, dissociate, unravel, and align in the direction of the flow. These changes allow the protein molecules to recombine and cross-link through specific linkages (Areas, 1992).

7.1 Collagen and gelatin

Collagen is a fibrous protein found in hides, tendon, cartilage, bone, and connective tissue. Two major components consisting of 2 types of covalent cross-linked chain pairs have been identified: α (M_w = 100 kDa) and β (M_w = 200 kDa) (Lacroix and Cooksey, 2005). A unique characteristic in collagen is the occurrence of glycine in every 3rd amino acid residue throughout most of its structure (Krochta, 1997). Hydrolysis of collagen produces gelatin with molecular weights from 3 to 200 kDa depending on the raw material used and the handling conditions (Lacroix and Cooksey, 2005). Bergo and Sobral (2007) studied the effect of plasticizer content on the physical properties of pigskin gelatin films. Tensile strength and elastic modulus of gelatin films were decreased, while the elongation at break was increased with glycerol content increasing from 0% to 45%. Composite edible/degradable films produced with hydrocolloids and lipids can result in better functionality than films produced with the components, especially with respect to their barrier properties. Bertan et al. (2005) reported gelatin and triacetin films were more permeable to water vapor than composite films of gelatin/triacetin and fatty acids. The addition of hydrophobic substances, as stearic and palmitic acids, to the gelatin and triacetin films favored an increase in oxygen permeability. Sobral et al. (2001) found that increasing

sorbitol content from 15 g to 65 g sorbitol/100 g protein in bovine hide and pigskin gelatin edible films increased both the puncture deformation and water vapor permeability, while decreased the puncture force.

7.2 Myofibrillar proteins

Fish has been the main source of myofibrillar proteins for edible film formation. Regardless of the source (mammalian or fish), the main proteins found in the muscle are myosin (500 kDa) and actin. These proteins are obtained after removing other components such as blood, lipids, myoglobin, and collagen through a series of washing treatments. Fibrous proteins (myosin, F-actin) can form films with good mechanical properties, while globular proteins such as G-actin need to be unfolded first ((Lacroix and Cooksey, 2005). The sarcoplasmatic proteins have the capacity to form a continuous matrix and may be supposed that edible films can be produced by the mixture of these proteins with myofibrillar proteins. Paschoalick et al. (2003) reported increasing glycerol concentration from 15-65 g glycerol/100g protein in muscle protein-based films from Nile Tilapia provoked linear reduction of puncture force and an increase on both the elongation at break and water vapor permeability. Dynamic mechanical thermogram indicated that increasing glycerol content caused depression on both the storage and loss moduli values but increased the tan δ. Garcia and Sobral (2005) studied the effect of the thermal treatment of the film-forming solution (FFS) on the mechanical properties of films based on muscle proteins of Tilapia. The films based on FFS treated at 65°C/30 min were more resistant and more rigid, than the films treated at 40°C/30 min. Sabato et al. (2007) found that the radiation from electron beam to muscle protein-based films caused a slightly increase on its tensile strength characteristic at 100 kGy, while elongation value at this dose had no reduction.

7.3 Corn zein

Zein is the prolamin protein fraction found in corn gluten. It has a molecular weight of 18 to 45 kDa and is soluble in 60% to 70% ethanol. The poor solubility of zein in water is due to its high content of hydrophobic amino acid residues leucine, alanine, and proline (Kokini et al., 1994). Lamination of a hydrophilic polymer with a hydrophobic one can result in the decrease of WVP without significant detriment to oxygen barrier properties as shown by Pol et al. (2002). The compression-molded soy protein films were either single- or double-coated with corn zein. Compared to the base soy film, WVP values were reduced by 20% for single-coated and by 50% for double coated samples. Rakotonirainy and Padua (2001) pressed together 1 to 4 layers of oleic acid-plasticized zein films previously formed by solution casting. Although the fusion-lamination eliminated cracks and pinholes, there was no significant decrease in WVP, suggesting that water vapor molecules migrated through the laminated structure regardless of voids and defects.

7.4 Wheat gluten

Wheat gluten is composed of the water-insoluble prolamin and glutelin protein fractions known as gliadin and glutenin, respectively. The molecular weight of gliadin is in the range of 20 to 50 kDa, while glutenin has an average molecular weight of 250 kDa. The high molecular weight in glutenin can be attributed to the presence of intermolecular disulfide bonds, joining individual protein chains and resulting in a larger polymer ((Krochta, 1997). The amino acid composition of gluten determines its properties. An important characteristic is its high content of glutamic acid (about 37%) existing mainly as glutamine. Another very important amino acid in gluten, although it is contained in a small quantity is the sulfur amino acid cystine.

Cystine residues link together to peptide chains (intermolecular bonding) or portions of the same polypeptide chain (intramolecular bonding) (Klomklao *et al*., 2007). Disulfide bonds played an important role during the formation of films. Films from wheat gluten have good strength and oxygen and carbondioxide barrier properties. Mangavel et al. (2002) compared the prolamin composition effects on mechanical properties of cast wheat gluten films. Mechanical properties revealed that glutenin films were more resistant than gluten and gliadin films. Hernandez-Munoz et al. (2004) studied the effect of cross-linking using aldehydes on properties of glutenin-rich films. Water vapor permeability values decreased by around 30% when formaldehyde, glutaraldehyde or glyoxal were incorporated. DSC curves showed the shifts of glass transition temperature of cross-linked films when cross-linking agents were used.

7.5 Soy proteins

The most common way to classify soy proteins is based on their sedimentation rate in fractional ultracentrifugation. Larger Svedberg (S) numbers indicate a larger protein. Soy protein fractions include 2S, 7S, 11S, and 15S. The main constituents of soy protein are the 7S (conglycinin) and 11S (glycinin) fractions. β-conglycinin has a molecular weight of 180 kDa and is rich in asparagines, glutamine, leucine, and arginine residues. Unlike glycinin, which contains 20 intramolecular disulfide bonds, disulfide cross-linking in conglycinin is limited (Krochta, 1997). Tang et al. (2005) studied the effect of transglutaminase treatment on the properties of cast films of soy protein isolate (SPI). The treatment by 4 units per SPI (U g^{-1}) of microbial transglutaminase (MTGase) increased the tensile strength by 10-20% and decreased the elongation at break and transparency. The MTGase treated films of SPI had a rougher surface and more homogeneous or compact cross-section compared to

the controls. Schmidt and Soldi (2006) reported that addition of polycaprolactone-triol decreased the temperature of thermal degradation and maximum degradation rate from 292 °C (pure SPI films) to 264 °C and 331 °C (pure SPI films) to 280 °C, respectively.

7.6 Milk proteins

Milk proteins can be divided into casein and whey protein. Casein represents 80% of the total milk protein and consist of α-, β-, κ- casein with molecular weights between 19 and 25 kDa. The low cysteine levels in casein result in little disulfide cross-linking and an open random-coil structure. The high praline content results in better emulsifying properties compared to whey protein (Khwaldia et al., 2004). Whey protein comprises 20% of the milk protein and is the protein that remains soluble after casein has been precipitated at pH 4.6. Whey protein includes β-lactoglobulin (M_w = 18 kDa), α-lactalbumin (M_w = 14 kDa), bovine serum albumin (M_w = 18 kDa), immunoglobulins, and proteose-peptones. Whey proteins are globular and heat labile in nature (Krochta, 1997). Audic and Chaufer (2005) studied the effect of plasticizers and crosslinking on the properties of sodium caseinate (NaCAS) films. Film treated with formaldehyde and plasticized with triethanolamine increased the water resistance of films. Increasing the plasticizer content, both glycerol and triethanolamine, from 25% to 100% (w/w) in NaCAS films decreased the maximum tensile stress. Oses et al. (2009) found that 40% glycerol and 50% sorbitol are saturation values for plasticizer content in whey protein isolate films. Glycerol was found to be a more efficient plasticizer than sorbitol. Films plasticized with glycerol were found to be more flexible and weaker and to have higher moisture content than films with the same amount of sorbitol.

8. Some factors affecting properties of protein-based film

8.1 pH

Solubilizing process directly affected the property of film. Protein solubility depends on the pH. The net charge of the protein molecule at any given pH is a consequence of the ionization status of all amino acids. At pI, protein molecules having large dipoles attract themselves through the countercharged domains and tend to coalesce and precipitate. This is a general rule that proteins are least soluble at a pI. At pH above or below the isoelectric point, electrostatic repulsion between protein molecules increased, leading to unfolding of protein molecules. Furthermore, increased ionic strength of film-forming solutions under acidic and alkaline conditions seemed to improve the protein solubility (Lin and Park, 1998). Generally, globular proteins must be denatured by heat, acid, base and/or solvent to form more extended structures that are required for film formation (Krochta, 2002). Quinn et al. (2003) investigated the formation of films from unheated whey protein isolate (WPI) solutions adjusted to pH 11, comparison with films preheated and pH adjustment of 7. Unheated films had significantly lower percent elongation, tensile strength, and Young's modulus than heated films, while the film solubility and dispersion in water of films were higher. Hamaguchi et al. (2007) reported that muscle fish protein films had the increased TS when FFS was adjusted to acidic and alkaline pHs, while no influenced on WVP of fish muscle protein films by the pH value of the film-forming solutions. Chinabhark et al. (2005) compared the pH effect of acidic (pH 3) and alkaline (pH 11) conditions on the properties of bigeye snapper surimi films. Acidic condition rendered the films with the greather EAB with the higher yellowness, while alkaline condition favored the protein degradation. Both conditions had no effect on WVP of the films.

8.2 Temperature and moisture

Proteins are also sensitive to changes in temperature, which can degrade proteins, and moisture, which acts as a plasticizer. High temperatures and low moisture contents can result in protein degradation during thermoplastic processing (Hernandez-Izquierdo and Krochta, 2008). The characteristics of protein-based films are determined by the nature of protein-protein interactions. Film-forming ability may be influenced by amino acid composition, distribution and polarity, ionic cross-links between amino and carboxyl groups, hydrogen bonding groups, and intramolecular and intermolecular S-S bonds (Gennadios and Weller, 1991). The intermolecular forces that promote cohesion in heat-denatured films also involve intermolecular S-S and hydrophobic bond among the unfolded protein stands (Perez-Gago et al. 1999). The degree of protein denaturation and unfolding as heating time and temperature are increased probably affects the degree and nature of protein-protein cross-linking. Perez-Gago and Krochta (2001) found that increasing film-forming solution time and temperature from 70°C to 80°C/20 min could be promote the tensile stress of films (from 5 to 14 MPa, respectively), showed that whey protein films became stiffer, stronger and more stretchable. Oxygen permeability was lower for films made from heat-denatured whey protein than film made from native whey protein. Iwata et al. (2000) who reported that fish water-soluble protein films had the improved mechanical properties by heat denaturization of protein. Thermal treatment temperature between 55ºC and 90 ºC for 15 min increased TS and EAB of film. Garcia and Sobral (2005) reported the films based on film-forming solution (FFS) from muscle proteins (without the stroma proteins) preheated at 65°C for 30 min were more resistant and more rigid than the films treated at 40°C for 30 min. The combined effect of temperature and relative humidity on the functional properties is traditionally

interpreted in terms of disruptive water-polymer hydrogen bonding in a hydrophilic network (Gordtard et al., 1993). Cuq et al. (1997) found that increasing the film water content from 5 to 45 g (g of water per 100 g of dry matter) involved a non-linear decrease in glass transition temperature of fish myofibrillar protein-based films from 63 to 25 °C (for onset temperature) and 78 to 41°C (for end-point temperature). Oses et al. (2009) stored films made from whey protein isolate in cabins at 50% or 75% relative humidity (RH) and at room temperature. Tensile properties of whey protein isolate films after reaching moisture equilibrium at 75% RH for 1 week had lower than films stored at 50% RH. In addition, the exposure of the films to a higher RH made films less stiff due to the water via the plasticizing effect.

8.3 Plasticizers

Plasticizers are generally added to the protein matrix to improve processability and to modify the properties of the final structure. As opposed to internal plasticizers, which are copolymerized or reacted with the polymer, external plasticizers consist of low molecular weight, low volatility substances that interact with the polymer chains producing swelling (Sothornvit and Krochta, 2005). To reduce the brittleness and improve processability of protein-based materials, addition of plasticizers is generally required (Pommet et al., 2005). The plasticizing effect of small polar molecules such as glycerol and water has been described in terms of insertion and positioning within the 3-dimentional protein network. The type and amount of plasticizer used can have an effect on protein reactivity. A variety of common plasticizers were used in edible films such as glycerol, polyethylene glycol (PEG), sorbitol, propylene glycol (PG) and ethylene glycol (EG), monosaccharide disaccharide or oligosaccharide, lipids and their derivatives (Gontard *et al*., 1993). In general, addition of plasticizer level especially polyols, decreased the mechanical

resistance and increased in the elasticity and water vapor permeability of the films. (Pascholiack *et al*., 2003).

Gueguen et al. (1998) used glycerol, triethylene glycol, and tetraethylene glycol as plasticizers to improved the hydrophobicity of films from pea proteins. Sobral et al. (2005) found that the increase of glycerol concentration caused reduction in the color difference of films due to the dilution effect of glycerol. A mechanical property of films from muscle proteins was affected by the plasticizer concentration. Increasing glycerol content from 15% to 65% in muscle protein film decreased tensile strength and puncture force, while increased percent elongation. Vanin et al. (2005) prepared gelatin-based films with 4 different plasticizers (glycerol, ethylene glycol, diethylene glycol and propylene glycol) at various concentrations (10-30 g/100 g gelatin). The glycerol showed the greater plasticizing effect and efficiency on the mechanical properties. The glass transitions temperature of gelatin-based films were shifted to lower temperature with the increase of plasticizer concentration and related to an increase in WVP with consequent increase of free volume of the proteinic network. A study on the influence of plasticizer content on the water sorption isotherms and water vapor permeability of chicken feather keratin films showed that increasing the plasticizer concentration caused a decrease in barrier properties and favored water adsorption by the polymeric network, increasing the moisture content of films (Martelli et al, 2006).

8.4 Cross-linking agents

Protein chains possess reactive side groups which can be potentially modified via chemical, physical or enzymatic cross-linking to enhance the functional properties of protein-based films (Hernandez-Munoz et al., 2004). Aldehyde which bond very quickly to proteins are usually used to cross-link protein, but aldehyde have

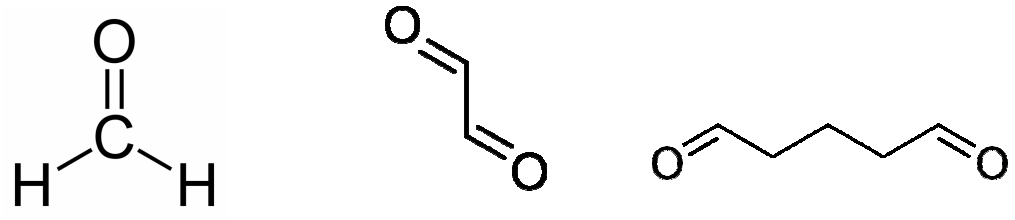
toxicity which may not be tolerable in many fields (Cho et al., 2007). Though some enzyme such as transglutaminase and natural cross-linking agent have been reported as cross-linking agent for protein-based films (Tang et al., 2005; Ou et al., 2005; Chambi and Grosso, 2006; Jiang et al., 2007).

Transglutaminase is a kind of enzyme, which can catalyze the cross-linking reactions between proteins and thus modify the several selective properties of protein films (Tang et al., 2005). Tranglutaminase catalyzes the introduction of ε-(γ-glutamyl)-lysine cross-links into proteins via an acyl transfer reation. The γ-carboxyamide group of glutamine serves as the acyl donor and the ε-amino group of lysine serves as the acyl receptor (Karaishi et al., 2001). The introduction of cross-linkages by the enzyme transglutaminase in the formation films from casein and gelatin was reported by Chambi and Grosso (2005). Transglutaminase induces the formation of high molecular mass polymers, resulting in changes in the mechanical properties and water vapor permeability of the films. Casein-gelatin blend films (especially portion of 75:25) showed a decrease in WVP from 7.17 to 5.06 g mm/m^2 d kPa, while increasing the elongation from 27.23% to 56.79%, after the action of the enzyme. To improve the properties of soy protein isolate(SPI) films, Jiang et al. (2007) investigated the effect of MTGase concentration and some processing parameters, such as pH of the film-forming solution, drying temperature and additional reaction before casting on the modification of SPI films. Increasing enzyme concentration from 4 to 60 U g^{-1} (of SPI) gradually decreased both TS and EAB values. The authors found that TS values increased by about 20-27%, while EAB values decrease by 12-25%, when films prepared at alkaline pH value (pH 8-10).

Aldehyde such as glutaraldehyde, glyoxal or formaldehyde have been used to cross-link proteins (Bigi et al., 2001; Marquie, 2001; Hernandez-Munoz et al.,

2004; de Carvalho et al., 2004). Formaldehyde is the simplest of cross-linking agents which has the broadest reaction specificity. This cross-linking agent contains a single functional group but it can react bifunctionally and therefore crosslink between ε-amino acid of lysine and formaldehyde to form aminomethylol derivatives (Hernandez-Munoz et al., 2004). The reactions are very quick reversible at highly alkaline pH (Means and Feeney, 1968). Glutaraldehyde is more specific than formaldehyde. It can react with lysine, cysteine, histidine and tryrosine (Tae, 1983). Most glutaraldehyde-induced protein cross-linking reaction are carried out al highly alkaline with α,β-unsaturated molecules predominating. The aldehyde functions of these molecules react with primary amines to form imines (Schiff bases) stabilized by resonance (Korn et al., 1972). Protein cross-linking by glyoxal involves lysine and arginine side chain groups at alkaline pH (Marquie, 2001). Bigi et al. (2001) studied the influence of glutaraldehyde concentrations ranging from 0.05 to 2.5 wt% on the stability of gelatin films. In the glutaraldehyde concentration range from 0.1 to 1.0 wt%, the extent of crosslinking increases from 60% to about 100%. A crosslinking degree of about 85%, obtained using 0.25% glutaraldehyde, is enough to prevent gelatin in buffer solution and to provoke a significant reduction of the swelling in physiological solution. Hernandez-Munoz et al. (2004) found that increasing cross-linking agents (glutaraldehyde, glyoxal, and formaldehyde) content from 2 to 8% (g/100 g dry protein) in glutenin-rich films decreased water vapor permeability around 30% and increased tensile strength by 63, 34, and 200%, respectively. Glutaraldehyde and glyoxal could be improve the mechanical properties of films but gave an undesirable yellow brownish coloration. de Carvalho and Grosso (2004) reported the melting points of gelatin based films incorporated with formaldehyde and glyoxal increased from 65.06°C (native film) to 77.4 and 87.09°C, respectively. SEM

images of the internal structures of films modified with formaldehyde or glyoxal shows a loss of fibillar orientation with an apparent decrease in free volume, while an orientation in the form of fibers with the presence of discontinuous zones was observed in the native film.



