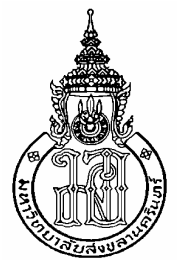

Discoloration and Its Prevention of Protein Film from Red Tilapia Muscle

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A Thesis Submitted in Partial Fulfillment of the Requirements for the
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ชื่อวิทยานิพนธ์	การเปลี่ยนแปลงสีของฟิล์มโปรตีนจากกล้ามเนื้อปลาทับทิมและการป้องกัน
ผู้เขียน	นายภควรรษ ทองนวลจันทร์
สาขาวิชา	วิทยาศาสตร์และเทคโนโลยีอาหาร
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บทคัดย่อ

จากการศึกษาคุณลักษณะและการติดตามการเปลี่ยนแปลงสีของฟิล์มโปรตีนจากกล้ามเนื้อปลาทับทิม พบว่าฟิล์มจากกล้ามเนื้อปลาที่ผ่านการล้างซึ่งเตรียมที่พีเอช 3 และ 11 มีค่าการทนต่อแรงดึงสูงกว่าฟิล์มซึ่งเตรียมจากกล้ามเนื้อปลาที่ไม่ผ่านการล้าง ($P < 0.05$) ฟิล์มจากกล้ามเนื้อปลาที่ผ่านและไม่ผ่านการล้างซึ่งเตรียมที่พีเอช 3 มีค่าการทนต่อแรงดึงสูงกว่าฟิล์มซึ่งเตรียมที่พีเอช 11 ($P < 0.05$) ฟิล์มจากกล้ามเนื้อปลาที่ผ่านการล้างและเตรียมที่พีเอช 3 มีค่าการทนต่อแรงดึงสูงสุด ในขณะที่ฟิล์มจากกล้ามเนื้อปลาที่ไม่ผ่านการล้างและเตรียมที่พีเอช 11 มีค่าการทนต่อแรงดึงต่ำสุด และมีค่าระยะยืดดึงเมื่อขาดสูงสุด ($P < 0.05$) ฟิล์มจากกล้ามเนื้อปลาที่ผ่านการล้างและเตรียมทั้งที่พีเอช 3 และ 11 มีค่า TBARS ต่ำกว่าฟิล์มจากกล้ามเนื้อปลาที่ไม่ผ่านการล้าง อย่างไรก็ตามค่า TBARS ของฟิล์มซึ่งเตรียมที่พีเอช 3 สูงกว่าฟิล์มซึ่งเตรียมที่พีเอช 11 ระหว่างการเก็บรักษาเป็นระยะเวลา 20 วันที่อุณหภูมิห้อง (28-32 องศาเซลเซียส) พบว่าฟิล์มมีสีเหลืองขึ้น แสดงจากค่า b^* และ ΔE^* ที่สูงขึ้น ฟิล์มซึ่งเตรียมที่พีเอช 11 มีค่า b^* และ ΔE^* สูงกว่าฟิล์มซึ่งเตรียมที่พีเอช 3 โดยเฉพาะสำหรับฟิล์มที่เตรียมจากกล้ามเนื้อปลาที่ไม่ผ่านการล้าง อย่างไรก็ตามฟิล์มจากกล้ามเนื้อปลาที่ผ่านการล้างและเตรียมที่พีเอช 3 มีค่า b^* และ ΔE^* สูงกว่าฟิล์มซึ่งเตรียมที่พีเอช 11 ($P < 0.05$) โดยทั่วไปฟิล์มมีค่าการทนต่อแรงดึงสูงขึ้น แต่มีค่าการซึมผ่านไอน้ำ ค่าการละลายของฟิล์มและโปรตีนลดลงภายหลังการเก็บรักษาเป็นระยะเวลา 20 วัน ($P < 0.05$)

เมื่อเก็บรักษาฟิล์มซึ่งเตรียมจากกล้ามเนื้อปลาทับทิมที่ไม่ผ่านการล้างที่พีเอช 3 และ 11 ภายใต้สภาวะ 100% N_2 พบว่าฟิล์มมีค่า TBARS และ b^* และ ΔE^* ต่ำสุดในระหว่างการเก็บรักษาเป็นเวลา 20 วันที่อุณหภูมิห้อง เมื่อเปรียบเทียบกับฟิล์มที่เก็บในสภาวะบรรยากาศปกติและ 100% O_2 ฟิล์มซึ่งเตรียมที่พีเอช 3 และเติมสารต้านอนุมูลอิสระ (Trolox และ catechin) ที่ทุกระดับความเข้มข้น (100, 200 และ 400 ppm) มีค่า TBARS และ b^* และ ΔE^* ต่ำสุดในระหว่างการเก็บรักษา ($P < 0.05$) แสดงถึงการชะลอการเกิดปฏิกิริยาออกซิเดชันของไขมันและการเกิดสีเหลือง

ในฟิล์ม แต่อย่างไรก็ตามฟิล์มที่พีเอช 11 และเติมสารต้านอนุมูลอิสระมีค่า TBARS ไม่แตกต่างจากชุดควบคุม และมีการเพิ่มของค่า b^* และ ΔE^* ในฟิล์มดังกล่าว

เมื่อเปรียบเทียบคุณลักษณะของฟิล์มจากโปรตีนไอโซเลทจากกล้ามเนื้อปลาที่ผ่านการล้างและการละลายด้วยด่าง กับฟิล์มที่เตรียมจากเนื้อที่ผ่านการล้าง พบว่าฟิล์มจากโปรตีนไอโซเลทซึ่งเตรียมที่พีเอช 3 และ 11 มีค่าการทนต่อแรงดึงและค่าระยะยืดดึงเมื่อขาดสูงกว่าฟิล์มที่เตรียมจากกล้ามเนื้อปลาที่ผ่านการล้าง ($P < 0.05$) ฟิล์มจากโปรตีนไอโซเลทซึ่งเตรียมที่พีเอช 3 มีค่าการทนต่อแรงดึงสูงสุด ในขณะที่ฟิล์มจากกล้ามเนื้อปลาที่ผ่านการล้างซึ่งเตรียมที่พีเอช 11 มีค่าการทนต่อแรงดึงต่ำสุด ($P < 0.05$) อย่างไรก็ตามฟิล์มจากโปรตีนไอโซเลทที่เตรียมทั้งสองพีเอชมีค่าการซึมผ่านไอน้ำต่ำกว่าฟิล์มจากกล้ามเนื้อปลาที่ผ่านการล้าง ($P < 0.05$) เมื่อเปรียบเทียบฟิล์มที่เตรียมที่ พีเอชเดียวกันฟิล์มจากโปรตีนไอโซเลทมีค่า TBARS ต่ำกว่าฟิล์มจากกล้ามเนื้อปลาที่ผ่านการล้าง ($P < 0.05$) และฟิล์มจากทั้งโปรตีนไอโซเลทและกล้ามเนื้อปลาที่ผ่านการล้างและเตรียมที่พีเอช 3 มีค่า TBARS สูงกว่าฟิล์มที่เตรียมที่พีเอช 11 ($P < 0.05$) จากตัวอย่างฟิล์มทั้งหมด พบว่าฟิล์มจากโปรตีนไอโซเลทซึ่งเตรียมที่พีเอช 3 มีความโปร่งใสของฟิล์มสูงสุดและไม่มีการเปลี่ยนแปลงสีเหลืองของฟิล์มระหว่างการเก็บรักษาเป็นระยะเวลา 20 วันที่อุณหภูมิห้อง เมื่อเปรียบเทียบกับฟิล์มอื่นๆ ($P < 0.05$) ในทางตรงกันข้ามฟิล์มจากกล้ามเนื้อปลาที่ผ่านการล้างที่เตรียมที่พีเอช 3 มีการเพิ่มขึ้นของค่า b^* และ ΔE^* สูงกว่าฟิล์มอื่น

จากการศึกษาเปรียบเทียบคุณลักษณะของฟิล์มจากโปรตีนไอโซเลทจากกล้ามเนื้อปลาทับทิมซึ่งเตรียมที่พีเอช 3 และเติมสารต้านอนุมูลอิสระ (Trolox) ที่ระดับ 100 ppm กับฟิล์มจากกล้ามเนื้อปลาที่ไม่ผ่านการล้าง ระหว่างการเก็บรักษาเป็นเวลา 40 วัน ที่อุณหภูมิห้อง พบว่าฟิล์มโปรตีนไอโซเลทที่เติมสารต้านอนุมูลอิสระมีค่าการทนต่อแรงดึงและค่าระยะยืดดึงเมื่อขาดสูงกว่าแต่มีค่าการซึมผ่านไอน้ำต่ำกว่าเมื่อเปรียบเทียบกับฟิล์มจากกล้ามเนื้อปลาที่ไม่ผ่านการล้าง ($P < 0.05$) ฟิล์มโปรตีนไอโซเลทที่เติมสารต้านอนุมูลอิสระมีค่า TBARS ต่ำกว่าฟิล์มจากกล้ามเนื้อปลาที่ไม่ผ่านการล้างระหว่างการเก็บรักษา ($P < 0.05$) นอกจากนี้ฟิล์มโปรตีนไอโซเลทที่เติมสารต้านอนุมูลอิสระมีความโปร่งใสของฟิล์มสูงกว่าและไม่มีการเปลี่ยนแปลงสีเหลืองของฟิล์มระหว่างการเก็บรักษาเป็นระยะเวลา 40 วัน โครงสร้างของฟิล์มทั้งจากโปรตีนไอโซเลทและจากกล้ามเนื้อปลาที่ไม่ผ่านการล้างยึดกันด้วย พันธะไฮโดรเจน อันตรกิริยาไฮโดรโฟบิก พันธะไดซัลไฟด์ และพันธะโควาเลนท์ที่ไม่ใช่พันธะไดซัลไฟด์ จากสเปกตรา FTIR พบว่าฟิล์มโปรตีนไอโซเลทที่เติมสารต้านอนุมูลอิสระมีปริมาณไขมันน้อยกว่า และมีแอมพลิจูดของ Amide B ต่ำกว่าฟิล์มจากกล้ามเนื้อปลาที่ไม่ผ่านการล้าง นอกจากนี้ฟิล์มโปรตีนไอโซเลทที่เติมสารต้านอนุมูลอิสระมีโครงสร้างที่แข็งแรงกว่าฟิล์มจากกล้ามเนื้อปลาที่ไม่ผ่านการล้าง โดยสังเกตจากค่า Td ที่สูงกว่า ซึ่งบ่งชี้อันตรกิริยา

ระหว่างโมเลกุลที่สูงกว่า และยังมีมีผิวหน้าและเนื้อฟิล์มที่เรียบกว่า ภายหลังการเก็บรักษาเป็นเวลา 40 วันพบว่าฟิล์มทั้งสองมีค่าการทนต่อแรงดึงและ Td สูงขึ้น แต่มีค่าระยะยืดดึงเมื่อขาด ค่าการซึมผ่านไอน้ำและค่าการละลายลดลง ดังนั้นฟิล์มจากโปรตีนไอโซเลทที่มีปริมาณไขมันและโปรออกซิแดนท์ต่ำ และเติมสารต้านอนุมูลอิสระ มีสมบัติเชิงกลและสมบัติทางกายภาพของฟิล์มที่ดีขึ้น ตลอดจนไม่มีการเปลี่ยนสีเหลืองของฟิล์มระหว่างการเก็บรักษา เมื่อใช้แผ่นฟิล์มโปรตีนไอโซเลทที่เติมสารต้านอนุมูลอิสระเก็บรักษาผงเนื้อปลาโอแห้ง พบว่าตัวอย่างผงเนื้อปลาโอแห้งมีค่า TBARS และ PV-value ต่ำกว่า แต่มีความชื้น และค่า b^* สูงกว่า เมื่อเปรียบเทียบกับผงเนื้อปลาโอแห้งที่ปิดผนึกด้วยฟิล์ม PE และ PP ดังนั้นฟิล์มโปรตีนไอโซเลทจากเนื้อปลาที่เติมสารต้านอนุมูลอิสระสามารถนำไปใช้เป็นแผ่นฟิล์มที่ย่อยสลายได้โดยปราศจากการเปลี่ยนแปลงสีและสามารถป้องกันการเกิดปฏิกิริยาออกซิเดชันของไขมันในผลิตภัณฑ์อาหารได้อย่างมีประสิทธิภาพ

Thesis Title	Discoloration and its prevention of protein-based film from red tilapia muscle
Author	Mr. Phakawat Tongnuanchan
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ABSTRACT

Protein-based films prepared from red tilapia washed and unwashed mince solubilized at pH 3 and 11 were characterized and monitored for discoloration during storage. Tensile strength (TS) of films from washed mince was greater than that of films prepared from unwashed mince for both pH used ($P < 0.05$). TS of films prepared at pH 3 was higher than that of films prepared at pH 11 for both washed and unwashed mince ($P < 0.05$). Film from washed mince with pH 3 showed the highest TS, while that from unwashed mince with pH 11 had the lowest TS with the highest elongation at break (EAB) ($P < 0.05$). Films from washed mince had the lower value of thiobarbituric acid reactive substances (TBARS) than did those from unwashed counterpart, regardless of pH used. Nevertheless, TBARS was much higher in films prepared at acidic pH, compared with those prepared at alkaline pH. During storage of 20 days at room temperature (28-32°C), films became yellowish as evidenced by the increases in b^* and ΔE^*-values. Films prepared at pH 11 showed the higher b^* and ΔE^*-values than did those prepared at pH 3, especially for those from unwashed mince. However, films prepared from washed mince at pH 3 showed higher b^* and ΔE^*-values than did those prepared at pH 11 ($P < 0.05$). Films generally had the increase in TS but the decreases in water vapor permeability (WVP), film solubility and protein solubility after 20 days of storage ($P < 0.05$).

Films prepared from unwashed mince at both pH 3 and 11 and stored under atmospheric containing 100% N_2 had the lowest TBARS value with the concomitant lowest b^* and ΔE^*-values during the storage of 20 days at room temperature ($P < 0.05$), when compared with other films kept in air and 100% O_2 atmosphere. Films prepared at pH 3 and incorporated with antioxidants (Trolox and catechin) at all levels (100, 200 and 400 ppm) had the lowest TBARS value, b^* and

ΔE^*-values during storage, indicating the retardation of lipid oxidation and yellow discoloration in films. Nevertheless, films prepared at pH 11 had no difference in TBARS values, in comparison with control film, regardless of antioxidant inorporation. Coincidentally, the increases in b^* and ΔE^*-values were observed in those films.

Films from fish protein isolate (FPI) prepared by prior washing followed by alkaline solubilization process (ASP) from red tilapia muscle were characterized, in comparison with films from washed mince ($P < 0.05$). Films from FPI had higher tensile strength (TS) and elongation at break (EAB) than did those from washed mince for both pH (3 and 11) used for film preparation ($P < 0.05$). Film from FPI prepared at pH 3 showed the highest TS, while that from washed mince prepared at pH 11 had the lowest TS ($P < 0.05$). Nevertheless, films from FPI had lower WVP than those from washed mince for both pH used ($P < 0.05$). At the same pH used for film preparation (3 or 11), films from FPI showed the lower TBARS values than did those from washed mince ($P < 0.05$). Nevertheless, films from both FPI and washed mince had the higher TBARS values when pH 3 was used for film preparation, compared with pH 11 ($P < 0.05$). Among all films, those from FPI prepared at pH 3 had the highest transparency and no yellow discoloration was observed during the storage of 20 days at room temperature, in comparison with other films ($P < 0.05$). Conversely, film from washed mince prepared at pH 3 had the higher increase in b^*-value and ΔE^*-value than other films.

Films from FPI of red tilapia muscle prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT film) were characterized in comparison with film prepared from unwashed mince (UWM) during storage of 40 days at room temperature. FPIT film had higher tensile strength (TS) and elongation at break (EAB) but lower water vapor permeability (WVP) than UWM film ($P < 0.05$). During the storage, FPIT film had much lower TBARS value than UWM film ($P < 0.05$). Furthermore, FPIT was more transparent and had no yellow discoloration, as evidenced by no change in b^* and ΔE^*-values, during the storage of 40 days. Both UWM and FPIT films were stabilized mainly by hydrogen bond, hydrophobic interaction, disulfide bond and non-disulfide covalent bond. FTIR spectra indicated that FPIT film contained the lower amount of lipids with the lower amplitude of

amide B band, compared with UWM film. Higher degradation temperature (Td) was observed in FPIT film, indicating a greater protein-protein interaction in film matrix. FPIT film had smoother surface and cross-section than UWM film. After 40 days of storage, both films had the increase in TS and Td but lower EAB, WVP and protein solubility. Thus, film from FPI with lowered lipid and prooxidant contents and incorporated with antioxidant had the improved mechanical and physical properties without yellow discoloration. When FPIT films were used to cover dried fish powder, lower TBARS and PV values were observed but moisture and yellowness were higher, compared to those covered with PE and PP films. Therefore, FPIT film can be used as biodegradable film for effective retardation of lipid oxidation of food products without its yellow discoloration.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Biopolymer edible films and coatings have increasingly attracted the attention, mainly owing to the large variety of applications. Biodegradability is one of the greatest benefits of edible films and coatings along with edibility (Debeaufort *et al.*, 1998). Many functions of edible films and coatings are similar to those of synthetic packaging films; however edible film and coating materials can be chosen according to specific food applications, the types of food products, and the major mechanisms of quality deterioration (Guilbert, 2002; Petersen *et al.*, 1999). Edible films and coatings can readily improve the physical strength of food products, reduce particle clustering, and improve visual and tactile features on product surfaces (Cisneros-Zevallos *et al.*, 1997; Cuq *et al.*, 1995). It can also protect food products from moisture migration, microbial growth on the surface, light-induced chemical changes, oxidation of nutrients etc. (Kester and Fennema, 1986). Most commonly, edible films and coatings function as barriers against oils, gases or vapors, and as carriers of active substances, such as antioxidants, antimicrobials, colors and flavors (Guilbert and Gontard, 1995; Kester and Fennema, 1986; Krochta and De Mulder-Johnston, 1997; Miller *et al.*, 1998).

The main film-forming materials are biopolymers, such as proteins, polysaccharides, lipids and resins. They can be used alone or in combinations (Cuq *et al.*, 1995; Gennadios *et al.*, 1994b; Krochta *et al.*, 1994). Proteins are commonly used as film-forming materials but understanding of the precise physical and chemical mechanisms of protein interactions continues to evolve (Li and Lee, 1996; Pommet *et al.*, 2005; Redl *et al.*, 1999). Fish muscle is the main source of myofibrillar and sarcoplasmic protein for biopolymeric film formation (Artharn *et al.*, 2007; Banerjee, 2006; Chinabhark *et al.*, 2007; Iwata *et al.*, 2000; Shiku *et al.*, 2003). It has been known that plasticized protein based-films have good oxygen, carbon dioxide and

lipid barrier properties but their predominantly hydrophilic nature results in poor water vapor permeability characteristics (Gennadios *et al.*, 1994b; Lacroix and Cooksey, 2005). However, the protein-based films from fish muscle become yellowish when stored for a long, particularly films from dark fleshed fish (Artharn *et al.*, 2009; Benjakul *et al.*, 2008; Cuq *et al.*, 1996a). Yellow color of myofibrillar protein-based film was mainly caused by Maillard reaction (Artharn *et al.*, 2009; Benjakul *et al.*, 2008). Lipid oxidation may play a role in yellow discoloration of fish muscle protein film by providing the carbonyl groups involved in Maillard reaction. Carbonyl compounds such as aldehydes or ketones, which were lipid oxidation products, were possibly associated with protein via the Maillard reaction. Such a discoloration directly limits the application of fish muscle protein-based film. The appropriate development of fish muscle protein-based film possessing the good mechanical and physical properties without the yellow discoloration should be promising to obtain the biopolymeric film, which can replace non-biodegradble synthetic counterpart. The information gained can be applicable for lowering yellow discoloration of fish muscle protein based film or films from other origins. Therefore, the better utilization of fish protein-based film can be achieved.

1.2 Review of literature

1.2.1 Biodegradable film

Biodegradable film and coating have been received the increasing attention owing to their biocompatibility and alternative packaging to synthetic polymers or plastics. Almost food packagings are generally made from plastics, which are non-biodegradable synthetic polymers and have the negative impact on environment (Kester and Fennema, 1986; Krochta and De Mulder-Johnston, 1997).

Biodegradable or compostable packaging is preferable to recyclable packaging because recyclable packaging, though better than non-recyclable packaging, still requires external energy to be provided to bring about the recycling process. Nevertheless, biodegradable or compostable packaging is difficult to be recycled (Cuq *et al.*, 1995; Guilbert and Gontard, 1995; Guilbert, 2002). Over the last decade, there has been a widespread interest in films made from renewable and natural polymers which can degrade naturally and more rapidly than petroleum-based plastics. Among all biopolymers, proteins have been paid increasing attention as a potential material for biodegradable films and coating.

Biopolymeric materials used for biodegradable films can be divided into 4 categories: biopolymer hydrocoilloids (proteins and polysaccarides), lipids, resins and composites (Krochta *et al.*, 1994). Physical and chemical characteristics of the biopolymers greatly influence the properties of resulting films and coatings (Sothornvit and Krochta, 2000). In general, plasticizers are required to increase the flexibility of film by lowering the extension between polymers. Films can be incorporated with other additive for different purposes (Table 1).

Table 1. Materials used for edible films and coatings.

Functional compositions	Materials
Film-forming materials	Proteins: myofibrillar protein, whey protein, casein, wheat gluten, soy protein, collagen, gelatin, corn zein, egg protein, pea protein, rice bran, sunflower, cottonseed protein, peanut protein, serum albumin, keratin, porcine plasma protein. Polysaccharides: starch, modified starch, modified cellulose (CMC, MC, HPC, HPMC), alginate, carrageenan, pectin, pullulan, chitosan, gellan gum, xanthan gum. Lipids: waxes (beeswax, paraffin, carnauba wax, candelilla wax, rice bran wax), resins (shellac, terpene), acetoglycerides.
Plasticizers	Glycerin, propylene glycol, sorbitol, sucrose, polyethylene glycol, corn syrup, water.
Functional additives	Antioxidants, antimicrobials, nutrients, nutraceuticals, pharmaceuticals, flavors, colors.
Other additives	Emulsifiers (lecithin, Tweens, Spans), lipid emulsions (edible waxes, fatty acids), cross-linkers (aldehyde, phenolic compounds).

*CMC, carboxy methylcellulose; MC, methylcellulose; HPC, hydroxypropyl cellulose; HPMC, hydroxypropyl methylcellulose.

Source: Adapted from Han *et al.* (2005)

1.2.1.1 Proteins as film forming material

Proteins are thermoplastic heteropolymers containing 20 amino acids. They are macromolecules with specific amino acid sequences and there are limitless number of sequential arrangements with a wide range of interactions and chemical reactions (Pommet *et al.*, 2003; Stevens, 1999). All structures of proteins can be easily modified by heat, pressure, irradiation, mechanical treatment, acids, alkalines, metal ions, salts, chemical hydrolysis, enzymatic treatment and chemical cross-linking (Han *et al.*, 2005; Krochta, 2002). Proteins are commonly used as film-forming materials. The most distinctive characteristics of proteins compared to other film-forming materials are conformational denaturation, electrostatic charges and amphiphilic nature. Many factors can affect the conformation, charge density and hydrophilic-hydrophobic balance of proteins, thereby influencing the physical and mechanical properties of prepared films and coatings. In addition, properties of protein based-films depend on various factors such as the source of protein, pH of protein solution, plasticizers film thickness, preparation conditions, formation process and additives incorporated into the film forming solutions (Benjakul *et al.*, 2008; Cuq *et al.*, 1996b; Park and Chinnan, 1995; Sobral *et al.*, 2005). Protein used as film-forming materials are derived from both animal and plant sources, such as animal tissues, milks, eggs, grains and oilseeds (Krochta, 2002).

1.2.1.1.1 Myofibrillar protein

Fish proteins including myofibrillar protein and sarcoplasmic proteins have been used as film-forming materials (Chinabhark *et al.*, 2007; Iwata *et al.*, 2000; Shiku *et al.*, 2003). Myofibrillar proteins are salt soluble proteins, comprising 54% of total protein. Generally, acid or alkaline solubilization is required for preparation of film forming-solution. Films prepared from myofibrillar proteins are flexible and semi transparent and their mechanical properties are altered with pH used for solubilization (Chinabhark *et al.*, 2007; Hamaguchi *et al.*, 2007; Shiku *et al.*, 2003). The removal of undesirable components and increasing concentration of myofibrillar proteins by washing could improve the properties of films (Artharn *et al.*, 2007).

1.2.1.1.2 Soy protein

Soy protein has been used for preparation of biodegradable films. Soy proteins are composed of a mixture of albumins and globulins, 90% of which are storage proteins with globular structure. The 7S (β-conglycinin) and 11S (glycinin) globulins are the major proteins in soy protein (Kinsella, 1979; Kumar *et al.*, 2002). Globulins are protein fractions in which the subunits are associated via hydrophobic and hydrogen-bonding (Thanh and Shibasaki, 1976). Temperature (over 70 °C), pressure and alkaline condition (pH ≈ 10) have been used to unfold globular structure. Those factors affect the formation of new intra- and inter-molecular bonding, such as hydrogen bonds or electrostatic or hydrophobic interaction of protein in film matrix (Fukushima, 1969; Hermansson, 1978; Thanh and Shibasaki, 1976). Soy protein films have received considerable attention due to their excellent film forming abilities, low cost and barrier properties against oxygen permeation, but they have poor mechanical properties and heat sealability, compared to synthetic polymer (Rhim *et al.*, 2006).

1.2.1.1.3 Corn Zein

Zein is the alcohol-soluble protein found in corn endosperm, and it is the by-product of the corn wet milling industry. It has a molecular weight of 18-45 kDa. Zein is a relatively hydrophobic and thermoplastic material; this hydrophobicity is related to its high content of non-polar amino acids as leucine, alanine and proline (Shukla and Cheryan, 2001). The packaging films made from an alcohol-soluble protein like corn zein have relatively high barrier properties, compared to films from other proteins. Although it has excellent film forming and gas barrier properties, the classical brittleness and flexibility problems of zein film is a great limitation for their use as a free standing film and as a coating material (Arcan and Yemenicioglu, 2011).

1.2.1.1.4 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 20-50 kDa, while glutenin has an average molecular weight of 250 kDa (Kokini *et al.*, 1994; Krochta and De Mulder-Johnston, 1997). Wheat gluten contains more than 75% protein. Wheat protein film show very efficient

oxygen barrier in comparison with some non-protein edible coatings and plastic packaging materials. Wheat gluten films are usually prepared by solution casting method using water and/or ethanol as cosolvent and polyols as plasticizers (Gennadios *et al.,* 1994a).

1.2.1.1.5 Milk protein

In general, milk protein can be divided into whey protein and casein. 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 proteins include ß-lactoglobulin (MW = 18 kDa), α-lactalbumin (MW = 14 kDa), bovine serum albumin (MW = 66 kDa), immunoglobulins, and proteose-peptones. Whey proteins are globular and heat labile in nature (Krochta and De Mulder-Johnston, 1997). Whey protein films are transparent and flexible. They possess films with good oxygen, aroma and oil barrier properties (McHugh and Krochta, 1994a). However, whey protein film exhibits relatively poor tensile properties and exhibits a poor moisture barrier property (Krochta *et al.*, 1994).

Casein represents 80% of total milk protein and consists of α, ß and 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 proline content results in better emulsifying properties compared to whey protein (Khwaldia *et al.*, 2004). Casein has also been used for film forming material because its inexpensive, readily available, non-toxic and highly stable (Abu Diak *et al.*, 2007). It has been reported that casein film exhibited high tensile strength. Moreover, β-casein films are expected to have lower water vapor permeability than other milk protein films because β-casein is the most hydrophobic protein in milk (McHugh and Krochta, 1994b).

1.2.1.1.6 Gelatin

Gelatin obtained by partial degradation of collagen has gained more attention as a new material for edible films (Jongjareonrak *et al.,* 2006). Hydrolysis of collagen produces gelatin with molecular weights from 3 to 200 kDa depending on the raw material used and the extraction conditions (Lacroix and Cooksey, 2005).

Gelatin edible films, with high puncture strength, low puncture deformation and high water vapor permeability, prepared from bovine and porcine skin were reported (Sobral *et al.*, 2001). Fish skin gelatin can be used as film forming material, but properties of film vary depending on the source of gelatin, plasticizer and other factors. Mechanical properties of film from brownstripe red snapper skin gelatin were higher than those of bigeye snapper skin gelatin film at any protein and plasticizer concentrations tested (Jongjareonrak *et al.*, 2006). Nevertheless, heat activated metallo- and/or serine-proteinases/collagenases were present in fish skin gelatin and decreased the mechanical properties of gelatin film via the hydrolysis of the peptide or protein chain, particularly during preparation process (Jongjareonrak *et al.*, 2006). The incorporation of herb extracts into gelatin film from cuttlefish skin effectively improve the mechanical properties and water barrier property of film, particularly the oxidized form of those extracts (Hoque *et al.*, 2011b).

1.2.1.1.7 Egg white

Egg white is a complex protein system made up of a solution of globular proteins containing ovomucin (Woodward, 1990). Ovalbumin, which constitutes more than half of egg white protein by weight, is the only fraction that contains free sulfhydryl (SH) groups. Other proteins, such as ovotransferrin, ovomucoid and lyzozyme contain disulfide (S-S) bonds (Mine, 1995). Preparation of egg white protein films involves denaturation of egg white protein in aqueous solution by alkaline solubilization or heat treatment (Gennadios *et al.*, 1996). At alkaline pH and heating treatment, protein chains further unfold and disulfide bonds in protein molecules are reduced to SH groups, thereby facilitating dispersion of the protein. It has been suggested that, during the gelation and drying steps, SH groups are converted to inter- and intramolecular S-S covalent cross-links through oxidation and sulfhydryl-disulfide interchange reactions (Gennadios *et al.*, 1996; Mine, 1992). The disulfide bonds are considered important in film formation for proteins containing cysteine and/or cystine amino acids (Gennadios *et al.*, 1994b; Okamoto, 1978). Di Pierro *et al.* (2007) reported chitosan–ovalbumin film prepared by transglutaminase-mediated cross-linking exhibited low degree of swelling and solubility at wide range of pH and also improved mechanical and water vapor barrier properties.

1.2.1.2 Polysaccharides as film forming material

Polysaccharides including starch, non-starch carbohydrates, gums and fibers can be used as film forming material (Guilbert, 1986; Guilbert, 2002). The sequence of polysaccharides is simple compared to proteins. However, the conformation of polysaccharide structures is more complicated and unpredictable. Most carbohydrates are neutral, while some gums are mostly negatively charged. Although this electrostatic neutrality of carbohydrates may not affect significantly the properties of formed films and coatings, the occurrence of relatively large numbers of hydroxyl groups or other hydrophilic moieties in the structure indicate that hydrogen bonds may play significant roles in film formation and characteristics (Han *et al.*, 2005).

1.2.1.3 Lipids as film forming material

Lipids and resins are also used as film-forming materials, but they are not polymers and, evidently, "biopolymers" is a misnomer for them. Nevertheless, they are edible, biodegradable and cohesive biomaterials. Most lipids and edible resins are soft-solids at room temperature and possess characteristic phase transition temperatures. They can be fabricated to any shape by casting and molding systems after heat treatment, causing reversible phase transitions between fluid, soft-solid and crystalline solid. Because of their hydrophobic nature, films or coatings made from lipid film-forming materials have very high water resistance and low surface energy (Han *et al.*, 2005). Lipids can be combined with other film-forming materials, such as proteins or polysaccharides, as emulsion particles or multi-layer coatings in order to increase the resistance to water penetration (Gennadios *et al.*, 1997; Perez-Gago and Krochta, 2002).

1.2.1.4 Composite film forming materials

Biopolymer composites can modify film properties and create desirable film structures for specific applications. Similar to multi-layered composite plastic films, biopolymer films can be produced as multiple composite layers, such as protein coatings (or film layers) on polysaccharide films, or lipid layers on protein/polysaccharide films. This multi-layered film structure optimizes the

characteristics of the final film. Composite films can also be created by mixing two or more biopolymers, yielding one homogeneous film layer (Debeaufort *et al.*, 1998; Were *et al.*, 1999; Yildirim and Hettiarachchy, 1997). Various biopolymers can be mixed together to form a film with unique properties that combine the most desirable attributes of each component (Wu *et al.*, 2002).

1.2.1.5 Plasticizers

Plasticizers are required for edible films and coatings, especially for polysaccharides and proteins. Those films are often brittle and stiff due to extensive interactions between polymer molecules (Krochta, 2002). Plasticizers are low molecular weight agents incorporated into the polymeric film-forming materials, which decrease the glass transition temperature of the polymers. They are able to position themselves between polymer molecules and to interfere with the polymer-polymer interaction to increase flexibility and processability (Guilbert and Gontard, 1995; Krochta, 2002). Plasticizers increase the free volume of polymer structures or the molecular mobility of polymer molecules (Sothornvit and Krochta, 2000). These properties imply that the plasticizers decrease the ratio of crystalline region to the amorphous region and lower the glass transition temperature (Guilbert *et al.*, 1997; Krochta, 2002). The addition of plasticizers affects not only the elastic modulus and other mechanical properties, but also the resistance of edible films and coatings to permeation of vapors and gases (Sothornvit and Krochta, 2000; Sothornvit and Krochta, 2001). Most plasticizers are very hydrophilic and hygroscopic. Therefore, they can attract water molecules and form a large hydrodynamic plasticizer-water complex. For protein and polysaccharide edible films, plasticizers disrupt inter- and intra-molecular hydrogen bonds, increase the distance between polymer molecules, and reduce the proportion of crystalline to amorphous region (Krochta, 2002). Water molecules in the films function as plasticizers. Water is actually a very good plasticizer, but it can easily be lost by dehydration at a low relative humidity (Guilbert and Gontard, 1995). Therefore, the addition of hydrophilic chemical plasticizers to films can reduce water loss through dehydration, increase the amount of bound water, and maintain a high water activity.

There are two main types of plasticizers (Sothornvit and Krochta, 2000; Sothornvit and Krochta, 2001):

1. Agents capable of forming many hydrogen bonds, thus interacting with polymers by interrupting polymer-polymer bonding and maintaining the farther distance between polymer chains.

2. Agents capable of interacting with large amounts of water to retain more water molecules, thus resulting in higher moisture content and larger hydrodynamic radius.

Owing to the hydrophilic nature of water, biopolymers and plasticizers, and due to the abundantly existing hydrogen bonds in their structures, it is very difficult to separate these two mechanisms. Sothornvit and Krochta (2001) suggested that several factors affect plasticizing efficiency of plasticizers, including size and shape of plasticizer molecules, number of oxygen atoms and their spatial distance within the structure of the plasticizers and water-binding capacity. Besides the effect of hydrogen bonding, repulsive forces between molecules of the same charge or between polar and non-polar polymers can increase the distance between polymers, thus achieving the function of plasticization in the case of charged polymeric film structures. Therefore, compared to neutral polymer films (e.g. starch films), the flexibility of charged polymer films (e.g. soy protein, carboxymethyl cellulose or alginate films) may be affected more significantly by altering pH and salt addition at the same water activity level. Four theories have been proposed to explain the mechanism of the plasticizer effect (di Gioia and Guilbert, 1999; Sears and Darby, 1982 ; Sothornvit and Krochta, 2005) shown as follows:

1. Lubricity theory – a plasticizer is considered as a lubricant to facilitate the movements of the macromolecules over each other.

2. Gel theory – a plasticizer disrupts the polymer–polymer interactions including hydrogen-bonds and van der Waals and ionic forces.

3. Free volume theory – a plasticizer may depress the glass transition temperature by increasing polymer free volume and mobility of polymeric chains. The fundamental concept underlying these theories is that a plasticizer can interpose itself between the polymer chains and decrease the forces holding the chains together.

4. Coiled spring theory – plasticizing effects from the point of review of tangled macromolecules.

1.2.1.6 Additives

Edible films and coatings can carry various active agents, such as emulsifiers, antioxidants, antimicrobials, nutraceuticals, flavors and colorants, thus enhancing food quality and safety, up to the level where the additives do not interfere with physical and mechanical properties of films (Baldwin *et al.*, 1995; Baldwin *et al.*, 1997; Guilbert *et al.*, 1996; Han, 2002; Han, 2003; Howard and Gonzales, 2001; Kester and Fennema, 1986). Because of the various chemical characteristics of these active additives, film composition should be modified to keep a homogeneous film structure when heterogeneous additives are incorporated into the film-forming materials (Debeaufort *et al.*, 1998). Emulsifiers are surface active agents of amphiphilic nature and are able to reduce the surface tension of the water-lipid interface or the water-air surface. Emulsifiers are essential for the formation of protein or polysaccharide films containing lipid emulsion particles. They also modify surface energy to control the adhesion and wettability of the film surface (Krochta, 2002). Although many biopolymers possess certain levels of emulsifying capacity, it is necessary to incorporate emulsifiers into film-forming solutions to produce lipid-emulsion films. In the case of protein films, some film-forming proteins have sufficient emulsifying capacity due to their amphiphilic structure.

Antioxidants and antimicrobial agents can be incorporated into film-forming solutions to achieve active packaging or coating functions (Han, 2002; Han, 2003). They provide additional active functions to the edible film and coating system to protect food products from oxidation and microbial spoilage, resulting in quality improvement and safety enhancement. When nutraceutical and pharmaceutical substances are incorporated into edible films and coatings, the system can be used for drug delivery purposes (Han, 2003). Incorporated flavors and colorants can improve the taste and the visual perception of quality, respectively. Direct surface application of active substances by spraying or dipping is not highly effective because the active substances can react with food components, evaporate or diffuse into the food, thereby showing reduced antimicrobial activity. As a result, large antimicrobial

concentrations are required (Han and Floros, 1998; Ouattara *et al.*, 2000; Quintavalla and Vicini, 2002). Instead, the incorporation of antimicrobial agents to packaging materials slows down their release and helps keeping high concentrations of the active compounds on the product surface for extended periods of time. The incorporation of partially purified lysozyme from hen egg white by precipitation of non-enzyme protein into zein film showed antimicrobial effect on *Bacillus subtilis* and *Lactobacillus plantarm* (Mecitoglu *et al.*, 2006). The grape seed extract, nisin and EDTA incorporated soy protein edible film is effective to variable degrees in inhibiting the growth of *L. monocytogenes*, *E. coli* and *S. typhimurium* (Sivarooban *et al.*, 2008).

To improve the film properties, several methods such blending with other biopolymers (Prodpran *et al.*, 2007) or chemical such glyoxal, caffeic acid, feruric acid or tannin acid (Cao *et al.*, 2007; Hernandez-Munoz *et al.*, 2004; Nuthong *et al.*, 2009b) and enzymatic modifications such as transglutaminases have been developed (Chambi and Grosso, 2006; De Carvalho and Grosso, 2004; Di Pierro *et al.*, 2007). However, synthetic protein cross-linking agents can be associated with possible toxicity. Thus, the use of cross-linking agents from natural source has been investigated to improve the mechanical properties of protein films (Nuthong *et al.*, 2009a). The improved properties of these materials confirm that chemical and enzymatic approach could be a useful tool for preparing edible film for food coating and pharmaceutical applications.

1.2.2 Functions and advantages of edible films

1.2.2.1 Edibility and biodegradability

The most beneficial characteristics of edible films and coatings are their edibility and inherent biodegradability (Guilbert *et al.*, 1996; Krochta, 2002). To maintain edibility, all film components (i.e. biopolymers, plasticizers and other additives) should be food-grade ingredients and all process facilities should be acceptable for food processing (Guilbert *et al.*, 1996). With regard to biodegradability, all components should be biodegradable and environmentally safe. Human toxicity

and environmental safety should be evaluated by standard analytical protocols by authorized agencies (Han *et al.*, 2005).

Table 2. Properties of some protein films and synthetic plastics.*

Properties	Inferior	Poor	Good	Excellent
Tensile strength**				
(MPa)	< 1	1-10	10-100	>100
	PPC: Gly	FMP : Gly	WPI : Gly	OPP
		PPP : Gly	CZ : Gly	WDC
		WPI : Sor	Gelatin : Gly	PET
		EWP : Gly	FPI : Gly	PE
		SPI : Gly	FMP : PVA : Gly	
		CZ : PEG	PP	
		WC : Gly	PS	
		WPI : BW : Gly	PVC	
		SPI : FA : Gly	LDPE	
		Pea protein : Gly	HDPE	
			PE	
			OPP	
			PVDC	
Elongation **				
(%)	< 1	1-10	10-100	>100
	PS	WPI : BW : Gly	WPI : Gly	CZ : Gly
		Gelatin : Gly	WPI : Sor	WC : Gly
			FMP : Gly	SPI : FA : Gly
			FPI : Gly	HAPS : Gly
			EWP : Gly	LDPE
			EWP : PEG	HDPE
			SPI : Gly	PPP: Gly
			CZ : PEG	FMP : PVA :
			Pea protein : Gly	Gly
			OPP	
			PET	
			PE	
			PVC	

(Continued)

Table 2. (Continued)

Properties	Inferior	Poor	Good	Excellent
Oxygen permeability				
(cm^3 µm m^{-2} d^{-1} kPa^{-1})	>1000	1000- 100	100-10	<10
	LDPE	Shellac	CZ : Gly	WG : Gly
		Beeswax	WPI : Gly	SPI : Gly
		Most waxes	EWP : Gly	WPI : Sor
		HDPE	PE	EVOH
		PP		PVDC
Water vapor				
permeability	>10	10-1	1-0.1	< 0.1
(g mm m^{-2} d^{-1} kPa^{-1})	PPP : Gly	WPI : BW: Sor	EVOH	BW
	Gelatin : Gly	WPI : BW: Gly	Shellac	Paraffin wax
	EWP : Gly	WC : Gly		Most waxes
	FMP : Gly	WC: BW : Gly		PVDC
	FPI : Gly			EVOH
	FMP : PVA :			LDPE
	Gly			HDPE
	WPI : Sor			PVC
	WPI : Gly			PET
	WG : Gly			
	SPI : Gly			
	PPC : Gly			
	SPI : FA : Gly			
	CZ: Gly			

* Abbreviations : Gly = glycerol; Sor = sorbitol; FMP = fish myofibrillar protein; FPI = fish protein isolate; EWP = egg white protein; SPI = soy protein isolate; WPI = whey protein isolate; CZ = corn zein; WG = wheat gluten; PEG = polyethylene glycol; PE = polyester; PS = polystyrene; PP = polypropylene; PVC = polyvinyl chloride; PVDC = polyvinylidene chloride; PET = polyethylene terephthalate; PPP = porcine plasma protein; PVA = polyvinyl alcohol; LDPE = low-density polyethylene; HDPE = high-density polyethylene; OPP = oriented polypropylene; EVOH = ethylene vinylalcohol; BW = beeswax; FA = fatty acids;

****** Films were conditioned for 48 h at 25 °C and 50 % RH prior to testing.

Source: Adapted from Han *et al.* (2005)

1.2.2.2 Physical and mechanical protection

Edible films and coatings protect packaged or coated food products from physical damage caused by mechanical impact, pressure, vibrations and other mechanical factors. Standardized mechanical examinations of commercial film structures are also applied to edible film and coating structures. Such tests include tensile strength, elongation-at break, elastic modulus, compression strength, puncture strength, stiffness, tearing strength, burst strength, abrasion resistance, adhesion force, folding endurance and others (Han *et al.*, 2005). Table 2 shows the tensile properties of various edible films and common plastic films. Edible films have lower tensile strength than common plastic films, while their elongation-at-break varies widely (Han *et al.*, 2005). Some edible films have elongation values comparable to those of common plastic films (Guilbert, 1986; Guilbert, 2002). Many edible film and coating materials are very sensitive to moisture (Guilbert and Gontard, 1995; Guilbert *et al.*, 1996; Krochta, 2002). At higher relative humidity conditions, their physical strength is lower than that at lower relative humidity since absorbed moisture actions as a plasticizer. Temperature is also an important variable affecting the physical and mechanical properties of edible films and coatings (Guilbert *et al.*, 1997; Miller *et al.*, 1998; Wu *et al.*, 2002). The physical strength of materials dramatically decreases when temperature increases above the glass transition temperature. High relative humidity and large amounts of plasticizers lower the glass transition temperature of film-forming materials (Irissin-Mangata *et al.*, 2001).

1.2.2.3 Migration, permeation and barrier function

The quality of most food products deteriorates via mass transfer phenomena, including moisture absorption, oxygen invasion, flavor loss, undesirable odor absorption and the migration of packaging components into the food (Debeaufort *et al.*, 1998; Kester and Fennema, 1986; Krochta, 2002; Miller *et al.*, 1998). These phenomena can occur between food and the atmospheric environment, food and packaging materials or among heterogeneous ingredients in the food product itself (Krochta, 1997). Atmospheric oxygen penetration into foods causes oxidation of food ingredients; inks, solvents and monomeric additives in packaging materials can migrate into foods; essential volatile flavors of beverages and confections may be

absorbed into plastic packaging materials; and pielpizza crusts absorb moisture from fillings/toppings, leading to the loss of crispiness. Edible films and coatings may wrap these food products or be located between heterogeneous parts of food products to prevent these migration phenomena and preserve quality (Guilbert *et al.*, 1997; Krochta, 2002). To characterize the barrier properties of edible films and coatings, the transmission rates of specific hazardous migrants should be determined using stand-alone edible films. Most research has dealt with water vapor permeability, oxygen permeability, carbon dioxide permeability, flavor permeability and oil resistance of edible films. Table 2 shows oxygen permeability and water vapor permeability values of edible films and common plastic films. Edible films possess a wide range of oxygen permeability values. Certain edible films are excellent oxygen barriers. Except for lipid-based materials, the water vapor permeability of most edible films is generally higher than that of common plastic films. All barrier properties of edible films and coatings are affected greatly by film composition and environmental conditions (relative humidity and temperature) (Gontard *et al.*, 1996; Grondahl *et al.*, 2004). Plasticizers in edible film-forming materials reduce glass transition temperatures and increase the permeability of most migrants. Oxygen permeability is very sensitive to relative humidity (Guilbert *et al.*, 1997; Mate and Krochta, 1998). At higher relative humidity conditions, oxygen permeability increases substantially. Therefore, it is very important to maintain low relative humidity environments to maximize the effectiveness of edible films as gas barriers (Han *et al.*, 2005).

Temperature is also an important factor of migration (Amarante and Banks, 2001; Guilbert *et al.*, 1997; Wu *et al.*, 2002). A temperature increase provides more energy to the migrating substances and increases the permeability. At temperatures far distant from the phase transition, changes of migration coefficients such as permeability and diffusivity follow the Arrhenius equation (Guilbert *et al.*, 1997; Miller *et al.*, 1998).

1.2.3 Film forming mechanisms

An edible film is essentially a dried and extensively interacting polymer network of a three-dimensional gel structure. Despite the film-forming process, whether it is wet casting or dry casting, film-forming materials should form a spatially rearranged gel structure with all incorporated film-forming agents, such as biopolymers, plasticizers, other additives and solvents in the case of wet casting (Rhim and Ng, 2007). Biopolymers as film-forming materials are generally gelatinized to produce film-forming solutions. Further drying of the hydrogels eliminates excess solvents from the gel structure. Whey protein films are produced from whey-protein gels by dehydration after heat-set or cold-set gel formation. This does not mean that the film-forming mechanism during the drying process is only the extension of the wet-gelation mechanism. The film forming mechanism during the drying process may differ from the wet-gelation mechanism, though wet gelation is the initial stage of the film-forming process. There could be a critical stage of a transition from a wet gel to a dry film, which relates to a phase transition from a polymer-in-water (or other solvents) system to a water-in-polymer system (Han *et al.*, 2005).

Figure 1 describes potential chemical and physical approaches to the modification of film forming mechanisms by altering film-forming raw materials, varying film-forming processing conditions and applying treatments on formed films. Potential chemical methods of modifying the film-forming mechanisms of protein-based films include pH changes, salt addition, heat denaturation, solvent changes, chemical modification of the side chains of peptides, cross-linking and hydrolysis of peptides (Yildirim and Hettiarachchy, 1997; Were *et* al., 1999), irradiation of peptides (Lacroix and Cooksey, 2005), and the addition of foreign proteins (Denavi *et al.*, 2009; Mecitoglu *et al.*, 2006). For polysaccharide-based films several chemical modifications are available, including salt addition, solvent changes, heat gelatinization, pH changes, chemical modification of hydroxyl groups, cross-linking of polysaccharides, hydrolysis of polysaccharides and the addition of foreign polysaccharides.

Physical modifications of edible films and coatings include lamination, formation of composites, addition of particles or emulsions, perforation, over-coating, annealing heat curing (Gennadios *et al.*, 1996; Micard *et al.*, 2000; Miller *et al.*, 1997), orientation, radiation (Gennadios *et al.*, 1998; Micard *et al.*, 2000) and ultrasound treatment (Banejee *et al.*, 1996).

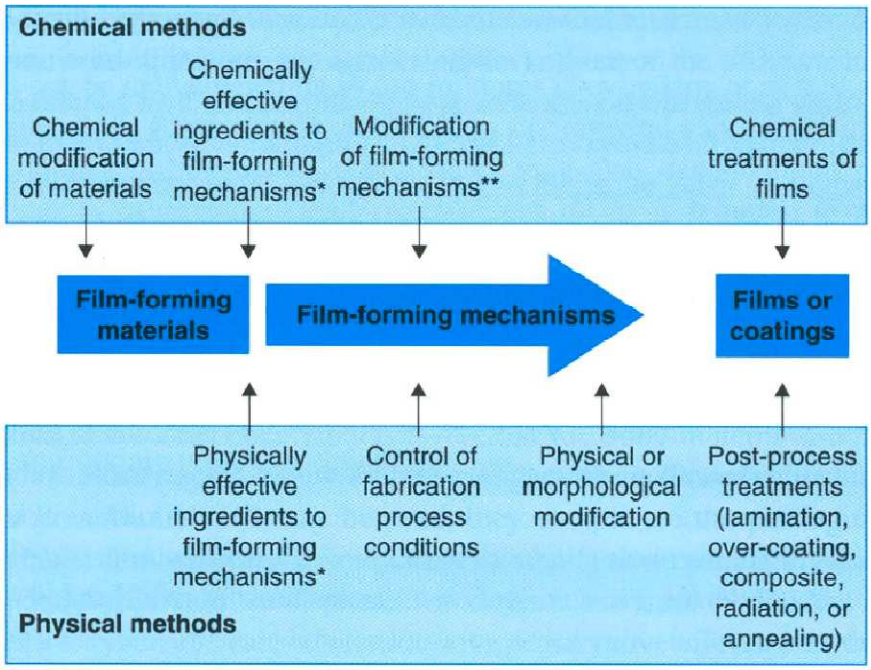


Figure 1. Various ways for modifying the characteristics of edible films and coatings.

* indicates the addition of chemically or physically active ingredients, which may enhance or interfere with the film-forming mechanisms

** includes any chemical cross-linking, chemical substitution of side chains to create hydrophobic interactions or electrostatic interactions and other extra mechanisms caused by chemical modifications

Source: Han *et al.* (2005)

Physico-chemical properties of proteins determine the behavior of proteins during preparation, processing, storage and consumption. These properties are not only important to facilitate processing, but also to determine the quality of the final product (Ralston and Osswald, 2008). The determination of most physical and mechanical characteristics of film structures is related to physical chemistry parameters, which include mechanical strength, elasticity, moisture and gas permeation, cohesion of polymers, film adhesion onto food surfaces, surface energy, surface roughness/smoothness, light transmittance, color (opaque/gloss), viscosity, thermoplastic characteristics and others (Sothornvit and Krochta, 2000). Cohesion of film-forming materials is a very important parameter that influences the mechanical strength of films, especially homogeneously continuous film structures (Guilbert *et al.*, 1996). Cohesion is the attractive force between molecules of the same substance (Anonymous, 1992). If the film-forming materials contain heterogeneous ingredients that are not compatible with the main biopolymers, the cohesion of the film forming materials decreases and the film strength weakens. When the use of new biopolymers or additives is investigated, the compatibility of all film-forming ingredients should be maintained to obtain strong cohesion (Han *et al.*, 2005). Plasticizers are the agents reducing the cohesion of film-forming polymers (Guilbert *et al.*, 1996). Adhesion of film-forming materials is an important parameter, practically, for film casting and coating processes (Guilbert *et al.*, 1996). Adhesion is the attractive force between the surface molecules of different substances, such as between coating materials and food surfaces (Anonymous, 1992). A low adhesion force results in incomplete coatings on the surface, or easy peel-off of the coating layers from the surface. The surface energy of film forming materials (surface tension of the film-forming solution), the solid surface energy of uncoated product and that of the dried film should be determined to achieve strong adhesion. A larger difference of the surface energy of a coating material from the uncoated product surface lowers the work of adhesion and results in a poor coating performance (Guilbert *et al.*, 1996). Surface active agents, such as emulsifiers and other amphiphilic chemicals in the film forming solution reduce the surface tension of the coating solution, thus decreasing the difference between the solid surface energy and the surface tension of the coating solution and ultimately increasing the work of adhesion (Guilbert, 2002; Han *et al.*, 2005).

1.2.4 Film formation processes

There are two categories of film formation processes; dry and wet (Guilbert *et al.*, 1997) (Figure 2). The dry process of edible film production does not use liquid solvents, such as water or alcohol. Molten casting, extrusion and heat pressing are good examples of dry processes. For the dry process, heat is applied to the film-forming materials to increase the temperature to above the melting point of the film-forming materials, to cause them to flow. Therefore, the thermoplastic properties of the film-forming materials should be identified in order to design film-manufacturing processes. It is necessary to determine the effects of plasticizers and any other additives on the thermoplasticity of the film-forming materials (Guilbert *et al.*, 1997; Krochta, 2002). The wet process uses solvents for the dispersion of film-forming materials, followed by drying to remove the solvent and form a film structure. For the wet process, the selection of solvents is one of the most important factors. Since the film-forming solution should be edible and biodegradable, only water, ethanol and their mixtures are appropriate as solvents (Krochta, 2002). All the ingredients of film-forming materials should be dissolved or homogeneously dispersed in the solvents to produce film-forming solutions (Cuq *et al.*, 1995; Gennadios *et al.*, 1994b; Guilbert and Gontard, 1995; Han and Floros, 1997; Han *et al.*, 2005). The film-forming solution should be applied to flat surfaces using a sprayer, spreader or dipping roller and dried to eliminate the solvent, forming a film structure. Phase separation of incompatible ingredients from the film-forming solution is not generally desirable unless the phase separation is intentionally designed for the formation of a bi-layer film structure. To produce a homogeneous film structure avoiding phase separation, various emulsifiers can be added to the film-forming solution (Krochta, 2002). The solvent compatibility of ingredients is very important to develop homogeneous edible film and coating systems carrying active agents. All ingredients, including active agents as well as biopolymers and plasticizers, should be homogeneously dissolved in the solvent to produce film-forming solutions. Most film-forming solutions possess much higher surface tension than the surface energy of dried films, since they contain excessive amounts of water or ethanol (Han *et al.*,

2005). During the solvent drying process, the film-forming solution is concentrated and its surface energy is decreased due to the loss of solvent.

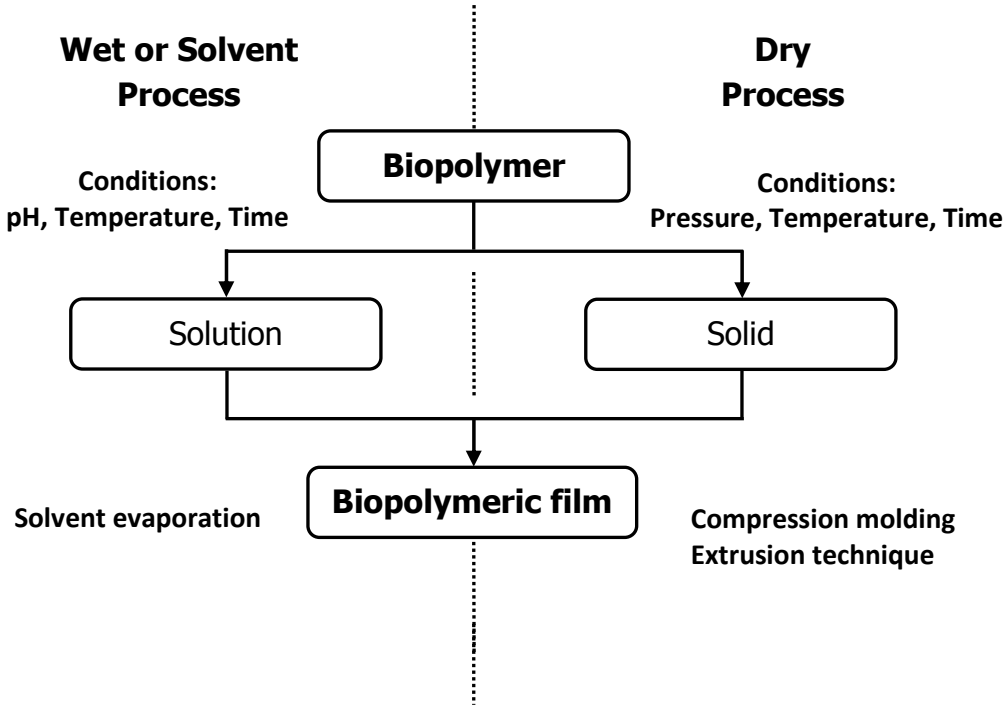


Figure 2. Processing methods: wet (or solvent) and dry process.
Source: Adapted from Guerrero *et al.* (2010)

1.2.5 Discoloration of protein-based film during storage

Generally, myofibrillar fish protein-based film becomes yellowish when being stored for a long time (Artharn *et al.*, 2009; Cuq *et al.*, 1996a). Somanathan *et al.* (1992) reported that triethanolamine-treated casein films became brown in color and were considerably less resistant after 1 year of storage at 25 °C and 65% relative humidity (RH). The browning rate (*b**-value/week) of myofibrillar protein-based film from Atlantic sardine increased with increasing relative humidity and temperature (Cuq *et al.*, 1996b). Artharn *et al.* (2009) reported the increase in *b**-value of film from round scad muscle during storage at 25 °C for 8 weeks. Additionally, Chinabhark *et al.* (2007) also found the increase in *b**-value of protein-based film from bigeye snapper surimi during storage of 60 days. Cuq *et al.* (1996a)

reported that fish myofibrillar protein-based films from Atlantic sardines showed yellow discoloration during storage for 8 weeks at 58.7% RH and 20 °C, which could be associated with the non-enzymatic browning reactions between protein and reducing sugars produced by partial hydrolysis of saccharose (as a plasticizing agent) introduced in the formulation of film. Discoloration of film is possibly via the Maillard reaction and directly limits the application of fish protein-based film.

1.2.6 Lipid oxidation

Lipid oxidation is a chain reaction by which unsaturated fatty acids react with the molecular oxygen to undergo autoxidation. The reaction can be influenced by both internal factors (fatty acid composition, concentration of pro-oxidants, endogenous ferrous iron, heme proteins (myoglobin and hemoglobin) and enzymes) and external factors (pH, temperature and oxygen consumption) (Chan *et al.*, 1997; Grunwald and Richards, 2006; Nawar, 1996). The autoxidation includes several steps of reactions (Michael, 2001).

Initiation

At this step, a fatty acid radical is produced. The most notable initiators in living cells are reactive oxygen species (ROS), such as $OH^{\cdot}$ and H_2O_2, which combines with a hydrogen atom to make water and a fatty acid radical.

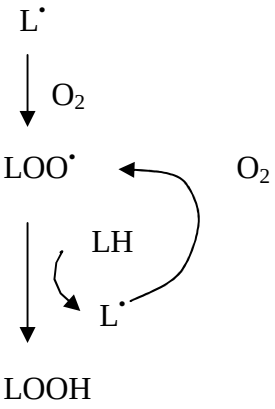
$$LH + X^{\cdot} \rightarrow L^{\cdot} + XH$$

$X^{\cdot}$ = oxidizing agent (Fe^{2+}, $OH^{\cdot}$, H_2O_2)

$L^{\cdot}$ = alkyl radical

Propagation

The fatty acid radical is not a very stable molecule, so it reacts readily with molecular oxygen, thereby creating a peroxyl-fatty acid radical. This is an unstable species that reacts with another free fatty acid, producing different fatty acid radicals and lipid peroxide, or cyclic peroxide if it reacts with itself. This cycle continues, as the new fatty acid radical reacts in the same way.



$$LOOH \rightarrow LO^{\bullet} + OH^{\bullet}$$

$$2LOOH \rightarrow LO^{\bullet} + LOO^{\bullet} + H_2O$$

$$M^{n+} + LOOH \rightarrow LO^{\bullet} + OH^{\bullet} + M^{(n+1)+}$$

$$M^{(n+1)+} + LOOH \rightarrow LOO^{\bullet} + H^{+} + M^{n+}$$

$LO^{\bullet}$ = alkoxyl radical

$LOO^{\bullet}$ = peroxyl radical

LOOH = hydroperoxide

M^{n+} = metal ion

Termination

This step is the final step of reaction. When a radical reacts with a non-radical, it always produces another radical, which is why the process is called a "chain reaction mechanism". The radical reaction stops when two radicals react and produce a non-radical species. This happens only when the concentration of radical species is high enough for collision of two radicals.

$$L^{\bullet} + L^{\bullet} \rightarrow L\text{-}L$$

$$L^{\bullet} + LOO^{\bullet} \rightarrow LOOL$$

$$LOO^{\bullet} + LOO^{\bullet} \rightarrow LOOL + O_2$$

Generally, fish muscle contains high levels of polyunsaturated fatty acids (PUFAs) as well as the presence of potential activators, such as heme pigments and trace metals (Richards *et al.*, 2002). Fatty and medium-fatty species are very susceptible to loss of nutritional quality and shortening of shelf life because of lipid oxidation. Oxidation process leads to the formation of free radicals and lipid hydroperoxides, primary products of oxidation which break down to secondary lipid oxidation compounds such as alcohols, aldehydes and ketones (Hultin, 1994). During storage at room temperature (28-30 °C), the increase in lipid oxidation was observed in dried fish powder from round scad (Artharn *et al.*, 2009) and even in frozen storage (below -18 °C) of horse mackerel patties (Giménez *et al.*, 2011).

Lipid oxidation under low pH system could be related with the enhanced autoxidation of hemoglobin at reduced pH (Tsuruga *et al.*, 1998). Low pH lowers oxygenation of hemoglobins and deoxy-form could be generated, thereby enhanced the lipid oxidation (Richards and Hultin, 2000). It has been known that in fish muscle contained pro-oxidants such as hemoglobin, myoglobin and free irons (Richards and Hultin, 2000). Chaijan *et al.* (2006) found that the oxidation of myoglobin of sardine and mackerel muscles became intense with increasing storage time.