Formaldehyde Glyoxal Glutaraldehyde

Figure 2 Comparison of chemical structure of formaldehyde, glyoxal and glutaraldehyde

Polyphenol-protein cross-linking reactions (Strauss and Gibson, 2004)

1. The diphenol moiety of a phenolic acid or other polyphenol is readily oxidized to an orthoquinone by enzymatic in plant tissue or by molecular oxygen.

2. The quinone forms a dimmer in a side reaction or reacts with amino or sulfhydryl side chains of polypeptide to form covalent C-N or C-S bonds with the phonolic ring, with regeneration of hydroquinone.

3. The latter can be reoxidized and bind a second polypeptide, resulting in a cross-link.

4. Alternatively, two quinines that each carrying one chain can dimerize and producing a cross-link.

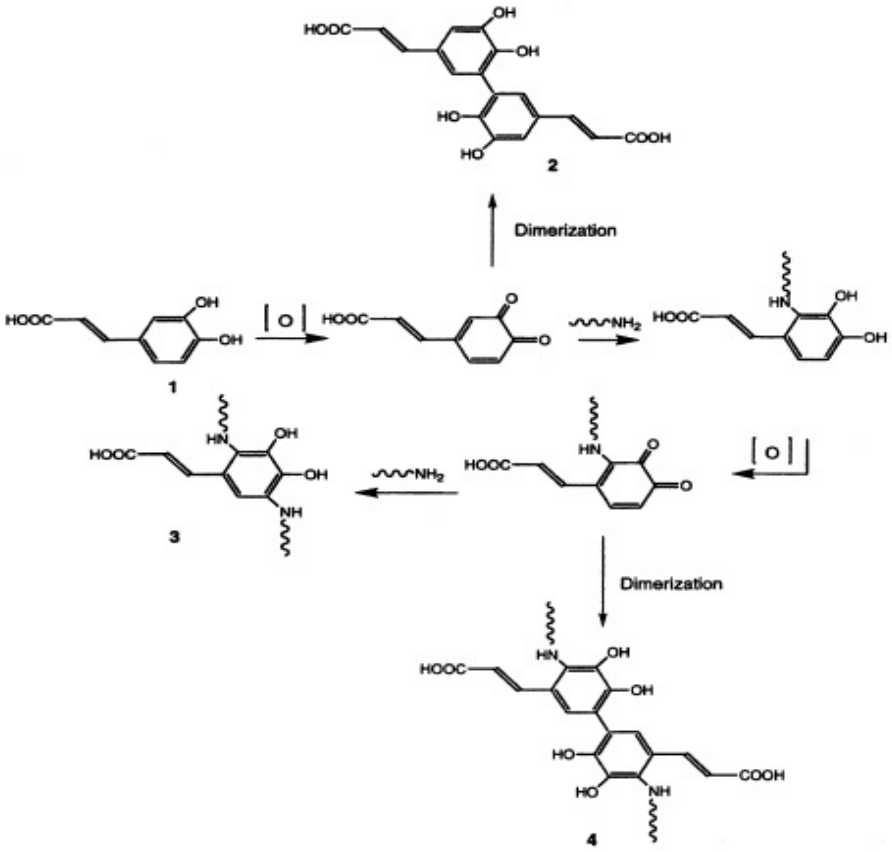
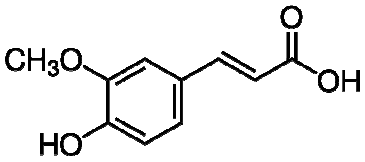


Figure 3 Reaction of phenolic acid with amino side chain of polypeptide

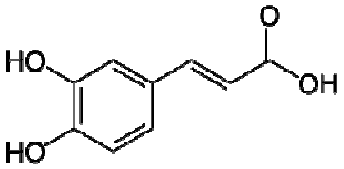
Source: Strauss and Gibson (2004)

The plant phenols are aromatic secondary metabolites that embrace a considerable range of substances possessing an aromatic ring bearing one or more hydroxy substituents (Ryan and Robards, 1998). Ferulic acid is a ubiquitous phenolic acid of low toxicity in the plant kingdom, it has such functions as antioxidant, antimicrobial, anti-cancer, lowers cholesterol, cross-linking activities etc. (Cao et al., 2007). Ferulic acid and its oxide can react with some amino acids in proteins such as tyrosine, lysine, cysteine and cross-link protein molecules (Ou and Kwok, 2004). Tannic acid is a typical hydrolysable tannins which obtained from galls of Rhus chinensis. It has in common multiple phenolic functionalities, high molecular weights, and the ability to bind protein and to act as antioxdants (Henson et al., 2004). Caffeic

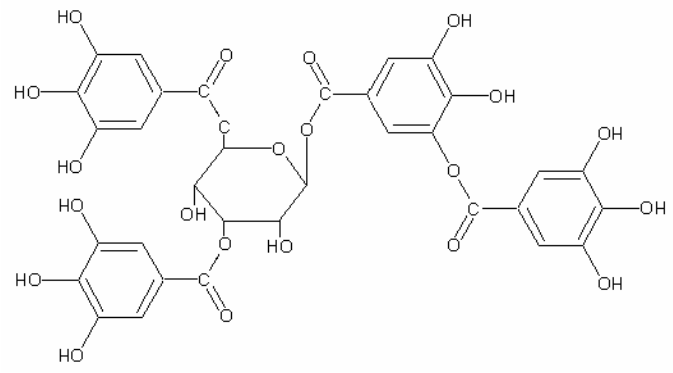
acid (3,4-dihydroxycinnamic acid) is one of the most common phenolic acids. It has antioxidant, antitumor and anti-inflammatory properties (Jiang et al., 2005).



Ferulic acid



Caffeic acid



Tannic acid

Figure 4 Comparison of chemical structure of ferulic acid, caffeic acid and tannic acid

Orliac et al. (2002) found that plant tannins gave films produced from sunflower protein isolate with properties similar to those obtained with aldehydes, but without the toxicity. Different concentrations of ferulic acid were added to film-forming solutions when preparing soy protein isolate-based edible films by Ou et al. (2005). Increasing ferulic acid content from 0 to 150 mg/100 g of film-forming solution increased tensile strength of films from 1.472 to 1.638 MPa, while decreased tensile strength when the level of ferulic acid was increased to 200 mg/100 g. The oxidized ferulic acid quinone is much greater than that of its native ferulic acid form in decreased the oxygen permeability of film. Adding 100 mg/100 g ferulic acid to SPI film forming solution decreased the permeabilty of O_2, CO_2 and N_2 by 18.8%, 18.1% and 14.9% respectively. Cao et al. (2007) compared the cross-linking effects of ferulic acid and tannic acid concentrations and pH values of film-forming solution on the mechanical properties of gelatin films. Tensile strength (TS) of films incorporated with ferulic acid (10 mg/g gelatin) increased from 85.51 to 95.61 MPa, while TS of tannic acid (40 mg/g gelatin)-modified films increased only to 88.26 MPa. The

maximum tensile strength value of ferulic acid cross-linked gelatin films was obtained at pH 7. As for tannic acid treated films, the maximum value was found at pH 9.

9. Application of protein-based films to extent shelf life of food products

Nowadays, vacuum packagings, nitrogen sweep before packing, metallized films, antioxidants, etc., are some of the different methods used in the food industry to increase the shelf life of foods with a high content of PUFA. The use of edible coating and biodegradable films has become an alternative (Krochta, 1997). Lipid oxidation or oxidative rancidity is one of the main causes of decay in foods rich in polyunsaturated fatty acids (PUFA) leading to the appearance of strange flavors and smells which make these food unacceptable to customers and shortens their shelf life (Baker et al., 1994).

Edible/biodegradable wheat gluten, soy protein, carrageenan and chitosan films and coating were used on precooked beef patties. Wu et al. (2000) reported carrageenan film decreased moisture loss but not as effective as polyvinyl chloride film. Wheat gluten, soy protein, and carrageenan coating and carrageenan film reduced thiobarbituric acid-reactive substances and hexanal values compared to unpackaged patties. Wheat gluten, soy protein and chitosan films were not effective in controlling lipid oxidation. Whey protein isolate (WPI) films acting as oxygen barrier can be used to delay lipid oxidation in foods with high content in poly unsaturated fatty acid (PUFA). Oses et al. (2008) found that WPI films delayed the rancidity in vegetable oil. Films with sorbitol were more effective than films with glycerol. Both plasticizer content and film thickness affected its protective capacity. The thickest films with the lowest plasticizer content provided the greatest protection against lipid oxidation. Films provided much better protection when working at lower RH. Liu et al. (2007) compared the potential for using pectin (containing 2.5% and 5% corn oil

and olive oil) and gelatin/sodium alginate blend-based (containing 2.5% corn oil and olive oil) casings in the manufacture of breakfast pork sausages. Increasing storage time from days 2 to 6, sausage meat stuffed into gelatin/sodium alginate blend castings had higher water content compared to sausage meat held in pectin castings. Gelatin/sodium alginate blend castings exhibited a greater ability to enhance product brightness but were poor in maintaining product redness while the reverse was true for pectin casting. Casing manufactured using corn oil had more desirable color attributes than those using olive oil. Lipid oxidation developed in both sausages by casting with pectin or gelatin/sodium alginate blend as storage time increased. Sausages held in gelatin/sodium alginate blend castings had lower TBARS compared to those held in pectin casting.

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CHAPTER 2

MATERIALS AND METHODS

1. Chemicals / materials

1.1 Chemical reagents

Sodium azide, sodium dodecylsulfate (SDS), β-mercaptoethanol (βME), tannic acid and caffeic acid were purchased from Sigma (St. Louis, MO, USA). Sodium citrate, glycerol and malondialdehyde tetraethylacetal were obtained from Merck (Darmstadt, Germany). Acrylamide, *N,N,N′,N′*-tetramethylethylenediamine (TEMED), bis-acrylamide were obtained from Fluka (Buchs, Switzerland). 2-thiobarbituric acid was purchased from Sigma-Aldrich (Vienna, Austria). Trichloroacetic acid was obtained from Riedel-dehaeun (Seelze, Germany).

Formaldehyde (37% solution) and glutaraldehyde (25% solution) with the laboratory grade were supplied from Fluka (Buchs, Germany). Glyoxal (40% solution) was purchased form Fluka (Steinheim, Switzerland). Ferulic acid, α-chymotrypsin and pepsin were procured from Sigma (Steinheim, Germany).

1.2 Raw materials

Porcine blood was obtained from a slaughterhouse of Hat Yai municipality, Songkhla province, Thailand. Sodium citrate solution was added as an anticoagulant to obtain the final concentration of 3.8% (w/v). The sample was kept in ice and transported to the Department of Food Technology, Prince of Songkla University, within 1 h.

2. Instruments

Instruments	Model	Company/Country
Refrigerated centrifuge	RC-5B plus	Sorvall, California, USA
pH meter	CG 842	Schott, Mainz, Germany
Homogenizer	T25 basic	IKA labortechnik, Selangor, Malaysia
Magnetic stirrer	Ro 15 power	IKA labortechnik, Stanfen, Germany
Electrophoresis apparatus	Mini-Protean II	Bio-Rad Laboratory Inc., California, USA
Micrometer	GT-313-A	Gotech testing machines Inc., Taichung, Taiwan
Universal testing machine	LR 30 K	LLOYD Instruments Ltd., Fareham, England
Environmental chamber	KBF 115	WTB Binder, Tuttlingen, Germany
Spectrophotometer	UV-16001	Shimadzu, Kyoto, Japan
CIE colorimeter	Color Flex	HunterLab Reston, Virginia, USA
Attenuated total reflectance-fourier transform infrared (ATR-FTIR) spectrometer	Equinox 55	Bruker Co., Ettlingen, Germany
Thermo-gravimetric analyzer	TGA7	Perkin Elmer, Norwalk, CT, USA
Differential scanning calorimeter	DSC 7	Perkin Elmer, Michigan, USA

Scanning electron microscope	JSM-5800LV	JEOL, Tokyo, Japan

3. Preparation of porcine plasma protein

Upon arrival, blood was dialyzed against 10 volumes of cold distilled water with 5 changes of dialysis medium. The dialysate was then centrifuged at 1,000xg for 30 min at 4°C using a refrigerated centrifuge (Sorvall®, Model RC-B Plus centrifuge Newtown, CT, USA) to separate plasma from blood cell. The plasma obtained was lyophilized using a freeze drier (Model Dura-Top™ μP/Dura Dry™ μP, FTS® System, Inc., Stone Ridge, New York, USA) and the plasma powder was stored at -20°C until use.

4. Compositional analysis of porcine plasma protein

Moisture and ash contents of porcine plasma protein powder were measured (AOAC, 1999). The plasma powder was determined for protein content using Kjeldahl method with the method No. of 940.25 (AOAC, 1999). Protein patterns were determined by SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970).

5. Effect of protein and glycerol contents on the properties of porcine plasma protein-based films

5.1 Preparation of film-forming solution

Plasma powder was added with distilled water to obtain the final protein concentration of 2 and 3% (w/v). Protein content of plasma powder determined by Kjeldahl method (AOAC,

1999) was 80.99 ± 0.69%. The mixture was stirred for 30 min at room temperature (28-30°C). Glycerol was added at 50, 60 and 70% (w/w) of protein content as a plasticizer. The solution obtained was referred to as film-forming solution (FFS).

5.2 Film casting and drying

FFS (5 g) was cast onto a rimmed silicone resin plate (50x50 mm) and air-blown for 12 h at room temperature. The film was dried in an environmental chamber (WTB Binder, Tuttlingen, Germany) at 25°C and 50% relative humidity (RH) for 24 h. Finally, films were manually peeled off and used for analyses.

5.3 Determination of film properties

5.3.1 Film thickness

The thickness of film was measured using a micrometer (Gotech, Model GT-313-A, Gotech testing machines Inc, Taichung, Taiwan). Nine random locations around each film of nine film samples were used for thickness determination.

5.3.2 Mechanical properties

Prior to testing, films were conditioned for 48 h at 50±5% relative humidity (RH) at 25°C. Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata *et al*. (2000) with a slight modification using the Universal Testing Machine (Lloyd

Instrument Ltd., Hampshire, UK). Five samples (2x5 cm) with initial grip length of 3 cm were used for testing. Cross-head speed was set at 30 mm/min.

5.3.3 Water vapor permeability (WVP)

WVP was measured using a modified ASTM method (American Society for Testing and Materials, 1989) as described by Shiku *et al*. (2004). The film was sealed on an aluminum permeation cup containing silica gel (0% RH) with silicone vacuum grease. A rubber gadget was placed around the cup to hold the film in place. The cup was placed at 30°C in a desiccator containing the distilled water. The cup was weighed at 1 h intervals over a 10 h period. WVP of the film was calculated as follows:

$$\text{WVP (g m}^{-1}\text{s}^{-1}\text{ Pa}^{-1}) = wlA^{-1}t^{-1}(P_2\text{-}P_1)^{-1}$$

where w is the weight gain of the cup (g); l is the film thickness (m); A is the exposed area of film (m^2); t is the time of gain (s); (P_2-P_1) is the vapor pressure difference across the film (Pa). Five films were used for WVP testing.

5.3.4 Color and film transparency

Color of film was determined using a CIE colorimeter (Hunter associates laboratory Inc., Reston, VA, USA) and expressed as L*-, a*- and b*-values. The transparency of film was calculated by the following equation (Han and Floros, 1997):

$$\text{Transparency value} = -\log T_{600}/x$$

where T_{600} is the fractional transmittance at 600 nm and x is the film thickness (mm). The greater value represents the lower transparence.

5.3.5 Film solubility and protein solubility

Film solubility was determined according to the method of Gennadios, *et al*. (1998). The conditioned film samples (2x4 cm) were weighed and placed in 50 ml centrifuge tube containing 10 ml of distilled water with 0.1% (w/v) sodium azide. The mixture was shaken at a speed of 250 rpm using a shaker (Heidolph Incubator 1000, Schwabach, Germany) at 30°C for 24 h. Undissolved debris was removed by centrifugation at 3,000x*g* for 20 min. The pellet was dried at 105°C for 24 h and weighed. The weight of solubilized dry matter was calculated by substracting the weight of unsolubilized dry matter from the initial weight of dry matter and expressed as the percentage of total weight.

To determine the protein solubility, the protein concentration in the supernatant was determined using the Biuret method (Robinson and Hodgen, 1940). Protein solubility was expressed as the percentage of total protein in the film, which was solubilized with 0.5 M NaOH at 30°C for 24 h.

5.3.6 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of porcine plasma powder and the films were determined by SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). Porcine plasma powder (0.5 g) was solubilized in 9 ml of 5% SDS. The mixture was stirred for 10 min to completely solubilize the proteins. To solubilize the films prior to SDS-PAGE analysis, films were mixed with 20 mM Tris HCl (pH 8.8) containing 2% SDS and 8 M urea in the presence and the absence of 2% βME. The mixture was homogenized at 13,000 rpm for 1 min

(IKA Labortechlink homogenizer, Selangor, Malaysia). The homogenate was stirred continuously for 24 h at room temperature (28-30°C). Then, the sample was centrifuged at 7,500x*g* for 10 min at room temperature using a Biofuge primo Centrifuge (Sorvall, Hanau, Germany). Proteins (15 μg) determined by the Biuret method (Robinson and Hodgen, 1940) were loaded onto the gel and subjected to electrophoresis at a constant current of 15 mA per gel using a Mini-Protean II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 12 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h.

6. Effect of pH levels of FFS on the properties of porcine plasma protein-based films

FFS containing protein at 3% (w/v) and glycerol at 70% (w/w) of protein was adjusted to different pHs (2-11) using 1 M HCl or 1 M NaOH. FFS obtained was subjected to casting and drying as previously described. All films were analyzed as described in section 5.3.1-5.3.3. pH of FFS, which yielded the film with the highest mechanical properties, was used for further study.

7. Effect of heating temperatures of FFS on the properties of porcine plasma protein-based films

Protein solution (3% w/v) was preheated at 40, 55 and 70 ºC for 30 min. The solution was then cooled with running water for 10 min. Glycerol was added at 70% (w/w) of protein content. The pH of solution was adjusted to 3 and 10 with 1 M HCl or 1 M NaOH. Thereafter, the

resulting FFS was cast and dried as previously described. All films were analyzed as described in section 5.3.1-5.3.3. The film obtained from this section with the highest mechanical properties was used for further analyses.