1.2.6.1 Factor affecting lipid oxidation in muscle

1.2.6.1.1 Heme proteins

It has been known that heme proteins (hemoglobin and myoglobin) are endogenous catalysts of fish muscle lipid oxidation (Hultin, 1994). The most abundant heme compounds found *in vivo* are myoglobin and hemoglobin. Hemoglobin (Hb) is the main pigment in red blood cells and myoglobin (Mb) is the main pigment in the muscle cell. The wide distribution of heme compounds in biological systems and especially the high concentration of hemoglobin in red blood cells and myoglobin in tissues has led to assumption that heme-compound-catalyzed lipid peroxidation is a basic pathological reaction *in vivo* and a deteriorative reaction in muscle foods (Apte and Morrissey, 1987; Kaschnitz and Hatefi, 1975; Rhee, 1988). Muscle-pigment-initiated lipid oxidation, leading to oxidative deterioration, has been

extensively reported in meats, as lipid peroxidation in muscle foods is the reaction which often limits storage (Richards *et al.*, 1998).

1.2.6.1.2 pH

The system under different pH conditions can have an impact on pro-oxidative properties of hemoglobin and the susceptibility of the muscle to lipid oxidation. After low pH treatment, washed cod muscle became slightly more susceptible to lipid oxidation, while alkaline treatment slightly protected the muscle from lipid oxidation mediated by trout hemoglobin (Kristinsson and Hultin, 2004). The exposure of trout Hb (hemoglobin) to low pH increased its prooxidative properties (Kristinsson and Hultin, 2004). Longer unfolding times and a lower pH led to less refolded Hb and increased prooxidative activity (Vareltzis and Hultin, 2007). The high oxidation state myoglobin species, that is, perferryland ferryl-myoglobin, are produced *in vivo* and are major prooxidative candidates in muscle-based foods (Carlsen *et al.*, 2005). Low pH favors the protonation of the ferryl species, which exhibit great instability and can be considered as possessing a radical-like nature (Reeder and Wilson, 2001). Richards and Hultin (2000) reported that there was a rapid trout Hb-mediated lipid oxidation of washed cod muscle at pH 3.5, while there was a considerable lag phase and a slower rate of oxidation at pH 7.8. Vareltzis and Hultin, (2007) reported that citric acid and calcium chloride were able to inhibit lipid oxidation of microsomal suspensions.

1.2.6.1.3 Fatty acids

Fish muscle contains saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Oxidation in fish oils has a significant impact on fatty acids (Dahl and Malcata, 1999). Moreover, fish lipids are rich in unsaturated fatty acids, and it is well recognized that oxidation of the lipid fraction of fish muscle is a major cause of deterioration in fatty fish (Brannan and Erickson, 1996; Harris and Tall, 1994). PUFAs are very susceptible to oxidation even under mild ambient conditions and are easily incorporated into the chain mechanism of lipid peroxidation to yield free and peroxy radicals, which may accelerate lipid oxidation. In general, docosahexaenoic acid C22:6($n-3$) (DHA) was usually more

abundant than eicosapentaenoic acid C20:5($n-3$) (EPA) (up to 2–3 times) (Kolakowska *et al.*, 2002). The high content of DHA was coincidental with the high content of phospholipids, which normally contain a high amount of polyunsaturated fatty acids (Chaijan *et al.*, 2006). Since triglycerides and phospholipids underwent hydrolysis into free fatty acids, free PUFA and MUFA possibly further undergo oxidation to a greater extent than SFA (Chaijan *et al.*, 2006).

1.2.6.1.4 Washing process

Washing process has been used widely in the surimi industry to remove small molecular-weight proteins such as sarcoplasmic proteins, fat, blood and heme proteins (hemoglobin and myoglobin) and to concentrate myofibrillar proteins, which are most likely essential for gel network formation (Morioka *et al.*, 1997; Park *et al.*, 1997). Heme proteins known as potentials pro-oxidants, can be removed by washing. Washing process with cold water could removed myoglobin from shot-bodied mackerel muscle by 61%, compared with control (unwashed) (Chaijan *et al.*, 2010). Washing process could remove some lipids as well as heme proteins, thereby reducing lipid oxidation (Hultin and Kelleher, 2000). Sarcoplasmic proteins (e.g. hemoglobin and myoglobin) might have contributed to the enhancement of Maillard browning reaction in acid or alkali treated gels (Kim *et al.*, 2003). Eymard *et al.* (2009) reported the decrease in primary oxidation products (lipid hydroperoxides) and secondary oxidation products (volatiles) in washed mince from horse mackerel (*Trachurus trachurus*) by increasing washing steps during storage.

1.2.6.2 Use of antioxidant

An antioxidant is a chemical compounds capable of inhibiting the oxidation of lipids. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions that damage cells. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions (Frankel, 1998; Sies, 1997).

Antioxidants present in foods and other biological materials have attracted considerable interest because of their safety and potential nutritional and

therapeutic effects. Because extensive and expensive testing of food additives is required to meet safety standards, synthetic antioxidants have generally been eliminated from many food applications. Synthetic antioxidants like butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate, tertiary butylhydroquinone (TBHQ), trihydroxybutyrophenone, nordihydroguairetic acid and ethoxyquin have been reported to be effective in reduction of lipid oxidation (Shahidi *et al.*, 1987). However, use of these types of antioxidants is controlled because of their carcinogenic potential and toxicity (Chen *et al.*, 1992; Sherwin, 1990; Whysner and Williams, 1996). The increasing interest in the search for natural replacements for synthetic antioxidants has led to the antioxidant evaluation of a number of plant sources.

Natural phenolic compounds have been proved to be effective in preventing rancidity of many lipid systems, in particular fish oils (Medina *et al.*, 1999) and minced fish muscle or surimi (Fagbenro and Jauncey, 1994; Ikawa, 1998). Tannic acid exhibited the highest antioxidative activity, compared with the others (catechin, caffeic acid and ferulic acid) and could prevent lipid oxidation effectively in menhaden oil-in-water emulsion as well as in fish mince whereas ferulic acid seemed to possess the lowest preventive effect on lipid oxidation (Maqsood and Benjakul, 2010). The inhibition of mackerel (Banerjee, 2006) tilapia and gray mullet gill lipoxygenase (LOX), which is initiator of auto-oxidation of fatty acids, by green tea extract was reported (Liu and Pan, 2004). Treatment with tea extract improved oxidative stability in ground white mackerel meat samples (He and Shahidi, 1997). Soybean meal (SBM) extracts were effective in retarding lipid oxidation in refrigerated trout mince (D'Souza and Skonberg, 2010).

1.2.7 Maillard reaction

The Maillard reaction has been named after the French chemist Louis Maillard who first described it but it was only in 1953 that the first coherent scheme was put forward by Hodge (1953) (Figure 3). At an early stage, a reducing sugar, like glucose, condenses with a compound possessing a free amino group (of an amino acid or in proteins mainly the ε-amino group of lysine, but also the α-amino groups of terminal amino acids) to give a condensation product N-substituted glycosylamine, which rearranges to form the Amadori rearrangement product (ARP). The subsequent degradation of the Amadori product is dependent on the pH of the system. At pH 7 or below, it undergos mainly 1,2-enolisation with the formation of furfural (when pentoses are involved) or hydroxymethylfurfural (HMF) (when hexoses are involved). At pH >7 the degradation of the Amadori compound is thought to involve mainly 2,3 enolisation, where reductones, such as 4-hydroxy-5-methyl-2,3-dihydrofuran-3- one (HMF^{one}) and a variety of fission products, including acetol, pyruvaldehyde and diacetyl are formed. All these compounds are highly reactive and take part in further reactions (Martins and Van Boekel, 2003). Carbonyl groups can condense with free amino groups, which results in the incorporation of nitrogen into the reaction products (Ames, 1990; Sensidoni *et al.*, 1999). Dicarbonyl compounds will react with amino acids with the formation of aldehydes and α-aminoketones. This reaction is known as the Strecker degradation. Subsequently, in an advanced stage, a range of reactions takes place, including cyclizations, dehydrations, retroaldolizations, rearrangements, isomerizations and further condensations, which ultimately, in a final stage, lead to the formation of brown nitrogenous polymers and co-polymers, known as melanoidins (Martins *et al.*, 2001).

Due to high content of polyunsaturated fatty acid in fish muscle, lipid oxidation more likely takes place. This is associated with the increases in yellowness of muscle. Thanonkaew *et al.* (2006) reported the increase in *b**-value (yellowness) of squid muscle during frozen storage. Phospholipid has been involved in Maillard reaction by providing amine group in reaction with aldehydic oxidation products (Thanonkaew *et al.*, 2006).

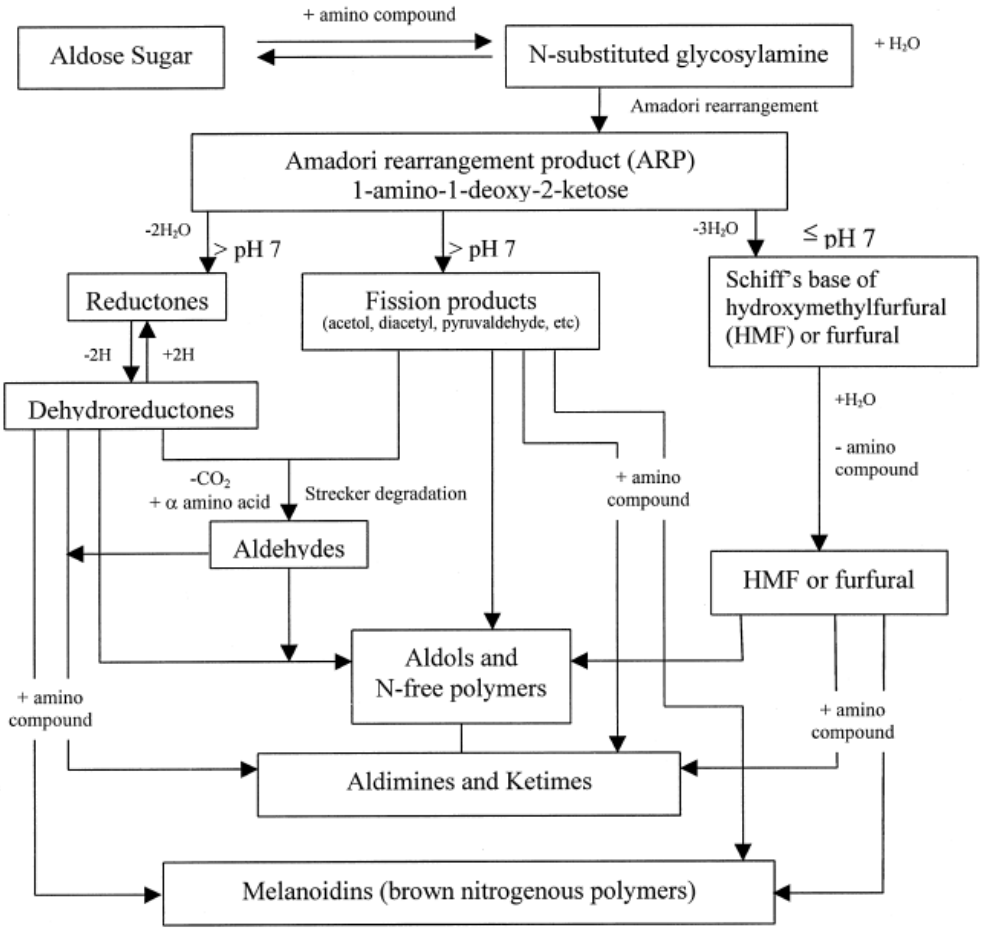


Figure 3. Maillard reaction scheme.

Source: Martins *et al.* (2001)

1.2.8 Fish protein isolate

The production of protein isolates with improved stability and functionality from fish by-products and low value underutilized fish species are of great interest in the fish industry. The pH-shift process is a one method for making functional protein isolate from underutilized muscle protein resources (Hultin and Kelleher, 2000) (Figure 4). An acid or alkaline solubilization process is applied to solubilize muscle proteins at either high or low pH. The solubilization of proteins makes it possible to remove unwanted high-density components, such as bones, scales, connective tissues, cell membranes; and so on and low-density components, such as neutral lipids, by centrifugation. The solubilized muscle proteins are then

collected and recovered by precipitating them at their isoelectric point (Kristinsson and Hultin, 2003; Kristinsson *et al.*, 2005; Undeland *et al.*, 2002). Although different amount of membrane lipids can be removed during acid and alkali-aided protein isolation, the residual membrane phospholipids still readily undergo oxidation in the presence of strong pro-oxidants like Hb, which limits their effective utilization for surimi production and as injection or tumbling marinades to improve water holding capacity of fish fillets (Nolsøe and Undeland, 2009; Raghavan and Hultin, 2009). The use of neutral antioxidants, may offer a novel approach to enhance the oxidative stability of fish protein isolates. Marmon and Undeland (2010) reported protein isolates from herring (*Clupea harengus*) prepared by both acid and alkaline pH-shift processes had a significantly whiter color and higher protein and lower in ash, Ca, Mg and lipid contents than the starting material. The removal of pigments such as melanin and heme proteins during pH-shift processing would thus be of great interest to increase the possibilities to utilize protein isolates originating from darkmuscle fish species (Marmon and Undeland, 2010). Higher protein yields, and greater lipid and pigment reductions of protein isolate from tilapia muscle were achieved with the acid-alkaline-aided processes than with the conventional washing process (Rawdkuen *et al.*, 2009). Chaijan *et al.* (2010) reported that gel of short-bodied mackerel (*Rastrelliger brachysoma*) protein isolate prepared using alkaline-aided process showed the higher breaking force than conventional surimi, possibly due to the partial denaturation of protein after alkaline treatment. Some sulfhydryl groups exposed underwent oxidation during heat treatment and disulfide bond was formed in gel network. In contrast, gel strength and deformation of protein isolated using acid- and alkaline-aided processes from Pacific whiting and tilapia were lower than conventional surimi. Fish proteins were extremely denatured during pH-shift process, particularly in acidic condition (Choi and Han, 2002; Rawdkuen *et al.*, 2009), resulting in poor gelation.

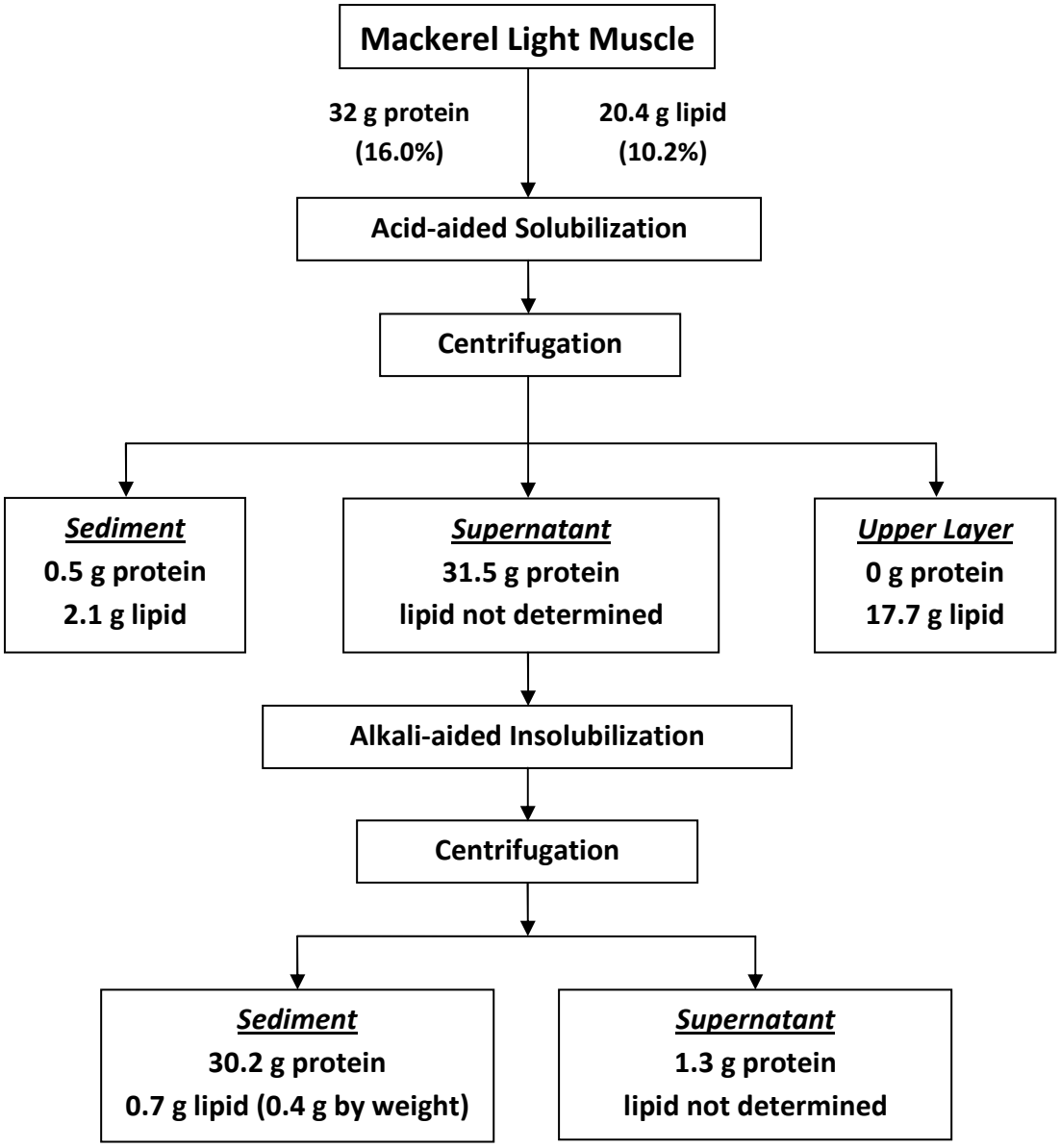


Figure 4. Scheme of new process for producing surimi from mackerel light muscle. Fish protein isolate-total protein recovery, 94.4%; total lipid removed, 96.7%.

Source: Hultin and Kelleher (1997)

Objectives

1. To study the effect of washing and pH on the properties and yellow discoloration of protein-based film from red tilapia and to elucidate the role of lipid oxidation on discoloration of resulting films.
2. To investigate the effect of oxygen and antioxidants on lipid oxidation and yellow discoloration of protein-based film from red tilapia mince during extended storage.
3. To investigate the properties of film from protein isolate from red tilapia prepared at acidic and alkaline condition and the yellow discoloration of film during storage.
4. To study the properties of protein-based film from red tilapia protein isolate incorporated with antioxidant during extended storage in comparison with film from unwashed mince.
5. To study the protective role of protein-based film from red tilapia protein isolate in prevention of lipid oxidation in dry fish powder during the extended storage.

CHAPTER 2

ROLES OF LIPID OXIDATION AND pH ON PROPERTIES AND YELLOW DISCOLORATION DURING STORAGE OF FILM FROM RED TILAPIA MUSCLE PROTEIN

2.1 Abstract

Protein-based films prepared from red tilapia washed and unwashed mince solubilised at pH 3 and 11 were prepared and characterised. Tensile strength (TS) of films from washed mince was greater than that of films prepared from unwashed mince for both pH used ($P < 0.05$). TS of films prepared at pH 3 was higher than that of films prepared at pH 11 for both of washed and unwashed mince ($P < 0.05$). Film from washed mince with pH 3 showed the highest TS, while that from unwashed mince with pH 11 had the lowest TS with the highest elongation at break (EAB) ($P < 0.05$). Films from washed mince had the lower value of thiobarbituric acid reactive substances (TBARS) than did those from unwashed counterpart, regardless of pH used. Nevertheless, TBARS was much higher in films prepared at acidic pH, compared with those prepared at alkaline pH. During storage of 20 days at room temperature, films became yellowish as evidenced by the increases in b^* and ΔE^*-values. Films prepared at pH 11 showed the higher b^* and ΔE^*-values than did those prepared at pH 3, especially for those from unwashed mince. However, films prepared from washed mince at pH 3 showed higher b^* and ΔE^*-values than did those prepared at pH 11 ($P < 0.05$). Films generally had the increase in TS but the decreases in water vapor permeability (WVP), film solubility and protein solubility after 20 days of storage ($P < 0.05$). Therefore, lipid oxidation more likely played a role in yellow discoloration of fish muscle protein film, mainly by providing the carbonyl groups involved in Maillard reaction, while pH regulated the rate of reaction.

2.2 Introduction

Biodegradable packagings from biopolymers have been received increasing attention because they are environmentally friendly alternative materials for non-biodegradable synthetic polymers. Biopolymers can be classified into four groups: polysaccharide, protein, polyester and ethers (Shiku *et al.*, 2003). Among these materials, proteins have been extensively studied because of their relative abundance, good film-forming ability and nutritional qualities (Gennadios *et al.*, 1994b; Krochta, 2002). Fish proteins including myofibrillar and sarcoplasmic protein have been used as film-forming materials (Chinabhark *et al.*, 2007; Iwata *et al.*, 2000; Paschoalick *et al.*, 2003; Shiku *et al.*, 2003; Shiku *et al.*, 2004). Nevertheless, myofibrillar fish protein-based film becomes yellowish when being stored for a long time (Cuq *et al.*, 1996a). Furthermore, Somanathan *et al.* (1992) reported that triethanolamine-treated casein films became brown in color and were considerably less resistant after 1 year of storage at 25 °C and 65% relative humidity (RH). The browning rate (*b**-value/week) of myofibrillar protein-based film increased with increasing relative humidity and temperature (Cuq *et al.*, 1996a). Discoloration of film is possibly via the Maillard reaction.

The Maillard reaction involved in the formation of brown pigments comprises the condensation between an amine group of free amino acid (such as amino acids, peptides, protein) and carbonyl group of reducing sugars, aldehyde, ketone, etc. (Jing and Kitts, 2004). Fish muscle contains a large amount of the polyunsaturated fatty acid, especially ω3 fatty acids, which are susceptible to oxidation (Chen-Huei and Yih-Ming, 1998). Lipid oxidation associates with the formation of unstable hydroperoxides, which are decomposed to the secondary products such as aldehydes, etc (Boyd *et al.*, 1993; White, 1991). Those aldehydes could serve an excellent source of carbonyl for glycation process. Due to the large surface of film, those fatty acids could undergo oxidation with ease and this may cause the yellow discoloration of fish protein-based film. Generally, fish muscle is not soluble and acid or alkaline solubilization is required for preparation of film forming-solution. At acidic or alkaline pH, the indigenous proteinases would be activated and hydrolyse the proteins, providing of free amino group for Maillard reaction. It has

been reported that different fish contained different types of proteinases. Additionally, pH may determine the rate of yellow discoloration of fish protein-based film. Therefore, the objectives of this investigation were to study the effect of washing and pH on the properties and yellow discoloration of protein-based film from red tilapia and to elucidate the role of lipid oxidation on discoloration of resulting films.

2.3 Materials and method

2.3.1 Chemicals

Glycerol, sodium chloride (NaCl), sodium dodecylsulfate (SDS), thiobarbituric acid, malondialdehyde bis (dimethyl acetal) and β-mercaptoethanol (βME) were purchased from Sigma (St. Louis, MO, USA). Acrylamide, *N,N,N', N'*-tetramethylethylenediamine (TEMED) and bis-acrylamide were obtained from Fluka (Buchs, Switzerland).

2.3.2 Preparation of unwashed and washed mince

Fresh tilapia (400–500 g/fish) were purchased from a local market in Hat Yai, Songkhla province, Thailand. Fish were kept in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 30 min. Upon the arrival, fish were immediately washed, filleted and minced to uniformity, using a mincer with a hole diameter of 0.5 cm. Washed mince was prepared according to the method of Toyohara *et al*. (1990) with slight modifications. Fish mince was homogenised with 5 volumes of cold 0.05 M NaCl (2–4 °C) at a speed of 13,000 rpm for 2 min, using an IKA Labortechnik homogeniser (Selangor, Malaysia). The washed mince was filtered through two layer of cheese-cloth. The washing process was repeated twice. Mince and washed mince obtained were stored on ice until used for analysis or for film preparation.

2.3.3 Chemical analyses of unwashed and washed mince

2.3.3.1 Proximate analysis

Protein, ash, fat and moisture contents of unwashed and washed mince were determined according to AOAC method (2000) with the analytical of moisture, protein, fat and ash content respectively.

2.3.3.2 Heme iron content

The heme iron content was determined by using the acidified acetone extraction method of Hornsey (1956). The absorbance was read at 640 nm and the heme iron content was calculated using a molar extraction coefficient of 4800 M^{-1} cm^{-1}.

2.3.3.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of washed and unwashed mince were determined by SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). Samples (3 g) were solubilised in 27 ml of 5% SDS. The mixture was homogenised for 1 min at a speed of 13,000 rpm using an IKA homogenizer and incubated at 85 °C for 1 h to dissolve total proteins. 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. Molecular weights of proteins were estimated from protein markers.

2.3.4 Preparation of film-forming solution

The film-forming solution (FFS) from washed and unwashed mince was prepared according to the method of Chinabhark *et al*. (2007). The washed and

unwashed mince (200 g) was added with 3 volumes of distilled water and homogenised at 13,000 rpm for 1 min. The protein concentration of the mixture was fixed at 2% (w/v). Glycerol, used as a plasticiser was added at 50% (w/w) of protein. The mixtures were stirred gently for 30 min at room temperature. Subsequently, the pH of mixture was adjusted to 3 or 11, using 1 M HCl and 1 M NaOH, respectively, to solubilise the protein. FFS obtained was filtered through a layer of cheese-cloth to remove undissolved debris. The filtrate was used for film casting.

2.3.5 Film casting and drying

To prepare the film, FFS (4 g) was cast onto a rimmed silicone resin plate (50 x 50 mm^2) and air-blown for 12 h at room temperature prior to further drying at 25 °C and 50±5% RH for 24 h in an environmental chamber (WTB Binder, Tuttlingen, Germany). The resulting films were manually peeled off and used for analyses.

2.3.6 Determination of film properties

2.3.6.1 Film thickness

The thickness of film was measured using a micrometer (Gotech, Model GT-313-A, Gotech testing machines Inc, Tawai). Five random locations around each film of ten film samples were used for average thickness determination.

2.3.6.2 Mechanical properties

Prior to testing the mechanical properties, films were conditioned for 48 h at 25 °C and 50±5% RH. 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, Hampshire, UK) equippted with tensile load cell of 100 N. Ten samples (2 × 5 cm^2) with initial grip length of 3 cm were used for testing. Cross-head speed was set at 30 mm/min.

2.3.6.3 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 dried silica gel (0% RH) with silicone vacuum grease and a rubber gasket to hold the film in place. The cups were placed in a desiccator containing the distilled water at 30 °C. The cups were weighed at 1 h intervals over a 10 h period. WVP of the film was calculated as follows:

$$WVP\ (\mathrm{gm^{-1}s^{-1}Pa^{-1}}) = wlA^{-1}t^{-1}(P_2 - P_1)^{-1}$$

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). WVP was expressed as $gm^{-1}\ s^{-1}\ Pa^{-1}$. A total of four films were used for WVP testing.

2.3.6.4 Color

Color of the film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., VA, USA). Using D_{65} (day light) and a measure cell with opening of 30 mm. The color of the films was expressed as b^* and the difference of color (ΔE^*) was calculated as follows:

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differentials between the color parameter of the samples and the color parameter of the white standard (L^* = 93.59, a^* = - 0.98, b^* = 0.35) used as the film background.

2.3.6.5 Light transmittance and transparency value

Light transmittance of films was measured in ultraviolet and visible range (200–800 nm) using UV–vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) as described by Shiku *et al*. (2004). The transparency value of film was calculated using 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 transparency value represents the lower transparency of film.

2.3.6.6 Film solubility and protein solubility

Film solubility was determined according to the method of Gennadios *et al*. (1998). The conditioned film samples (2×5 cm^2) were weighed and placed in a 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 (Heidolth Inkubator 10000, Schwabach, Germany) at 30 °C for 24 h. Undissolved debris was removed by centrifugation at 3000g for 20 min using a centrifuge (model J-E Avanti, Beckman Coulter, Inc., Palo Alto, CA, USA). The pellet was dried at 105 °C for 24 h and weighed. The weight of solubilised dry matter was calculated by substracting the weight of unsolubilised dry matter from the initial weight of dry matter and expressed as a percentage of the total initier dry matter 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 solubilised with 0.5 M NaOH at 30 °C for 24 h.

2.3.6.7 Protein pattern

Protein patterns of films were determined using SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). To solubilise 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 homogenised at 13,000 rpm for 1 min. The homogenate was stirred continuously for 24 h at room temperature (28–30 °C). Then, the sample was centrifuged at 7500g for 10 min at room temperature using a centrifuge (model J-E Avanti, Beckman Coulter, Inc., Palo Alto, CA, USA). The supernatant was subjected to SDS-PAGE as described previously.

2.3.6.8 Determination of TBARS

TBARS value of film was determined according to the method of Buege and Aust (1978). Film (0.05 g) was mixed with 2.5 ml of TBA solution (0.375 g of thiobarbituric acid, 15 g of trichloroacetic acid, and 0.875 ml of hydrochloric acid mixed thoroughly in 100 ml of distilled water). The mixture was heated for 10 min in a boiling water bath (95–100 °C) to develop pink color, cooled with tap water and centrifuged at 7500xg for 10 min. Absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using malonaldehyde bis (dimethyl acetal) (MDA) at concentrations ranging from 0 to 10 mM. TBARS value in each sample was expressed as mg MDA/kg dried sample using the standard curve.

2.3.7 Effect of storage time on changes in properties of film from red tilapia muscle

Films from washed and unwashed mince were prepared at pH 3 and 11 as described above. After drying, films were placed in a humidity control chamber (50% RH) and stored at room temperature (28-32 °C). Samples were taken for all analyses, except light transmittance, transparency value, color and TBARS value were monitored every 5 days for totally 20 days.

2.3.8 Effect of pH on protein degradation in film forming solution

The film-forming solutions from both washed and unwashed mince were prepared under acidic (pH 3) and alkaline (pH 11) conditions, as described previously. The solution was allowed to stand at room temperature and taken for analysis at 0, 3, 6, 9 and 12 h. At the time designated, the solution was neutralized

using either 1 N NaOH or 1 N HCl. Then, the neutralized solution was mixed with 5% SDS at a ratio of 1:1 (v/v). The mixture was incubated at 85 °C for 15 min and centrifugation at 3500g for 5 min. The supernatants were subjected to SDS–PAGE analysis (Buege and Aust, 1978) and α-amino content determination.

2.3.8.1 α-amino group content

The α-amino group content was determined according to the method of Benjakul and Morrissey (1997). Properly diluted samples (125 µl) were mixed thoroughly with 2.0 ml of 0.20 M phosphate buffer, pH 8.2, followed by the addition of 1.0 ml of 0.01 % TNBS solution. The mixtures were then placed in a water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 ml of 0.1 M sodium sulfite. The mixtures were cooled down at ambient temperature for 15 min. The absorbance was measured at 420 nm using a double beam spectrophotometer (model UV-1800, Shimadzu, Kyoto, Japan) and α-amino group content was expressed in terms of L-leucine.

2.3.8 Statistical analysis

All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test. For pair comparison, T-Test was used. (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL).

2.4 Results and Discussion

2.4.1 Chemical compositions

Chemical compositions of washed and unwashed mince of red tilapia muscle are shown in Table 3. Protein was found as a major constituent for washed mince and unwashed mince (13.19% and 17.25% wet weight, respectively). The flesh of fish normally contains 11-24% crude protein, depending on the species, the type of muscle, etc. (Sikorski *et al.*, 1990). Based on dry weight, washed mince and unwashed mince contained 84.27% and 79.31% protein content, respectively. The result indicated that washing could remove other soluble components, leading to the concentrated proteins.