8. Effect of aldehyde compounds on the properties of films

Formaldehyde, glutaraldehyde and glyoxal at different concentrations (1, 2 and 3% (w/w) of protein content) were added into FFS. The pH of solution was adjusted to 10 with 1 M HCl or 1 M NaOH, followed by heating at 70°C for 30 min. FFS obtained was subjected to casting as previously described. Film without addition of aldehyde compounds was used as the control. All films were analyzed as described in sections 5.3.1-5.3.4. The film sample obtained from this section with the highest mechanical properties was used for further analyses.

9. Effect of phenolic compounds on the properties of films

9.1 Effect of types and concentration of phenolic compounds

Tannic acid, caffeic acid and ferulic acid at different concentrations (1, 2 and 3% (w/w) of protein content) were added into FFS (section 8). The pH of solution was adjusted to 10 with 1 M HCl or 1 M NaOH. FFS obtained was subjected to casting as previously described. The control film was prepared from FFS (pH 10) preheated at 70°C for 30 min without addition of phenolic compounds. All films were analyzed as described in sections 5.3.1-5.3.4. The film samples obtained from this section with the highest mechanical properties was used for further analyses.

9.2 Effect of pH and oxygenation on the properties of film incorporated with phenolic compound

FFS was mixed with 3% caffeic acid (based on protein content). FFS containing caffeic acid was adjusted to different pHs (7-10) using 1 M HCl or 1 M NaOH. FFS of each pH was separated into two portions; one portion was oxygenated for 30 min, while another portion was not oxygenated. Thereafter, the resulting FFS was cast and dried. All films were analyzed as described in sections 5.3.1-5.3.4. The film sample obtained from this section with the highest mechanical properties was used for further analyses.

10. Characterization of porcine plasma protein-based films

Plasma powder was added with distilled water to obtain the final protein concentration of 3% (w/v) and stirred for 30 min at temperature of 28-30°C**.** The solution was added with glycerol at a level of 70% (w/w) of protein content as a plasticizer. Film forming solution (FFS) (5 g) was cast onto a rimmed silicone resin plate (50x50 mm), air-blown and dried as previously described. Finally, film was manually peeled off and was referred to as 'control film'.

Another portion of plasma solution (3%, w/v) was preheated at 70°C for 30 min, following by cooling using running water for 10 min. The pH of solutions was then adjusted to 10 with 1 M NaOH. Glycerol was added at 70% (w/w) of protein content as a plasticizer. FFS obtained was subjected to casting as previously described and the resulting film was referred to as 'pretreated film'.

10.1 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) (as described in section 5.3.6)

10.2 Hydrolysis by protease

Hydrolysis of film by protease was determined according to the method of Yildirim and Hettiarachchy (1998) with a slight modification. Ground film samples (0.25 g) were suspended in 50 ml of ∝-chymotrypsin solution (40 μg/ml in 40 mM Tris-HCl buffer, pH 7.6) or pepsin solution (40 μg/ml in Mcllvaine buffer, pH 2.0). The suspension was then incubated at 37°C for 2 h. At 0, 30, 60, 90 and 120 min, the samples (2.5 ml) were taken and 0.5 ml of cold 50% (w/v) trichloroacetic acid (TCA) was added to terminate the enzymatic reaction. The mixture was kept at 4°C for 18 h and centrifuged at 7,500x*g* for 30 min. Peptide and amino acid content in the supernatant was determined by the Biuret method (Robinson and Hodgen, 1940).

10.3 Solubility of film in various solvents

Solubility of films in various solvents was determined as described by Chawla *et al*. (1996) with some modifications. The solvents used included 20 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS (S1), 20 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS and 8 M Urea (S2) and 20 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS, 8 M Urea and 2% (v/v) βME (S3)

Film samples (0.4 g) were homogenized in various solvents at a speed of 13,000 rpm for 1 min using a homogenizer (IKA Labortechnik, Selangor, Malasia). The homogenate was

heated in boiling water (100°C) for 2 min and stirred at room temperature for 4 h. The resulting homogenate was centrifuged at 7,500x*g* for 30 min using a microcentrifuge (MIKRO20, Hettich Zentrifugan, Germany). The supernatant (10 ml) was added with 2.6 ml of cold 50% (w/v) trichloroacetic acid (TCA) to precipitate the proteins. The mixture was kept at 4°C for 18 h and centrifuged at 7,500x*g* for 30 min. The precipitate was washed with 10% TCA and solubilized in 10 ml of 0.5 M NaOH. The protein content was determined using the Biuret test (Robinson and Hodgen, 1940). To obtain the total amount of protein in the films, films were solubilized in 0.5 M NaOH. The solubility was reported as the percentage of total protein.

10.4 Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy

Prior to analysis, films were conditioned in a desiccator containing silica gel for 7 days at room temperature to obtain the most dehydrated films. FTIR spectra of films incorporated with glyoxal or caffeic acid with and without oxygenation were recorded using a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI) equipped with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) at room temperature. For spectra analysis, film sample was placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra in the range of 400-4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were rationed against a background spectrum recorded from the clean

empty cell at 25°C. The spectra obtained were used to determine possible interactions of functional groups between porcine plasma protein molecules with glyoxal or caffeic acid.

10.5 Differential scanning calorimetry (DSC)

Transition temperature (T_m) and the respective enthalpy (ΔH_m) were determined by a differential scanning calorimeter (DSC7, Perkin Elmer, Norwalk, CT, USA) as described by Bertan *et al*. (2005). The instrument was calibrated using Indium as a standard. Films were conditioned over silica gel at 25°C for 3 weeks before testing. Dry samples (2-5 mg) were placed in a hermetically sealed aluminum pan and heated at 10°C/min between 20 and 200°C. The maximum peak temperature of the endotherm was taken as the transition temperature or melting point.

10.6 Thermo-gravimetric analysis (TGA)

Prior to testing, films were conditioned over silica gel at 25°C for 3 weeks. Prepared films were scanned using a thermo-gravimetric analyzer (TGA7, Perkin Elmer, Norwalk, CT, USA) from room temperature to 800°C at a rate of 10°C/min (Lodha and Netravali, 2005). Nitrogen was used as the purge gas at a flow rate of 20 ml/min. The temperature at which slope of the weight loss versus temperature curve started to increase was considered as the temperature of the degradation (Td) phenomenon.

10.7 Film morphology

Morphologies of surface and cross-section of the film samples without and with 3% caffeic acid incorporated were visualized using a scanning electron microscope (SEM) (JSM-5800LV, JEOL, Tokyo, Japan) at an accelerating voltage of 10 kV. Prior to surface morphology visualization, the film samples were mounted on brass stub and sputtered with gold in order to make the sample conductive, and photographs were taken at 7,500x magnification. For cross-section, fractured films were mounted around stubs using double sided adhesive tape, then coated with gold and observed at the same magnification.

11. Uses of porcine plasma protein-based film for keeping dried fish powder

11.1 Preparation of bags from porcine plasma protein-based films

Porcine plasma protein-based film and porcine plasma protein-based film incorporated with 3% caffeic acid with prior oxygenation were prepared as previously described. To prepare the bag, two films were sealed for three sides using the hand sealer (AIE-500 Impulse Hand Sealer, AIE, USA).

11.2 Preparation of fish powder

Mackerel muscle was washed and filleted. The fillets were subjected to drying using a hot-air oven with the air velocity of 1.5 m/s at 60°C for 8 h. The dried fish were powderized using the blender until the uniformity was obtained.

11.3 Changes of quality of fish powder packaged in porcine plasma protein bags during storage

Fish powder (5 g) was transferred to the different film bags with the size 50x50 mm. These film bags were sealed with the hand sealer (AIE-500 Impulse Hand Sealer, AIE, USA). Polyethylene bag was also used to pack the samples. The samples were stored at 28-30°C at 60±10% relative humidity (RH). The samples were taken every 3 days for analyses up to 24 days.

11.4 Analyses

-Moisture content of fish powder was determined according to the method of AOAC (1999).

-Thiobarbituric acid-reactive substances (TBARS) of fish powder were determined as described by Buege and Aust (1978). Samples (0.1 g) were homogenized with 9.9 ml of TBARS solution (0.375% TBA, 15% TCA, and 0.25 N HCl). The mixture was heated for 10 min in boiling water (95-100°C) to develop a pink color. Then the mixture was cooled with running water and centrifuged at 3,600x*g* for 10 min. The absorbance of the supernatant was measured at 532 nm, using a model UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan). TBARS value was calculated from the standard curve of malonaldehyde (0-50 mg/L) and expressed as mg malonaldehyde/ 100 g sample.

- Color of the fish powder was reported as L* a* and b* as previously described in section 5.3.4.

12. Statistical analysis

A factorial design was used in the experimental sections 5, 6, 7, 8 and 9. Complete randomized design (CRD) was used throughout the study. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

CHAPTER 3

RESULTS AND DISCUSSION

1. Chemical compositions of porcine plasma protein

Porcine plasma contained 4.37 $\pm$ 0.09% moisture, 80.99 $\pm$ 0.69% protein and 16.34 $\pm$ 1.03% ash contents. Electrophoretic study revealed that albumin (65 kDa) and globulin (56 kDa) constituted as the major proteins in porcine plasma powder when determined under reducing condition (Figure 5). Albumin and globulin were the dominant proteins in porcine plasma (Howell and Lawrie, 1983). Under non-reducing condition, proteins with apparent MW of 58, 60, 84 and 122 kDa were noticeable. The result suggested that disulfide bond most likely played a role in stabilizing porcine plasma protein, mainly tertiary structure.

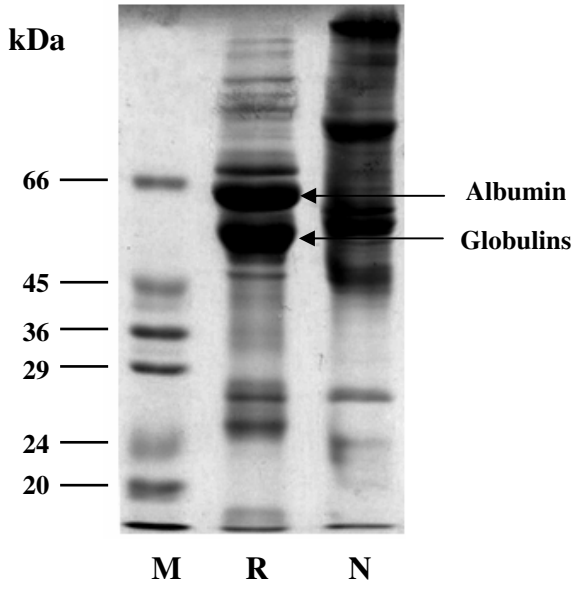

Figure 5. Protein pattern of porcine plasma protein powder under reducing (R) and non-reducing (N) condition. M : protein marker.

2. Effect of protein and glycerol contents on the properties of porcine plasma protein-based films

2.1 Thickness

Generally, a higher thickness was found in films prepared from FFS containing 3% protein, compared with those from 2% protein counterpart ($p<0.05$) (Table 4). At 2% protein content, no differences in thickness were found among films containing different levels of glycerol ($p>0.05$). For films prepared from FFS with 3% protein, that incorporated with 70% glycerol exhibited the highest thickness ($p<0.05$). In the presence of sufficient plasticizer, the aggregation of protein molecules became lessen. As a consequence, entangled and random structure was most likely formed and associated with the protruded network. This was evidenced by the increase in film thickness.

2.2 Mechanical properties

At the same glycerol content, tensile strength (TS) of films prepared from FFS with 3% protein was greater than that prepared from FFS containing 2% protein ($p<0.05$) (Table 4), except for films added with 70% glycerol, which similar TS was obtained between both protein contents. Among all films tested, that containing 3% protein together with 50 or 60% glycerol had the highest TS (2.11 and 2.48 MPa). Proteins at the higher amount might aggregate intermolecularly to a greater extent, compared with the lower amount, resulting in the stronger interaction as evidenced by the higher TS. Elongation at break (EAB) of films prepared at 2% or 3% protein increased when the plasticizer content increased ($p<0.05$). Film prepared at 3% protein and 70% glycerol had the greatest EAB (18.33%). Glycerol at a higher level decreased the protein-protein interactions, resulting in the increased mobility of polypeptide chains. This allowed the films less resistant and more extensible. The reduction of TS and the tendency of increasing EAB with the increase of glycerol content are typical behaviors of protein-based films (Gontard *et al*., 1993; Cuq *et al*., 1995).

2.3 Water vapor permeability

Water vapor permeability (WVP) of films from FFS containing 3% protein and glycerol (60 or 70%) was higher than that of other films ($p<0.05$) (Table 4). A higher amount of protein was probably associated with a higher amount of polar groups in

porcine plasma, which could absorb more water from the surrounding atmosphere. Due to the hydrophilic nature of glycerol used as a plasticizer, film containing a greater amount of glycerol became more hydrophilic. This resulted in the higher WVP of resulting film. Usually, the increase of plasticizer concentration causes an increase in WVP of hydroscopic films, due to the reorganization of the protein network, with consequent increase of free volume (Cuq *et al*., 1997). However, Vanin *et al*. (2005) found no differences in WVP of gelatin-based film containing different levels of glycerol (10-30%).

Table 4. Effect of protein and glycerol contents on thickness, mechanical properties and water vapor permeability of porcine plasma protein-based films.

Protein (w/v) : Glycerol (w/w)	Thickness[#] (mm)	TS[#] (MPa)	EAB[#] (%)	WVP[#] ($x10^{-10}gm^{-1}s^{-1}Pa^{-1}$)
2% : 50%	$0.046\pm0.005^{c\Delta}$	1.44 ± 0.11^{c}	4.16 ± 1.02^{d}	1.97 ± 0.106^{b}
2% : 60%	0.045 ± 0.003^{c}	1.85 ± 0.42^{b}	5.09 ± 0.88^{cd}	2.07 ± 0.09^{b}
2% : 70%	0.045 ± 0.005^{c}	1.54 ± 0.14^{c}	12.50 ± 1.33^{b}	2.08 ± 0.05^{b}
3% : 50%	0.059 ± 0.008^{b}	2.11 ± 0.19^{a}	3.79 ± 1.18^{d}	2.16 ± 0.35^{b}
3% : 60%	0.062 ± 0.007^{b}	2.48 ± 0.53^{a}	9.31 ± 0.52^{c}	2.68 ± 0.25^{a}
3% : 70%	0.066 ± 0.008^{a}	1.77 ± 0.36^{bc}	18.33 ± 1.56^{a}	2.67 ± 0.17^{a}

[#]Mean ± SD from five determinations.
[Δ]The different superscripts in the same column indicate the significant differences ($p<0.05$).

2.4 Color and transparency

L*-, a*- and b*- values of porcine plasma protein-based films prepared from FFS containing different concentrations of proteins and plasticizers are shown in Table 5. Film with 2% protein and 70% glycerol had the greater L*-value but lower b*-values than other films ($p<0.05$). L*-value of film with 2% protein increased with increasing glycerol content ($p<0.05$). However, no differences in L*-value was noticeable in film containing the higher protein content (3%) as glycerol content increased ($p>0.05$). For b*-value, the decrease in b*-value was found in both films containing 2% and 3% protein when glycerol at 70% was used ($p<0.05$). At the same protein content, films incorporated with 70% glycerol were more transparent than those with the lower glycerol contents ($p<0.05$). The decrease in protein interaction caused by the presence of plasticizer might enhance the light transmission through the

film. Film containing higher protein content (3%) had the higher thickness, in which light could not be transmitted effectively.

Table 5. Effect of protein and glycerol contents on color and transparency of porcine plasma protein-based films.

Protein (w/v) : Glycerol (w/w)	L*[#]	a*[#]	b*[#]	Transparency[#]
2% : 50%	87.16±0.33$^{c\Delta}$	-2.37±0.04^{a}	7.47±0.13^{b}	1.46±0.03^{a}
2% : 60%	87.94±0.27^{b}	-2.47±0.04^{b}	7.37±0.10^{b}	1.45±0.02^{a}
2% : 70%	88.44±0.08^{a}	-2.39±0.02^{a}	5.73±0.14^{c}	1.41±0.01^{b}
3% : 50%	86.95±0.33^{c}	-2.67±0.04^{c}	8.32±0.16^{a}	1.39±0.01^{b}
3% : 60%	87.19±0.11^{c}	-2.75±0.06^{d}	8.37±0.15^{a}	1.30±0.01^{c}
3% : 70%	87.31±0.15^{c}	-2.76±0.03^{d}	7.11±0.45^{b}	1.25±0.01^{d}

[#]Mean ± SD from five determinations.
[Δ]The different superscripts in the same column indicate the significant differences ($p<0.05$).

2.5 Film solubility and protein solubility

Film solubility and protein solubility of porcine plasma protein-based film containing different protein and glycerol contents are shown in Table 6. At the same protein content, no differences in film solubility and protein solubility were found in films with all levels of glycerol used ($p>0.05$). In general, albumin and globulin were the major protein components, which are water soluble in nature. Additionally, glycerol was the hydrophilic plasticizer. Very high protein and film solubility of porcine plasma protein-based film suggested that proteins were interacted each other or with plasticizer by weak bonds, such as hydrogen bond, hydrophobic or ionic interaction, which were easy to break down. This contributed to the high solubility of those films in water.

2.6 Protein pattern of protein-based film

Protein patterns of films containing different protein and glycerol contents are shown in Figure 6. Under either reducing or non-reducing condition, protein patterns of all films were similar to those of porcine plasma powder (Figure 5), in which albumin (65 kDa) and globulin (56 kDa) constituted as the major proteins. The result indicated that the bonds stabilizing the film matrix were mainly weak bonds including

ionic interaction, hydrogen bond and hydrophobic interaction, which were destroyed by SDS and urea. Furthermore, intermolecular cross-linking of protein via disulfide bond and non-disulfide covalent bond was negligible. This was reconfirmed by the high solubility of film (Table 6). Covalent cross-links were more likely less soluble in the water.

Table 6. Effect of protein and glycerol contents on film solubility and protein solubility of porcine plasma protein-based films.

Protein (w/v) : Glycerol (w/w)	Film solubility[#] (%)	Protein solubility[#] (%)
2% : 50%	$96.98\pm0.49^{b\Delta}$	$97.57\pm0.88^{a\Delta}$
2% : 60%	97.61 ± 0.41^{ab}	97.74 ± 1.79^{a}
2% : 70%	97.93 ± 0.91^{ab}	97.97 ± 0.58^{a}
3% : 50%	97.50 ± 0.43^{ab}	97.61 ± 1.65^{a}
3% : 60%	97.80 ± 0.49^{ab}	97.66 ± 1.44^{a}
3% : 70%	98.74 ± 0.65^{a}	98.72 ± 0.38^{a}

[#]Mean ± SD from five determinations.
[Δ]The different superscripts in the same column indicate the significant differences ($p<0.05$).