After washing, the fat content was decreased. This implied that washed mince was more likely less susceptible to lipid oxidation. Coincidentally, heme protein content was lowered in washed mince. Heme protein has been shown to be pro-oxidant in muscle food (Brannan *et al.*, 2001; Brannan and Decker, 2001). Therefore, washing played a role in lowering lipid substrate and prooxidative heme protein. In general, washing process can remove fat and undesirable materials, such as blood, pigments and odorous substances and increase the concentration of myofibrillar protein (Lanier and Lee, 1992). Ash content in washed mince was also slightly decreased, compared with that found in unwashed mince.

Protein patterns of washed mince (WM) and unwashed mince (UWM) are depicted in Figure 5. Myosin heavy chain (MHC), actin and tropomyosin were found as the dominant proteins in washed and unwashed mince. After washing, band intensity of myofibrillar proteins (MHC and actin) was increased. The myofibrillar or salt soluble proteins constitute 54% of total proteins (Wahyuni *et al.*, 1998). They are insoluble in water, but become soluble by adjusting the pH to very acidic or alkaline pH. Under reducing condition, high MW cross-link became disappeared with the coincidental increase in MHC and actin band intensity. The result suggested that those proteins or cross-links were mainly stabilized by disulphide bond. Disulphide bond is another bond involving in muscle protein stabilization in muscle (Saxena and Wetlaufer, 1970).

Table 3. Chemical composition of washed and unwashed mince of red tilapia

Composition (% wet weight)	Washed mince	Unwashed mince
Moisture	85.73 ± 0.39[a]	80.24 ± 0.05[b*]
Protein	13.19 ± 0.66[b]	17.25 ± 0.17[a]
Fat	1.08. ± 0.05[b]	1.52 ± 0.03[a]
Ash	0.49 ± 0.05[b]	1.07 ± 0.04[a]
Heme iron (mg/100g dried sample)	0.05 ± 0.01[b]	0.08 ± 0.00[a]

* Mean ± SD (n=3).

Different lowercase superscripts in the same row indicate the significant differences ($P < 0.05$).

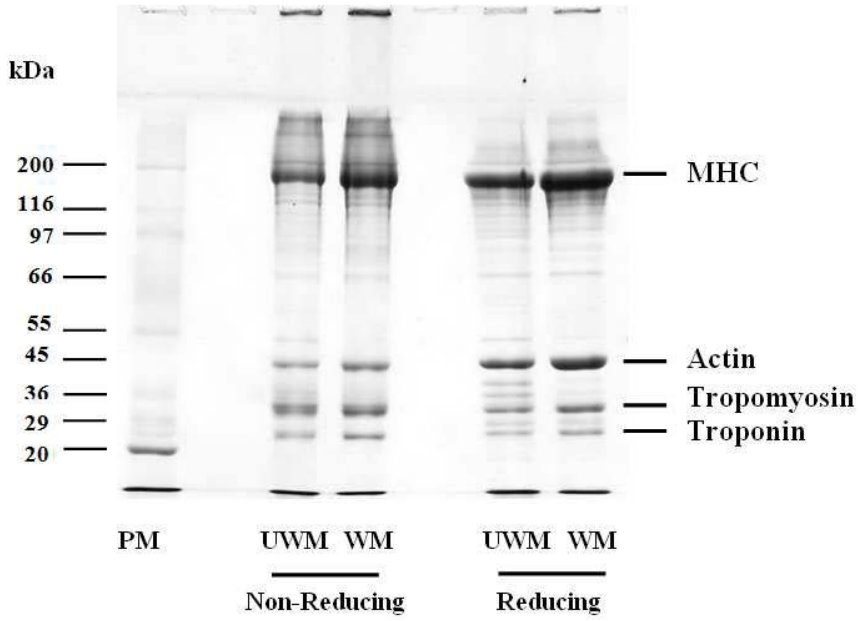


Figure 5. Protein patterns of unwashed mince (UWM) and washed mince (WM) from red tilapia muscle under reducing and non-reducing conditions. PM: protein marker, MHC: myosin heavy chain.

2.4.2 Properties of protein based film from washed and unwashed mince of red tilapia

2.4.2.1 Mechanical properties

Mechanical properties and thickness of films from washed and unwashed mince of red tilapia prepared at acidic (pH 3) and alkaline (pH 11) condition at day 0 and 20 of storage are shown in Table 4. Films from washed and unwashed mince prepared with different pH levels had the similar thickness ($P > 0.05$). At the same pH used for film preparation, TS of films from washed mince was higher than that of films from unwashed mince ($P < 0.05$). This result suggested that the removal of the water soluble protein and low-molecular-weight protein such as sarcoplasmic protein from the flesh as well as the concentration of myofibrillar proteins by washing process resulted in the improved TS of the films. Washed mince containing a greater content of myofibrillar proteins, which had the fibrillar structure, and high molecular weight and rendered the films with the stronger network (Artharn *et al.*, 2008). Iwata *et al*. (2000) observed that the reduction of the sarcoplasmic protein concentration from 4% to 2% in FFS caused a significant increase in TS of films. For unwashed mince, sarcoplasmic proteins were also present. Those proteins and fat in mince might have the lower film forming ability, compared with myofibrillar proteins. As a result film from unwashed mince showed the lower TS than did that from washed mince. High TS is generally necessary for edible films in order to withstand the normal stress encountered during their application, subsequent shipping, and the food handling. However, flexibility of edible films, i.e., EAB should be adjusted according to the intended application of edible films. The main interactions involved in the structure of both sarcoplasmic and myofibrillar protein films are intermolecular covalent bonds with the secondary hydrophobic and hydrogen interactions (Choi and Han, 2002; Rangavajhyala *et al.*, 1997; Rhim *et al.*, 2002; Shiku *et al.*, 2003). Additionally, disulphide bond formation plays an important role in the development of the fish protein film (Iwata *et al*., 2000). When comparing the effect of pH on TS of resulting films, TS of films prepared at pH 3 was higher than that of films prepared at pH 11 for both washed and unwashed mince ($P < 0.05$). Film from washed mince with pH 3 showed the highest TS, while that from unwashed

mince with pH 11 had the lowest TS ($P < 0.05$). Acidic pH might favour the solubilisation and subsequent alignment of protein molecules, in the way which interjunctions with strong bonds were formed.

For EAB, films prepared from unwashed mince had the higher EAB than did those from washed mince, regardless of pH used ($P < 0.05$). Nevertheless, pH had no impact on EAB of film ($P > 0.05$). This result was in accordance with Hamaguchi *et al.* (2007) who reported that pH level had no effect on EAB of protein films from blue marlin (*Makaira mazara*). In general, the higher TS of film was concomitant with the lower EAB. The higher EAB reflected the increased flexibility. The increased EAB of film prepared from unwashed mince was more likely caused by the presence of weaker bonds stabilising film matrix.

Films prepared at pH 3 from washed and unwashed mince generally had the increases in TS after 20 days of storage ($P < 0.05$). However, film prepared at pH 11 had no changes in TS after storage ($P > 0.05$). Additionally, it was found that no changes in EAB of all films were observed after storage ($P > 0.05$). The increase in TS of film prepared at pH 3 after 20 days of storage was probably due to the enhanced interaction between protein molecules, leading to the increased rigidity of resulting film.

2.4.2.2 Water vapor permeability (WVP)

WVP of films from washed and unwashed mince prepared at different pH levels (pH 3 and 11) at day 0 and 20 of storage is shown in Table 4. At day 0, film from washed mince and prepared at pH 3 had the lowest WVP ($P < 0.05$). Nevertheless, WVP of other films was not different ($P > 0.05$). The lowest WVP of the film from washed mince prepared at pH 3 was in agreement with the highest TS (Table 4). The film with strong interaction of protein molecules in the matrix more likely had the compactness. This resulted in the lower migration of moisture through the film. Chinabhark *et al*. (2007) reported that films from bigeye snapper (*Priacanthus tayenus*) surimi prepared at pH 3 and 11 ($P > 0.05$), had no differences in WVP when the same protein content was used. Iwata *et al*. (2000) observed no differences in WVP of fish water soluble protein (FWSP) films from blue marlin prepared at pH 4 and 10 ($P > 0.05$), but WVP of the films prepared at pH 12 showed

Table 4. Properties of films from washed mince (WM) and unwashed mince (UWM) of red tilapia prepared at pH 3 and 11 at day 0 and 20 of storage.

Storage time (Days)	Materials	pH levels	TS (MPa)	EAB (%)	WVP (x 10^{-11} $gm^{-1}s^{-1}Pa^{-1}$)	Thickness (mm)
0	WM	3	$7.07 \pm 0.45^{a,B}$	$54.38 \pm 4.67^{b,A}$	$4.47 \pm 0.33^{a,A}$	$0.030 \pm 0.005^{a*}$
		11	$5.69 \pm 0.12^{b,A}$	$56.48 \pm 2.17^{b,A}$	$5.05 \pm 0.28^{a,A}$	0.029 ± 0.002^{a}
	UWM	3	$3.60 \pm 0.46^{c,B}$	$60.30 \pm 6.43^{ab,A}$	$5.05 \pm 0.29^{a,A}$	0.031 ± 0.003^{a}
		11	$3.12 \pm 0.09^{d,A}$	$63.02 \pm 3.81^{a,A}$	$5.07 \pm 0.23^{a,A}$	0.030 ± 0.004^{a}
20	WM	3	$7.64 \pm 0.30^{a,A}$	$53.47 \pm 5.03^{b,A}$	$3.58 \pm 0.16^{bc,B}$	0.032 ± 0.003^{a}
		11	$5.85 \pm 0.52^{b,A}$	$56.01 \pm 3.51^{ab,A}$	$3.82 \pm 0.11^{b,B}$	0.032 ± 0.002^{a}
	UWM	3	$4.46 \pm 0.52^{c,A}$	$57.79 \pm 2.25^{ab,A}$	$3.49 \pm 0.13^{c,B}$	0.035 ± 0.004^{a}
		11	$3.31 \pm 0.21^{d,A}$	$60.43 \pm 4.63^{a,A}$	$4.32 \pm 0.21^{a,B}$	0.033 ± 0.004^{a}

*Mean $\pm$ SD (n=3).

Different lowercase superscripts in the same column under the same storage time indicate significant differences ($P < 0.05$). Different uppercase superscripts in the same column under the same material and pH levels used indicate significant differences ($P < 0.05$).

slightly higher value ($P < 0.05$) (Hamaguchi *et al*., 2007). Thus, the differences in pH FFS did not apparently influence WVP (Kester and Fennema, 1986; Krochta and Johnston, 1997; Miller and Krochta, 1997). Although edible films obtained from protein exhibit the good mechanical properties, but their water vapor barrier characteristics are usually poor because of their hydrophilic nature (Guilbert, 1986; Kester and Fennema, 1986). Fish muscle is hydrophilic associated with polar amino acids. Paschoalick *et al.* (2003) reported that the muscle protein of Nile tiapia had polar ionic amino acids (aspartic acid, glutamic acid, arginine and lysine) at high concentration. After 20 days of storage, WVP of all films decreased ($P < 0.05$). This result suggested that film matrix became denser due to the alignment or further cross-linking during the extended storage. It was noted that for films prepared from unwashed mince, those prepared at pH 11 had the greater WVP than those prepared at pH 3 ($P < 0.05$). However, no difference in WVP was observed between films from washed mince prepared at different pH at 20 days of storage ($P > 0.05$). Thus, both washing and pH had the influence on WVP of resulting film after extended storage.

2.4.2.3 Film solubility and protein solubility

Film solubility of films from washed and unwashed mince prepared at different pH levels at day 0 and 20 of storage is shown in Table 5. The solubility of films from unwashed mince was higher than that of film from washed mince for both pH 3 and 11 ($P < 0.05$). Washing process could remove the sarcoplasmic or water-soluble proteins as well as some water-soluble components in fish muscle, leading to the lowered solubility of film from washed mince. Sarcoplasmic proteins might not cross-link with the myofibrillar proteins effectively, resulting in the weakened attachment between protein molecules in film matrix (Artharn *et al*., 2008). For both films from washed and unwashed mince, films prepared at pH 3 had the higher solubility than those prepared at pH 11 ($P < 0.05$). Alkaline condition might favor the formation of high MW cross-links, resulting in the lowered solubility. In alkaline environment, it is possible that disulphide bonds in the myofibrillar proteins are reduced to sulfhydryl group, which will reform disulphide bonds to yield the film structure upon casting and drying of the FFS (Shiku *et al*., 2003). Films prepared at pH 11 from both washed and unwashed mince had the lower film solubility and protein solubility than those prepared at pH 3, especially at the beginning of the storage (0 day). Also, glycerol used as plasticiser might bind with film matrix to a higher extent. Thus, glycerol was less leached out. After 20 days of storage, film solubility and protein solubility of all films were decreased ($P < 0.05$). Films prepared at pH 3 from both washed and unwashed mince had the decrease in the film solubility and protein solubility at the higher rate than those prepared at pH 11. The decreases in both film solubility and protein solubility reflected the enhanced interaction between protein molecules during extended storage.

Table 5. Film solubility and protein solubility of films from washed mince (WM) and unwashed mince (UWM) of red tilapia prepared at pH 3 and 11 at day 0 and 20 of storage.

Storage time (Days)	Materials	pH levels	Film solubility (%)	Protein solubility (%)
0	WM	3	$80.76 \pm 1.84^{b,B}$	$75.43 \pm 1.73^{b,B*}$
		11	$35.16 \pm 1.52^{d,B}$	$12.58 \pm 0.34^{c,B}$
	UWM	3	$86.25 \pm 1.90^{a,B}$	$80.22 \pm 2.10^{a,B}$
		11	$38.70 \pm 0.37^{c,B}$	$12.60 \pm 0.32^{c,B}$
20	WM	3	$16.86 \pm 3.10^{c,A}$	$6.63 \pm 0.13^{b,A}$
		11	$19.70 \pm 1.28^{bc,A}$	$6.35 \pm 0.14^{b,A}$
	UWM	3	$27.07 \pm 1.16^{a,A}$	$12.62 \pm 0.67^{a,A}$
		11	$22.40 \pm 1.88^{b,A}$	$6.98 \pm 0.07^{b,A}$

*Mean $\pm$ SD (n=3).

Different lowercase superscripts in the same column under the same storage time indicate significant differences ($P < 0.05$). Different uppercase superscripts in the same column under the same material and pH levels used indicate significant differences ($P < 0.05$).

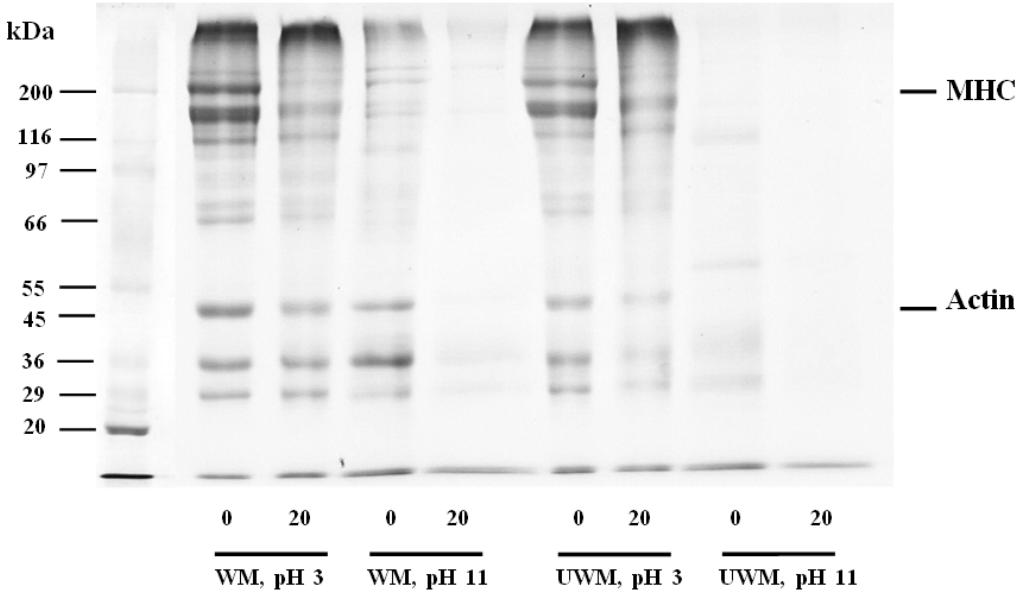
2.4.2.4 Protein pattern

Protein patterns of films from washed and unwashed mince prepared at pH 3 and 11 at day 0 and 20 of storage determined under reducing and non reducing conditions are presented in Figure 6. For films from washed mince, some protein bands with MW lower than MHC were observed when pH 3 was used. On the other hand, most of proteins with MW of 97-200 kDa disappeared in film prepared at pH 11. Similar protein pattern was found between films from washed and unwashed mince prepared at pH 3. Nevertheless, for films prepared at pH 11, most of protein bands disappeared. The disappearance of the protein band might be due to cross-linking of protein induced by alkaline pH. It was noted that Maillard reaction is favored at alkaline pH (Benjakul *et al.*, 2005). The glycation more likely occurred at

alkaline pH, leading to polymerisation of protein molecules in the film matrix. When protein patterns were determined under reducing condition, more protein bands were obtained, especially for film from unwashed mince prepared at pH 11. Actin and tropomyosin bands were more regained, suggesting the role of disulphide bonds in film stabilisation.

After 20 days of storage, most of protein bands were decreased in intensity. It was obvious that the rate of decrease was more pronounced when pH 11 was used. The similar result was observed when SDS-PAGE was performed under reducing condition. The result indicated that the cross-linking was more enhanced, especially at pH 11, when the storage time increased. This result suggested that some proteins, especially actin, were cross-linked preferably by disulphide bond. SH groups in muscle proteins underwent oxidation, in which disulphide bonds were formed. SH groups of proteins in FFS could be exposed at different degrees when varying pH was used for protein solubilisation. As a result, those reactive groups might undergo oxidation at different extents.

(A)



(B)

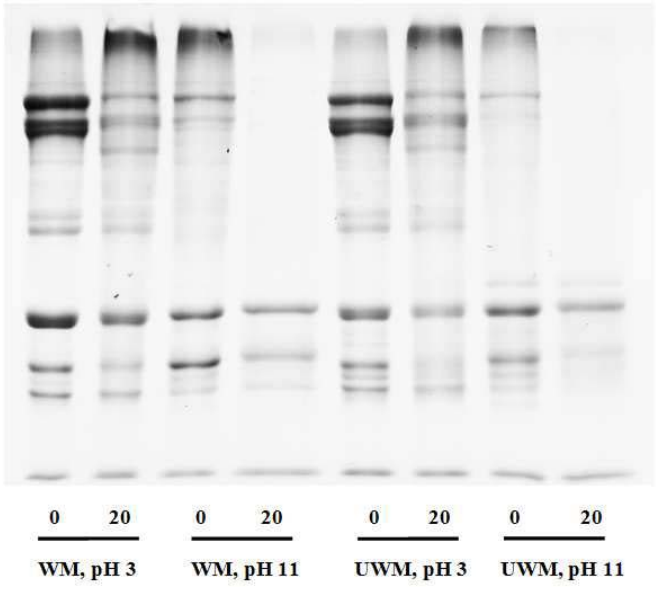


Figure 6. Protein patterns under non-reducing (A) and reducing (B) conditions of films from washed mince (WM) and unwashed mince (UWM) of red tilapia prepared at pH 3 and 11 at day 0 and 20 of storage.

2.4.2.5 TBARS

TBARS values of films from washed and unwashed mince prepared at pH 3 and 11 during storage at room temperature for 20 days are depicted in Figure 7. After casting and drying, films from washed mince had the lower TBARS than did those from unwashed mince for both pH used ($P < 0.05$). Nevertheless, TBARS value was much higher in films prepared at acidic pH, compared with those prepared at alkaline pH. Even though washing process could remove some lipid as well as heme proteins such as hemoglobin and myoglobin, which were involved in lipid oxidation (Chan *et al.*, 1997; Koizumi *et al.*, 1987), membrane lipids as well as some prooxidants were still retained. As a result, lipid oxidation still occurred. Lipid oxidation mediated by hemoglobin might be accelerated as evidenced by increased TBARS. Low pH lowers hemoglobin oxygenation of hemoglobins and deoxy-form could be generated, thereby promoting lipid oxidation (Richards and Hultin, 2000). Acceleration of lipid oxidation by pH reduction could be due to enhanced auto-oxidation of hemoglobin at reduced pH (Tsuruga *et al.*, 1998). Films which were thin and possessed a large surface area might be prone to oxidation owing to the increased exposure to oxygen. The result suggested that lipid oxidation took place during casting and drying. All film samples had the highest TBARS value at day 0 (after complete drying). During drying in an environmental chamber, the oxidation took place rapidly due to the lowered water content, in which lipids were more exposed to atmospheric oxygen. Thereafter, a slight decrease in TBARS values of films prepared at pH 3 were observed at day 5. No further change in TBARS value was found during 5-20 days of storage ($P > 0.05$). For films prepared at pH 11, the gradual decrease in TBARS value was found during 20 days of storage ($P < 0.05$) for both films from washed and unwashed mince. This indicated that those aldehyde compounds might involve in Maillard reaction at a higher extent at pH 11. Furthermore, those volatile oxidation products could be lost during the extended storage. Moreover, the lipid oxidation products could interact with protein in the film, in which the stronger film network could be obtained as indicated by the increased TS and decreases in EAB, WVP and film solubility. 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). TBARS

value in the protein system come from the lipid oxidation products such as aldehyde or ketone, which could interact with TBA solution and pink color was developed. Normally, TBARS assay is a method used to measure secondary lipid oxidation products, specifically aldehydes (Jardine *et al.*, 2002). Due to greater oxidation of lipid from fish muscle protein film for an extended period, lipid oxidation products generated such substances as aldehydes or ketones might react well with reducing sugar or amine group of free amino acid (such as amino acids, peptides and protein) via Maillard reaction. Primary and secondary lipid oxidation products may react with biological amino constituents such as proteins, peptides, and free amino acids (Aubourg *et al.*, 1998; Pokorny, 1977). Aldehydes could create more change in the conformation of myosin, which a greater accessibility of the aldehyde and the possible reactivity with the amino acid side chains acting as nucleophiles. Aldehyde reacts with free amino groups, such as the α-amino group of lysine, of proteins, leading to intra or inter-molecular cross-links, that give rise to protein polymerization (Kikugawa *et al.*, 1988; Stapelfeldt and Skibsted, 1994).

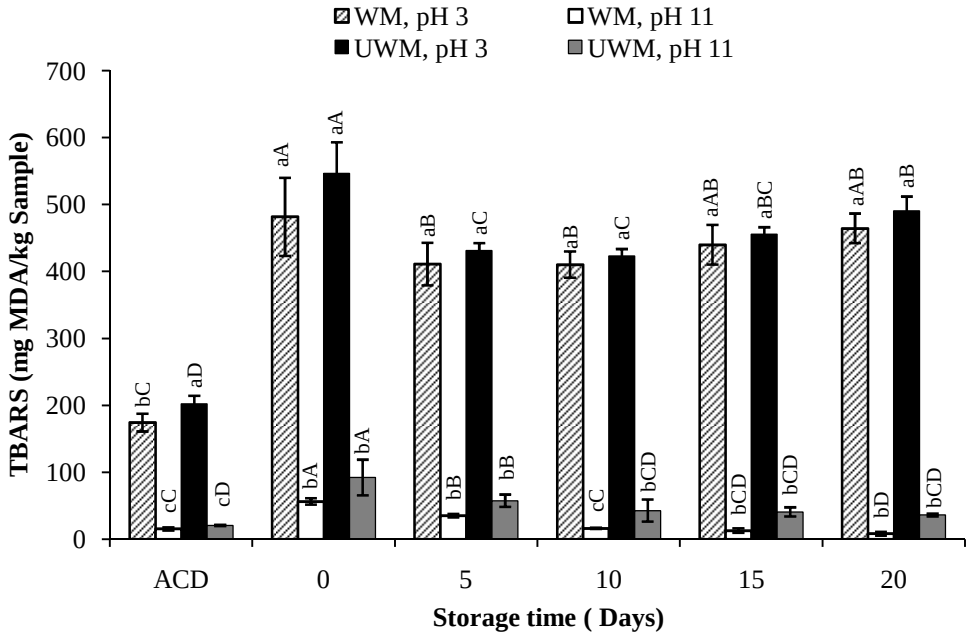


Figure 7. Changes in TBARS of films from washed mince (WM) and unwashed mince (UWM) of red tilapia prepared at pH 3 and 11 during the storage of 20 days. ACD: after casting and drying. Bars represent the standard deviation (n=3). The different lowercase letters on the bars within the same storage time indicate significant differences ($P < 0.05$). Different uppercase letters on the bars within the same material and pH levels used indicate the significant differences ($P < 0.05$).

2.4.2.6 Color

Color expressed as b^*-value (yellowness) and ΔE^* (total color difference) of protein films from washed and unwashed mince prepared at pH 3 and 11 during storage for 20 days are depicted in Figure 8. At day 0, the difference in b^* and ΔE^*-values was observed among all films. Film from unwashed mince prepared at pH 11 had the highest b^* and ΔE^*-value, while that from washed mince prepared at the same pH (pH 11) showed the lowest b^* and ΔE^*-value ($P < 0.05$). The result indicated that during casting and drying, discoloration took place, mainly via Maillard reaction. In films from unwashed mince, lipid oxidation occurred to some extent, leading to the formation of carbonyl compound, which underwent Maillard reaction with amino group of free amino, peptide as well as protein in unwashed mince to a

higher degree, especially at alkaline pH. Nevertheless, film prepared from unwashed mince at pH 11 might have the low lipid oxidation, compared with pH 3. As a consequence, the lower rate of Maillard reaction was obtained. During storage, all films became yellowish as evidenced by the increases in b^* and ΔE^*-values. Films prepared from unwashed mince showed the higher b^* and ΔE^*-values than did those prepared from washed mince ($P < 0.05$). Film prepared from washed mince at pH 3 showed the higher b^* and ΔE^*-values than did that prepared at pH 11 ($P < 0.05$). This was in agreement with the higher TBARS in films prepared at pH 3. The result suggested that the discoloration of fish muscle protein films might be governed by non-enzymatic browning reaction. Lipid oxidation more likely played a role in yellow discoloration of fish muscle film, mainly by providing the carbonyl groups involved in Maillard reaction. Washing process could remove some lipid as a substrate of lipid oxidation and some pigments such as hemoglobin and myoglobin. Furthermore, heme pigments in unwashed mince are the prooxidant and as a source of iron. Kanner *et al.* (1987) reported that free ionic iron is the major catalyst for lipid oxidation in meat products. Films from unwashed mince had the higher TBARS with the concomitant higher b^* and ΔE^*-values, compared with those from washed mince. Nevertheless, film from unwashed mince prepared at alkaline condition (pH 11) had the higher b^* and ΔE^*-values than that from unwashed mince prepared at pH 3, even though the former had much lower TBARS value. Thus, alkaline pH was very effective in enhancing the Maillard reaction rather than carbonyl precursor. Higher pH favours the reductone formation over furfural production from the Amadori products, leading to color development (Bates *et al.*, 1998). The results were in agreement with those of Cuq *et al*. (1996a) who reported fish myofibrillar protein-based films from Atlantic sardines showed yellow discoloration during storage for 8 weeks at 58.7% RH and 20 °C, which could be associated with the non-enzymatic browning reactions between protein and reducing sugars produced by partial hydrolysis of saccharose (as a plasticizing agent) introduced in the formulation of film. Therefore, both available precursor as well as pH of FFS played a role in the development of yellow color in the films from washed and unwashed mince.

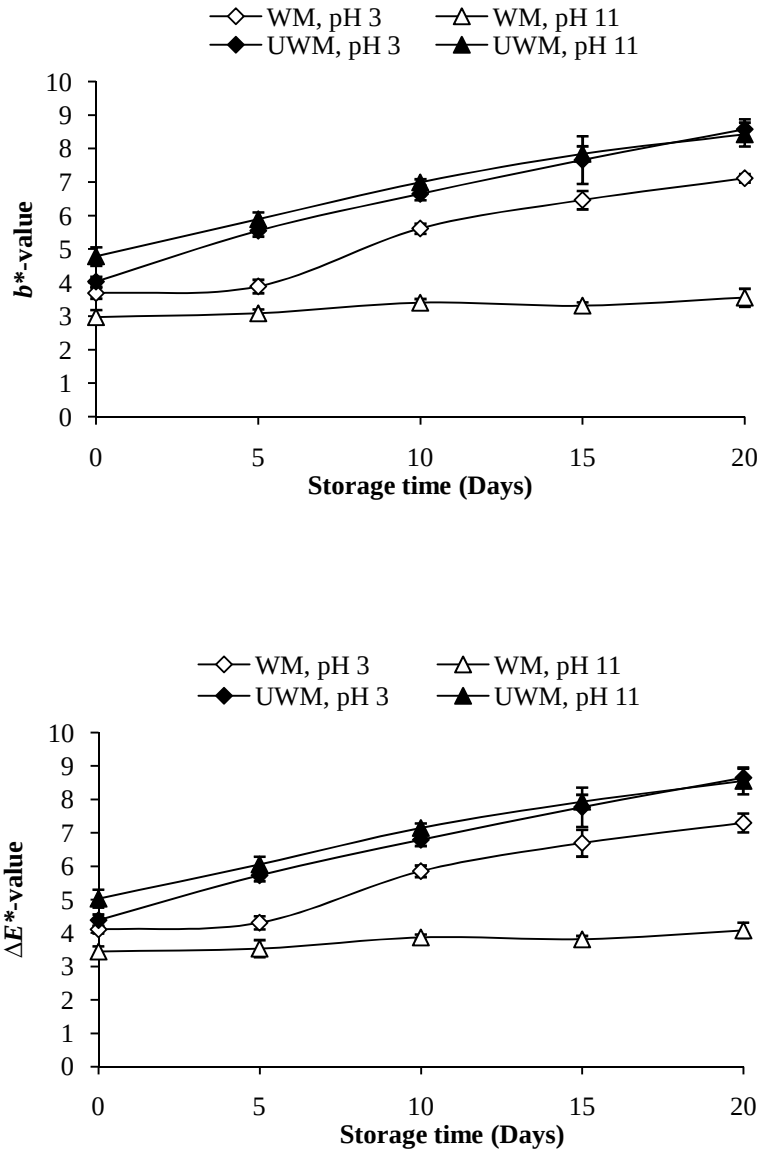


Figure 8. Changes in b^* and ΔE^*-values of films from washed mince (WM) and unwashed mince (UWM) of red tilapia prepared at pH 3 and 11 during the storage of 20 days. Bars represent the standard deviation (n=3).

2.4.2.7 Light transmittance and film transparency

Changes in light transmission at selected wavelengths from 200 to 800 nm in UV and visible ranges and transparency value of protein films from washed and unwashed mince prepared at pH 3 and 11 during storage for 20 days were dependent on pH of FFS and washing process. All films from washed and unwashed mince

prepared in acidic and alkaline conditions had a good barrier property in the UV-ranges (200-280 nm) (Table 6). These results were in agreement with Benjakul *et al.* (2008) who observed that films prepared from unwashed mince and washed mince exhibited the lower transmission to light in UV range. During storage, films from unwashed mince both at pH 3 and 11 had the decreases in light transmission in UV-visible range (200-800). This was in accordance with the increase in *b**-value of films. Cross-linking of protein via the Maillard reaction might contribute to the increased compactness of film matrix, in which the barrier property to light transmission was gained.

Based on transparency value (Figure 9), films prepared at acidic condition (pH 3) from both washed and unwashed mince were more transparent than those prepared at alkaline condition (pH 11) as evidenced by the lower transparency value. Higher transparency value indicated that the film had the lower transparency. Film from unwashed mince prepared at pH 11 had the higher transparency value than that from washed mince. Film from unwashed mince prepared at pH 11 was more turbid than other films. During storage, there was no changes in transparency value of all films (p>0.05).