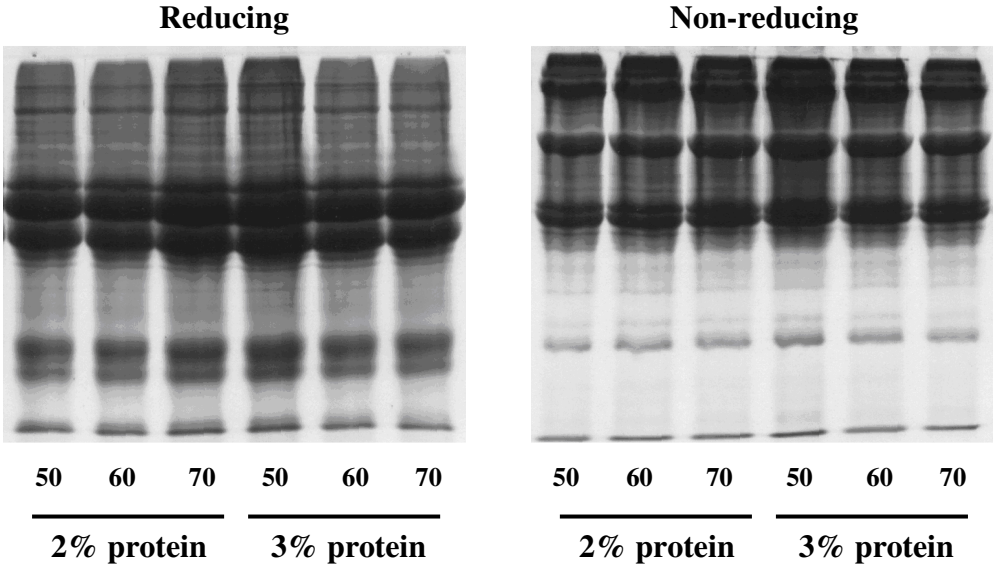

Figure 6. Protein patterns of porcine plasma protein film, prepared with different protein and glycerol contents under reducing and non-reducing condition. Numbers designated glycerol content (% (w/w) of protein).

3. Effect of pH levels of FFS on the properties of porcine plasma protein-based films

3.1 Mechanical properties

TS and EAB of porcine plasma protein-based films prepared from FFS with various pHs are shown in Table 7. The lowest TS of films were observed at pHs 6 and 7 and the lowest EAB was found at pH 7. TS became higher when FFS was in acidic and alkaline pH ranges. Similar result was found for EAB, in which EAB increased continuously with decreasing or increasing pHs. However, slight decrease in EAB was noticeable at pH 11 ($p<0.05$). Hamaguchi *et al*. (2007) reported that muscle fish protein films had the increased TS when FFS was adjusted to acidic and alkaline pHs. At pH above or below the isoelectric point, electrostatic repulsion between protein molecules increased, leading to unfolding of protein molecules. Stretched protein molecules underwent interaction with more interjunction zone. This resulted in the increases in both TS and EAB. Additionally, the unfolding of proteins induced the exposure of hydrophobic domain, in which subsequent interaction between proteins via hydrophobic interaction could be enhanced. The charged residues of protein molecules at very acidic or alkaline pHs also were able to form ionic interaction between protein chains. TS of porcine plasma protein film prepared at pH 3 had the highest value ($p<0.05$), whereas the greatest EAB was obtained for film prepared at pH 10 ($p<0.05$).

3.2 Water vapor permeability

WVP of porcine plasma protein-based films prepared from FFS with different pHs is presented in Table 7. WVP of films prepared at pH 7 was highest and decreased slightly when the pH value was in the ranges of 5-6 ($p<0.05$). No differences in WVP were observed between films prepared at pH 2 to 4 and 8 to 11. Salaman and Williamson (1971) reported that the pI of plasma bovine albumin was 4.86 and the isoelectric region of γ-globulin was 6.60 (Deval *et al*., 2004). The decrease in WVP was found in film prepared from FFS with acidic or alkaline pHs, in which proteins unfolded to a larger extent. As a result the interaction between protein molecules occurred more effectively and the more compact and stronger film network could be found. The charged residues might interact together, leading to the neutralization of charges. This could lower WVP of film.

3.3 Thickness and other characteristics

Thickness of films varied slightly with pHs. It ranged from 0.060 to 0.068 mm. pHs of FFS had no impact on film and protein solubility as well as protein patterns of resulting films (data not shown).

Table 7. Effect of pH levels of FFS on thickness, mechanical properties and water vapor permeability of porcine plasma protein-based films.

pH level	Thickness[#] (mm)	TS[#] (MPa)	EAB[#] (%)	WVP[#] ($x10^{-10}gm^{-1}s^{-1}Pa^{-1}$)
control[†]	$0.065\pm0.003^{b\Delta}$	1.68 ± 0.17^{b}	17.96 ± 1.60^{ef}	2.65 ± 0.06^{d}
2	0.066 ± 0.001^{b}	1.79 ± 0.10^{ab}	31.24 ± 1.30^{c}	2.65 ± 0.04^{d}
3	0.064 ± 0.002^{bcd}	1.96 ± 0.05^{a}	30.12 ± 0.79^{c}	2.64 ± 0.02^{d}
4	0.063 ± 0.002^{cd}	1.45 ± 0.19^{c}	24.34 ± 1.89^{d}	2.61 ± 0.04^{d}
5	0.068 ± 0.004^{a}	1.34 ± 0.11^{cd}	24.69 ± 1.17^{d}	2.75 ± 0.04^{c}
6	0.060 ± 0.001^{d}	1.24 ± 0.05^{d}	19.69 ± 0.89^{e}	2.83 ± 0.06^{b}
7	0.065 ± 0.001^{bc}	1.25 ± 0.09^{d}	16.92 ± 1.09^{f}	3.02 ± 0.03^{a}
8	0.063 ± 0.003^{bcd}	1.63 ± 0.07^{b}	24.88 ± 1.57^{d}	2.63 ± 0.03^{d}
9	0.066 ± 0.004^{bc}	1.65 ± 0.08^{b}	30.39 ± 1.31^{c}	2.68 ± 0.03^{d}
10	0.065 ± 0.002^{b}	1.66 ± 0.05^{b}	38.94 ± 1.72^{a}	2.64 ± 0.05^{d}
11	0.062 ± 0.001^{cd}	1.67 ± 0.05^{b}	36.46 ± 0.92^{b}	2.62 ± 0.04^{d}

[†]Control film was prepared from FFS containing 3% protein and 70% glycerol without pH adjustment.
[#]Mean ± SD from five determinations.
[Δ]The different superscripts in the same column indicate the significant differences ($p<0.05$).

4. Effect of heating temperatures of FFS on the properties of porcine plasma protein-based films

4.1 Mechanical properties

Films prepared from FFSs with pHs 3 or 10 and preheated at different temperatures (40, 55 and 70°C) possessed different properties (Table 8). At the same pH used, TS of films prepared from FFS preheated at different temperatures was not different ($p>0.05$). However, film from preheated FFS had the lower TS than the control film ($p<0.05$). On the other hand, EAB of films increased with increasing

preheating temperature ($p<0.05$). Preheating temperature of 70°C yielded the film with the highest EAB at both pHs 3 and 10 ($p<0.05$). Thermal pretreatment together with pH adjustment to very acidic or alkaline ranges could unfold protein, in which more extended chains could be formed. As a consequence, cross-linked proteins in a way, which enhanced the elongation of film, could occur. For films without preheating at both pHs 3 and 10, TS was greater, while EAB was lower, compared with films subjected to preheating ($p<0.05$). Thus, heating contributed to the enhanced unfolding of proteins. At acidic or alkaline conditions, the modification of charge could be formed after the unfolding induced by heat took place. As a result, repulsive force was more pronounced in film from FFS with preheating and pH adjustment. Garcia and Sobral (2005) reported that films prepared from FFS of Nile Tilapia muscle treated at 65ºC/30 min presented higher values of deformation at break than films prepared with same protein content treated at 40ºC/30 min. The results were in accordance with Iwata *et al*. (2000) who reported that fish water-soluble protein films had the improved mechanical properties by heat denaturization of protein. Thermal treatment temperature between 55ºC and 90ºC for 15 min increased TS and EAB of film.

At the same temperature used, TS of films prepared at the acidic (pH 3) and alkaline (pH 10) conditions was not different ($p>0.05$). However, EAB of films prepared at pH 10 was higher than that prepared at pH 3 ($p<0.05$). It was proposed that at strongly alkaline pH, proteins containing acidic amino acids as major constituents attained an overall negatively charged residue, leading to complete protein unfolding. Subsequently, intermolecular association of protein molecules could be formed in the way, which elongation could be improved. Krochta (1997) specified that the mechanical properties of protein film might be influenced by the degree of chain extension and the nature of sequence of amino acid residues. Therefore, preheating of FFS under alkaline conditions effectively unfolded globular protein, leading to stretched chains, which could form the film with high extensibility and flexibility.

4.2 Water vapor permeability

WVP of films prepared at pH 3 with different preheating temperatures was higher than that of films prepared at pH 10 (Table 8) ($p<0.05$). At acidic pHs, amino acid residue in plasma protein possibly became more amphiphilic than those found at alkaline pH. This could facilitate the water adsorption of the film prepared at acidic pH. Without preheating, films from FFS with pH 3 or 10 had the lower WVP than those with preheating ($p<0.05$). At the same pH used, increasing temperature caused the increase in WVP of resulting film. Heat pretreatment could provide energy to break down intra and inter-molecular bonds, leading to the more exposure of charged amino acid to the medium. However, Perez-Gago *et al*. (1999) found no difference in the WVP of films prepared from heated and unheated whey protein isolate (WPI) in pH ranges of 6 - 8. Quinn *et al.* (2003) reported that films prepared from heated WPI had non-significantly lower WVP than those prepared from unheated WPI.

Table 8. Effect of heating temperatures of FFS on thickness, mechanical properties and water vapor permeability of porcine plasma protein-based films.

pH level / temperature	Thickness[#] (mm)	TS[#] (MPa)	EAB[#] (%)	WVP[#] ($x10^{-10}gm^{-1}s^{-1}Pa^{-1}$)
Control[†]	$0.064\pm0.001^{bc\Delta}$	1.69 ± 0.17^{b}	18.70 ± 1.90^{g}	2.84 ± 0.04^{c}
pH3 / no heat	0.064 ± 0.002^{bc}	1.96 ± 0.05^{a}	30.12 ± 0.79^{f}	2.64 ± 0.02^{d}
pH3 / 40ºC	0.066 ± 0.002^{bc}	1.19 ± 0.04^{c}	33.86 ± 1.83^{e}	2.99 ± 0.07^{b}
pH3 / 55ºC	0.069 ± 0.001^{a}	1.26 ± 0.05^{c}	35.67 ± 3.26^{de}	3.24 ± 0.06^{a}
pH3 / 70ºC	0.067 ± 0.001^{ab}	1.33 ± 0.18^{c}	42.48 ± 3.40^{bc}	3.04 ± 0.01^{b}
pH10 / no heat	0.065 ± 0.002^{bc}	1.66 ± 0.05^{b}	38.94 ± 1.72^{cd}	2.64 ± 0.05^{d}
pH10 / 40ºC	0.060 ± 0.002^{c}	1.34 ± 0.05^{c}	43.13 ± 1.67^{b}	2.66 ± 0.02^{d}
pH10 / 55ºC	0.059 ± 0.003^{c}	1.25 ± 0.10^{c}	44.19 ± 1.63^{b}	2.69 ± 0.09^{d}
pH10 / 70ºC	0.064 ± 0.002^{bc}	1.32 ± 0.08^{c}	65.26 ± 1.70^{a}	2.88 ± 0.04^{c}

[†]Control film was prepared from FFS containing 3% protein and 70% glycerol without pH adjustment and preheating.
[#]Mean ± SD from five determinations.
[Δ]The different superscripts in the same column indicate the significant differences ($p<0.05$).

4.3 Thickness and other characteristics

For both pHs, film had the slight increase in the thickness as the preheating temperature increased ($p<0.05$). The different alignment of unfolded protein molecules might govern the various thicknesses of resulting films. No differences in film solubility, protein solubility and protein pattern were found among all films, regardless of pHs and preheating temperatures used (data not shown). Thus, the different mechanical properties as well as WVP of different films might be determined by the alignment pattern of proteins, degree of unfolding as well as bonding stabilizing the film matrix.

5. Effect of aldehyde compounds on the properties of porcine plasma protein-based films

5.1 Mechanical properties and thickness

TS and EAB of the control film and films added with different types and levels of aldehydes are shown in Table 9. Addition of all aldehydes into the film-forming solution yielded the films with an increased TS ($p<0.05$). The increases in TS of resulting films suggested the occurrence of new covalent bonds between protein molecules, especially albumin and globulin, mediated by formaldehyde, glutaraldehyde and glyoxal. Increases in TS have been reported in sunflower protein isolate films treated with formaldehyde, glyoxal, and glutaraldehyde (Orliac *et al*., 2002), gelatin based films treated with glyoxal and formaldehyde (Carvalho and Grosso, 2004), glutenin-rich films added with formaldehyde, glyoxal, and glutaraldehyde (Hernandez-Munoz *et al*., 2004). TS of all films increased when the concentration of aldehydes increased ($p<0.05$). Incorporation of glutaraldehyde, glyoxal and formaldehyde at a level of 3% into porcine plasma films resulted in the increase in TS of resulting film by 68, 154 and 90%, respectively. At the same level of aldehyde used, the higher TS was found in films treated with glyoxal, probably due to the broad specificity of this chemical in reacting with amino acid side chain groups. Cross-linking of proteins by glyoxal is mainly via lysine and arginine side chain groups at alkaline pH (Marquie, 2001).

Addition of aldehyde to the film-forming solution rendered the films with an increased EAB, in comparison with the control film ($p<0.05$). EAB increased as the

level of all aldehydes, except formaldehyde, increased up to 2% (Table 9). With the addition of 3% glyoxal or 3% formaldehyde, EAB of resulting films decreased ($p<0.05$). The lowered EAB was in agreement with the development of a more rigid structure as shown by the increased TS. Film added with 2% glyoxal had the increase in EAB by 98%, compared with the control film. EAB of films treated with 2% glutaraldehyde and 2% formaldehyde increased by 59 and 73%, respectively, in comparison with the control film. Orliac *et al*. (2002) reported that TS of sunflower protein isolate films treated with 1.5% glutaraldehyde increased from 2.8 to 5.2 MPa with no loss in elongation. Slightly increased elongation capacity was obtained when treated with 2% glyoxal.

The increase in EAB of film treated with the appropriate aldyhydes at the proper level might be associated with the increase in chain length of protein molecules connected together by the cross-linkers. However, the excessive amount of aldehydes resulted in the formation of large aggregate, particularly in form of bundles, which lacked ability to form the uniform and fine film network.

5.2 Water vapor permeability

Influences of type and concentration of aldehyde incorporated on WVP of films are shown in Table 9. It was noted that the increase in WVP was observed in film treated with glutaraldehyde at 2 or 3%, in comparison with the control ($p<0.05$). More pronounced aggregation of protein in polymeric matrix might influence the moisture diffusion coefficient within the porcine plasma protein network. Although aldehydes can make the network rigid, they tend to increase the distance between the protein chains (Orliac *et al.*, 2002). As a result, WVP of glutaraldehyde treated film was higher than the control film. The cross-linked protein induced by glyoxal or formaldehyde at a level of 3% also increased WVP of resulting film. Furthermore, the incorporation of plasticizer also resulted in an increase in the free volume of the system, favoring solvent mobility and stimulating an increase in water diffusion in the polymeric matrix (Sobral *et al*., 2001). The results suggested that the structural changes in the protein network by the chemical modifications could not reduce the free volume of the system.

Table 9. Effect of types and concentrations of aldehyde compounds on thickness, mechanical properties and water vapor permeability of porcine plasma protein- based films.

Aldehyde compounds/ concentration (w/w of protein)	Thickness[#] (mm)	TS[#] (MPa)	EAB[#] (%)	WVP[#] ($x10^{-10}gm^{-1}s^{-1}Pa^{-1}$)
Control	$0.060\pm0.001^{b\Delta}$	1.49 ± 0.019^{i}	63.09 ± 2.70^{f}	2.98 ± 0.08^{c}
Glutaraldehyde / 1%	0.062 ± 0.001^{ab}	1.83 ± 0.162^{h}	76.11 ± 7.58^{e}	3.04 ± 0.05^{bc}
Glutaraldehyde / 2%	0.062 ± 0.002^{ab}	2.11 ± 0.085^{g}	100.15 ± 5.97^{cd}	3.17 ± 0.02^{a}
Glutaraldehyde / 3%	0.063 ± 0.001^{ab}	2.51 ± 0.146^{e}	90.11 ± 6.07^{d}	3.13 ± 0.02^{a}
Glyoxal / 1%	0.063 ± 0.002^{ab}	2.89 ± 0.261^{c}	109.09 ± 6.68^{bc}	3.16 ± 0.04^{a}
Glyoxal / 2%	0.065 ± 0.005^{a}	3.49 ± 0.235^{b}	124.74 ± 7.53^{a}	3.03 ± 0.06^{bc}
Glyoxal / 3%	0.061 ± 0.001^{ab}	3.78 ± 0.279^{a}	93.02 ± 8.06^{d}	3.09 ± 0.07^{ab}
Formaldehyde / 1%	0.062 ± 0.001^{ab}	2.28 ± 0.218^{f}	105.93 ± 3.28^{bc}	3.01 ± 0.02^{bc}
Formaldehyde / 2%	0.063 ± 0.001^{ab}	2.68 ± 0.263^{d}	109.20 ± 6.09^{b}	2.96 ± 0.06^{c}
Formaldehyde / 3%	0.062 ± 0.002^{ab}	2.83 ± 0.288^{c}	69.13 ± 2.91^{f}	3.03 ± 0.04^{bc}

Control film was prepared from FFS (pH 10) preheated at 70ºC for 30 min without addition of aldehyde compounds.

[#]Mean ± SD from five determinations.

[Δ]The different superscripts in the same column indicate the significant differences ($p<0.05$).

5.3 Color and transparency

L* (lightness), a* (redness/greenness) and b* (yellowness/blueness) values of film from porcine plasma protein without and with the addition of aldehydes are shown in Table 10. Addition of all aldehydes resulted in the decrease in L*-value ($p<0.05$). Lightness of film was less affected by formaldehyde, glutaraldehyde and glyoxal. With increasing concentration of glutaraldehyde and glyoxal, the decrease in lightness was observed ($p<0.05$). However, no difference in lightness was found when formaldehyde ranging from 1 to 3% was incorporated ($p>0.05$). Film modified with glutaraldehyde had the higher a*- and b*-values, compared with the control film ($p<0.05$). Addition of formaldehyde at levels greater than 1% resulted in the increased a*-value ($p<0.05$), but had no effect on b*-value ($p>0.05$). For film modified by glyoxal, b*-value increased, but a*-value decreased ($p<0.05$). In general, the changes in a*- and b*-values of resulting film were dependent on concentrations used. From

the result, it indicated that both glutaraldehyde and glyoxal might undergo Maillard reaction with free amino groups to form yellowish pigment more effectively than formaldehyde. Similar results have been reported for glutenin-rich films treated with glutaraldehyde and glyoxal (Hernandez-Munoz *et al*., 2004).

The film became less transparent when all aldehydes were added at higher level as indicated by the higher transparency value (Table 10). At a concentration of 3%, glyoxal had the lowest transparency than those incorporated with formaldehyde or glutaraldehyde ($p>0.05$). Those pigments formed might play a role in absorbing the light to some extent as indicated by the lowered transmission.

Table 10. Effect of types and concentrations of aldehyde compounds on color and transparency of porcine plasma protein-based films.

Aldehyde compounds/ concentration (w/w of protein)	L*[#]	a*[#]	b*[#]	Transparency[#]
Control	$87.51\pm0.34^{a\Delta}$	-2.02 ± 0.08^{e}	6.66 ± 0.71^{e}	1.29 ± 0.00^{d}
Glutaraldehyde / 1%	83.42 ± 0.45^{e}	0.36 ± 0.08^{b}	18.16 ± 0.68^{d}	1.29 ± 0.00^{cd}
Glutaraldehyde / 2%	80.71 ± 0.74^{f}	1.28 ± 0.10^{a}	25.37 ± 1.28^{b}	1.29 ± 0.00^{cd}
Glutaraldehyde / 3%	78.35 ± 0.47^{g}	1.33 ± 0.11^{a}	29.14 ± 0.75^{a}	1.30 ± 0.01^{b}
Glyoxal / 1%	85.62 ± 0.63^{c}	-3.71 ± 0.09^{f}	17.04 ± 0.75^{d}	1.27 ± 0.00^{e}
Glyoxal / 2%	84.65 ± 0.49^{d}	-4.30 ± 0.10^{g}	24.10 ± 0.33^{c}	1.26 ± 0.00^{f}
Glyoxal / 3%	84.62 ± 0.50^{d}	-4.18 ± 0.10^{g}	24.32 ± 0.25^{c}	1.33 ± 0.00^{a}
Formaldehyde / 1%	86.61 ± 0.49^{b}	-1.66 ± 0.03^{c}	6.55 ± 0.31^{e}	1.28 ± 0.01^{d}
Formaldehyde / 2%	86.84 ± 0.40^{b}	-1.82 ± 0.06^{d}	6.33 ± 0.31^{e}	1.29 ± 0.00^{cd}
Formaldehyde / 3%	86.56 ± 0.43^{b}	-1.83 ± 0.08^{d}	6.34 ± 0.10^{e}	1.30 ± 0.01^{b}

Control film was prepared from FFS (pH 10) preheated at 70ºC for 30 min without addition of aldehyde compounds.

[#]Mean $\pm$ SD from five determinations.

[Δ]The different superscripts in the same column indicate the significant differences ($p<0.05$).

6. Effect of phenolic compounds on the properties of porcine plasma protein-based films

6.1 Effect of phenolic compounds with different types and concentrations on the properties of porcine plasma protein-based films

6.1.1 Mechanical properties and thickness

TS and EAB of porcine plasma protein-based films incorporated with tannic acid, caffeic acid and ferulic acid at 1, 2 and 3% are presented in Table 11. TS of film added with tannic acid or caffeic acid increased with increasing concentrations used ($p<0.05$). However, no changes in TS of resulting film were noticeable when ferulic acid at levels of 1 or 2% was added ($p>0.05$). Increase in TS of film incorporated with 3% ferulic acid was found, compared with the control film ($p<0.05$). TS of the films increased by 123.3, 194.3 and 19.5% when tannic acid, caffeic acid and ferulic acid at a concentration of 3% were incorporated, respectively. From the result, it was suggested that tannic acid and caffeic acid showed the superior enhancing effect on the film strength to ferulic acid, most likely due to their multidentate mechanism. Phenolic compound could react with more than one protein site and led to cross-links between proteins (Haslam, 1989). The major advantages of phenolic compound over the aldehydes used are that they are non-toxic and entirely biodegradable. At pH 10, phenolic compounds could be converted to quinone, a protein cross-linker, in which new covalent cross-links could be formed. Quinones react with amino or sulfhydryl side chains of polypeptides to form covalent C-N or C-S bonds (Strauss and Gibson, 2004). Increases in TS were reported in ferulic acid-treated soy protein isolate films (Ou *et al*., 2005), and starch-chitosan blend film cross-linked by ferulic acid (Mathew and Abraham, 2008). Cao *et al*. (2007) reported that the gelatin films treated with ferulic acid had the maximum TS at pH 7, whereas tannic acid treated film had the maximum TS at pH 9.

Addition of all phenolic compounds resulted in the increases in EAB, compared with the control (without phenolic compound) ($p<0.05$). For tannic acid treated film, EAB of film increased with increasing concentration of tannic acid ($p<0.05$). When the concentrations of caffeic acid and ferulic acid were higher than 2%, the decreases in EAB were observed ($p<0.05$). The results indicated that phenolic

compound at concentration greater than 2% could be an excessive concentration, in which a number of intermolecular cross-links could be formed. This led to the development of a more rigid structure as evidenced by the lowered EAB. From the result, the marked increase in both TS and EAB was found in film treated with 2% caffeic acid. Both caffeic acid and tannic acid are large molecules with several functional groups, which can polymerize protein with a longer chain length. This might contribute to the increase in EAB of resulting films. Orliac *et al*. (2002) reported that sunflower protein isolate films treated with 1-2% gallic acid had an increase in EAB, while EAB was decreased when gallic acid greater than 3% was used. Therefore, the mechanical properties of resulting films treated with phenolic compound at alkali pH were governed by types and concentrations of phenolic compounds used. Addition of all phenolic compounds at all concentrations used had no pronounced impact on thickness of resulting films (Table 11).

6.1.2 Water vapor permeability

Effect of types and concentrations of phenolic compounds on WVP of films is shown in Table 11. Addition of tannic acid, caffeic acid and ferulic acid at all levels increased WVP of resulting films ($p<0.05$). This might be due to the presence of polar groups of phenolic compounds incorporated. For the same type of phenolic compounds, the concentration in range of 1-3% had no effect on WVP of films ($p>0.05$). Nonetheless, films incorporated with tannic acid tended to possess the higher WVP than those treated with caffeic acid and ferulic acid. Ferulic acid did not affect WVP of soy protein isolate films even at higher levels (Ou *et al*., 2005). Cao *et al*. (2007) reported that tannic acid slightly increased WVP of gelatin films.

6.1.3 Color and transparency

L*-, a*- and b*-values of porcine plasma films treated with different types and concentration of phenolic compounds are shown in Table 12. Decreases in L*-value and increases in a*- and b*-values were observed when the films were treated with all phenolic compounds ($p<0.05$). Films treated with tannic acid and caffeic acid had higher a*- and b*-values than did those treated with ferulic acid ($p<0.05$). Yellowness and redness of film treated with both compounds were mostly from the color of compounds. Only slight decreases in L*-value with slight increases in a*- and b*-values of films treated with ferulic acid were observed. Film had the

increased redness and yellowness when higher concentrations of tannic acid and caffeic acid were used. Gelatin films treated with ferulic acid were transparent and colorless when prepared at pHs 6-9 and became white and opaque after pH of FFS was adjusted to pH 10 (Cao *et al*., 2007). Films from porcine plasma treated with 3% caffeic acid had the lowest transparency (highest value) ($p<0.05$) (Table 12). This was in accordance with the highest increase in a*-value and the lowest L*-value. Film with yellowness/redness could retard the transmission of light both in UV and visible range (data not shown).

Table 11. Effect of types and concentrations of phenolic compounds on thickness, mechanical properties and water vapor permeability of porcine plasma protein-based films.

Phenolic compounds/ concentration (w/w of protein)	Thickness[#] (mm)	TS[#] (MPa)	EAB[#] (%)	WVP[#] ($x10^{-10}gm^{-1}s^{-1}Pa^{-1}$)
Control	$0.068\pm0.001^{bc\Delta}$	1.23 ± 0.06^{f}	67.08 ± 3.84^{g}	2.80 ± 0.01^{d}
Tannic acid / 1%	0.070 ± 0.001^{bc}	1.73 ± 0.02^{d}	74.88 ± 2.88^{f}	3.15 ± 0.05^{a}
Tannic acid / 2%	0.069 ± 0.001^{bc}	2.24 ± 0.09^{c}	95.42 ± 4.70^{d}	3.05 ± 0.05^{ab}
Tannic acid / 3%	0.072 ± 0.001^{a}	2.71 ± 0.08^{b}	114.79 ± 4.47^{c}	3.16 ± 0.01^{a}
Caffeic acid / 1%	0.069 ± 0.001^{bc}	2.17 ± 0.09^{c}	115.42 ± 2.17^{c}	3.07 ± 0.10^{ab}
Caffeic acid / 2%	0.069 ± 0.002^{bc}	2.77 ± 0.03^{b}	146.12 ± 2.55^{a}	2.99 ± 0.10^{bc}
Caffeic acid / 3%	0.069 ± 0.001^{bc}	3.62 ± 0.10^{a}	125.00 ± 3.18^{b}	2.99 ± 0.07^{bc}
Ferulic acid / 1%	0.067 ± 0.001^{c}	1.25 ± 0.02^{f}	78.21 ± 2.07^{f}	3.03 ± 0.09^{bc}
Ferulic acid / 2%	0.067 ± 0.001^{c}	1.24 ± 0.07^{f}	89.56 ± 3.34^{e}	2.93 ± 0.03^{c}
Ferulic acid / 3%	0.068 ± 0.001^{bc}	1.47 ± 0.02^{e}	73.94 ± 1.79^{f}	2.97 ± 0.03^{bc}

Control film was prepared from FFS (pH 10) preheated at 70ºC for 30 min without addition of phenolic compounds.

[#]Mean $\pm$ SD from five determinations.

[Δ]The different superscripts in the same column indicate the significant differences ($p<0.05$).

Table 12. Effect of types and concentrations of phenolic compounds on color and transparency of porcine plasma protein-based films.

Phenolic compounds/ concentration (w/w of protein)	L*[#]	a*[#]	b*[#]	Transparency[#]
Control	$87.44\pm0.68^{a\Delta}$	-1.86 ± 0.07^{f}	6.80 ± 0.27^{i}	1.24 ± 0.01^{d}
Tannic acid / 1%	78.40 ± 0.78^{d}	-0.79 ± 0.09^{e}	33.64 ± 0.64^{e}	1.26 ± 0.03^{cd}
Tannic acid / 2%	73.59 ± 0.34^{e}	3.18 ± 0.15^{d}	42.09 ± 0.61^{b}	1.28 ± 0.03^{bc}
Tannic acid / 3%	65.57 ± 0.39^{f}	10.90 ± 0.57^{b}	53.06 ± 0.39^{a}	1.30 ± 0.01^{c}
Caffeic acid / 1%	72.78 ± 0.86^{e}	2.91 ± 0.20^{d}	28.06 ± 0.82^{f}	1.30 ± 0.01^{b}
Caffeic acid / 2%	62.05 ± 1.49^{g}	6.93 ± 0.76^{c}	36.42 ± 1.21^{d}	1.39 ± 0.01^{a}
Caffeic acid / 3%	53.10 ± 1.60^{h}	12.03 ± 0.66^{a}	40.15 ± 1.85^{c}	1.40 ± 0.01^{a}
Ferulic acid / 1%	84.93 ± 0.36^{b}	-2.29 ± 0.06^{fg}	8.59 ± 0.20^{h}	1.26 ± 0.01^{cd}
Ferulic acid / 2%	82.72 ± 0.57^{c}	-2.28 ± 0.09^{fg}	8.97 ± 0.41^{gh}	1.28 ± 0.01^{bc}
Ferulic acid / 3%	82.31 ± 0.26^{c}	-2.47 ± 0.07^{g}	9.54 ± 0.43^{g}	1.28 ± 0.01^{bc}

Control film was prepared from FFS (pH 10) preheated at 70ºC for 30 min without addition of phenolic compounds.

[#]Mean ± SD from five determinations.

[Δ]The different superscripts in the same column indicate the significant differences ($p<0.05$).

6.2 Effect of pH levels and oxygenation on the properties of porcine plasma protein-based films

6.2.1 Mechanical properties and thickness

The effects of pHs of FFS containing 3% caffeic acid yielding the resulting film with the highest TS (Table 11) are shown in Table 13. Without prior oxygenation, FFS rendered the film with higher TS and EAB ($p<0.05$) as pH increased from 7 to 10. The highest TS and EAB of resulting film were obtained at pH 10. At pH greater than 8, the resulting film exhibited the higher EAB, compared to the control film (without prior pH adjustment and phenolic compounds). Improved TS of porcine plasma films, prepared under alkaline conditions, might be partially associated with the unfolding of porcine plasma proteins in the FFS by increasing the electrostatic repulsion between protein molecules. Unfolded proteins possessed the increased functional groups available for cross-linking induced by phenolic compounds added. Caffeic acid

underwent conversion to quinone to some degree at pH 10 and quinone could act as protein cross-linker.

At the same pH used, TS of resulting films prepared with prior oxygenation was higher than those of film without oxygenation. The increases in TS were coincidental with the lowered EAB. Oxygenation could induce the transformation of caffeic acid to quinone effectively. Quinone formed most likely reacted with amino or sulfhydryl side chains of polypeptides to form the rigid network as evidenced by the increased TS with the concomitant loss in EAB. Therefore, pH was a prime factor determining the mechanical property of film containing caffeic acid.

Thickness of all films prepared at different pHs with and without prior oxygenation ranged from 0.068 to 0.077 mm. At pH 10, oxygenation resulted in the slight increase in thickness of resulting film. Quinones formed at pH 10 might cross-link unfolded proteins and were localized between protein molecules. This might result in slightly increased thickness.

6.2.2 Water vapor permeability

Effects of pH levels and oxygenation of FFS containing 3% caffeic acid on WVP of resulting films are shown in Table 13. For all pHs used, oxygenation of FFS had no impact on WVP of resulting films ($p>0.05$). WVP of all films were not different from that of control film. Additionally, pHs showed no effect on WVP of resulting films, irrespective of oxygenation. Cao *et al*. (2007) found that ferulic acid and tannic acid did not affect WVP of gelatin film. Therefore, film treated with 3% caffeic acid could possess the improved mechanical properties without the changes in WVP.

6.2.3 Color and transparency

L*-, a*- and b*-values of porcine plasma films containing 3% caffeic acid prepared at different pHs with and without oxygenation are shown in Table 14. The control film had the greatest L*-value but lowest a*- and b*-values ($p<0.05$), compared with films treated with caffeic acid, regardless of oxygenation. Under the same oxygenation condition, L*-value of film decreased, while a*- and b*-values increased with increasing pHs ($p<0.05$). At the same pH except for pH 10, the oxygenation lowered L*-value, whereas increased a*- and b*-values of film containing 3% caffeic acid ($p<0.05$). No differences in transparency were observed between films prepared with and without prior oxygenation when the same pH was

used ($p>0.05$). The films became less transparent when pH decreased from 10 to 7 as evidenced by the higher transparency value. Decrease in transparency was in accordance with the increases in a*- and b*-values and lowered L*-value. The result indicated that alkali pH enhanced the redness and yellowness of film treated with caffeic acid. This might affect the color and appearance of resulting film.

Table 13. Effect of pH levels and oxygenation on thickness, mechanical properties and water vapor permeability of porcine plasma protein-based films treated with 3% caffeic acid.

pH/oxygenation[ɔ]	Thickness[#] (mm)	TS[#] (MPa)	EAB[#] (%)	WVP[#] ($x10^{-10}gm^{-1}s^{-1}Pa^{-1}$)
Control	$0.069\pm0.001^{f\Delta}$	1.34 ± 0.04^{h}	63.40 ± 3.23^{d}	2.99 ± 0.03^{bcde}
pH7	0.071 ± 0.001^{e}	1.98 ± 0.13^{g}	39.16 ± 3.83^{f}	3.05 ± 0.04^{abcd}
pH7/oxygenated	0.068 ± 0.001^{f}	2.46 ± 0.10^{f}	17.87 ± 1.98^{h}	2.96 ± 0.07^{cde}
pH8	0.074 ± 0.001^{cd}	2.57 ± 0.09^{f}	49.22 ± 4.03^{e}	3.12 ± 0.06^{a}
pH8/oxygenated	0.075 ± 0.001^{bc}	2.78 ± 0.07^{e}	32.78 ± 2.05^{g}	3.07 ± 0.05^{abc}
pH9	0.077 ± 0.001^{a}	2.90 ± 0.01^{d}	84.34 ± 3.98^{c}	3.03 ± 0.10^{abcd}
pH9/oxygenated	0.077 ± 0.001^{ab}	3.08 ± 0.02^{c}	54.36 ± 4.21^{e}	3.10 ± 0.05^{ab}
pH10	0.069 ± 0.001^{f}	3.52 ± 0.02^{b}	126.39 ± 3.24^{a}	2.95 ± 0.07^{de}
pH10/oxygenated	0.073 ± 0.001^{de}	3.71 ± 0.04^{a}	93.27 ± 2.89^{b}	2.90 ± 0.06^{e}

Control film was prepared from FFS (pH 10) preheated at 70ºC for 30 min without caffeic acid addition.

[ɔ]Oxygenation was carried out by bubbling oxygen gas into FFS containing 3% caffeic acid for 30 min.

[#]Mean $\pm$ SD from five determinations

[Δ]The different superscripts in the same column indicate the significant differences ($p<0.05$).

7. Characterization of porcine plasma protein-based films

7.1 Protein patterns

Protein patterns of films prepared from porcine plasma protein without and with the modification with glyoxal or caffeic acid are shown in Figure 7. The differences in protein patterns were observed between those run under reducing (Figure 7A) and non-reducing (Figure 7B) conditions. The result indicated that protein film matrix was partially stabilized by disulfide bond. Furthermore, protein in

plasma might consist of subunits associated together with disulfide bond. In the presence of βME, those cross-links or subunits might be dissociated. Under reducing condition, both albumin (65 kDa) and α-, β-globulin (56, 48 kDa) in the control film and film from FFS with pH 10 and preheating constituted as the major components.