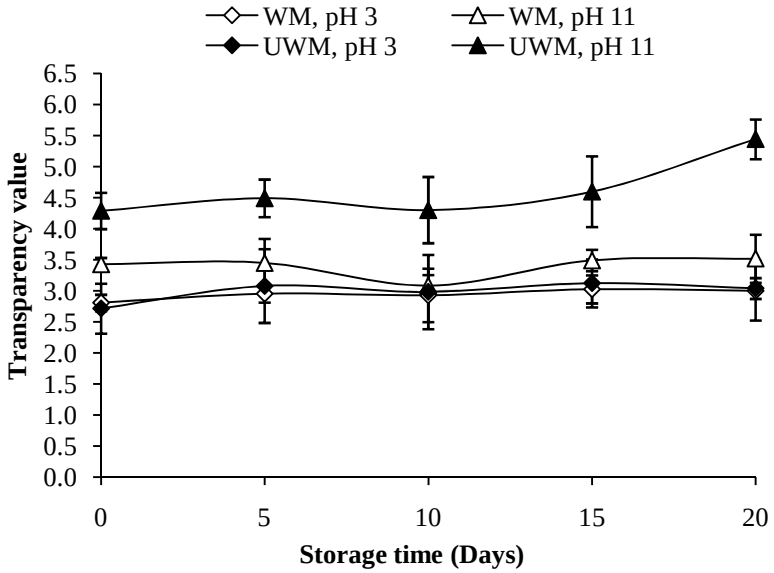


Figure 9. Changes in transparency value of films from washed mince (WM) and unwashed mince (UWM) of red tilapia prepared at pH 3 and 11 during the storage of 20 days. Bars represent the standard deviation (n=3).

Table 6. Light transmittance (%) of films from washed mince (WM) and unwashed mince (UWM) of red tilapia prepared at pH 3 and 11 during 20 days of storage.

Samples	pH	Storage time	Light transmittance (%) at different wavelength (nm)							
		(Days)	200	280	350	400	500	600	700	800
WM	3	0	0.00	0.81	63.79	72.60	78.97	81.16	82.06	83.10
		5	0.00	0.25	57.18	69.09	77.73	80.36	81.41	82.18
		10	0.00	0.33	57.87	70.35	79.24	81.69	82.75	83.43
		15	0.00	0.63	55.66	68.81	78.37	81.01	82.13	82.89
		20	0.00	0.35	53.88	67.79	78.08	80.74	81.87	82.60
	11	0	0.00	0.54	58.73	67.33	74.54	77.34	79.27	80.62
		5	0.00	0.48	58.11	67.97	75.47	78.16	79.91	81.14
		10	0.00	0.54	61.18	71.49	78.56	80.73	82.09	83.04
		15	0.00	0.64	56.90	67.56	75.63	78.58	80.51	81.90
		20	0.00	0.50	56.07	67.40	75.41	78.05	79.75	80.93
UWM	3	0	0.00	2.95	67.55	75.98	81.68	83.47	84.35	85.71
		5	0.00	0.54	62.19	72.14	78.97	80.93	81.92	82.59
		10	0.00	0.29	59.15	70.28	78.00	80.06	81.04	81.70
		15	0.00	0.35	57.07	68.96	77.56	79.70	80.62	81.25
		20	0.00	0.33	55.04	67.79	77.31	79.51	80.45	81.05
	11	0	0.00	1.16	50.22	62.48	71.22	73.98	75.65	76.83
		5	0.00	0.59	46.17	60.74	71.09	74.20	75.99	77.25
		10	0.00	0.86	63.57	73.77	75.53	76.24	77.47	78.30
		15	0.00	0.61	42.19	57.44	68.58	71.72	73.39	74.59
		20	0.00	0.40	36.13	50.41	61.20	64.65	66.68	68.16

2.4.3 Effect of pH on protein degradation in film forming solution

2.4.3.1 α-amino content

Protein degradation in film forming solution (FFS) from unwashed and washed mince prepared under acidic (pH 3) and alkaline (pH 11) conditions during storage for 12 h was monitored in the term of α-amino group content as depicted in Figure 10. The α-amino group content of FFS with pH 3 and 11 from washed and unwashed mince gradually increased as the exposure time increased up to 12 h ($P < 0.05$). However, FFS of unwashed mince prepared at pH 3 generally exhibited the higher rate of increase than those prepared at pH 11, particularly with increasing exposure time. Therefore, hydrolysis was more favorable under acidic condition. Acidic condition might enhance the activity of acidic proteases, particularly cathepsin. Cysteine proteases, especially cathepsin D and cathepsin L, had an optimum range of 3.0-4.5 and 3.0-6.0, respectively (Kang and Lanier, 2000; Makinodan *et al.*, 1982). Under acidic or alkaline conditions, proteins were partially unfolded via electrostatic repulsion between protein molecules. Those unfolded proteins might easily be hydrolyzed by acidic and alkaline proteases. Nevertheless, no difference was observed between FFS from washed mince prepared at pH 3 and pH 11 ($P > 0.05$). This result suggested that washing mince with saline solution (50 mM NaCl) could remove sarcoplasmic proteases to some degree. Therefore, the washing process was able to decrease the protein degradation in FFS mediated by acidic proteases. Nevertheless, the protein degradation still occurred to some extent in FFS from washed mince at both pH 3 and 11 when the exposure time increased. The result suggested that the remaining proteases still played a role in degradation of proteins in FFS. It was noted that the lower α-amino group content was observed in FFS from washed mince at time 0. This indicated the lower amino acids, peptides and low molecular weight proteins, which were removed by washing process.

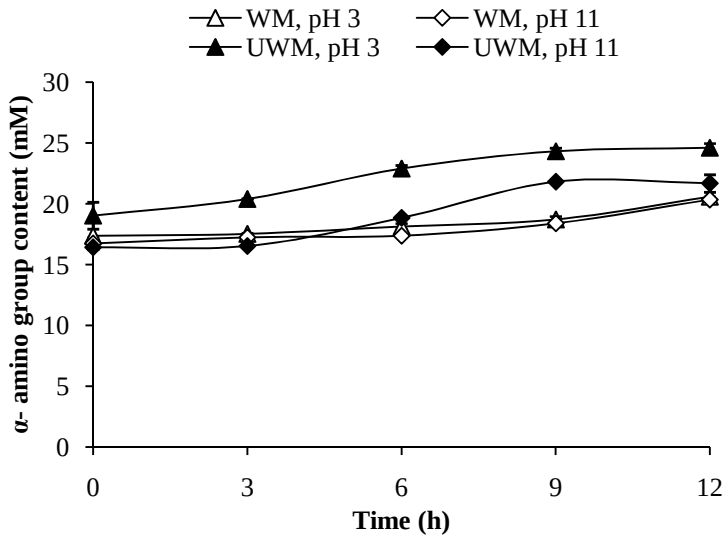


Figure 10. Changes in α-amino group content of film forming solution from washed mince (WM) and unwashed mince (UWM) of red tilapia prepared at pH 3 and 11 with different exposure times.

2.4.3.2 Protein pattern

Protein patterns under reducing and non-reducing conditions of FFS from unwashed and washed mince prepared under acidic (pH 3) and alkaline (pH 11) conditions during 12 h of exposure are presented in Figure 11 and 12, respectively. Protein patterns of all FFS samples were similar. Myosin heavy chain (MHC) constituted as the dominant protein. Actin, tropomyosin and troponin were also found in FFS. FFS from washed mince had slightly higher MHC band intensity than those from unwashed mince, regardless of pH used. During washing process, sarcoplasmic proteins were mostly removed, leading to the increase in concentration of myofibrillar proteins including MHC and actin. For both FFS prepared from unwashed and washed mince, the degradation was more pronounced in FFS with acidic pH as evidenced by the decrease in MHC band intensity as well as the coincidental appearance of proteins with MW of 130-150 kDa. The degradation suddenly took place after preparation of FFS (time 0). Degradation of proteins increased as the exposure time increased up to 12 h. This suggested that the degradation of proteins occurred in FFS during preparation of FFS, casting and drying. This result was in agreement with the higher α-amino group content of FFS with pH 3. Thus, protein

hydrolysis caused by endogenous proteases was greater under acid condition. Washing process could remove proteolytic enzymes from mince, resulting in the lower rate of degradation of proteins in FFS from washed mince, compared with those from unwashed mince.

Under alkaline condition, much lower degradation of proteins in FFS for both washed and unwashed mince was obtained. However, MHC and actin were hydrolyzed to some degree as the exposure time increased. It has been reported that alkaline condition favored the formation of protein cross-linking via disulfide bonds in fish protein based film (Shiku *et al.*, 2003). After casting and drying of FFS from both washed and unwashed mince prepared under alkaline condition, films had the lower solubility, possibly caused by high molecular weight polymerized proteins via disulfide bond, which were strong and insoluble. Those polymerized proteins were more likely resistant to hydrolysis by indigenous proteases. Free amino groups generated via degradation in both FFS prepared at pH 3 and 11 could serve as the precursor for Maillard reaction in the presence of lipid oxidation products. As a result, films could turn to yellow mediated by Maillard reaction. Although the less degradation was observed in FFS prepared at pH 11, the higher yellowness (b^*-value) was obtained in film with alkaline condition. This was because the Maillard reaction was more favorable at alkaline pH (Benjakul *et al.*, 2005).

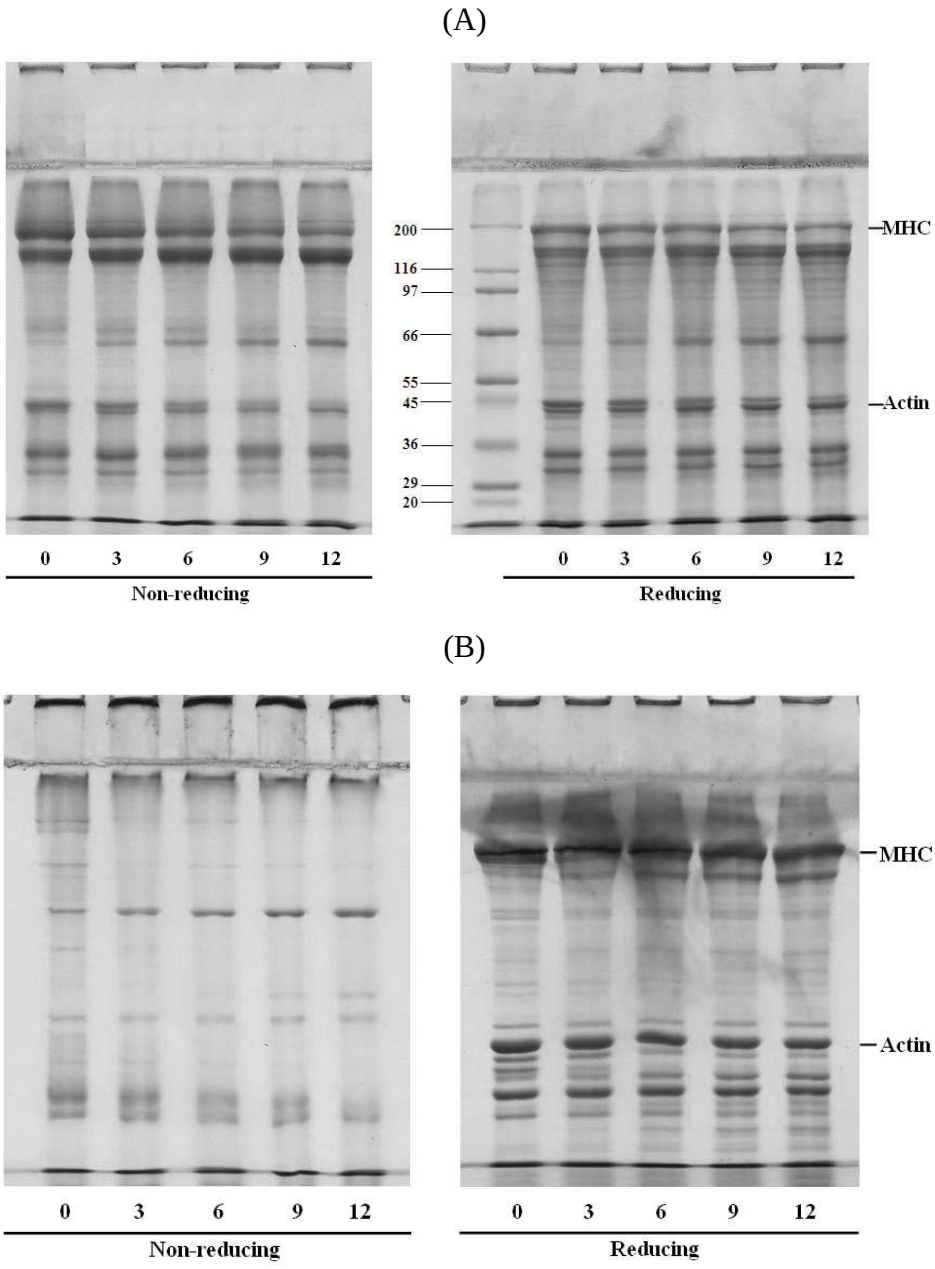


Figure 11. Protein patterns of acidic (A) and alkaline (B) film-forming solution from unwashed mince from red tilapia muscle with different exposure times. MHC: myosin heavy chain. The numbers designate the exposure time (h).

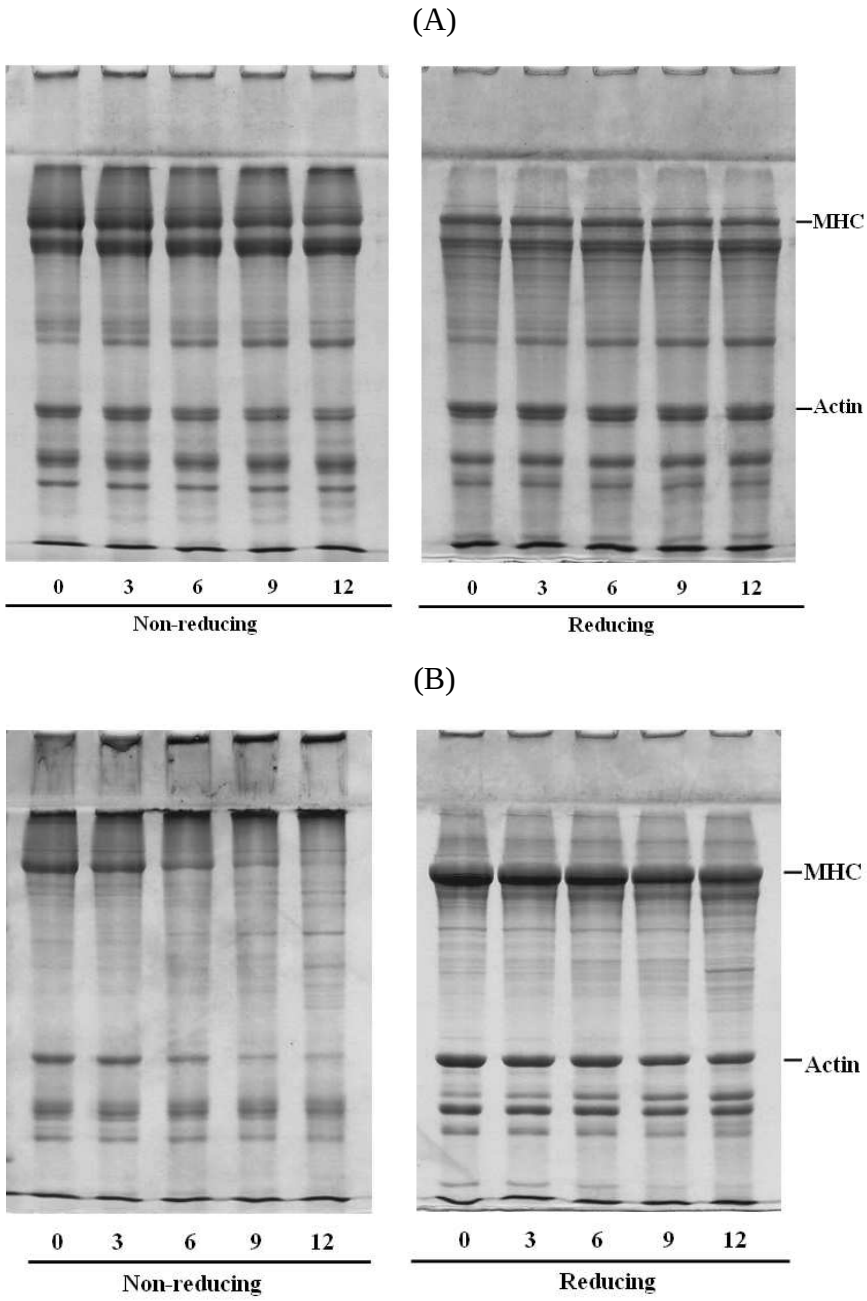


Figure 12. Protein patterns of acidic (A) and alkaline (B) film-forming solution from washed mince from red tilapia muscle with different exposure times. MHC: myosin heavy chain. The numbers designate the exposure time (h).

2.5 Conclusion

Mechanical and physical properties of film from red tilapia muscle were affected by mince washing process and pH level of FFS. These factors also affected the yellow discoloration of film mediated by Maillard reaction during storage. Lipid oxidation and alkaline pH more likely played a role in yellow discoloration of fish muscle film, mainly by providing the carbonyl groups involved in Maillard reaction, which was enhanced at alkaline pH (Figure 13).

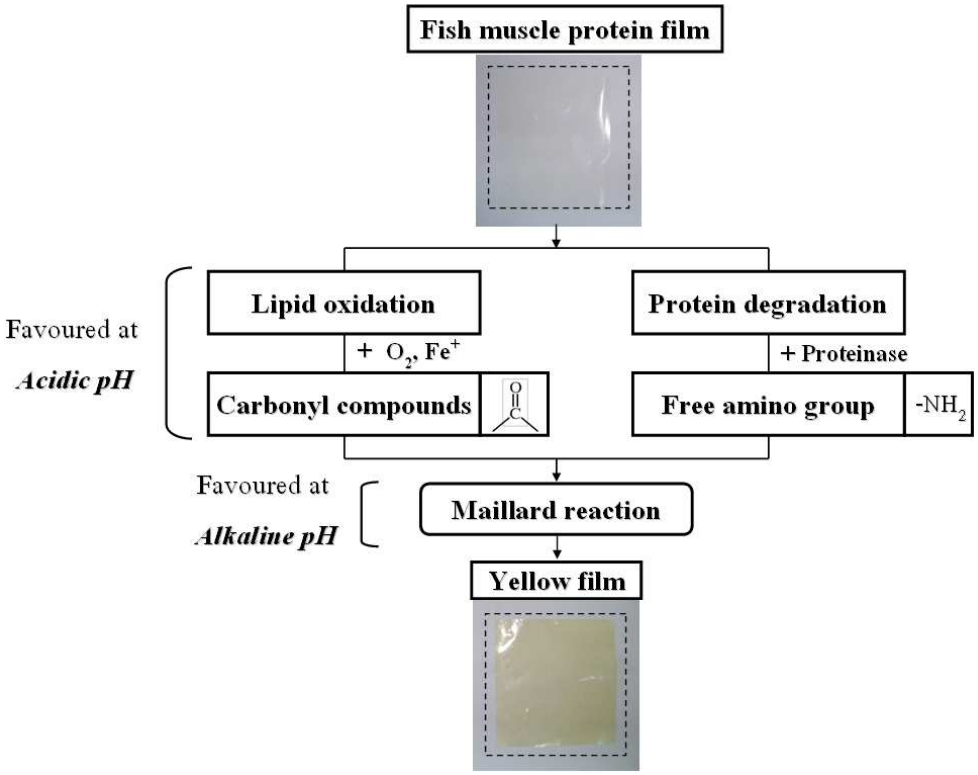


Figure 13. Proposed scheme of yellow discoloration of film fish muscle protein during the extended storage.

CHAPTER 3

EFFECTS OF OXYGEN AND ANTIOXIDANTS ON THE LIPID OXIDATION AND YELLOW DISCOLORATION OF FILM FROM RED TILAPIA

3.1 Abstract

The effects of oxygen and antioxidants (Trolox and catechin at level of 100, 200 and 400 ppm) on lipid oxidation and yellow discoloration of film from red tilapia mince prepared at acidic (pH 3) and alkaline (pH 11) condition were investigated during 20 days of storage. During storage, both films prepared at pH 3 and 11, and kept under atmospheric containing 100% N_2 had the lowest TBARS value with the concomitant lowest b^* and ΔE^*-values ($P < 0.05$) during storage, when compared with other films kept in air and 100% O_2 atmosphere. No differences in light transmittance and transparency value were observed between films stored at different atmospheres ($P > 0.05$). Films prepared at pH 3 and incorporated with antioxidants (Trolox and catechin) at all levels (100, 200 and 400 ppm) had the lowest TBARS value, b^* and ΔE^*-values during storage, indicating the retardation of lipid oxidation and yellow discoloration in films. Nevertheless, films prepared at pH 11 had no difference in TBARS values, in comparison with control film, regardless of antioxidant inorporation. Coincidentally, the increases in b^* and ΔE^*-values were observed in those films. Therefore, lipid oxidation was the main factor inducing yellow discoloration of film exposed to oxygen and the incorporation of antioxidants in film prepared at acidic pH was able to prevent yellow discoloration of resulting film.

3.2 Introduction

Food packaging has been served as good containment, including protection against physical (shock, vibration, compression, temperature, UV, etc), chemical (gas, liquid, etc), biological (fungi, mold, microbial spoilage, etc) and environmental factors. Shelf life of food products can be extended with appropriate packaging. Normally, almost food packagings are made from plastics, which are non-biodegradable synthetic polymers and have the negative impact on environment. Therefore, the use of biodegradable materials can be the most effective solution to this problem. Biopolymer films and coating have been paid increasing attention because of their biodegradability and can be used as alternative material to synthetic packaging. Biopolymeric materials can be derived from polysaccharides, proteins, resins, lipids or blending of these materials. Protein is a neutral biopolymer obtained from animal or plant. Proteins from fish muscle have been used for a film-forming material because of their good film-forming ability and prevention of the migration of oxygen, carbon dioxide and lipids. However, protein-based films have poor water vapor permeability (Hamaguchi *et al.*, 2007; Paschoalick *et al.*, 2003; Shiku *et al.*, 2003; Shiku *et al.*, 2004).

Biopolymers are generally much less stable than most synthetic materials. The color discoloration of fish protein-based film occurs generally during extended storage and become limitation for application of those films (Artharn *et al.*, 2009; Cuq *et al.*, 1996a). Recently, Tongnuanchan *et al.* (2011) reported that lipid oxidation more likely played a role in yellow discoloration of fish muscle protein film, by providing the carbonyl compounds (aldehydes or ketones) involved in Maillard reaction. Fish muscle contains high levels of polyunsaturated fatty acids (PUFAs), which are prone to oxidation. Therefore, the incorporation of antioxidants into protein-based film could lower yellow discoloration of films. The objective of this study was to investigate the effect of oxygen and antioxidants on lipid oxidation and yellow discoloration of protein-based film from red tilapia mince during extended storage.

3.3 Materials and methods

3.3.1 Chemicals

Glycerol, catechin and 2-thiobarbituric acid were purchased from Sigma (St. Louis, MO, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Fluka (Buchs, Switzerland).

3.3.2 Fish collection and mince preparation

Fresh red tilapia (*Oreochromis niloticus*) (400–500 g/fish) were purchased from a local market in Hat Yai, Songkhla province, Thailand. Fish were kept in ice with a fish/ice ratio of 1:2 and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 30 min. Upon the arrival, fish were immediately washed, filleted and minced to uniformity using a mincer with a hole diameter of 0.5 cm. Mince obtained was stored on ice until used for analysis or for film preparation.

3.3.3 Preparation of film

Film-forming solution (FFS) from mince (protein content of 17.11 ± 0.52%, wet weight basis) was prepared according to the method of Chinabhark *et al*. (2007). Mince was added with 3 volumes of distilled water and homogenized at 13,000 rpm for 1 min using IKA Labortechnik homogenizer (Selangor, Malaysia). The protein concentration of the mixture as determined by Kjeldahl method (AOAC, 2000) was adjusted to 2% (w/v). Glycerol, used as a plasticizer, was added at 50% (w/w) of protein. The mixtures were stirred gently for 30 min at room temperature. The pH of mixture was adjusted to 3 and 11, using 1 M HCl and 1 M NaOH, respectively, to solubilize the proteins. FFS obtained was filtered through two layers of cheesecloth to remove undissolved debris.

To prepare the films, FFS (4 g) was cast onto a rimmed silicone resin plate (50 × 50 mm^2) and air-blown for 12 h at room temperature. The resulting films

were manually peeled off and were referred to as “after casting and drying, ACD”. Thereafter, films were conditioned in an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttlingen, Germany) at 25 °C and 50±5% RH for 24 h and the resulting films were considered as ‘films at day 0 of storage’.

3.3.4 Study on the effects of oxygen and antioxidants on yellow discoloration and properties of film

Films prepared at both pH (3 and 11) were inserted in nylon/LLDPE bag (10 × 15 cm^2) (Asian Foams, Hat Yai, Thailand) with the thickness of 0.08 mm and gas permeability of CO_2, N_2 and O_2 of 1.7×10^{-10}, 0.1×10^{-10} and 0.4×10^{-10} m^3 mm/cm^2 s cmHg, respectively (at 25 °C, 1 atm pressure) and was packed with a film/gas ratio of 1:3 (w/v) using a Henkovac type 1000 (Tecnovac, Italy). Prior to filling the gas, the bag was evacuated fully. Gases used included 100% O_2 and 100% N_2. Films kept in air were used as the control. To study the impact of antioxidants on yellow discoloration and properties of film, Trolox and catechin at different concentrations (100, 200 and 400 ppm) were incorporated into FFS with both pH (3 and 11). FFS were then stirred gently at room temperature for 10 min. FFS obtained was subjected to casting and drying as previously described. Films prepared at both pH without antioxidants were used as the control.

All film samples were stored at room temperature (28-32 °C). Samples were taken for determination of thiobarbituric acid reactive substrances (TBARS), color, light transmittance and transparency value every 5 days for totally 20 days.

3.3.5 Analyses

3.3.5.1 Thiobarbituric acid reactive substrances (TBARS)

TBARS value of films was determined according to the method of Buege and Aust (1978) Film samples (0.05 g) were mixed with 2.5 ml of TBA solution (0.375 g of thiobarbituric acid, 15 g of trichloroacetic acid, and 0.875 ml of HCl in 100 ml of distilled water). The mixtures were heated for 10 min in a boiling water bath (95–100 °C) to develop pink color, cooled with tap water and centrifuged

at 7500xg for 10 min. Absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using malonaldehyde bis (dimethyl acetal) (MDA) at concentrations ranging from 0 to 10 mM. TBARS value in each sample was expressed as mg MDA/kg dried sample.

3.3.5.2 Color

Color of films was determined using a CIE colorimeter (Hunter associates laboratory, Inc., VA, USA). D_{65} (day light) and a measure cell with opening of 30 mm were used. The yellowness of films was expressed as b^*-value and total difference of color (ΔE^*) was calculated as follows (Gennadios *et al.*, 1996):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differentials between the color parameter of the samples and the color parameter of the white standard (L^* = 93.45, a^* = - 0.65, b^* = 0.24).

3.3.5.3 Light transmittance and transparency value

Light transmittance of films was measured in ultraviolet and visible range (200–800 nm) using UV–vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) as described by Shiku *et al*. (2004). The transparency value of film was calculated using 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 transparency value represents the lower transparency of film.

3.3.6 Statistical analysis

All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980) Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL, USA).

3.4 Results and discussion

3.4.1 Effect of oxygen on lipid oxidation, yellow discoloration and properties of films

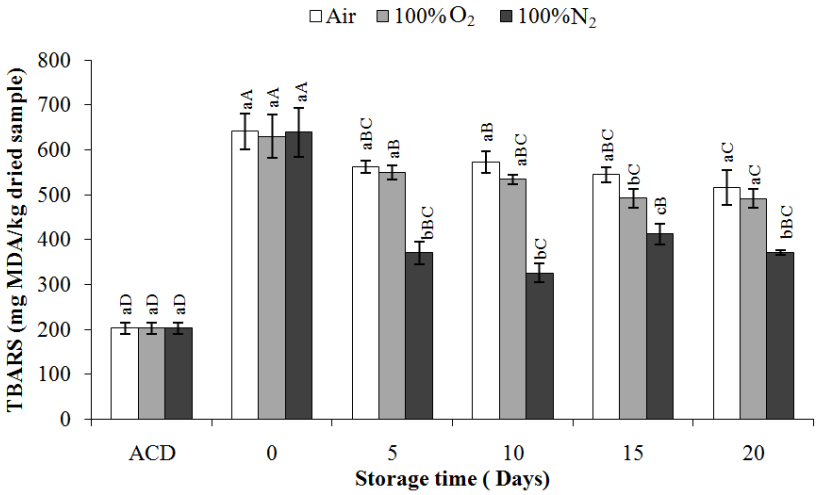
3.4.1.1 Lipid oxidation

Lipid oxidation of films from red tilapia mince prepared at pH 3 and 11 and stored in air and under different atmospheric conditions (100% O_2 and 100% N_2) during 20 days of storage was monitored and expressed as TBARS value (Figure 14). In general, lipid oxidation more likely occurred during film preparation as evidenced by the formation of TBARS after casting and drying (ACD). When comparing TBARS value between films prepared at pH 3 and 11, much higher TBARS value was observed in the former. During drying process, film had lowered moisture content and lipids were more exposed to atmospheric oxygen. This led to the enhanced lipid oxidation of resulting film. The result was in agreement with Tongnuanchan *et al.* (2011) who found that lipid oxidation in fish muscle protein-based film prepared at acidic condition was more pronounced than that prepared at alkaline pH. At acidic pH, heme proteins became more prooxidative and could accelerate lipid oxidation of films.

During the storage, the gradual decrease in TBARS value was observed in films prepared at pH 3 up to 20 days. However, the marked decrease was found in film stored under 100% N_2 atmosphere at day 5, followed by the negligible changes up to the end of storage (day 20). The decrease in TBARS was possibly caused by a loss of volatile lipid oxidation products. Furthermore, those compounds

might react with proteins in film matrix, leading to intra or inter-molecular cross-links (Kikugawa *et al.*, 1988; Saeed *et al.*, 1999; Tongnuanchan *et al.*, 2011). Under atmosphere containing oxygen, both in air or 100% O_2, lipid oxidation of fish protein-based film still occurred during storage, though some volatile compounds were lost. This was reflected by the higher TBARS values stored under O_2 atmosphere. Similar results were observed with films prepared at pH 11 during the storage under different atmospheric conditions. Systems that contain lipids can undergo oxidative deterioration by reacting with molecular oxygen. The reaction of lipids with previously activated molecular oxygen through a self-catalyzing mechanism is known as autoxidation (Wrolstad *et al.*, 2004). Two electron reactions of ground state dioxygen are thermodynamically unfavorable due to spin restinction of the two unpaired electrons. Stepwise activation through donation and acceptance of a single electron is energetically more favorable. The reaction yields several reactive oxygen reactive species (ROS): superoxide anion radical ($O_2^{\cdot -}$), perhydroxyl radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$) (Kanner *et al.*, 1987). ROS have the ability to react with a large variety of easily oxidizable cellular components, particularly proteins, lipids and lipoproteins (Fridovich, 1995). Moreover, the newly formed highly reactive free radicals react with oxygen and/or other fatty acids to form more free radicals and hydroperoxides in a chain reaction (Dembele *et al.*, 2010). Since fish mince contains a high amount of prooxidative heme proteins as well as free ions, the initiation step could also take place easily. In the presence of atmospheric oxygen, the propagation more likely occurred. As a result, a number of lipid oxidation products were formed as indicated by the formation of TBARS. Therefore, oxygen was proven to be a prime factor affecting lipid oxidation of fish muscle protein-based film, particularly with increasing storage time.