Table 14. Effect of pH levels and oxygenation on color and transparency of porcine plasma protein-based films treated with 3%caffeic acid.

pH/oxygenation[ɔ]	L*[#]	a*[#]	b*[#]	Transparency[#]
Control	87.05±0.79[aΔ]	-1.94±0.06[h]	6.54±0.31[h]	1.23±0.01[f]
pH7	77.19±0.73[b]	0.61±0.07[g]	24.93±0.89[f]	1.26±0.01[e]
pH7/oxygenated	76.25±0.78[c]	1.51±0.06[f]	23.10±0.82[g]	1.27±0.01[d]
pH8	70.53±0.53[d]	4.71±0.27[e]	26.75±0.97[e]	1.29±0.01[c]
pH8/oxygenated	68.79±0.50[e]	7.51±0.72[d]	30.62±0.64[d]	1.29±0.01[c]
pH9	59.40±0.99[f]	8.53±0.27[c]	36.22±0.51[c]	1.32±0.01[b]
pH9/oxygenated	57.37±0.66[g]	9.71±0.65[b]	37.78±0.81[b]	1.33±0.01[b]
pH10	55.66±0.98[h]	11.16±0.96[a]	41.15±0.68[a]	1.41±0.01[a]
pH10/oxygenated	54.44±0.72[i]	11.46±1.03[a]	41.90±0.78[a]	1.42±0.01[a]

Control film was prepared from FFS (pH 10) preheated at 70ºC for 30 min without caffeic acid addition.

[ɔ]Oxygenation was carried out by bubbling oxygen gas into FFS containing 3% caffeic acid for 30 min.

[#]Mean ± SD from five determinations

[Δ]The different superscripts in the same column indicate the significant differences ($p<0.05$).

The protein pattern of film prepared from FFS with pH 10 and preheated at 70°C was similar to that of control film. Under non-reducing condition, a slight difference was noticeable between both films. Band with MW of 250 kDa was found in the control film, but it was almost disappeared in film made from FFS with pH adjustment and preheating. Therefore, it was more likely that those proteins were either degraded or polymerized during film preparation. For film treated with glyoxal, the marked decrease in band intensity of both albumin and globulin was noticeable, in comparison with the control film. This was concomitant with the formation of large aggregate on the stacking gel. The polymerized proteins were also found under non-reducing condition. This indicated that glyoxal mainly induced the formation of non-

disulfide covalent bond. No differences in protein pattern and band intensity between films containing caffeic acid with and without prior oxygenation. However, band intensity of albumin in film modified with glyoxal was lower than that of both films treated with caffeic acid. This suggested that bondings which stabilized the protein cross-links of porcine plasma protein films induced by caffeic acid belonged to the covalent bond. Under non-reducing condition, the band intensity of major proteins was also noticeable when glyoxal or caffeic acid with and without prior oxygenation were added. This indicated that both components might induce the formation of disulfide bond to some extent. Nevertheless, the lowest band intensity was observed in film treated with glyoxal. It was reported that phenolic compound or oxidized counterpart could induce the formation of disulfide in proteins (Rawel *et al*., 2002).

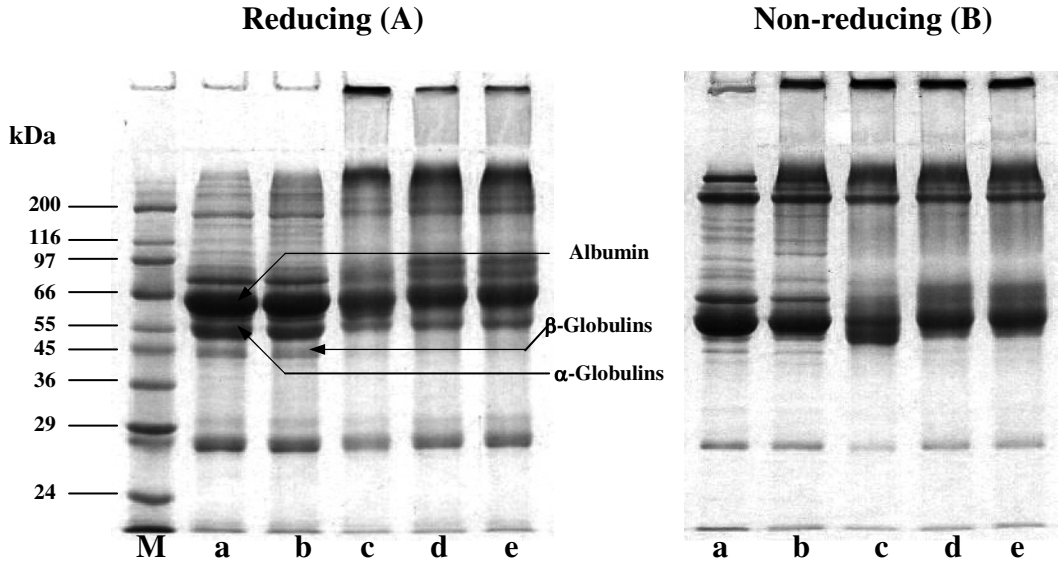

Figure 7. Protein patterns of porcine plasma protein film prepared with 3% protein (w/v) and 70% glycerol (w/w of protein content) without and with cross-linkers under reducing (A) and non-reducing condition (B): **M**, protein marker; **a**, the control; **b**, film prepared from FFS (pH 10) with preheating at 70°C for 30 min; **c**, film incorporated with 2% glyoxal; **e**, film incorporated with 3% caffeic acid; **f**, film incorporated with 3% caffeic acid with prior oxygenation.

7.2 Hydrolysis by protease

Hydrolysis of different porcine plasma protein-based films by α-chymotrypsin and pepsin as monitored by TCA-soluble peptide content is shown in Table 15. TCA soluble peptide content of all films hydrolyzed by α-chymotrypsin and pepsin increased as the hydrolysis time increased ($p<0.05$). For α-chymotrypsin hydrolysis, for the same hydrolysis time, the highest protein digestibility of film was observed with the control film ($p<0.05$).

Table 15. TCA soluble peptide content (mg/ml) of porcine plasma protein-based films without and with cross-linkers digested by α-chymotrypsin or pepsin.

Enzymes	Films	Hydrolysis times (min)				
		0	30	60	90	120
α-Chymotrypsin	Control	$1.73\pm0.01^{a*}$	1.86 ± 0.01^{a}	1.94 ± 0.01^{a}	1.97 ± 0.02^{a}	2.01 ± 0.01^{a}
	Pretreated (pH10, 70°C)	1.64 ± 0.01^{b}	1.71 ± 0.01^{b}	1.80 ± 0.03^{b}	1.88 ± 0.01^{b}	1.96 ± 0.01^{b}
	With 2% GLY	1.65 ± 0.01^{b}	1.68 ± 0.01^{c}	1.73 ± 0.01^{c}	1.76 ± 0.01^{c}	1.78 ± 0.01^{d}
	With 3% CA	1.65 ± 0.02^{b}	1.71 ± 0.01^{b}	1.71 ± 0.02^{c}	1.76 ± 0.03^{c}	1.83 ± 0.01^{c}
	With 3% CA (oxygenated)	1.65 ± 0.01^{b}	1.68 ± 0.03^{c}	1.72 ± 0.01^{c}	1.78 ± 0.03^{c}	1.83 ± 0.01^{c}
Pepsin	Control	$1.09\pm0.06^{a*}$	1.28 ± 0.03^{a}	1.53 ± 0.03^{a}	1.75 ± 0.03^{a}	1.85 ± 0.01^{a}
	Pretreated (pH10, 70°C)	0.92 ± 0.02^{b}	0.90 ± 0.03^{b}	0.92 ± 0.02^{b}	1.06 ± 0.04^{b}	1.13 ± 0.06^{b}
	With 2% GLY	0.95 ± 0.09^{b}	0.88 ± 0.01^{b}	0.78 ± 0.03^{d}	0.90 ± 0.09^{c}	0.92 ± 0.01^{d}
	With 3% CA	0.86 ± 0.03^{c}	0.88 ± 0.06^{b}	0.84 ± 0.01^{c}	0.88 ± 0.06^{c}	0.92 ± 0.02^{d}
	With 3% CA (oxygenated)	0.86 ± 0.09^{c}	0.90 ± 0.03^{b}	0.84 ± 0.06^{c}	0.88 ± 0.02^{c}	1.01 ± 0.03^{c}

*The different superscripts in the same column indicate the significant differences ($p<0.05$).
Control: FFS without heating and pH adjustment was directly subjected to casting.
GLY: Glyoxal, CA: Caffeic acid

Plasma proteins were hydrophilic and film matrix was stabilized by non-covalent bonds. As a consequence, the film was easily solubilized and readily hydrolyzed. On the other hand, the cross-linked proteins had the increases in resistance to hydrolysis by α-chymotrypsin as evidenced by the lowered increase in TCA soluble peptide content as the hydrolysis time increased. From the results, the

film incorporated with 2% glyoxal were hydrolyzed to the lowest extent by ∝-chymotrypsin, compared with other films ($p<0.05$). No differences in digestibility between film containing caffeic acid with and without oxygenation were obtained. For pepsin, the similar results were observed. However, the efficiency of ∝-chymotrypsin in hydrolysis of porcine plasma protein-based film was higher than that of pepsin when the same hydrolysis time was used generally cleaved peptide bonds in the either single or cross-linked proteins more effectively than pepsin.

7.3 Protein solubility

Protein solubility of different porcine plasma protein-based films in various solvents is shown in Table 16. The distribution and extents of inter- and intra-molecular interaction between proteins, such as ionic interaction, hydrogen bond, hydrophobic interaction, and disulfide bonds are associated with the development of a three-dimensional network of the films. The solubility of films in three different denaturing solutions was used to indicate the major associative force involved in the film formation. As solubilized by S1, the control film showed the highest solubility, followed by the film with pH adjustment and preheating. Film prepared at pH 10 with preheating at 70°C was solubilized in S1 at a lower extent than the control film. Therefore, the low solubility of this film suggested the presence of protein linkages, which were formed in the film matrix. The result indicated that hydrogen bonds were the main associative force for the formation of films of porcine plasma based film. SDS is able to destroy hydrogen bonds. Film treated with glyoxal showed the lowest solubility ($p<0.05$). Furthermore, films treated with caffeic acid also exhibited the low solubility. When S2 was used for film solubilization, the increases in solubility of all films increased. The highest increase was found in film prepared from FFS adjusted to pH 10 and preheating. The result suggested that the main force involved in the formation of film structure were hydrogen bond and hydrophobic interaction. With preheating, the exposure of hydrophobic portion was enhanced. As a result, hydrophobic interaction could be formed to a greater extent in this film. When S3 was used as solubilizing agent, the increase in solubility was noticeable, indicating the involvement of disulfide bond in film stabilization. Film incorporated with glyoxal had the lower solubility, followed by film modified with caffeic acid. The low

solubility in S3 indicated that non-disulfide covalent bond most likely stabilized the film network. From the results, it can be inferred that hydrogen bonds, hydrophobic interactions, ionic bonds and disulfide bonds played an important role in the formation of porcine plasma protein-based films. Nevertheless, the different bonds were involved when different chemicals or treatments were used.

Table 16. Protein solubility (%)[#] of porcine plasma protein-based films without and with cross-linkers.

Film	S1[*]	S2	S3
Control	94.11±0.76[aΔ]	97.65±1.51[a]	99.55±0.38[a]
Preheated (pH10, 70°C)	61.64±0.66[b]	84.61±0.13[b]	99.26±0.26[a]
With 2% GLY	2.81±0.15[d]	13.45±0.45[d]	27.76±0.30[d]
With 3% CA	9.45±0.20[c]	20.30±0.40[c]	59.70±0.40[b]
With 3% CA (oxygenated)	10.00±0.19[c]	20.20±0.28[c]	52.15±0.24[c]

[*](S1) 20 mM Tris-HCl, pH 8.0 containing 2% (w/v) SDS; (S2) 20 mM Tris-HCl, pH 8.0 containing 2% (w/v) SDS and 8 M Urea; (S3) 20 mM Tris-HCl, pH 8.0 containing 2% (w/v) SDS, 8 M Urea and 2% (v/v) β-mercaptoethanol.
[#]Mean ± SD from triplicate determinations.
[Δ]The different superscripts in the same column indicate the significant differences ($p<0.05$).
Control: FFS without heating and pH adjustment was directly subjected to casting. GLY: Glyoxal, CA: Caffeic acid

7.4 Infrared spectroscopy

FTIR spectra of different films are shown in Figure 8. In general, the bands situated at 3288, 1632 and 1548 cm^{-1}, corresponding to amide-III and free water, amide-II and amide-I, respectively (Bergo and Sobral, 2007). The amide-I arises from stretching of C=O of amide in protein; the amide-II arises from bending vibration of N-H groups and stretching vibrations of C-N groups. Amide-III is related to the vibrations in plane of C-N and N-H groups of bound amide or vibrations of CH_2 groups of glycine (Schmidt *et al.*, 2005). The peak situated around 1033 cm^{-1} might be related to the glycerol (Bergo and Sobral, 2007).

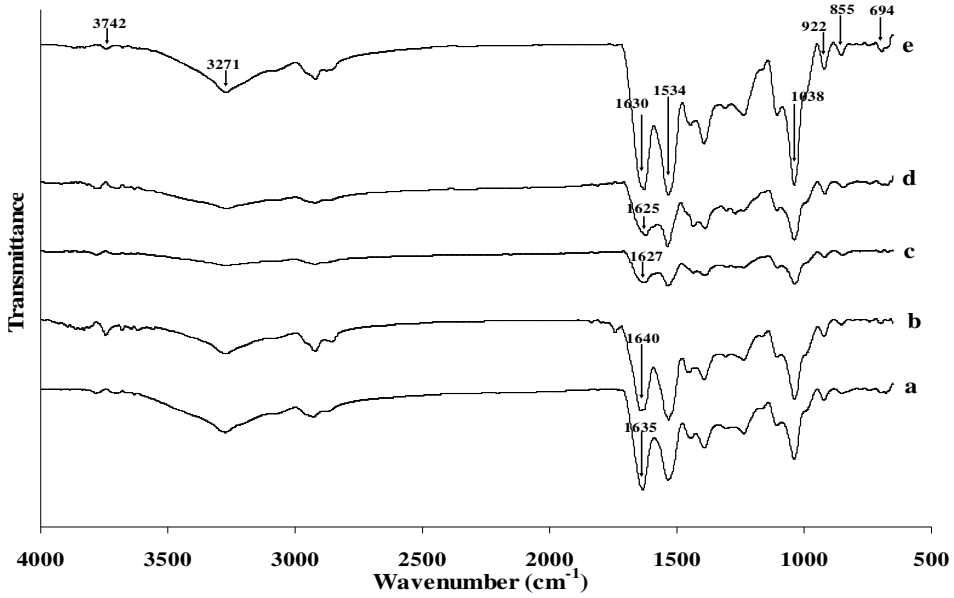

Figure 8. Infrared spectra of porcine plasma protein-based films without and with cross-linkers: **a**, the control; **b**, film prepared from FFS (pH 10) with preheating at 70°C for 30 min; **c**, film incorporated with 2% glyoxal; **d**, film incorporated with 3% caffeic acid; **e**, film incorporated with 3% caffeic acid with prior oxygenation.

From the results, the wave number of amide-I peak shifted from 1635 for control to 1627, 1630 and 1625 cm^{-1} for films incorporated with 2% glyoxal and 3% caffeic acid with or without oxygenation, respectively. It was noted that the amplitude of these peaks decreased markedly in film treated with caffeic acid without oxygenation and that treated with glyoxal. On the other hand, the higher peak amplitude of film treated with caffeic acid with oxygenation was observed, in comparison with other films. The results indicated that oxygenated caffeic acid might induce the protein cross-linking to a high extent. This led to the irregular surface (data not known), which contributed to the higher diffraction at the film surface. For film treated with caffeic acid without oxygenation, the smoother surface was noticeable (data not shown). In general, IR absorption depends on both the penetration depth of the IR beam and on the angle. The penetration depth of the beam in the ATR technique strongly depends on the diffraction index of the material. Therefore, the treatments used most likely had the impact on the diffraction index of the resulting film. The modification of protein network in film by protein cross-linkers was

mainly associated with the changes in functional group and conformation of proteins as indicated by the changes in FTIR spectra.

7.5 Differential scanning calorimetry (DSC)

Crosslinking also increased thermal stability of porcine plasma protein-based films, as shown by the shift of the transition temperature (T_m) to higher value. Figure 9 shows the typical DSC thermograms for the control film, film with pretreatment (adjusted pH to 10 and heated at 70°C), film incorporated with 2% glyoxal and film modified with 3% caffeic acid with and without oxygenation. The major endothermic transition peaks (peak II) were found at 173.5, 166.8, 176.2, 186.2 and 181.0°C, respectively. The peaks observed from DSC thermogram typically reflected the disruption of ordered phase of molecules in the film matrix. During heating, the initially ordered structure of films is gradually destroyed. This may be possible after the breaking-up of inter- and intra-molecular hydrogen bonds which are responsible for the maintenance of the polymeric chain order (alpha-helix, beta-structure) in protein films (Barreto *et al*., 2003). Cross-linking of molecules induced by chemically used might contributed to the increase in T_m. Cross-linking effect of caffeic acid was reflected by increasing TS to some degrees, owing to the decrease in molecular mobility by covalent bonds (Nuthong *et al*., 2009). Among all films, that prepared from FFS (pH 10) with preheating had the lowest T_m for the peak II (166.8°C), but showed the highest ΔH_m. Film incorporated with caffeic acid without and with oxygenation had the highest T_m than other films. However, film treated with caffeic acid without oxygenation showed the higher T_m but showed no difference in transition enthalpy (ΔH_m), in comparison with oxygenated counterpart. Hernandez-Munoz *et al*. (2004) reported that glutenin-rich films added with aldehyde as the reticulating agent, showed the increase in the transition temperature from 162.8 (control) to 176.2, 180.0 and 182.5°C for films cross-linked with 2% glyoxal, 2% glutaraldehyde and 2% formaldehyde, respectively. An increase in the transition temperature corresponded with the cross-linking effect was observed. The highest transition temperature found in film incorporated with 3% caffeic acid without oxygenation was associated with the lower protein solubility in S3 (Table 16) and the greater resistance to hydrolysis by protease (Table 15), compared to the control film. The similar result was also found for minor endothermic transition peak (peak I), in which film treated with

caffeic acid without oxygenation had the highest transition. Peak I might represent the transition of molecules with the lower ordered structure or those interacted each other via the weaker bonds.

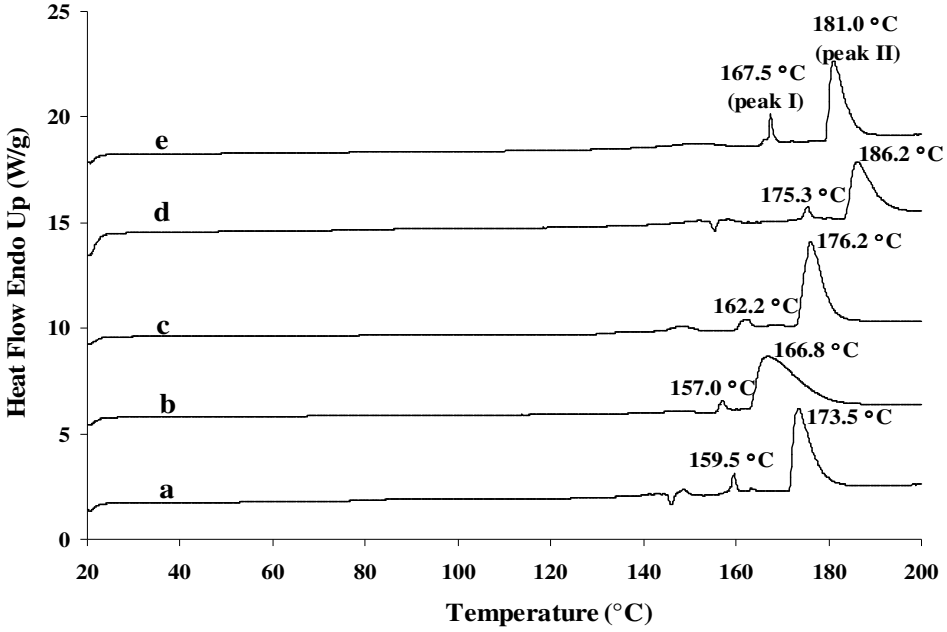

Figure 9. Differential scanning calorimetric thermogram of porcine plasma protein-based films without and with cross-linkers: **a**, the control; **b**, film prepared from FFS (pH 10) with preheating at 70°C for 30 min; **c**, film incorporated with 2% glyoxal; **d**, film incorporated with 3% caffeic acid; **e**, film incorporated with 3% caffeic acid with prior oxygenation.

7.6 Thermo-gravimetric analysis (TGA)

TGA scans of different porcine plasma protein-based films are shown in Figure 10 (Appendix B). All films exhibited three main stages of weight loss. The first stage observed up to approximately 52.9-66.9°C (Td_1) was possibly related to the loss of adsorbed and bound water. The second stage (Td_2) (170.0-203.8°C) and third stage (Td_3) (>288.8°C), were most likely associated with the degradation of porcine plasma protein and protein crosslinks. The result revealed that the degradation of porcine plasma protein-based films began at 170°C and the highest degradation rate was obtained at 319.6°C. The residue, at 800°C, was about 19.5%. For the film prepared from FFS (pH 10) with

preheating at 70°C, slightly higher temperature (172.8°C) of the second stage (Td_2) was noticeable. The higher Td_2 were found in films treated with glyoxal or caffeic acid. However, films treated with both caffeic acid with and without oxygenation showed the higher temperature of the second and third stages (Td_2 and Td_3) than those modified with glyoxal. The result indicated an increase in the thermal stability due to protein crosslinker. These results were not consistent with the previous reports on starch-chitosan blend films, in which the control and ferulic acid incorporated films had no significant difference in the thermal stability (Mathew and Abraham, 2008). Apparently, the more thermally-stable structures formed were due to the occurrence of cross-linking reactions (Schmidt *et al*., 2005). Furthermore, the highest decomposition temperature of film treated with caffeic acid with prior oxygenation (321.8°C) was lower than that treated with glyoxal (325.1°C). This might lead to the higher heat resistance of film containing glyoxal. Therefore, glyoxal and caffeic acid were able to improve the thermal stability of porcine plasma protein-based film.

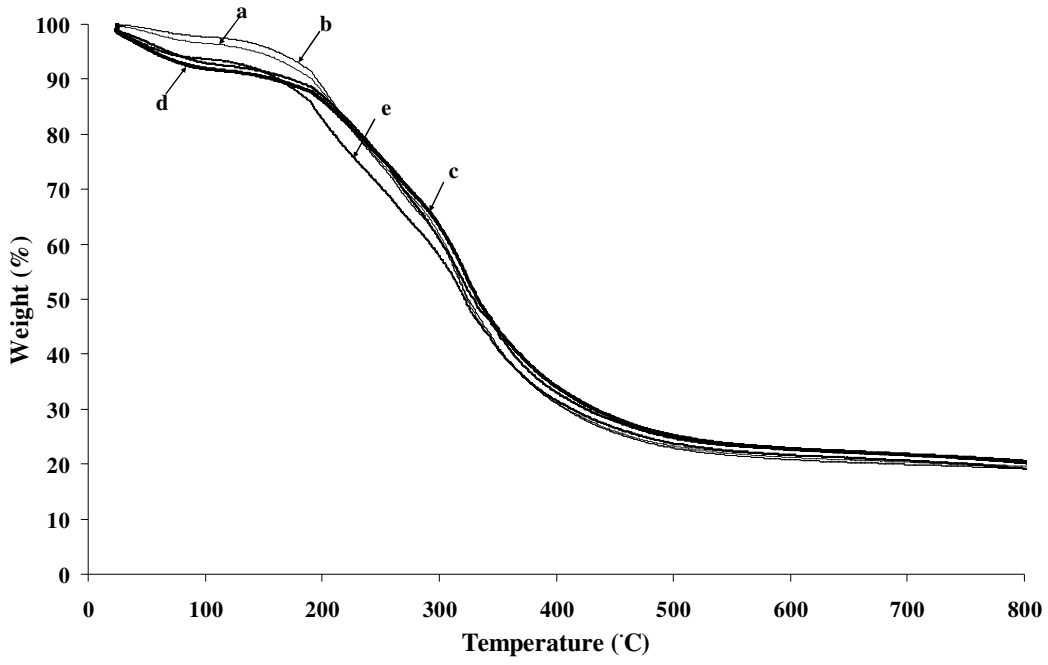

Figure 10. Thermogravimetric curves of porcine plasma protein-based films without and with cross-linkers: **a**, the control; **b**, film prepared from FFS (pH 10) with preheating at 70°C for 30 min; **c**, film incorporated with 2% glyoxal; **d**, film incorporated with 3% caffeic acid; **e**, film incorporated with 3% caffeic acid with prior oxygenation.

7.7 Morphology

SEM micrographs representing the morphology of the surface and cross-sections of porcine plasma protein-based films treated with and without 2% glyoxal or 3% caffeic acid are depicted in Figure 11. Surface of film treated with 2% glyoxal or 3% caffeic acid was smoother than control film. No cracks were found in the treated films, while the cracks were noticeable in the control film. For cross-section, more ordered matrix were observed in film treated with 2% glyoxal or 3% caffeic acid as indicated by the absence of irregular morphology. Glyoxal molecules might react with primary amines to form imines (Schiff bases) at alkaline pH (Marquie, 2001). Glyoxal modified film was denser and rougher surface than caffeic acid treated film, possibly due to the faster cross-linking of protein by the former cross-linker. Quinone formed at pH 10 possibly reacted with amino side chain on polypeptide chain, leading to the reduction of free volume in the polymer matrix. Caffeic acid incorporated films were more compact; however, sandy surface with some cracks was obtained. Surface and cross-section of oxygenated film was rougher than non-oxygenated film. Oxygenation could induce the conversion of caffeic acid to quinone more effectively. Quinone could induce the aggregation of protein via multidentate mechanism (Haslam, 1989). As a consequence, coarser surface and sandy cross-section were found in film containing 3% caffeic acid with prior oxygenation.

8. Changes of quality of fish powder packaged in porcine plasma protein bags during storage

8.1 Changes in moisture content of fish powder

Moisture content of dried fish powder packaged in bags produced from porcine plasma protein-based films incorporated without and with 3% caffeic acid with prior oxygenation or polyethylene (PE) bag during storage at 28-30°C is shown in Figure 12. In general, moisture content of dried fish powder packaged in bags produced from porcine plasma protein-based films increased continuously during 24 days of storage ($p<0.05$). However, the increase in moisture content of dried fish powder packaged in PE bag exhibited the much lower rate, compared with those packaged in bags produced from porcine plasma protein-based films.

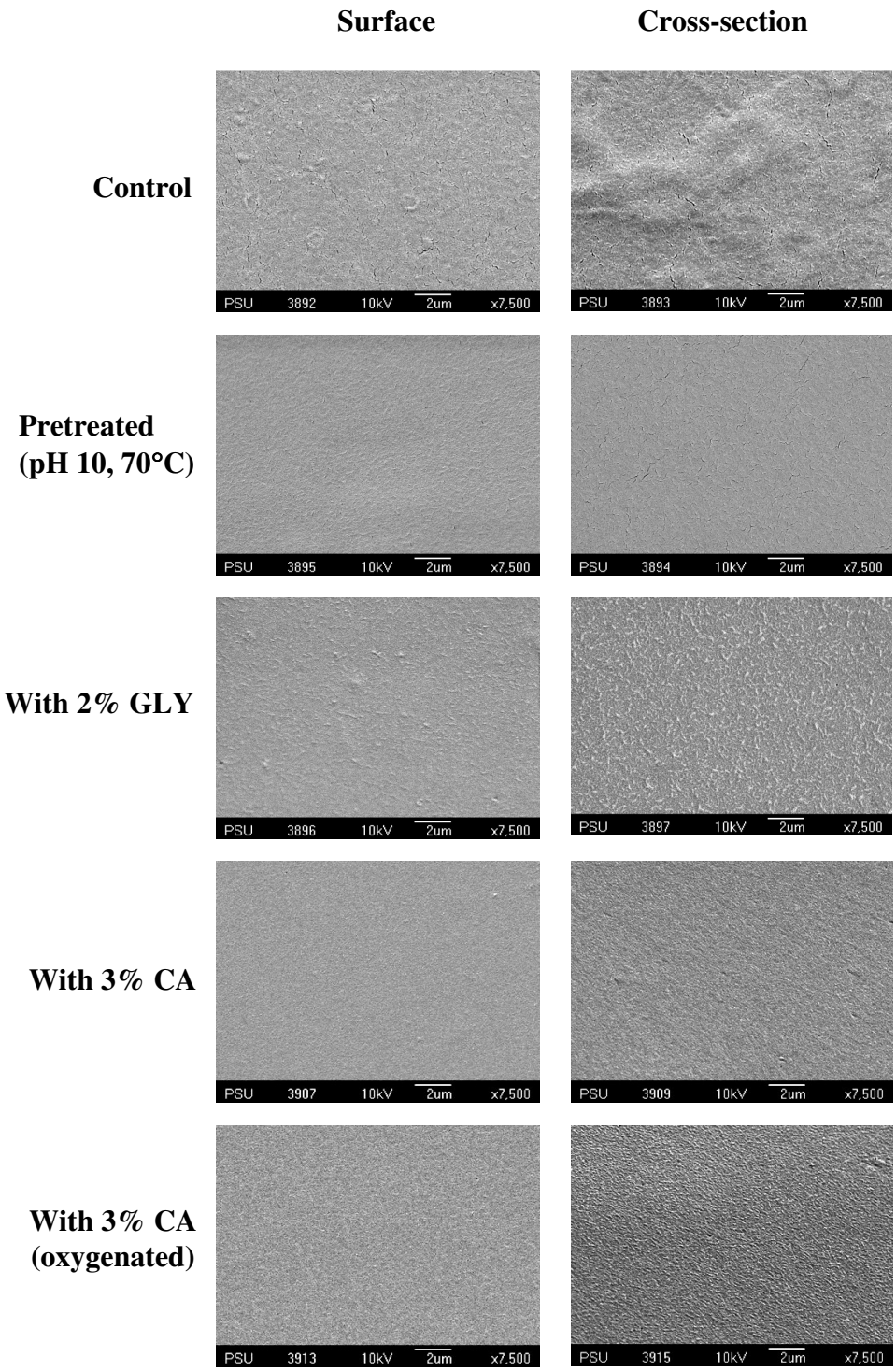

Figure 11. Micrographs of the surface and cross-section of the porcine plasma protein-based films treated with and without 2% glyoxal (GLY) or 3% caffeic acid (CA) with and without oxygenation. Control: FFS with no pH adjustment and preheating; Pretreated: FFS (pH 10) preheating at 70°C.

After 24 days of storage, samples packaged in bags produced from porcine plasma protein-based films bags, from film incorporated with 3% caffeic acid with prior oxygenation and PE bag had the moisture contents of 19.22, 18.88 and 4.79%, respectively. The addition of caffeic acid into the film forming solutions with prior oxygenation could not improve the water vapor barrier properties of films ($p<0.05$). These results were not consistent with the previous report on oxidized ferulic acid incorporated starch-chitosan blend films that showed the decrease of WVP when comparison with the blend film alone (Mathew and Abrahm, 2008). Ou *et al*. (2005) reported that oxidized ferulic acid did not significantly decrease the water vapor permeability of soy protein isolate films. Similar behavior was observed by Cao *et al*. (2007) who reported that gelatin based films incorporated with ferulic acid and tannic acid had a slight decrease in WVP.

8.2 Changes in color of fish powder

L*-, a*- and b*-values of dried fish powder packaged in bags produced from porcine plasma protein-based films without and with 3%caffeic acid with prior oxygenation and PE bag during storage at 28-30°C are displayed in Figure 13. Decreases in L*-value and the increases in a*- and b*-values of dried fish powder packaged in PE bag were lower than samples packaged in bags produced from porcine plasma protein-based films throughout the storage of 24 days. The increased b*-value suggested the formation of yellowish pigment, especially via Maillard reaction, which might be associated with increasing moisture content in dried fish powder during the extended storage. This was in agreement with the darker color as indicated by lowered L*-value. During 9-18 days of storage, b*-value of sample packaged in bag produced from porcine plasma protein-based film incorporated with 3% caffeic acid with prior oxygenation was lower than that of samples stored in plasma protein-based film bag without caffeic acid addition ($p<0.05$). The water activity affects rate and extent of nonenzymatic browning reaction and lipid oxidation of food products. Increased water activity enhances many reactions (Martinez and Labuza, 1968). Thanonkaew *et al*. (2007) reported that aldehydes from lipid oxidation could react with amines of proteins and peptides and contribute to the nonenzymatic browning.

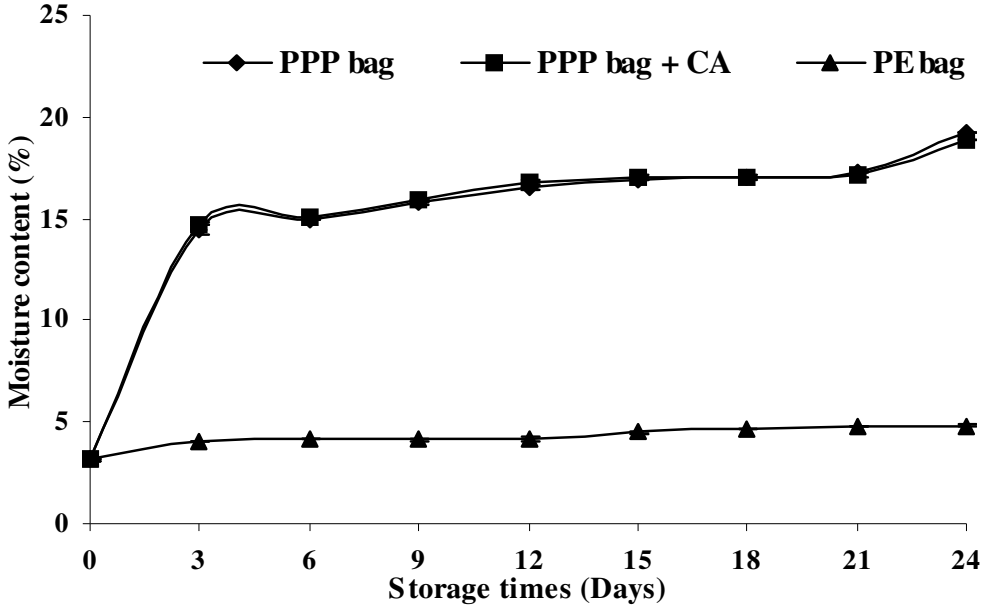

Figure 12. Changes in moisture content of fish powder packaged in bag made from porcine plasma protein-based films without (PPP bag) and with 3% caffeic acid with prior oxygenation (PPP bag + CA) and PE bag during storage at 28-30°C at RH of 60±10%. Bars represent standard deviation from triplicate determinations.

8.3 Changes in TBARS of fish powder

TBARS values of fish powder packaged in bags produced from porcine plasma protein-based films incorporated without and with 3%caffeic acid or PE bag during storage at 28-30°C are depicted in Figure 14. TBARS values of all samples increased continuously up to 24 days of storage ($p<0.05$). Sample kept in bags produced from film added with 3% caffeic acid with prior oxygenation showed the lowest TBARS value throughout the storage time. During the first 3 days of storage, no differences in TBARS value were observed between samples kept in PE and the sample stored in bag produced from porcine plasma protein-based films ($p<0.05$). Thereafter, sample kept in bag made from porcine plasma protein-based film showed the highest TBARS value up to 21 days. In general, good oxygen barrier ability is a characteristic of protein films. On the other hand, the greater oxygen permeability was found in synthetic films (Miller and Krochta, 1997).

The lipid oxidation can be initiated and accelerated by various mechanisms including the production of singlet oxygen, enzymatic and non-enzymatic generation of free radicals and active oxygen (Kubow, 1992). Different oxygen transmission rate between porcine plasma protein-based film and PE film might affect lipid oxidation differently. Oxygen molecules which could penetrate to fish powder packaged in PE bag could accelerate the lipid oxidation. Hydrophilic nature of porcine plasma protein films was associated with the low effectiveness as moisture barriers, as evidenced by the increase in moisture content of samples stored in bag made from porcine plasma protein films (Figure 12). The higher moisture content might facilitate the migration of reactants for lipid oxidation. It has been reported that the incorporation of hydrophobic compounds into film caused negative effects on oxygen transmission rate (Wessling *et al*., 2000). Homma and Fujimaki (1982) reported that the decrease in water activity could retard the rate of lipid oxidation and browning of Kori-tofu during storage. Due to the crosslinking of protein molecules in film incorporated with 3% caffeic acid, the decrease in the oxygen permeability of film was presumed. Additonally, caffeic acid might function as antioxidant. Flavonoids and hydrocinnamic acid derivatives were reported to scavenge free radicals in an activity-structure related manner by donating hydrogen atoms (Shahidi and Wanasundara, 1992). Chen and Ahn (1998) reported that caffeic acid (50μM) was able to inhibit the lipid oxidation of oil emulsions was induced by UV light and/or ferrous iron. Liu *et al*. (2007) studied the potential for using pectin (containing 2.5% and 5% corn oil and olive oil) and gelatin/sodium alginate blend-based (containing 2.5% corn oil and olive oil) casings in the manufacture of breakfast pork sausages. Sausages held in gelatin/sodium alginate blend castings had lower TBARS compared to those held in pectin casting.