(A)



(B)

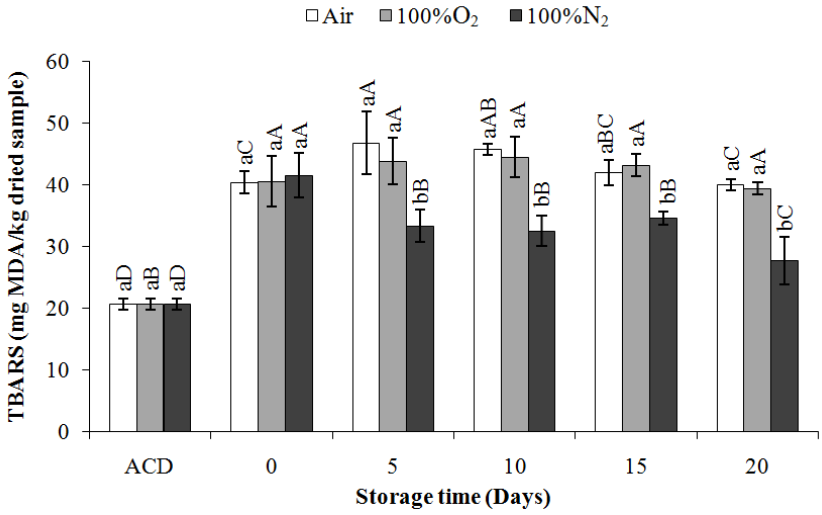
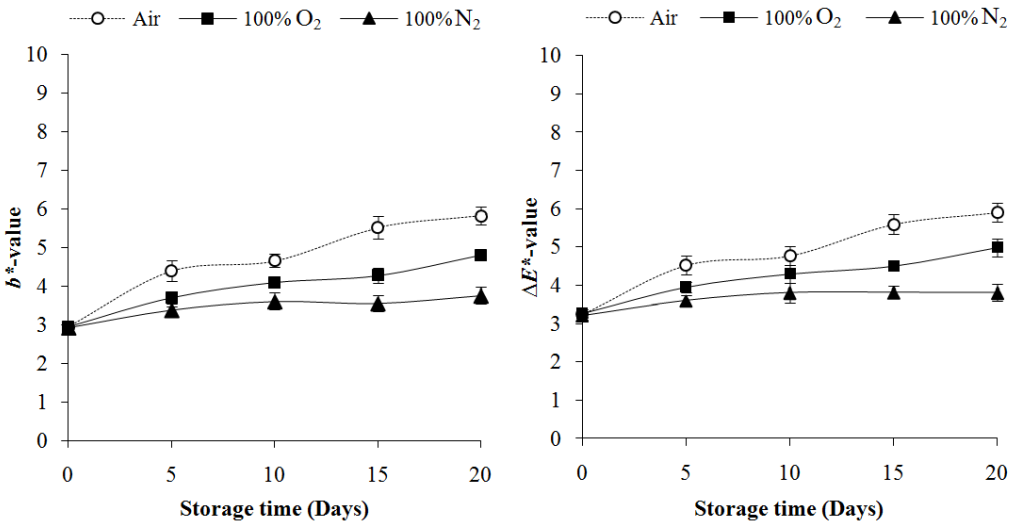


Figure 14. Changes in TBARS of films from red tilapia mince prepared at pH 3 (A) and 11 (B) stored in air and under different atmospheres (100% O_2 and 100% N_2) during 20 days of storage at room temperature. ACD: after casting and drying. Bars represent the standard deviation (n=3). The different lowercase letters on the bars within the same storage time indicate significant differences ($P < 0.05$). Different uppercase letters on the bars within the same atmospheric condition indicate the significant differences ($P < 0.05$).

3.4.1.2 Color of films

Figure 15 shows color, expressed as b^*-value (yellowness) and ΔE^*-value (total color difference) of films from red tilapia mince prepared at pH 3 (Figure 15A) and pH 11 (Figure 15B) stored under different atmospheric conditions during 20 days of storage. At day 0, films prepared at pH 11 showed the higher b^* and ΔE^*-values than those prepared at pH 3 ($P < 0.05$). Generally, yellowness of films increased during the extended storage ($P < 0.05$). During storage of 20 days, film prepared at pH 3 and kept in air showed the increase in b^* and ΔE^*-values with the highest rate, followed by those kept under 100% O_2. For film kept under 100% N_2, slight increases in both of b^* and ΔE^*-values were observed during the storage. Lipid oxidation was a potential factor inducing yellow discoloration of film by providing the carbonyl compounds involved in Maillard reaction. It was noted that films stored in air showed the higher yellowness, compared with those kept under 100% O_2. Films kept under 100% O_2 closed system might have the diluted O_2 level caused by volatile oxidation products in the headspace, while those in air were exposed to circulating air containing O_2. This result was in agreement with TBARS value in film (Figure 14). For film prepared at alkaline condition, similar results were obtained, compared with those of films prepared at acidic pH. Nevertheless, similar b^* and ΔE^*-values were found between films kept in air and stored under 100% O_2 atmosphere throughout the storage. The difference between films prepared at both pH was probably governed by different dissolvation of O_2 into the film at various pH. Therefore, yellow discoloration of fish protein-based film was directly affected by oxygen as well as pH used for film preparation.

(A)



(B)

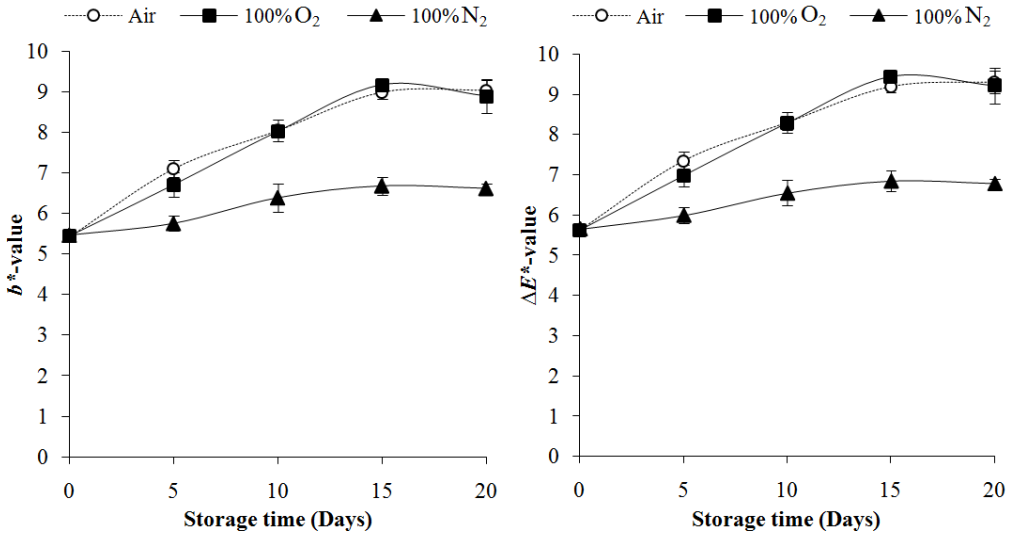


Figure 15. Changes in *b** and Δ*E**-values of films from red tilapia mince prepared at pH 3 (A) and 11 (B) stored in air and under different atmospheres (100% O_2 and 100% N_2) during 20 days of storage at room temperature. Bars represent the standard deviation (n=3).

3.4.1.3 Light transmittance and film transparency

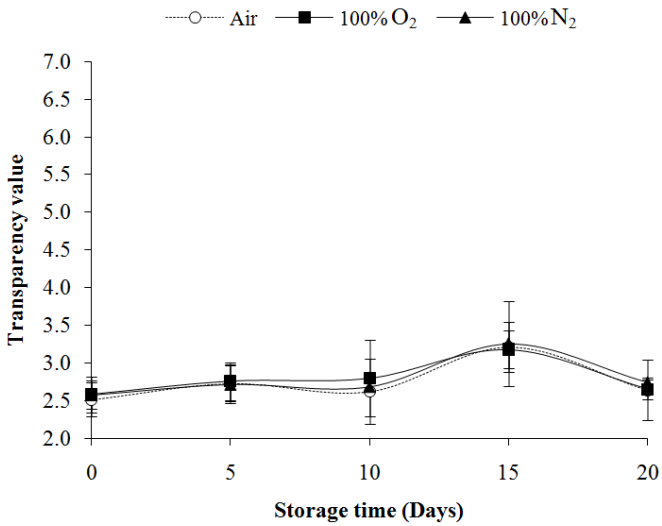
Transmission of UV and visible light at selected wavelength in the range of 200 - 800 nm of films from red tilapia mince prepared at pH 3 and 11 stored under different atmospheres during the storage of 20 days is shown in Table 7. Generally, fish muscle protein-based film had the good barrier to UV light as indicated by low transmission in the range of (200-280 nm) for films prepared at both pH and kept under all atmospheric conditions. Light transmission of visible range (350-800 nm) of film prepared at pH 3 and kept under different conditions ranged from 62.85 to 85.83%, whereas the lower values were found for film prepared at pH 11 (34.75-77.59%). This result was in agreement with Limpan *et al.* (2010) and Tongnuanchan *et al.* (2011) who reported that fish muscle protein-based film prepared under alkaline condition had the lower light transmission in visible range, compared with those prepared at acidic condition. In general, the decrease in light transmission in UV-visible range was observed in all samples with extended storage time. However, the rate of decrease was found to be different.

During storage, both films prepared at pH 3 and 11 and kept under 100% N_2 had the lowest decrease in transmission, particularly in the wavelength of 350-500 nm, when compared with those stored in air and 100% O_2. This result suggested that the increase in yellow discoloration of film might prevent transmission of light or pigments formed might absorb the light, resulting in the decrease in transmission of films during extended storage. The transparency value of film prepared at pH 3 and kept under all atmospheric conditions were lower than those prepared at pH 11 ($P < 0.05$) (Figure 16), indicating that the former was more transparent than the latter. No difference in transparency value was observed between the films prepared at pH 3 and 11 kept under different conditions ($P > 0.05$). No marked change in transparency values was observed in all films during 20 days of storage.

Table 7. Light transmittance of protein films from red tilapia mince prepared at pH 3 and 11 stored in air and under different atmospheres (100% O_2 and 100% N_2) during 20 days of storage at room temperature.

pH	Atmosphere	Storage time (Days)	Light transmittance (%) at different wavelength (nm)							
			200	280	350	400	500	600	700	800
3	Air	0	0.00	0.54	74.09	79.85	83.53	83.11	84.26	85.85
		5	0.00	0.82	71.58	77.82	82.33	83.75	84.49	85.01
		10	0.00	1.53	69.95	76.92	81.84	83.23	83.91	84.37
		15	0.00	2.69	70.88	77.17	81.65	82.94	83.61	84.05
		20	0.00	0.27	62.85	73.10	80.90	82.92	83.84	84.45
	100% O_2	0	0.00	0.54	74.06	79.82	83.51	83.09	84.25	85.83
		5	0.01	0.51	70.24	76.86	81.59	83.10	84.00	84.65
		10	0.00	0.74	68.57	76.50	81.70	83.15	83.94	84.50
		15	0.00	0.81	68.34	76.26	81.37	82.71	83.43	83.92
		20	0.00	0.74	65.61	75.73	81.53	83.59	84.34	84.86
	100% N_2	0	0.00	0.54	74.08	79.83	83.52	83.10	84.30	85.84
		5	0.01	1.48	74.44	79.13	82.65	83.87	84.58	85.08
		10	0.01	0.95	72.11	78.18	82.55	83.95	84.71	85.24
		15	0.00	1.03	70.37	77.92	81.45	82.87	83.62	84.13
		20	0.00	1.74	71.25	77.87	82.33	83.64	84.28	84.72
11	Air	0	0.00	1.18	50.77	62.63	71.22	74.05	75.80	77.05
		5	0.00	0.89	45.18	60.60	69.94	72.97	74.89	76.36
		10	0.00	0.64	42.67	58.21	67.28	70.16	72.03	73.47
		15	0.00	0.44	39.56	58.18	70.18	73.28	74.69	75.56
		20	0.00	0.22	34.82	53.36	66.25	70.20	71.43	72.58
	100% O_2	0	0.00	1.62	52.49	63.65	71.72	74.46	76.16	77.37
		5	0.00	0.35	43.53	59.58	68.93	71.71	73.40	74.64
		10	0.00	0.38	39.28	56.69	67.19	70.58	72.74	74.38
		15	0.00	0.60	43.48	60.45	69.99	72.61	74.16	75.25
		20	0.00	0.16	34.75	55.04	67.63	70.70	72.23	73.29
	100% N_2	0	0.00	1.27	53.28	63.84	71.69	74.50	76.29	77.59
		5	0.00	1.08	44.69	59.94	69.15	72.12	73.90	75.18
		10	0.00	1.01	41.06	58.85	69.83	73.12	74.80	75.93
		15	0.00	0.26	41.67	58.10	69.33	72.81	74.79	76.17
		20	0.00	1.02	42.31	61.15	71.75	73.04	75.43	76.36

(A)



(B)

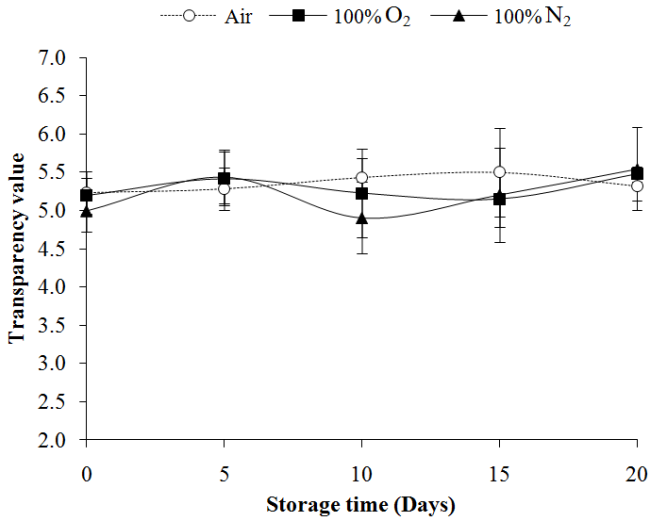


Figure 16. Changes in transparency value of films from red tilapia mince prepared at pH 3 (A) and 11 (B) stored in air and under different atmospheres (100% O_2 and 100% N_2) during 20 days of storage at room temperature. Bars represent the standard deviation (n=3).

3.4.2 Effect of antioxidants on lipid oxidation, yellow discoloration and properties of films

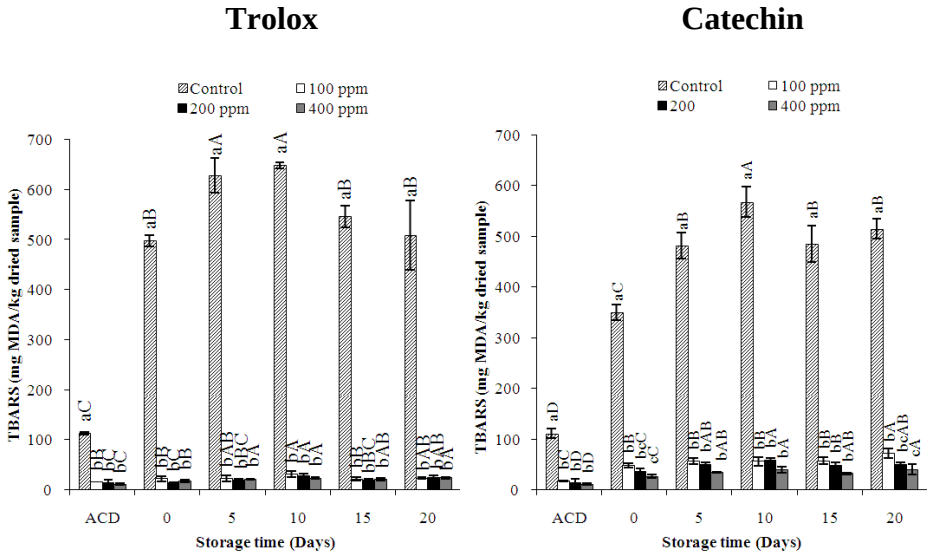
3.4.2.1 Lipid oxidation

TBARS values of films prepared at pH 3 and 11 and incorporated with antioxidants (Trolox or catechin) at different levels (100, 200 and 400 ppm) during 20 days of storage are shown in Figure 17. Fish lipids are susceptible to oxidation owing to high content of $\omega-3$ polyunsaturated fatty acids (PUFAs), such as eicosapentaenoic acid (20:5 $n-3$) and docosahexaenoic acid (22:6 $n-3$). For both films prepared at pH 3 and 11 without antioxidant (the control), the continuous increase in TBARS value was found within the first 10 days of storage ($P < 0.05$). Thereafter, the decrease in TBARS was observed until the end of storage, probably due to the loss in volatile lipid oxidation products with low molecular weights. Film prepared at pH 3 had the higher TBARS than those prepared at pH 11 ($P < 0.05$). When antioxidants were incorporated into films prepared at pH 3, much lower TBARS values were found in films during storage, compared with the control. Antioxidative activity of both antioxidants, Trolox and catechin, was in dose dependent manner. This result suggested that the incorporation of antioxidant more effectively decreased lipids oxidation in resulting film prepared at pH 3. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) is a synthetic water-soluble form of alpha-tocopherol (Vitamin E) and has been well known as potent antioxidant. In addition, Trolox exhibits activity toward the reactive oxygen species (ROS), particularly for superoxide radical anion (Triantis *et al.*, 2005). It also acts as free radical scavenger, which attenuates lipid peroxidation (Rezk *et al.*, 2004). Trolox has 2-4 fold higher antioxidant activity than butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA). It was more efficient than α-tocopherol, propyl gallate and ascorbyl palmitate to prevent oxidation of both vegetable and animal oils (Madhavi *et al.*, 1995). At the same level of antioxidant used, Trolox showed the higher efficiency in preventing lipid oxidation as evidenced by lower TBARS values. Tea catechin polyphenols have strong scavenging capacity for free radicals; they also possess metal-chelating capacity (Jo *et al.*, 2003; Tang *et al.*, 2002; Unno *et al.*, 2000; Wiseman *et al.*, 1997). Catechin reduced the formation of peroxides more

significantly than α-tocopherol, BHA and ascorbic acid in animal fats (Chen *et al.*, 1998) and fish system (He and Shahidi, 1997). Their scavenging efficiency depends on the concentration of phenols, the numbers and locations of the hydroxyl groups (Benavente-Garcia *et al.*, 1997). Therefore, the incorporation of Trolox or catechin in the range of 100-400 ppm was able to prevent lipid oxidant of film prepared at acidic pH.

For films prepared at pH 11, when both antioxidants, Trolox and catechin were incorporated, lipid oxidation still took place as indicated by the continuous increase in TBARS within the first 10 days of storage. The decrease in TBARS was observed in all samples at day 15 and 20 of storage. Antioxidants at all levels (100, 200 and 400 ppm) did not exhibited antioxidative activity in film prepared at pH 11. As a result, lipid oxidation in film could not be prevented. Trolox and catechin might not be stable and lost their activities under alkaline conditions. Under alkaline condition, polyphenols underwent decomposition, thereby losing the scavenging activity on superoxide anion (Brooks *et al.*, 1972; Leffler, 1993). Polyphenol also underwent oxidation under alkaline condition, quinones were formed (Balange and Benjakul, 2009). Thus, the incorporation of antioxidant into film prepared at alkaline (pH 11) condition was not able to prevent lipid oxidation in resulting film.

(A)



(B)

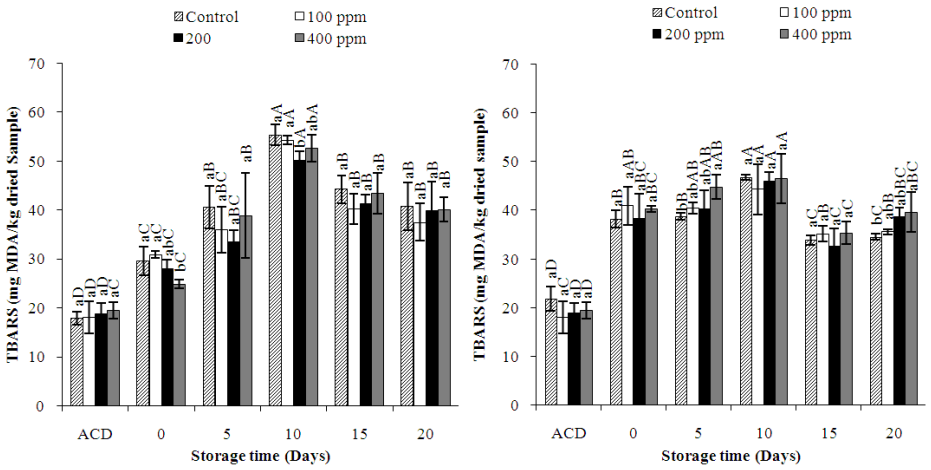


Figure 17. Changes in TBARS of films from red tilapia mince prepared at pH 3 (A) and 11 (B) incorporated without and with Trolox or catechin at different levels (100, 200 and 400 ppm) during 20 days of storage. ACD: after casting and drying. Bars represent the standard deviation (n=3). The different lowercase letters on the bars within the same storage time indicate significant differences ($P < 0.05$). Different uppercase letters on the bars within the same level of antioxidant indicate the significant differences ($P < 0.05$).

3.4.2.2 Color

The color expressed as b^*-value and ΔE^*-value of films prepared at pH 3 and 11 and incorporated with antioxidants, either Trolox or catechin, at different levels during 20 days of storage is shown in Figure 18 and 19, respectively. Films prepared at pH 3 and incorporated with Trolox at all levels used (100, 200 and 400 ppm) showed the lower b^* and ΔE^*-values than did the control film throughout the storage time. No changes in b^* and ΔE^*-values in film added with antioxidants were observed during storage ($P < 0.05$), while the continuous increases in both values were found in the control during storage. It was also noted that yellow discoloration took place during casting and drying. The lowered b^*-value and ΔE^*-value in films added with both antioxidants, were in accordance with the lower TBARS values of those films (Figure 17).

Levels of both antioxidants used in the present study had no impact on b^* and ΔE^*-values of films. It was postulated that antioxidant at 100 ppm was sufficient for inhibition of lipid oxidation, which was a major cause for yellow discoloration. From the result, both Trolox and catechin effectively prevented yellow discoloration of film prepared at pH 3.

For film prepared at pH 11 without antioxidant incorporated (the control), the lowest b^* and ΔE^*-values were obtained, when compared with those incorporated with Trolox and catechin ($P < 0.05$). Films had the higher increase in yellowness (b^*-value and ΔE^*-values) when higher concentrations of antioxidants were used. It was noted that the incorporation of both Trolox and catechin had impact on yellowness of film prepared at pH 11. This result suggested that the formation of yellow or brown color in film added with antioxidant under alkaline (pH 11) condition were more likely related with the conversion of phenolic compound to quinone. Quinones, an oxidized form of phenolic compounds, are brown or dark in color (Pati *et al.*, 2006). Zhang *et al*. (2010) reported that caffeic acid (CA) solution with pale yellow turned to dark brown when being oxidized under alkaline condition. This result was in agreement with Nuthong *et al.* (2009a) who reported that porcine plasma film had the increases in a^* and b^*-values, when incorporated with 3% caffeic acid oxidized under alkaline condition along with oxygenation. Furthermore, the incorporated antioxidant into films prepared at pH 11 was unable to retard the lipid

oxidation. When phenolic compounds were oxidized to quinone, it lost their electron donating ability (Leffler, 1993). During storage, films prepared at pH 11 with and without antioxidant had the increase in both b^* and ΔE^*-values. Cao *et al.* (2007) also reported that gelatin film incorporated with phenolic compounds (tannic acid and ferulic acid) at alkaline pH had the changes in color. Therefore, antioxidants had the negative effect on color of films prepared at alkaline condition.

3.4.2.3 Light transmittance and film transparency

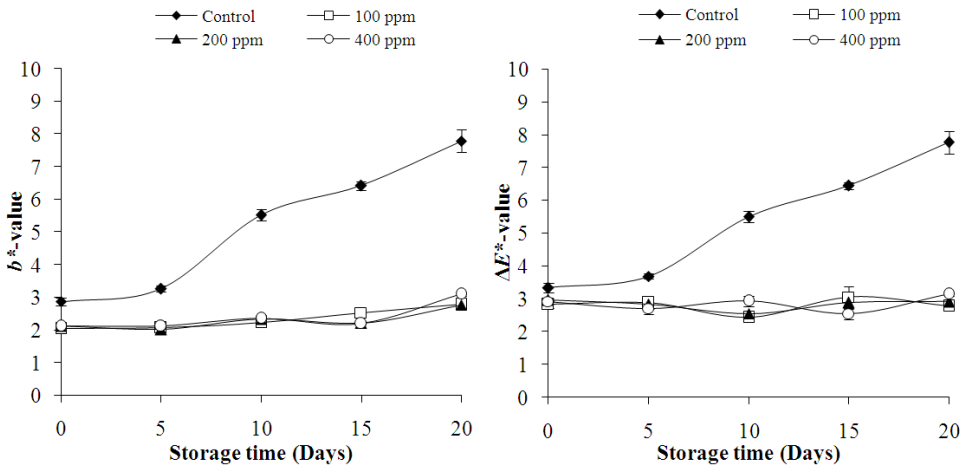
Transmission of UV and visible light in the range of 200 - 800 nm of films prepared at pH 3 and 11 and incorporated with Trolox and catechin at different concentrations during 20 days of storage is shown in Table 8 and 9, respectively. Films prepared at pH 3 had the excellent barrier to the light in UV range (200-280 nm), regardless of antioxidant incorporation and levels added. Protein-based film obtained from fish muscle (Chinabhark *et al.*, 2007; Hamaguchi *et al.*, 2007; Tongnuanchan *et al.*, 2011), fish skin gelatin (Jongjareonrak *et al.*, 2006) and whey protein (Fang *et al.*, 2002) had good UV light protection. In general, the light transmittance in visible range (350-800 nm) for film with and without antioxidant ranged from 61.71 to 85.50%. The incorporation of Trolox at different levels showed no effects on transmission in visible range of the resulting films. However, the incorporation of catechin at higher level resulted in slight decrease in light transmission at 400 and 500 nm. Moreover, no differences in transparency value were observed between films without and with antioxidant at all levels (Figure 20). During 20 days of storage, the decrease in light transmission was more pronounced in the control film. The decrease in light transmission was lowered in film added with antioxidant, especially at high level (400 ppm). Nevertheless, no changes in transparency values were observed during the storage of 20 days.

Films prepared at pH 11 with and without antioxidant also showed the excellent UV light barrier properties. Chinabhark *et al.* (2007) and Tongnuanchan *et al.* (2011) also reported that protein-based film from fish muscle had the higher UV light barrier capacity, regardless of pH used. In general, light transmittance in visible range (350-800 nm) of films with and without antioxidant prepared at pH 11 ranged from 9.24 to 78.81%. During storage, film prepared at pH 11 with and without

antioxidant had the decrease in light transmission in UV-visible range (200-800 nm). It was suggested that antioxidant had no effect on lowering the changes in light transmission. This result was associated with the increase in b^*-values during storage for all films, regardless of antioxidant incorporated.

Based on transparency value, no difference was found for films prepared at pH 11 with and without antioxidant ($P > 0.05$). Transparency value of film prepared at pH 11 incorporated with catechin increased as the level of catechin used increased, which was correlated with the increases in b^* and ΔE^*-values as well as the decrease in light transmission. Nevertheless, transparency value of films added with Trolox was not changed as the level of Trolox increased. Rattaya *et al.* (2009) reported the increases in transparency value of gelatin film incorporated with phenolic compound. The transparency value of films prepared at pH 3 was lower than those prepared at pH 11, irrespective of antioxidants and levels used. It indicated that films prepared at pH 3 were more transparent than those prepared at pH 11. This was in agreement with Limpan *et al.* (2010) and Tongnuanchan *et al.* (2011) who found that myofibrillar protein-based film prepared at acidic pH was more transparent than that prepared at alkaline pH ($P < 0.05$). Both Trolox and catechin in the range of 100-400 ppm had no impact on transparency value of films prepared at both pH during storage of 20 days.

(A)



(B)

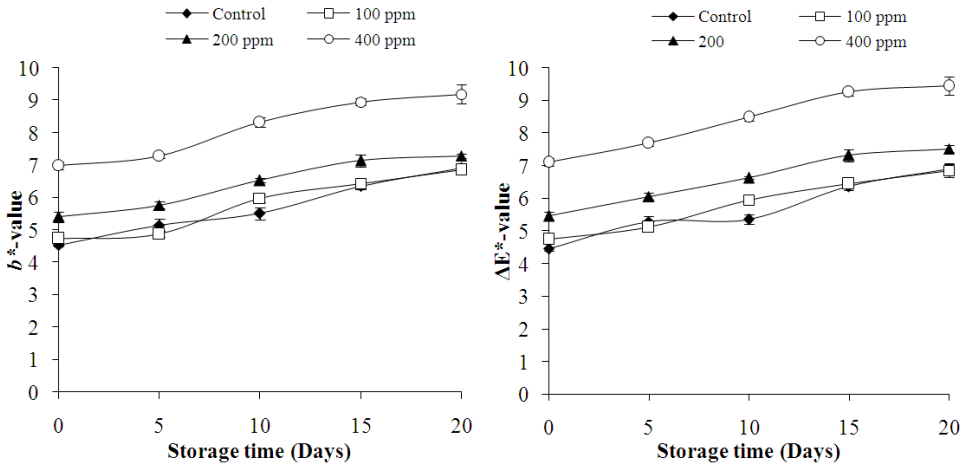


Figure 18. Changes in *b** and Δ*E**-values of films from red tilapia mince prepared at pH 3 (A) and 11 (B) incorporated without and with Trolox at different levels (100, 200 and 400 ppm) during 20 days of storage. Bars represent the standard deviation (n=3).