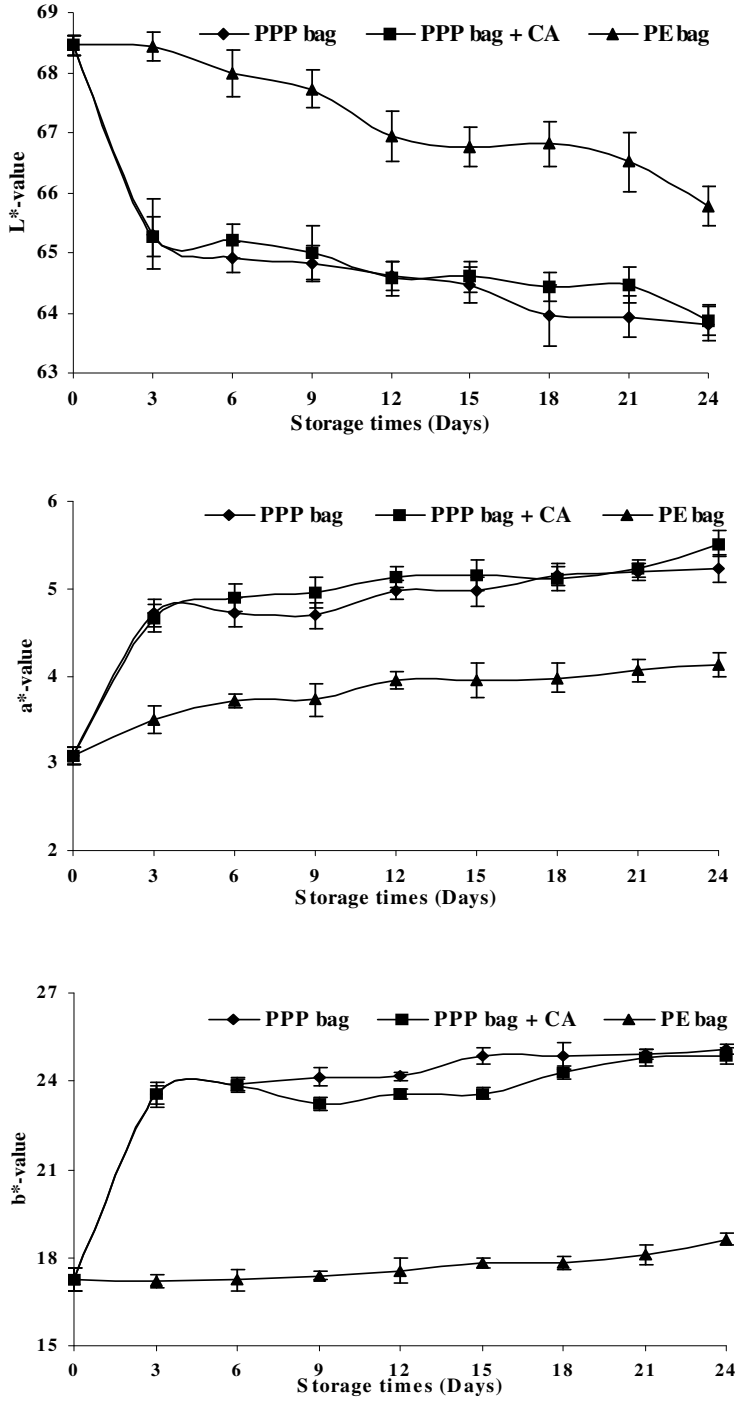

Figure 13. L*-, a*- and b*-values of fish powder packaged in bag made from porcine plasma protein-based films without (PPP bag) and with 3% caffeic acid with prior oxygenation (PPP bag + CA) and PE bag during storage at 28-30°C at RH of 60±10%. Bars represent standard deviation from triplicate determinations.

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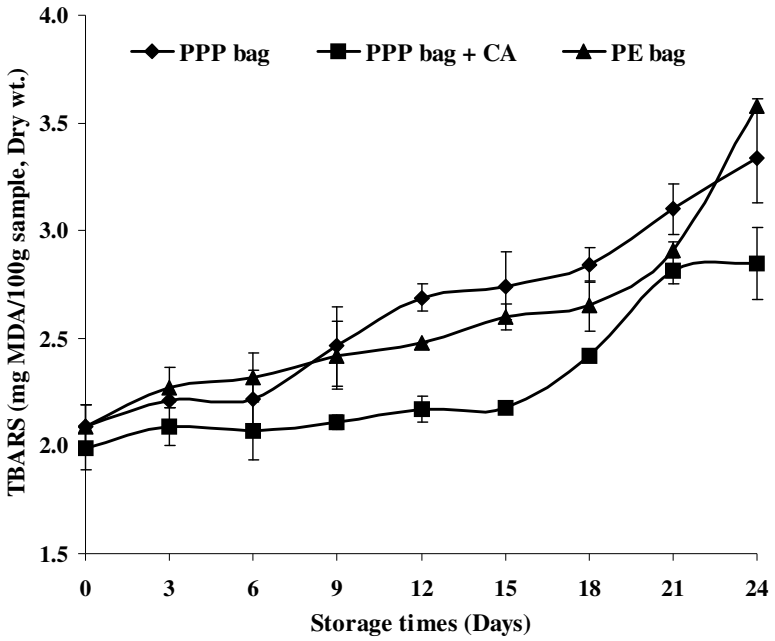

Figure 14. TBARS of fish powder packaged in bag made from porcine plasma protein-based films without (PPP bag) and with 3% caffeic acid with prior oxygenation (PPP bag + CA) and PE bag during storage at 28-30°C at RH of 60±10%. Bars represent standard deviation from triplicate determinations.

CHAPTER 4

CONCLUSIONS

1. Protein and glycerol contents affected properties of porcine plasma protein-based film. The increase of glycerol content enhanced EAB, L*-value and transparency of film, but decreased b*-value. FFS with higher protein content yielded the film with higher thickness and TS. Film and protein solubility were not significantly influenced by protein and glycerol content.

2. Mechanical properties of films were markedly affected by pHs and preheating temperature of FFS. WVP of film was slightly influenced by pH. Film with alkaline condition and preheated at higher temperature rendered the films with greater EAB. Thus, the appropriate pretreatment together with the use of proper glycerol content in FFS could be a promising means to improve the properties of resulting film.

3. Cross-linking of porcine plasma protein-based films with glyoxal, formaldehyde and glutaraldehyde enhanced the mechanical properties of films but slightly increased the water barrier properties. The glyoxal at a level of 2% showed the most effectiveness in improving the elongation at break of film but resulted in an undesirable yellowish coloration.

4. Phenolic compounds including tannic acid, caffeic acid and ferulic acid could improve the mechanical properties without the marked changes in water vapor barrier properties. Caffeic acid at a level of 3% was the most effective in increasing TS and EAB. Oxygenation of FFS at pH 10 yielded the film with a greater improvement of mechanical property associated with the increased protein aggregation. Film became more yellowish and reddish when tannic acid or caffeic acid was added.

5. Incorporation of cross-linking agents was associated with the increase in the resistance to enzymatic hydrolysis and the increase in degradation temperature and melting point of film. Nevertheless, due to the toxicity of glyoxal, non-toxic natural compound, such as caffeic acid or other natural phenolic compounds would be the recommendable cross-linker for preparation of edible protein-based film.

6. Porcine plasma protein-based films containing caffeic acid with prior oxygenation could retard the lipid oxidation and the formation of yellowish pigment of dried fish powder. However, it was not able to reduce the water migration into the sample.

Suggestions

1. Gas permeability of porcine plasma protein-based films should be further determined.

2. Improvement of water vapor barrier property of film should be carried out to prolong the shelf-life of hygroscopic foods.

3. Modification of proteins, in which the lower content of glycerol can be used, should be conducted to decrease the hydrophilicity of resulting film.

4. The removal or lowering of the indigenous undesirable smell of porcine plasma protein-based film should be further studied.

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APPENDIX A

ANALYTICAL METHODS

1. Determination of moisture content (AOAC, 2000)

Method

1. Dry the empty dish and lid in the oven at 105°C for 3 h and transfer to desiccator to cool. Weigh the empty dish and lid.
2. Weigh about 3 g of sample to the dish. Spread the sample to the uniformity.
3. Place the dish with sample in the oven. Dry for 3 h at 105°C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried sample.

Calculation

$$\text{Moisture content (\%)} = \frac{(W1-W2)}{W1} \times 100$$

where: W1 = weight (g) of sample before drying

W2 = weight (g) of sample after drying

2. Determination of protein content (AOAC, 2000)

Reagents

- Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) with 1 part of coppersulphate ($CuSO_4$)
- Sulfuric acid (H_2SO_4)
- 40% NaOH solution
- 0.2 N HCl solution
- 4% H_3BO_3
- Indicator solution: Mix 100 ml of 0.1% methyl red (in 95% ethanol) with 200 ml of 0.2% bromocresol green (in 95% ethanol)

Method

1. Place sample (0.5-1.0 g) in digestion flask.
2. Add 5 g Kjedahl catalyst and 200 ml of conc. H_2SO_4.

3. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently unit frothing ceases. Boil briskly until solution clears.
4. Cool and add 60 ml distilled water cautiously.
5. Immediately connect flask to digestion bulb on condenser and with tip of condenser immersed in standard acid and 5-7 indicator in receiver. Rotate flask to mix content thoroughly; then heat until all NH_3 is distilled.
6. Remove receiver, wash tip of condenser and titrate excess standard acid distilled with standard NaOH solution.

Calculation

$$\text{Protein content (\%)} = \frac{(A-B) \times N \times 1.4007 \times 6.25}{W}$$

Where: A = volume (ml) of 0.2 N HCl used for sample titration

B = volume (ml) of 0.2 N HCl used in blank titration

N = Normality of HCl

W = weight (g) of sample

14.007 = atomic weight of nitrogen

6.25 = the protein-nitrogen conversion factor for fish and its by-products

3. Determination of ash content (AOAC, 2000)

Method

1. Place the crucible and lid in the furnace at 550°C overnight to ensure that impurities on the surface of crucible are burned off.
2. Cool the crucible in the desiccator (30 min).
3. Weigh the crucible and lid to 3 decimal places.
4. Weigh about 5 g sample into the crucible. Heat over low Bunsen flame with lid half covered. When fumes are no longer produced, place crucible and lid in furnace.
5. Heat at 550°C overnight. During heating, do not cover the lid. Place the lid after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.

6. Weigh the ash with crucible and lid when the sample turns to gray. If not, return the crucible and lid to the furnace for the further ashing.

Calculation

$$\text{Ash content (\%)} = \frac{\text{Weight of ash}}{\text{Weight of sample}} \times 100$$

4. Biuret method for quantitation of protein (Robinson and Hodgen, 1940)

Reagents

1. Biuret reagent: combine 1.50 g $CuSO_4.5H_2O$, 6.00 g sodium potassium tartrate, and 500 ml distilled water in a beaker and stir, add while stirring 300 ml of 10% NaOH (w/v), transfer to plastic bottle for storage.
2. Distilled water
3. Standard reagent: 10 mg/ml bovine serum albumin (BSA)

Method

1. To each of seven disposable cuvette, add the following reagents according to the table.
2. Add 2.0 ml of the biuret reagent to each tube, and mix well.
3. Incubate the mixture at room temperature for 30-45 min, and then read the adsorbance of each tube at 540 nm.
4. For tube 1-5, plot the absorbance at 540 nm as a function of effective BSA concentration and calculate the best fit straight line from data. Then, using the average absorbance for the three sample of unknown read the concentration of sample from the plot.

Table A1. Experimental set up for the Biuret's assay.

Tube number	Water (µL)	10 mg/ml BSA (µL)	Effective BSA Concentration (mg/ml)
1	500	0	0
2	400	100	2
3	300	200	4
4	200	300	6
5	100	400	8
6	0	500	10
7	0	0	unknown

5. Thiobarbituric acid-reactive substance (TBARS) of lecithin liposome system (Frankel *et al*., 1997)

Reagent

1. TBA solution (15% TCA/ 0.375% TBA/ 0.025N HCl)
2. 0.2 % BHT

Method

1. Mix liposome sample (1 ml) with 20 µl of butylated hydroxytoluene (0.2%) and add 2 ml of TBA solution into the mixture.
2. Heat the mixtures for 10 min in a boiling water bath (95-100^{o}C) to develop pink color.
3. Cool with tap water and centrifuge for 20 min at 5,500x*g*.
4. Read the absorbance of the supernatant containing the pink chromogen at 532 nm and express TBARS as mg MDA/ml liposome.

6. Electrophoresis (SDS-PAGE) (Laemmli, 1970)

Reagent

1. 30% Arylamide - 0.8% bis Acrylamide
2. Sample buffer: Mix 30 ml of 10% of SDS, 10 ml of glycerol, 5 ml of β-mercaptoethanol, 12.5 ml of 50 mM Tris-HCl, pH 6.8, and 10 mg bromophenol blue. Bring the volumn to 100 ml with distilled water. Divide into 1 ml aliquots, and store at -20 °C.
4. 2% (w/v) Ammonium persulfate
5. 1% (w/v) SDS
6. TEMED (*N,N,N'N'*- tetramethylethylenediamine)
7. 0.5 M Tris-HCl, pH 6.8
8. 1.5 M Tris-HCl, pH 8.8
9. Electrode buffer: Dissolve 3 g of Tris-HCl, 14.4 g of glycine and 1 g of SDS in distilled water. Adjust to pH 8.3. Add distilled water to 1 liter to total volume.
10. Staining solution: Dissolve 0.05 g of Coomassie blue R-250 in 15 ml methanol. Add 5 ml of glacial acetic and 80 ml of distilled water.
11. Destaining solution: 30% methanol-10% glacial acetic acid

Method

Pouring the running gel:

1. Assemble the minigel apparatus according to the manufacture's detailed instructions. Make sure that the glass and other components are rigorously clean and dry before assembly.
2. Mix the separating gel solution by adding as defined in following Table.
3. Transfer the separating gel solution using a Pasture pipettes to the center of sandwich is about 1.5 to 2 cm from the top of the shorter (front) glass plate.
4. Cover the top of the gel with a layer of distilled water by squiting the distilled water against the edge of one of the spacers. Allow the resolving gel to polymerize fully (usually 30-60 min).

Pouring the stacking gel:

1. Pour off completely the layer of isobutyl alcohol.
2. Prepare a 4% stacking gel solution by adding as defined in Table.
3. Transfer stacking gel solution to tickle into the center of the sandwich along an edge of the one of the spacers.
4. Insert comb into the layer of stacking gel solution by placing one corner of the comb into the gel and slowly lowering the other corner in. Allow the stacking gel solution to polymerize 30 to 45 min at room temperature.

Table A2. Experimental set up for running and stacking gel.

Reagents	7.5% running gel	4% stacking gel
30% Acrylamide-bis	2.500 mL	0.665 mL
1.5 M Tria-HCl buffer, pH 8.8	2.500 mL	-
0.5 M Tris-HCl buffer, pH 6.8	-	1.25 mL
Distilled water	4.845 mL	3.00 mL
10% SDS	10 µL	50 µL
2% Ammonium persulfate	50 µL	25 µL
TEMED	5 µL	3 µL

Sample preparation:

1. Weigh 0.5 g of sample and completely dissolve with 5% SDS (w/v) in a final volume of 10 ml.
2. Centrifuge at 7,500x*g* for 5 min at ambient temperature and collect supernatant.

Loading the gel:

1. Dilute the protein to be 1:1 (v/v) with sample buffer in microcentrifuge tube and boil for 1 min at 100°C.
2. Remove the comb without tearing the edge of the polyacrylamide wells.
3. Fill the wells with electrode buffer.
4. Place the upper chamber over the sandwich and lock the upper buffer chamber to the sandwich. Pour electrode buffer into the lower buffer chamber. Place the sandwich attached to the upper buffer chamber into the lower chamber.

5. Fill the upper buffer chamber with electrode buffer so that the sample wells of the stacking gel are filled with buffer.
6. Use a 10-25 µL syringe with a flate-tipped needle; load the protein sample into the wells by carefully applying the sample as a thin layer at the bottom of the well.
7. Fill the remainder of the upper buffer chamber with additional electrode buffer.

Running the gel:

1. Connect the power supply to the anode and cathode of the gel apparatus and run at 50 V and 150 V.
2. After the bromophenol blue tracking dye has reached the bottom of the separating gel, disconnect the power supply.

Disassembling the gel:

1. Remove the upper buffer chamber and the attached sandwich.
2. Orient the gel so that the order of the sample well is known, remove the sandwich from the upper buffer chamber, and lay the sandwich on a sheet of absorbent paper or paper towels. Carefully slide the spacers out from the edge of the sandwich along its entire length.
3. Gently twist the spacer so that the upper glass plate pulls away from the gel. Remove the plate. The gel will stick to one of the plates.
4. Remove the gel from the lower plate. Place the plate with the gel attached into the shallow dish of fixing agent or dye and swishing the plate.

Staining the gel:

1. Place the gel in a small plastic box and cover with the staining solution. Agitate slowly for 3 h or more on a rotary rocker.
2. Pour off the staining solution and cover the gel with a solution of destaining solution I. Agitate slowly for about 15 min.
3. Pour off the destaining solution I and replace with flesh solution. Repeat until the gel is clear except for the protein bands.

APPENDIX B

Table B1. Thermogravimetric parameters for the thermal degradation of porcine plasma protein-based film.

Films	Td^a_1 (°C)	Td^a_2 (°C)	Td^a_3 (°C)	$T_{max}{}^b$ (°C)	Residue[c] (%)
Control	52.9	170.0	288.8	319.6	19.5
Pretreated (pH10, 70°C)	64.8	172.8	290.0	312.0	19.1
With 2% GLY	66.3	186.0	291.6	325.1	20.3
With 3% CA	66.9	201.6	299.2	317.2	19.9
With 3% CA (oxygenated)	66.1	203.8	300.0	321.8	19.0

[a] Temperature of degradation
[b] Temperature at maximum degradation rate
[c] Value determined at 800°C at a heating rate of 10°C min^{-1}
Control: FFS without heating and pH adjustment was directly subjected to casting.
GLY: Glyoxal, CA: Caffeic acid

VITAE

Name Mr. Pornpot Nuthong

Student ID 4882014

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biology)	Prince of Songkla University	2001

Work-Position and Address

Scientist

Scientific Equipment Center, Prince of Songkla University

List of Publication and Proceedings

Nuthong, P., Benjakul, S. and Prodpran, T. 2009. Effect of phenolic compounds on the properties of porcine plasma protein-based film. Food Hydrocolloids. 23: 736-741.

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