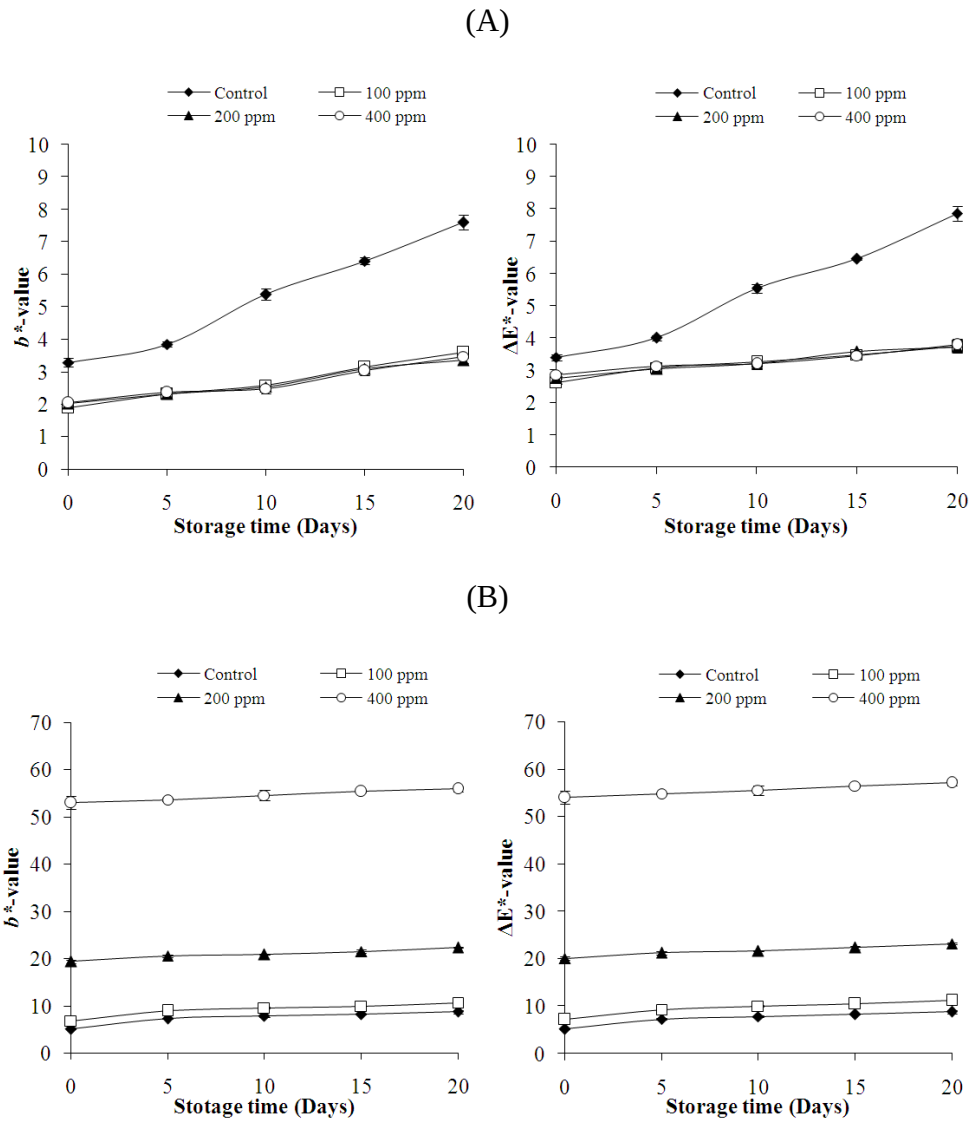


Figure 19. Changes in b^* and ΔE^*-values of films from red tilapia mince prepared at pH 3 (A) and 11 (B) incorporated without and with catechin at different levels (100, 200 and 400 ppm) during 20 days of storage. Bars represent the standard deviation (n=3).

Table 8. Light transmittance of films from red tilapia mince prepared at pH 3 and 11 incorporated without and with Trolox at different levels during 20 days of storage at room temperature.

pH	Trolox levels	Storage time	Light transmittance (%) at different wavelength (nm)							
	(ppm)	(Days)	200	280	350	400	500	600	700	800
3	Control	0	0.00	0.62	75.87	78.54	82.26	83.56	84.41	84.97
	(without Trolox)	5	0.01	0.29	68.61	75.99	81.29	83.05	83.98	84.62
		10	0.00	0.89	68.36	76.16	81.66	83.34	84.18	84.76
		15	0.00	0.96	66.66	76.45	81.90	83.45	84.23	84.77
		20	0.01	0.64	61.71	72.61	81.10	83.18	83.96	84.45
	100	0	0.01	1.37	75.58	78.34	81.88	83.29	84.21	84.85
		5	0.00	1.37	75.58	78.34	81.88	83.29	84.21	84.85
		10	0.00	2.22	75.96	78.80	82.08	83.38	84.23	84.84
		15	0.00	0.84	73.95	77.46	81.47	82.95	83.94	84.64
		20	0.00	0.91	74.00	77.81	81.75	83.19	84.09	84.73
	200	0	0.00	0.62	75.87	78.54	82.26	83.56	84.41	84.97
		5	0.01	0.92	75.98	78.63	82.13	83.46	84.32	84.92
		10	0.00	1.87	74.80	77.82	81.52	83.05	84.08	84.85
		15	0.00	0.79	73.63	77.21	81.39	82.95	84.01	84.74
		20	0.01	1.57	75.28	78.53	81.99	83.24	84.08	84.67
	400	0	0.00	1.55	75.38	78.19	81.63	82.99	83.86	84.49
		5	0.00	0.69	75.37	78.02	81.81	83.15	84.05	84.64
		10	0.00	1.25	75.34	78.12	81.66	82.86	83.62	84.65
		15	0.00	0.59	75.01	78.20	82.00	83.24	84.07	84.61
		20	0.00	0.37	73.28	77.14	81.55	82.95	83.85	84.45
11	Control	0	0.00	0.63	48.35	61.21	70.55	74.05	76.12	77.56
	(without Trolox)	5	0.00	0.77	45.84	59.46	70.35	74.45	75.96	77.26
		10	0.00	0.39	39.03	56.20	69.10	73.20	75.83	77.43
		15	0.00	0.42	35.31	53.42	67.00	72.26	74.44	76.27
		20	0.00	0.43	33.97	52.77	67.40	72.12	74.62	76.30
	100	0	0.00	0.50	52.98	63.66	71.72	74.61	76.35	77.53
		5	0.00	0.76	51.94	63.13	71.35	74.55	76.48	77.80
		10	0.00	0.32	46.78	61.00	70.64	74.02	75.94	77.25
		15	0.00	0.48	49.59	61.81	70.64	73.84	75.70	76.93
		20	0.01	0.31	44.67	60.06	70.29	73.65	75.51	76.76
	200	0	0.00	0.47	48.71	59.59	70.84	74.28	76.27	77.54
		5	0.00	0.27	45.96	59.34	70.45	74.16	76.26	77.63
		10	0.00	0.38	38.52	55.98	68.59	72.92	75.30	76.94
		15	0.00	0.49	42.49	59.05	70.41	73.96	75.84	76.97
		20	0.00	0.38	39.48	56.63	68.81	73.00	75.34	76.83
	400	0	0.00	0.63	48.03	59.80	71.38	74.50	76.30	77.17
		5	0.00	0.62	48.50	61.82	71.71	74.87	76.60	77.72
		10	0.00	0.35	48.15	61.80	71.12	74.06	75.62	77.11
		15	0.00	0.42	35.31	53.42	67.00	72.26	74.44	76.27
		20	0.00	0.28	34.13	54.72	68.20	72.32	74.50	75.85

Table 9. Light transmittance of films from red tilapia mince prepared at pH 3 and 11 incorporated without and with catechin at different levels during 20 days of storage at room temperature.

pH	Catechin levels	Storage time	Light transmittance (%) at different wavelength (nm)							
	(ppm)	(Days)	200	280	350	400	500	600	700	800
3	Control	0	0.00	1.60	76.69	79.33	82.58	84.03	84.98	85.66
	(without catechin)	5	0.00	1.13	77.39	79.82	82.67	84.01	84.88	85.52
		10	0.00	0.88	73.72	78.94	82.90	84.32	85.13	85.69
		15	0.00	1.64	69.67	77.04	82.34	84.01	84.89	85.51
		20	0.00	0.86	66.82	75.07	81.06	82.88	83.85	84.50
	100	0	0.00	1.81	76.29	79.02	82.37	83.82	84.78	85.45
		5	0.00	1.32	75.99	78.84	82.49	84.05	85.08	85.80
		10	0.00	0.85	76.60	79.33	82.29	83.67	84.56	85.17
		15	0.00	1.59	74.94	79.41	83.15	84.54	85.33	85.85
		20	0.01	1.22	75.85	79.21	82.34	83.75	84.61	85.21
	200	0	0.00	1.38	76.08	78.42	82.17	83.72	84.71	85.38
		5	0.00	1.74	76.35	79.01	82.27	83.64	84.56	85.19
		10	0.00	1.14	75.21	78.20	81.92	83.51	84.53	85.25
		15	0.00	0.22	73.56	77.18	81.82	83.60	84.72	85.44
		20	0.01	1.18	73.23	76.68	80.87	82.67	83.80	84.58
	400	0	0.01	1.48	76.39	77.12	81.69	83.39	84.37	85.04
		5	0.00	0.89	75.71	78.53	82.13	83.69	84.73	85.44
		10	0.00	0.90	75.90	78.78	82.26	83.70	84.61	85.24
		15	0.00	0.92	72.96	77.72	82.29	83.95	84.93	85.56
		20	0.01	1.09	73.60	77.12	81.17	82.94	84.03	84.77
11	Control	0	0.00	0.10	52.66	63.25	71.15	74.41	76.87	78.57
	(without catechin)	5	0.00	1.00	44.95	59.82	70.47	74.45	76.63	78.18
		10	0.00	0.12	44.13	56.61	70.22	74.30	76.47	77.95
		15	0.00	0.40	35.92	60.55	71.02	74.66	76.80	78.28
		20	0.01	1.68	36.53	59.32	69.96	73.88	76.24	77.92
	100	0	0.00	0.11	50.55	63.00	71.61	74.37	77.07	78.77
		5	0.00	1.43	40.92	55.87	69.90	74.72	76.76	78.18
		10	0.00	0.11	35.63	55.87	69.90	74.37	77.07	78.77
		15	0.00	1.46	43.29	62.49	71.49	74.80	76.91	78.38
		20	0.01	0.79	35.64	56.72	68.26	72.65	75.45	77.39
	200	0	0.00	0.04	31.43	51.88	67.37	72.50	75.69	77.67
		5	0.00	0.01	23.72	38.65	62.71	71.06	75.43	77.48
		10	0.00	0.01	19.59	39.56	63.90	72.13	76.24	78.20
		15	0.00	0.01	17.82	38.28	63.66	72.48	76.78	78.81
		20	0.01	0.04	17.55	26.88	57.96	71.39	75.63	77.80
	400	0	0.01	0.06	19.47	27.44	57.09	70.12	74.37	76.65
		5	0.00	0.11	19.52	31.14	58.78	70.57	74.47	76.64
		10	0.00	0.00	9.24	15.89	50.01	68.57	74.26	77.04
		15	0.01	0.00	9.94	18.24	52.10	69.41	74.82	77.52
		20	0.01	0.01	9.54	17.40	51.85	69.78	74.43	76.20

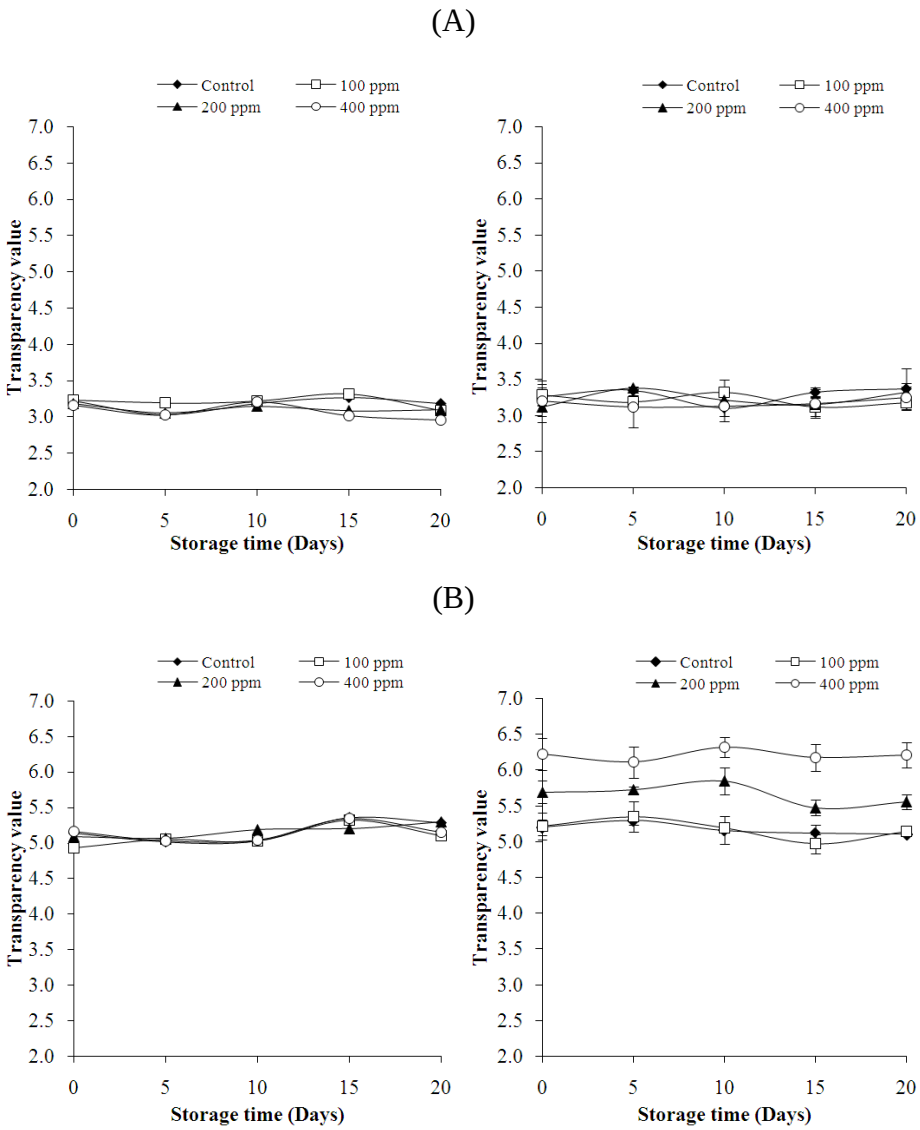


Figure 20. Changes in transparency value of films from red tilapia mince prepared at pH 3 (A) and 11 (B) incorporated without and with Trolox or catechin at different levels (100, 200 and 400 ppm) during 20 days of storage Bars represent the standard deviation (n=3).

3.5 Conclusions

Lipid oxidation of fish muscle protein-based film exposed to oxygen was the key factor affecting yellow discoloration during extended storage. The inhibition of lipid oxidation by incorporation of antioxidants (Trolox and catechin) in the range of 100-400 ppm was able to prevent discoloration, mainly via the retardation of lipid oxidation of film during the storage.

CHAPTER 4

CHARACTERISTICS OF FILM BASED ON PROTEIN ISOLATE FROM RED TILAPIA MUSCLE WITH NEGLIGIBLE YELLOW DISCOLORATION

4.1 Abstract

The properties of film from fish protein isolate (FPI) prepared by prior washing followed by alkaline solubilization process (ASP) from red tilapia muscle were monitored during the storage of 20 days at 50% RH and 25ºC, in comparison with those of films from washed mince. Lipid, heme iron and non-heme iron contents in FPI were decreased by 98.8, 36.8 and 91.9%, in comparison with those of mince ($P < 0.05$). Films from FPI had higher tensile strength (TS) and elongation at break (EAB) than those from washed mince for both pH (3 and 11) used for film preparation ($P < 0.05$). Film from FPI at pH 3 showed the highest TS, while that from washed mince at pH 11 had the lowest TS ($P < 0.05$). Nevertheless, films from FPI had higher WVP than those from washed mince for both pH used ($P < 0.05$). At the same pH used for film preparation (3 or 11), films from FPI showed the lower TBARS values than those from washed mince ($P < 0.05$). Nevertheless, films from both FPI and washed mince had the higher TBARS values when pH 3 was used for film preparation, compared with pH 11 ($P < 0.05$). Among all films, those from FPI prepared at pH 3 had the highest transparency and no yellow discoloration was observed during the storage of 20 days, in comparison with other films ($P < 0.05$). Conversely, film from washed mince prepared at pH 3 had the higher increase in b^*-value and ΔE^*-value than other films. Therefore, FPI could serve as a potential material for film preparation with lower contents of lipid and prooxidants, thereby preventing the yellow discoloration of the fish myofibrillar protein-based film during extended storage.

4.2 Introduction

Biodegradable packagings have been paid more increasing attention, especially those prepared from alternative materials for petrochemical and plastic products, which are non-biodegradable and have the negative impact on environment. Proteins are biopolymers capable of forming the film and their properties can be varied with proteinaceous materials. Myofibrillar and sarcoplasmic proteins from fish muscle have been widely used as film forming material (Artharn *et al.*, 2009; Chinabhark *et al.*, 2007; Limpan *et al.*, 2010; Paschoalick *et al.*, 2003). Apart from possessing the poor water barrier properties, yellow discoloration of myofibrillar protein based films is another drawback for applications. During extended storage, films from the muscle of Atlantic sardine (Cuq *et al.*, 1996a), round scad (Artharn *et al.*, 2009) and red tilapia (Tongnuanchan *et al.*, 2011), turned to be yellowish as indicated by the increases in b^* and ΔE^*-values. Yellow discoloration of myofibrillar film was mainly caused by Maillard reaction and directly limits the application of fish muscle protein-based film (Artharn *et al.*, 2009; Benjakul *et al.*, 2008; Tongnuanchan *et al.*, 2011). Lipid oxidation more likely played a role in yellow discoloration of fish muscle protein film, by providing the carbonyl groups involved in Maillard reaction (Tongnuanchan *et al.*, 2011). Therefore, fat removal can be a means to retard discoloration of film during storage. Conventional washing process has been used to concentrate myofibrillar proteins in fish mince by removing sarcoplasmic protein, fat, blood and pigment (Morioka *et al.*, 1997; Park *et al.*, 1997). Recently, Tongnuanchan *et al*. (2011) reported that washing process could decrease the yellow discoloration of film to some degree. Nevertheless, yellow discoloration associated with lipid oxidation still occurred. To overcome this problem, the removal of membrane lipid by pH-shift process might lower the oxidation in the films.

After the muscle is solubilized at low and high pH, the soluble proteins are separated from insoluble material, such as bones, skin, connective tissue, cellular membranes, and neutral storage lipids via high-speed centrifugation. Thereafter, the solubilized proteins are collected and recovered by isoelectric precipitation to give a highly functional and stable protein isolate (Hultin and Kelleher, 2000; Kristinsson and Ingadottir, 2006). The lowered lipids retained would undergo less oxidation. As a

consequence, the film produced from protein isolate may have the lower yellow discoloration. Additionally, the mechanical and other properties could be modified when protein isolate is used as material for film formation. However, no information regarding the use of fish protein isolate on properties of film, especially, yellow discoloration has been reported. The present study was undertaken to investigate the properties of film from protein isolate from red tilapia prepared at acidic and alkaline condition and the yellow discoloration of film during storage.

4.3 Materials and method

4.3.1 Chemicals

Glycerol, sodium chloride (NaCl), sodium dodecylsulfate (SDS), thiobarbituric acid, malondialdehyde bis (dimethyl acetal) and β-mercaptoethanol (βME) were purchased from Sigma (St. Louis, MO, USA). Acrylamide, *N,N,N',N'*-tetramethylethylenediamine (TEMED) and bis-acrylamide were obtained from Fluka (Buchs, Switzerland).

4.3.2 Fish sample

Fresh tilapia (400–500 g/fish) were purchased from a local market in Hat Yai, Songkhla province, Thailand. Fish were kept in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 30 min. Upon the arrival, fish were immediately washed, filleted and minced to uniformity using a mincer with a hole diameter of 0.5 cm.

4.3.3 Preparation of fish protein isolate from washed mince

Prior to the isolation of fish protein, mince was subjected to washing as per the method of Toyohara *et al*. (1990) with slight modifications. Fish mince was homogenised with 5 volumes of cold 0.05 M NaCl (2–4 °C) at a speed of 13,000 rpm for 2 min, using an IKA Labortechnik homogeniser (Selangor, Malaysia). The washed

mince was filtered through two layers of cheese-cloth. The washing process was repeated twice. Unwashed mince and washed mince obtained were stored on ice until used.

To prepare fish protein isolate (FPI), alkaline solubilization process was used as described by Hultin and Kelleher (2000) with slight modifications. Washed mince was added with cold distilled water at the ratio of 1:9 (w/v), followed by homogenisation for 1 min at the speed of 13,000 rpm. The pH of the homogenates was then adjusted to 11 using 2 M NaOH. The resulting mixture was centrifuged at 10,000xg for 20 min at 4 °C. The supernatant was collected and the pH was adjusted to 5.5 using 2 M HCl. The precipitate was then filtered through 4 layers of cheese-cloth. The retentate was dewatered by centrifugation at 12,000xg for 20 min at 4 °C. The final pH of the sample was adjusted to pH 7.0 using 2 M NaOH. The sample was referred to as 'fish protein isolate; FPI'. FPI was subjected to analyses and was used for film preparation.

4.3.4 Analyses of FPI

FPI was analysed in comparison with fish mince and washed mince as follows:

4.3.4.1 Determination of lipid content

Lipid content was determined by Soxhlet apparatus according to the method of AOAC (2000) with the analytical No. of 920.39B. Lipid content was expressed on a dry weight basis.

4.3.4.2 Measurement of total pigment and heme iron contents

Total pigment content was determined according to the method of Lee *et al*. (1999) and Cheng and Ockerman (2004) with a slight modification. The ground sample (2 g) was mixed with 9 ml of acid-acetone (90% acetone, 8% deionised water and 2% HCl). The mixture was stirred with a glass rod and allowed to stand for 1 h at room temperature. The mixture was filtered with a Whatman No. 42 filter paper (Whatman International Ltd., Maidstone, England). The absorbance of the filtrate was

read at 640 nm against an acid-acetone blank. The total pigment and heme iron contents were calculated as hematin using the following formula:

Total pigment content (mg/100 g dry sample) = $A_{640} \times 680$
Heme iron content (mg/100 g dry sample) = Total pigment (mg/100 g) × 0.0882

4.3.4.3 Measurement of non-heme iron content

Non-heme iron content was determined as per the method of Schricker *et al*. (1982). The sample (1.0 g) was weighed and transferred into a screw cap test tube and 50 ml of 0.39% (w/v) sodium nitrite were added. Four ml of a mixture of 40% trichloroacetic acid and 6 M HCl (ratio of 1:1 (v/v), prepared freshly) were added. The tightly capped tubes were placed in an incubator shaker (W350, Memmert, Schwabach, Germany) at 65 °C for 22 h and then cooled at room temperature for 2 h. The supernatant (400 µl) was mixed with 2 ml of the non-heme iron color reagent, a mixture of bathophenanthroline disulfonic acid, double-deionised water and saturated sodium acetate solution at a ratio of 1:20:20 (w/v/v), prepared freshly. After vortexing and standing for 10 min, the absorbance was measured at 540 nm. The non-heme iron content was calculated from iron standard curve. The iron standard solution ($Fe(NO_3)$ in HNO_3) with the concentration range of 0-2 ppm were used.

4.3.4.4 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of mince, washed mince and FPI were determined by SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). Samples (3 g) were solubilized in 27 ml of 5% SDS. The mixture was homogenised for 1 min at a speed of 13,000 rpm and incubated at 85 °C for 1 h to dissolve total proteins. 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. Molecular

weights of proteins were estimated from protein markers including myosin (206 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), serum albumin (66 kDa), glutamic dehydrogenase (55 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa) and trypsin (20 kDa).

Band intensity was measured using a densitometer. Quantitative analysis of protein band intensity was performed using a Model GS-700 Imaging Densitometer (Bio-Red Laboratories, Hercules, CA, USA) with Molecular Analyst Software version 1.4 (image analysis systems).

4.3.5 Preparation of film from washed mince and FPI

The film-forming solutions (FFS) from washed mince (15.22% protein, wet weight basis) and FPI (14.13% protein, wet weight basis) were prepared according to the method of Chinabhark *et al*. (2007). The samples were added with 3 volumes of distilled water and homogenised at 13,000 rpm for 1 min. The protein concentration of the mixture as determined by Kjeldahl method (AOAC, 2000) was adjusted to 2% (w/v). Glycerol, used as a plasticizer, was added at 50% (w/w) of protein. The mixtures were stirred gently for 30 min at room temperature. Subsequently, the pH of mixture was adjusted to 3 and 11, using 1 M HCl and 1 M NaOH, respectively, to solubilize the protein. FFS obtained was filtered through two layers of cheese-cloth to remove undissolved debris. The filtrate was used for film casting.

To prepare the film, FFS (4 g) was cast onto a rimmed silicone resin plate (50 × 50 mm^2) and air-blown for 12 h at room temperature prior to further drying at 25 °C and 50±5% RH for 24 h in an environmental chamber (WTB Binder, Tuttlingen, Germany). The resulting films were manually peeled off and used for analyses.

4.3.6 Determination of film properties

4.3.6.1 Film thickness

The thickness of film was measured using a micrometer (Gotech, Model GT-313-A, Gotech Testing Machines Inc, Tawai). Five random locations around each film of ten film samples were used for average thickness determination.

4.3.6.2 Mechanical properties

Prior to testing the mechanical properties, films were conditioned for 48 h at 25 °C and 50 ± 5% RH. 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, Hampshire, UK) equippted with tensile load cell of 100 N. Ten samples (2 × 5 cm^2) with initial grip length of 3 cm were used for testing. Cross-head speed was set at 30 mm/min.

4.3.6.3 Water vapor permeability (WVP)

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

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

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).

4.3.6.4 Color

Color of the film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., VA, USA). D_{65} (day light) and a measure cell with opening of 30 mm were used. The color of the films was expressed as *b**-value (yellowness) and total difference of color (ΔE^*) was calculated as follows (Gennadios *et al.*, 1996):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differentials between the color parameter of the samples and the color parameter of the white standard (L^* = 93.55, a^* = - 0.84, b^* = 0.37).

4.3.6.5 Light transmittance and transparency value

The light transmittance of films was measured at the ultraviolet and visible range (200–800 nm) using UV–vis spectrophotometer (Shimadzu UV-1800, Tokyo, Japan) as described by Shiku *et al.* (2004). The transparency value of film was calculated using 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 transparency value represents the lower transparency of film.

4.3.6.6 Film and protein solubilities

Film solubility was determined according to the method of Gennadios *et al*. (1998). The conditioned film samples (2 × 5 cm^2) were weighed and placed in a 50 ml centrifuge tube containing 10 ml of distilled water with 0.1% (w/v) sodium azide, used as antimicrobial agent. The mixture was shaken at a speed of 250 rpm using a shaker (Heidolth Inkubator 10000, Schwabach, Germany) at 30 °C for 24 h. Undissolved debris was removed by centrifugation at 3000xg 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 a percentage of the 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.

4.3.6.7 Protein pattern

Protein patterns of films were determined by SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). 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 homogenised at 13,000 rpm for 1 min. The homogenate was stirred continuously for 24 h at room temperature (28–30 °C). Then, the sample was centrifuged at 7500xg for 10 min at room temperature. The supernatant was subjected to SDS-PAGE as described previously.

4.3.6.8 Scanning electron microscopy (SEM)

Morphology of surface and cross-section of the film samples were visualised using a scanning electron microscope (SEM) (Quanta 400, FEI, Eindhoven, Netherlands). For cross-section, samples were fractured under liquid nitrogen prior to morphology visualisation. Then, the samples were mounted on bronze stub and sputtered with gold (Sputter coater SPI-Module, PA, USA) in order to make the sample conductive. The photographs were taken at an acceleration voltage of 15 kV.

4.3.7 Change in TBARS, color and solubility of film during the storage

Films from washed mince and FPI, prepared at pH 3 and 11, were stored at the room temperature (28-32 °C). Samples were taken for determination of thiobarbituric acid reactive substrances (TBARS), color and transparency value every

5 days for totally 20 days. Mechanical properties and WVP of the films were also tested at day 0 and 20 of storage.

TBARS value of film was determined according to the method of Buege and Aust (1978). Film samples (0.05 g) were mixed with 2.5 ml of TBA solution (0.375 g of thiobarbituric acid, 15 g of trichloroacetic acid, and 0.875 ml of HCl in 100 ml of distilled water). The mixtures were heated for 10 min in a boiling water bath (95–100 °C) to develop pink color, cooled with tap water and centrifuged at 7500xg for 10 min. Absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using malonaldehyde bis (dimethyl acetal) (MDA) at concentrations ranging from 0 to 10 mM. TBARS value in each sample was expressed as mg MDA/kg dried sample.

4.3.8 Statistical analysis

All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL).

4.4 Results and discussion

4.3.4 Chemical composition of mince, washed mince and FPI from red tilapia

4.4.1.1 Lipid content

Lipid content of FPI from red tilapia muscle was 0.12 % (dry weight basis), while mince and washed mince had the lipid content of 11.17% and 9.56%, respectively (Table 10). After washing process and preparation of FPI, lipid content was decreased by 14.41 % and 98.8%, respectively, in comparison with that found in mince. Rawdkuen *et al*. (2009) reported that the lipid reduction by 85.2 and 86.6% was obtained for FPI recovered using acid and alkaline solubilization process, respectively. Lipid reduction of 31.1 and 68.4% was found in protein recovered from Atlantic croaker muscle by acid- and alkaline-aided processes, respectively

(Kristinsson and Liang, 2006). Chaijan *et al*. (2010) reported that the lipid content of the protein isolate prepared by alkaline solubilization process with and without prewashing was not different but was lower, when compared with protein obtained from conventional washing process. Washing could remove lipids to some extent because membrane lipids were still retained. Additionally, lipoproteins associated with other muscle proteins might not be leached out easily. When alkaline-aided process was applied for washed mince, proteins were more likely dissociated. As a result, lipids and the membrane phospholipids could be liberated to a higher extent. After solubilization, these components were separated on the basis of density and solubility differences (Ingadottir and Kristinsson, 2005; Undeland *et al.*, 2002). Generally, neutral lipids are more easily removed in the acid and alkaline-aided processes than membrane phospholipids (Kristinsson and Liang, 2006). The higher lipid removal by the alkaline process may be due to the higher emulsification ability of the proteins at alkaline pH (Kristinsson and Hultin, 2003). Therefore, the effective removal of lipid could be achieved by the combined process between washing and alkaline solubilization process.

Table 10. Chemical compositions of mince, washed mince and FPI from red tilapia.

Composition	Mince	Washed mince	FPI
Lipid (% dry basis)	11.17 ± 0.46^{a}	9.56 ± 0.12^{b}	0.12 ± 0.01^{c}*
Total pigment (mg/100 g sample)	5.83 ± 0.69^{a}	3.52 ± 0.54^{b}	2.61 ± 0.20^{c}
Heme iron (mg/100 g sample)	0.51 ± 0.06^{a}	0.31 ± 0.05^{b}	0.19 ± 0.01^{c}
Non-heme iron (mg/100 g sample)	0.10 ± 0.01^{a}	0.15 ± 0.01^{a}	0.03 ± 0.02^{b}

*Mean $\pm$ SD (n=3).

Different lowercase superscripts in the same row indicate the significant differences ($P < 0.05$).

4.4.1.2 Total pigment, heme iron and non-heme iron content

Total pigment contents of mince, washed mince and FPI were 5.83, 3.52 and 2.61 mg/100g sample, respectively (Table 10). Washing using 0.05 M NaCl could remove 39.62% of total pigment from the red tilapia muscle and washing in

combination with alkaline solubilization process could remove 55.23% of total pigment. The pigments in muscle food are mainly myoglobin and hemoglobin (Pearson and Gillett, 1996). The result indicated that both of myoglobin and hemoglobin, water soluble globular proteins could be removed in washing process to a high extent. With the subsequent alkaline solubilization, the dissociation of muscle proteins might facilitate the release of heme protein into the medium. Amino acids of heme proteins packed into the interior of the molecule were predominantly hydrophobic in character, whilst those exposed on the surface of the molecule were generally hydrophilic. Alkaline solubilization process also resulted in the increased charged molecules, which become more soluble. Falk *et al*. (1998) reported that pIs of hemoglobins of 5 tilapia species of genera *Oreochromis* and *Serotherodon* ranged from 5.94–8.06 and they differed by their globin chains. At pH 5.5, used for muscle protein recovery, some hemoproteins might not be precipitated. As a result, the lower amount of total pigment was obtained in FPI.

Heme iron content in all samples was in accordance with total pigment content. The lowest heme iron content was found in FPI, whereas unwashed mince had the highest content ($P < 0.05$). Both hemoglobin and myoglobin contain iron in the prophyrin ring, called heme protein. Since the heme proteins, the major pigment in mince, were removed, the heme irons were simultaneously removed, thereby lowering heme iron content in washed mince. The lowest content of heme iron was also in agreement with the highest efficiency in pigment removal. For non-heme iron, the lowest content was obtained in FPI ($P < 0.05$). However, no difference was found between unwashed mince and washed mince ($P > 0.05$). During mincing, porphyrin ring might be disrupted to some degree and non-heme iron was released. Although washing might remove those free irons to some extent, the release of free iron from heme possibly occurred to some degree during washing. For FPI, washing along with alkaline solubilization could facilitate the removal of free irons associated with muscle protein. As a result, non-heme iron was lowered. The results indicated that FPI had lowered pigment as well as non-heme iron content, compared with unwashed and washed mince.

4.4.1.3 Protein pattern of mince, washed mince and FPI

Protein patterns of mince (M), washed mince (WM) and FPI are shown in Figure 21. For all samples, myosin heavy chain (MHC) was the dominant protein, followed by actin, tropomyosin and troponin, respectively. Band intensity of MHC from washed mince and FPI increased by 9.3 and 12.0%, when compared with that found in mince. No significant change in actin band intensity was observed, compared with that found in mince. Therefore, band intensity of MHC and actin was the highest in FPI, followed by washed mince and mince, respectively. During washing process, water soluble protein or sarcoplasmic proteins were mostly removed. This led to higher concentration of myofibrillar proteins including MHC, actin, etc, as evidenced by the increased band intensity of those proteins. For FPI, after washing and alkaline solubilization, the dissociation of actomyosin complex more likely took place. When the pH adjustment was made to 5.5, myofibrillar proteins were mainly precipitated, whereas other sarcoplasmic proteins might not be recovered. As a result, MHC and actin could become more concentrated. Therefore, those myofibrillar proteins in FPI could serve for strong film network formation.

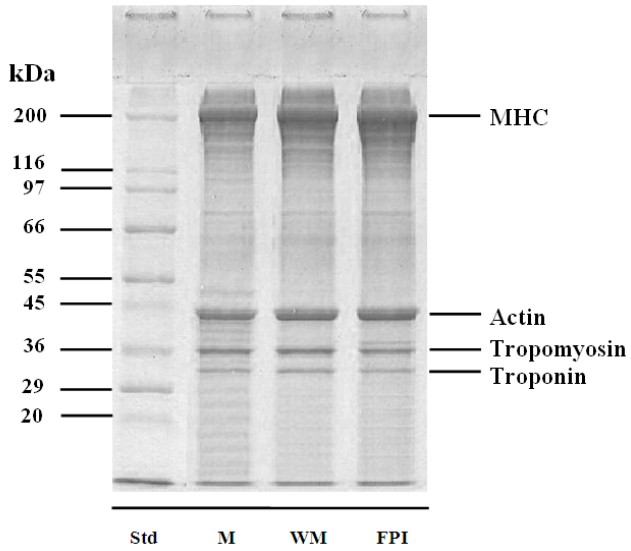


Figure 21. Protein patterns of mince (M), washed mince (WM) and FPI of red tilapia muscle under reducing condition. Std: Standard marker, MHC: myosin heavy chain.

4.4.2 Properties of protein based film from washed mince with and without subsequent alkaline solubilization process of red tilapia

4.4.2.1 Mechanical properties

Mechanical properties and thickness of films from washed mince and FPI from red tilapia prepared at acidic (pH 3) and alkaline (pH 11) conditions at day 0 and 20 of storage are shown in Table 11. The thickness of film from FPI was higher than that of film from washed mince, regardless of pH used ($P < 0.05$). This result suggested the difference in alignment of protein molecules in film network between FPI and washed mince. FPI had higher content of MHC, in comparison with washed mince. Those MHC with long chain might undergo inter-connection, in the way which protruded network was formed, whereas more compact network was developed in film from washed mince. At day 0, films from FPI had the higher TS than those from washed mince, irrespective of pH used ($P < 0.05$). No difference in TS was found between films prepared from washed mince at both pHs ($P > 0.05$). Nevertheless, the higher TS was obtained in films prepared from FPI at pH 3, compared with those prepared at pH 11 ($P < 0.05$). It has been reported that the unwanted high-density components such as connective tissues, cellular membranes, insoluble proteins and low-density components such as membrane lipids and neutral storage lipids could be removed from fish mince by alkaline solubilization process (Kristinsson and Liang, 2006). As a consequence, myofibrillar proteins in FPI become more concentrated in comparison with those found in washed mince. During alkaline solubilization process, the unfolding of protein more likely took place. Those proteins could be more extended in chain length during the preparation of film forming solution. Additionally, the reactive group such as sulfhydryl group as well as hydrophobic domains could be more exposed. Sulfhydryl and hydrophobic groups could undergo interaction via disulfide bond and hydrophobic-hydrophobic interaction in film matrix, respectively. The main interactions involved in the structure of myofibrillar protein films are intermolecular covalent bonds including disulfide bond (Benjakul *et al.*, 2009; Iwata *et al.*, 2000; Rhim *et al.*, 2002). Furthermore, the lipid reduction was one of the factors yielding the increased TS of FPI. The presence of lipids might interfere the cross-linking of protein for film network. The slight

difference in TS between films from FPI prepared at both pHs might be due to the differences in charged residues of protein, which determined the ionic interaction between adjacent molecules in forming the film network. Recently, Tongnuanchan *et al*. (2011) reported that films from both unwashed and washed mince of red tilapia prepared at pH 3 had higher TS than those films prepared at pH 11 ($P < 0.05$). Acidic pH might favour the solubilization and subsequent alignment of protein molecules, in the way which interjunctions with strong bonds were formed (Tongnuanchan *et al*., 2011).

For EAB, films prepared from FPI had much higher EAB than did those from washed mince, regardless of pH used ($P < 0.05$). Nevertheless, pH had no impact on EAB of films for both materials used ($P > 0.05$). This result was in agreement with Hamaguchi *et al*. (2007) who reported that pH level of film forming solution had no effect on EAB of protein films from blue marlin (*Makaira mazara*). The high EAB of films from FPI reflected their increased extensibility. Unfolded or stretched molecules of proteins in FPI in film-forming solution might facilitate the molecular entanglement, thereby allowing more molecular slippage of those proteins upon tensile deformation before breaking of the film. This resulted in the increased EAB.

After 20 days of storage, all films had no change in both TS and EAB ($P > 0.05$). Thus, the storage time of 20 days had no effect on mechanical properties of films from washed mince and FPI.

4.4.2.2 Water vapor permeability (WVP)

WVP of films from washed mince and FPI prepared at different pH levels (pH 3 and 11) at day 0 and 20 of storage is shown in Table 11. At the same storage time, WVP of films from FPI was lower than that of films from washed mince for both pHs used for film preparation ($P < 0.05$). Film from FPI prepared at pH 3 had lower WVP than did those prepared at pH 11 ($P < 0.05$), but no differences in WVP were found between films from washed mince prepared at pH 3 and 11 ($P > 0.05$). The lowest WVP of the film from FPI prepared at pH 3 was in agreement with the highest TS (Table 11). The lowered permeation of water vapor through the film prepared from FPI at pH 3 was probably determined by the stronger interaction of

protein molecules in the film network with the high compactness. Normally, fish muscle protein is hydrophilic in characteristics associated with polar amino acids. The

Table 11. Mechanical properties, water vapor permeability (WVP) and thickness of films from washed mince (WM) and FPI of red tilapia prepared at pH 3 and 11 at day 0 and 20 of storage.

Storage time (Days)	Samples	pH levels	TS (MPa)	EAB (%)	WVP (x 10^{-11} $gm^{-1}s^{-1}Pa^{-1}$)	Thickness (mm)
0	WM	3	6.98 ± 0.92^{cA}	47.93 ± 7.45^{cA}	4.46 ± 0.18^{aA}	$0.030 \pm 0.005^{bA*}$
		11	6.34 ± 0.45^{cA}	59.25 ± 10.24^{bcA}	4.58 ± 0.13^{aA}	0.029 ± 0.004^{bA}
	FPI	3	12.51 ± 0.72^{aA}	74.15 ± 8.54^{abA}	3.22 ± 0.16^{cA}	0.034 ± 0.002^{aA}
		11	11.25 ± 1.06^{bA}	80.39 ± 21.13^{aA}	3.70 ± 0.26^{bA}	0.035 ± 0.004^{aA}
20	WM	3	7.16 ± 1.07^{cA}	42.79 ± 6.36^{bA}	4.28 ± 0.13^{aA}	0.029 ± 0.002^{bA}
		11	6.40 ± 0.10^{cA}	49.01 ± 5.59^{bA}	4.39 ± 0.10^{aA}	0.028 ± 0.003^{bA}
	FPI	3	14.46 ± 2.43^{aA}	67.61 ± 13.53^{aA}	3.15 ± 0.14^{bA}	0.036 ± 0.003^{aA}
		11	12.28 ± 1.25^{bA}	71.83 ± 16.51^{aA}	3.31 ± 0.23^{bA}	0.035 ± 0.005^{aA}

*Mean $\pm$ SD (n=3).

Different lowercase superscripts in the same column under the same storage time indicate significant differences ($P < 0.05$). Different uppercase superscripts in the same column under the same material and pH levels used indicate significant differences ($P < 0.05$).

enhanced interaction via those reactive groups would lower the hydrophilicity of the resulting film. Paschoalick *et al*. (2003) reported that muscle protein of Nile tiapia had a high concentration of polar ionic amino acids (aspartic acid, glutamic acid, arginine and lysine). Protein films from fish muscle are usually poor in water vapor barrier properties (Hamaguchi *et al.,* 2007). Furthermore, the polar proteins or amino acids, especially from sarcoplasmic protein such as heme protein, were leached out more

effectively. As a consequence, water vapor barrier property of protein film from washed fish muscle was improved.

After 20 days of storage, no changes in WVP of all films were obtained ($P > 0.05$). This result suggested that film matrix was not affected by storage time of 20 days. Therefore, film from washed mince and FPI were stable in term of water vapor barrier property during the extended storage.

4.4.2.3 Film and protein solubilities

Film and protein solubilities of films from washed mince and FPI prepared at different pH levels (pH 3 and 11) at day 0 and 20 of storage are shown in Table 12. At day 0, film and protein solubilities of films from washed mince were higher than those of film from FPI when the same pH of film preparation was used. For the film prepared from same material, film and protein solubilities were much lower in film prepared at pH 11, in comparison with those prepared at pH 3 ($P < 0.05$). Washing process could remove the sarcoplasmic or water-soluble proteins, resulting in the lowered content of water soluble components. At pH 11, stronger film network could be formed via the stronger bonds as supported by higher TS (Table 11). Films prepared at alkaline condition, which favours the formation of disulfide bonds in film network (Shiku *et al.*, 2003), had the lowered film and protein solubility, compared with those prepared at pH 3. The strong protein-protein interaction led to the lowered solubility of film. At pH 3, the lower film and protein solubilities were found in film prepared from FPI, suggesting the greater cross-linking via the stronger bonds in FPI films. This reconfirmed that FPI rendered the film with more cross-linkings than washed mince. After 20 days of storage, film solubility of films from both washed mince and FPI prepared at pH 3 decreased sharply, while those from washed mince and FPI prepared at pH 11 had no changes in both film and protein solubilities after storage ($P > 0.05$). The result suggested that the molecular change in the film matrix, plausibly via cross-linking of proteins, was more pronounced in film prepared at pH 3 after 20 days of storage. Nevertheless, protein solubility was decreased after 20 days of storage. Thus, the proteins in film matrix previously formed might undergo further interaction, leading to the decrease in proteins, which could be solubilized in water. The amino group of protein possibly

reacted with the carbonyl groups of aldehyde or ketone from lipid oxidation via Maillard reaction, leading to the covalent cross-linking of protein, during storage. The cross-linked proteins in the film matrix were insoluble (Orliac *et al.*, 2002). Artharn *et al*. (2009) reported that protein film from round scad muscle was more rigid as the storage time increased.

Table 12. Film solubility and protein solubility of films from washed mince (WM) and FPI of red tilapia prepared at pH 3 and 11 at day 0 and 20 of storage.

Storage time (Days)	Samples	pH levels	Film solubility (%)	Protein solubility (%)
0	WM	3	73.15 ± 30.2^{aA}	71.98 ± 2.22^{aA}
		11	22.08 ± 2.52^{cA}	21.69 ± 0.73^{cA}
	FPI	3	56.01 ± 2.51^{bA}	54.22 ± 3.60^{bA}
		11	21.43 ± 1.10^{cA}	20.62 ± 2.80^{cA}
20	WM	3	16.72 ± 2.23^{aB}	13.62 ± 3.03^{aB}
		11	18.63 ± 3.56^{aA}	11.62 ± 3.38^{bB}
	FPI	3	15.32 ± 1.17^{aB}	14.78 ± 1.08^{abB}
		11	18.90 ± 1.10^{aA}	16.90 ± 1.36^{cB}

*Mean $\pm$ SD (n=3).

Different lowercase superscripts in the same column under the same storage time indicate significant differences ($P < 0.05$). Different uppercase superscripts in the same column under the same material and pH levels used indicate significant differences ($P < 0.05$).

4.4.2.4 Protein pattern

Protein patterns of films from washed mince and FPI prepared at pH 3 and 11 at day 0 and 20 of storage determined under reducing and non-reducing conditions are presented in Figure 22. After film preparation (day 0), films from washed mince and FPI prepared at pH 3 and pH 11 showed the slight differences in protein pattern. Generally, both MHC and actin disappeared almost completely in films prepared at pH 11. On the other hand, MHC and actin were maintained to a

higher extent in film prepared at pH 3. However, band intensity of both proteins was much lower in all films, compared with that found in initial materials, both washed mince and FPI. This suggested that partial degradation of proteins under alkaline condition on FFS preparation possibly occurred to some degree, leading to the formation of those proteins. The disappearance of the protein band might be also due to cross-linking of protein via weak bonds including hydrogen bond and hydrophobic interaction. When protein patterns were determined under reducing condition, some protein bands were more regained for film prepared from FPI, especially those prepared at pH 3. This result suggested the role of disulphide bonds in film stabilisation. Furthermore, the protein band with molecular weight below MHC was found in films prepared from both washed mince and FPI at pH 3, possibly caused by protein degradation induced at acidic pH (Chinabhark *et al.*, 2007).

After 20 days of storage, some protein bands were decreased in intensity with concomitant increase in band intensity of polymerised protein appeared on the stracking gel. This was more pronounced in film prepared at pH 3. The result agreed well with the pronounced decrease in film and protein solubilities of film prepared at pH 3 stored for 20 days (Table 12). However, the polymerised proteins were decreased in band intensity under reducing condition, especially those prepared at pH 3. This result suggested that proteins were cross-linked by disulphide bond during 20 days of storage. Nevertheless, no changes in band intensity of film prepared at pH 11 were found in films prepared at pH 11 stored for 20 days when determined under reducing and non-reducing conditions. This suggested that the cross-linking of protein in film under alkaline condition, taking place during storage, was mainly merdiated by non-disulfide covalent bonds. The glycation more likely occurred at alkaline pH (Benjakul *et al.*, 2005), leading to polymerisation of protein molecules in the film matrix. Therefore, during storage of 20 days, the cross-linking of protein still proceeded via different bondings, depending upon the pH of FFS as well as material used.

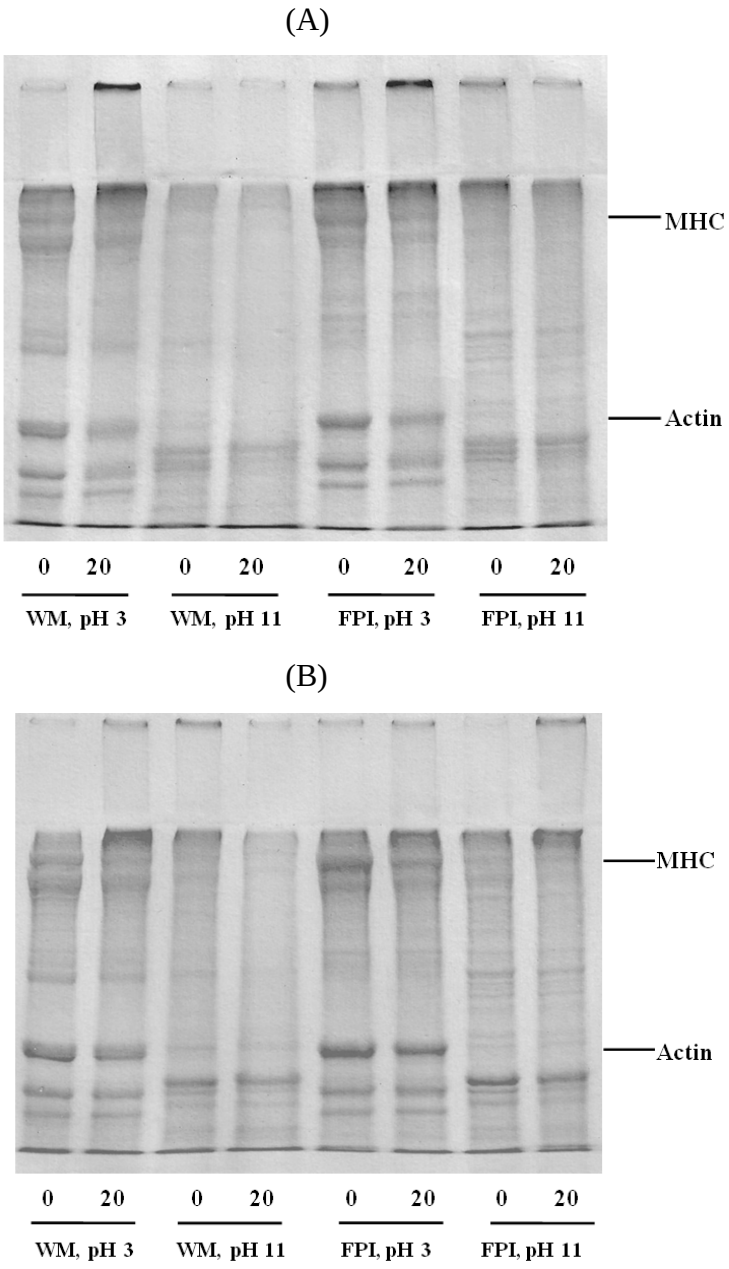


Figure 22. Protein patterns under non-reducing (A) and reducing (B) conditions of films from washed mince (WM) and FPI of red tilapia prepared at pH 3 and 11 at day 0 and 20 of storage.

4.4.2.5 TBARS

Lipid oxidation presented as TBARS value of films from washed mince and FPI prepared at pH 3 and 11 was monitored during storage at room temperature for 20 days (Figure 23). After casting and drying, TBARS were detected in films from washed mince and FPI. The higher TBARS value was found in films prepared at pH 3. This indicated that acidic condition favored the oxidation process of lipid retained in washed mince and FPI. Generally, TBARS values of films from washed mince were higher than those found in films from FPI, which contained the lower lipid content (Table 10). Thus, the combination of washing process, which could remove of heme protein, the potent prooxidant (Richards and Hultin, 2000), and alkaline solubilsation process, which could enhance the lipid removal effectively, was able to lower lipid oxidation of films. Despite of the removal of lipid and heme proteins by those two processes, the lipid oxidation still occurred, especially film prepared at pH 3. No differences in TBARS between films from washed mince and FPI prepared at pH 11 were obtained. Thus, lipid content and pH were found as the major factors affecting lipid oxidation of film from fish muscle. Also the oxidation took place during casting and drying. For both matarials, heme iron and non-heme iron were also found in both washed mince and FPI (Table 10), and might act as prooxidants. Washed mince added with hemoglobin showed the increased lipid oxidation when pH was decreased (Richards and Hultin, 2000; Undeland *et al.*, 2002). Hemoglobin and myoglobin are generally in the deoxy form at low pH, and this form exhibits the prooxidative activity (Maqsood and Benjakul, 2011). Low pH lowers oxygenation of hemoglobins and deoxy-form could be generated, thereby promoting lipid oxidation (Richards and Hultin, 2000). Acceleration of lipid oxidation by pH reduction could be due to enhanced autoxidation of hemoglobins at reduced pH (Tsuruga *et al.*, 1998). An increased prooxidative potential of hemoglobin at low pH has been demonstrated in other systems such as washed muscle system (Kristinsson and Hultin, 2004). On the other hand, the fish muscle system with hemoglobin at high pH did not develop any oxidation products, as measured by TBARS (Kristinsson and Hultin, 2004).

Substantial increase in TBARS for all films after complete drying (day 0), suggesting that lipid oxidation more likely occurred at the early stage of film

formation. Films, which were thin and possessed a large surface area, might be prone to oxidation owing to the increased exposure to oxygen (Tongnuanchan *et al.*, 2011). Thereafter, TBARS values of films were fluctuated up to 20 days of storage. The decrease in TBARS values was more likely due to the loss of volatile oxidation products during the extended storage. Moreover, the lipid oxidation products could interact with protein in the film. On the other hand, the increase in TBARS value was probably due to the higher rate of formation of secondary oxidation product, compared with that of losses. The oxidation of lipid might be associated with the change of film properties.

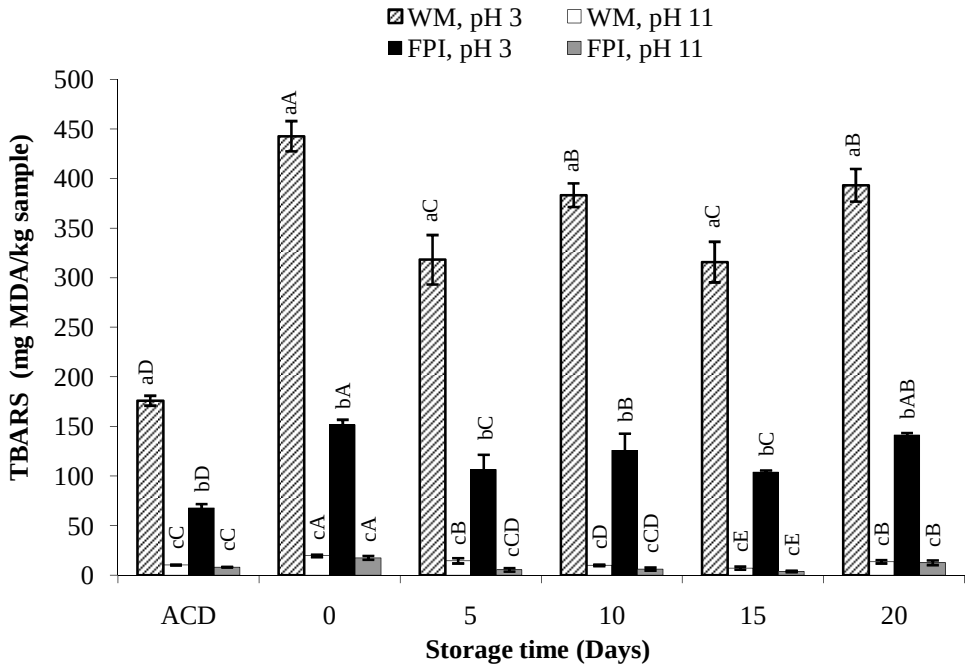


Figure 23. Changes in TBARS of films from washed mince (WM) and FPI of red tilapia prepared at pH 3 and 11 during the storage for 20 days. ACD: after casting and drying. Bars represent the standard deviation (n=3). The different lowercase letters on the bars within the same storage time indicate significant differences ($P < 0.05$). Different uppercase letters on the bars within the same material and pH level used indicate the significant differences ($P < 0.05$).

4.4.2.6 Color

The color expressed as b^*-value (yellowness) and ΔE^*-value (total color difference) of protein films from washed mince and FPI prepared at pH 3 and 11 during storage for 20 days is depicted in Figure 24. At day 0, films from FPI had lower b^*-value than other films ($P < 0.05$). Generally, film from washed mince prepared at pH 3 had the highest rate of increase in b^* and ΔE^*-values. For film from washed mince prepared at pH 11, the increase in b^*-value was found up to day 10 of storage ($P < 0.05$). Thereafter, no changes in b^*-value were found ($P > 0.05$). Film from FPI prepared at pH 3 also had the increased b^*-values within the first 10 days ($P < 0.05$). However, no changes in b^* and ΔE^*-values were obtained in film from FPI prepared at pH 11. This result reconfirmed the work of Tongnuanchan *et al*. (2011) who reported that film from unwashed and washed mince of red tilapia became yellow as evidenced by the increases in b^* and ΔE^*-values during storage. Artharn *et al*. (2009) reported that film from round scad muscle was more yellowish as indicated by the increase in b^*-values with increasing storage time. The increase in b^*-values was in agreement with lipid oxidation (Figure 23), which was dominant in films prepared at pH 3. Lipid oxidation more likely played a role in yellow discoloration of fish muscle film, mainly by providing the carbonyl groups involved in Maillard reaction (Tongnuanchan *et al.*, 2011). Washing process could remove some lipid as a main substrate of lipid oxidation and some prooxidant such as hemoglobin and myoglobin but some lipids, especially phospholipids, still remained in the washed mince and FPI. The membrane phospholipids are known to be the main substrate for oxidative reactions in fish muscle (Gandemer, 1999; Hultin, 1994). Their removal could lower the oxidation, therby retarding the formation of carbonyl compound for Maillard reaction.

Film prepared at pH 11 from FPI had the negligible change in b^* and ΔE^*-values during storage. Even though the alkaline pH favours Maillard reaction (Bates *et al.*, 1998), a smaller amount of lipid oxidation products, which acted as the precursor for Maillard reaction, were formed. Therefore, no changes in the yellowness were obtained in film from FPI prepared at alkaline pH.

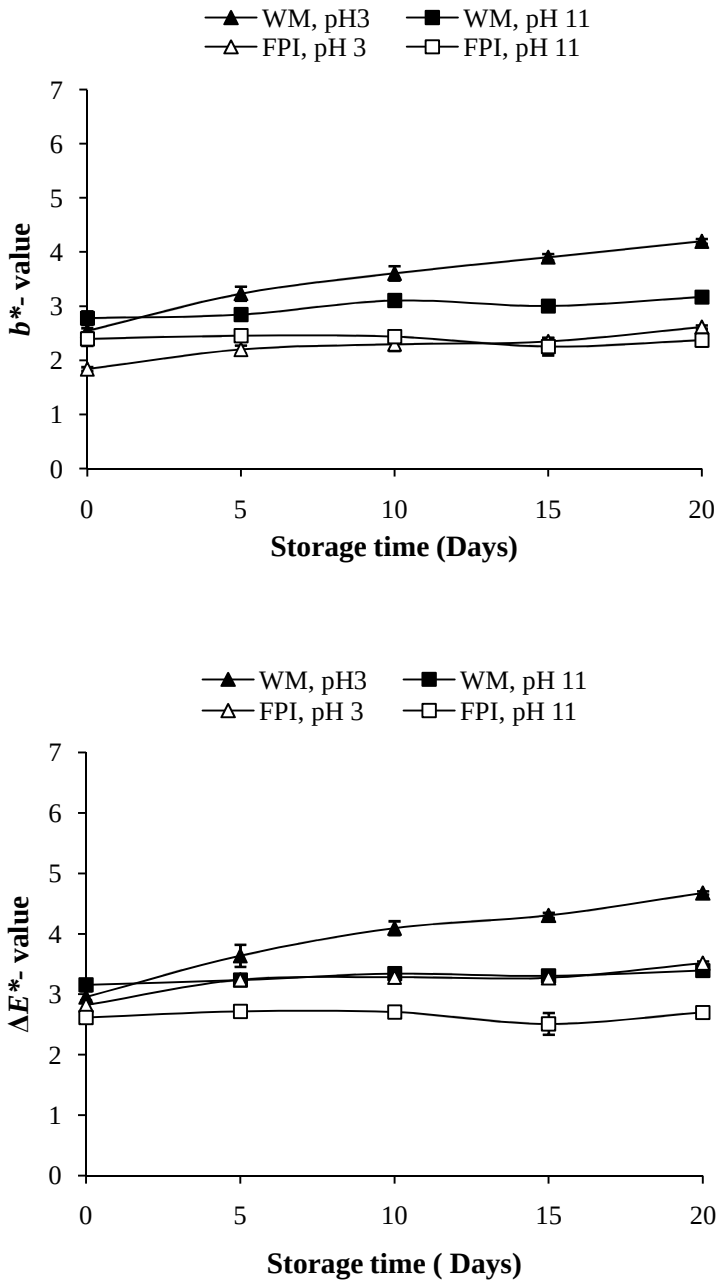


Figure 24. Changes in *b** (A) and Δ*E**-values (B) of films from washed mince (WM) and FPI of red tilapia prepared at pH 3 and 11 during the storage for 20 days. Bars represent the standard deviation (n=3).

4.4.2.7 Light transmittance and film transparency

The study on light transmission at selected wavelengths of 200 to 800 nm of films from washed mince and FPI prepared at pH 3 and 11 indicated that all films had the excellent barrier properties against UV light in the range of 200-280 nm (Table 13). Fish muscle protein film exhibited the good UV barrier properties (Benjakul *et al.*, 2008; Prodpran and Benjakul, 2005; Tongnuanchan *et al.*, 2011), owing to their high content of aromatic amino acids that absorb UV light (Hamaguchi *et al.*, 2007).

Films from washed mince and FPI prepared at pH 3 had lower transparency value than those film prepared at pH 11. At pH 3, film from washed mince had the higher transparency value than that from FPI (Figure 25), suggesting that the former was less transparent than the latter. This results were in agreement with Tongnuanchan *et al*. (2011) who reported that films prepared at acidic condition (pH 3) from both washed and unwashed mince of red tilapia were more transparent than those prepared at alkaline condition (pH 11) as indicated by the lower transparency value. However, there were no differences in transparency value between film from washed mince and FPI, when they were prepared at pH 11. No changes in transparency values were found in all samples during storage of 20 days.

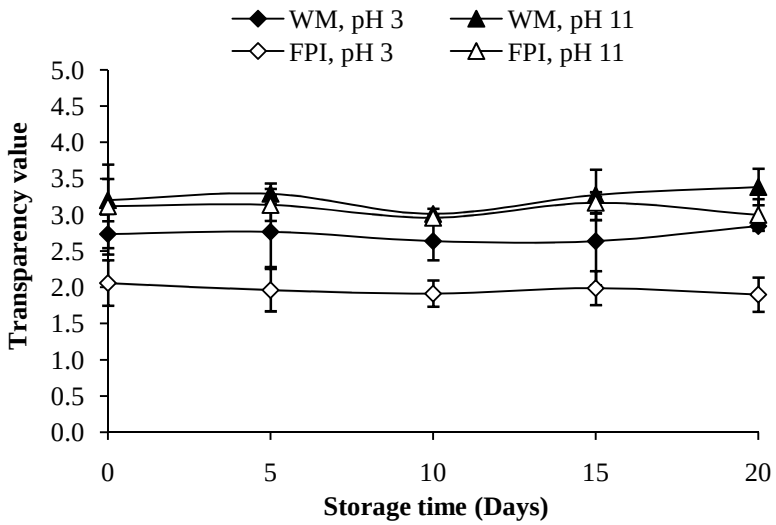


Figure 25. Changes in transparency value of films from washed mince (WM) and FPI of red tilapia prepared at pH 3 and 11 during the storage for 20 days. Bars represent the standard deviation (n=3).

Table 13. Light transmittance (%) of films from washed mince (WM) and FPI of red tilapia prepared at pH 3 and 11 at day 0 and 20 of storage.

Samples	pH	Storage time	Light transmittance (%) at different wavelength (nm)							
		(Days)	200	280	350	400	500	600	700	800
WM	3	0	0.00	1.06	72.51	78.60	82.97	84.66	85.63	86.33
		5	0.00	1.13	71.28	78.72	83.74	85.37	86.23	86.81
		10	0.00	1.44	70.52	78.49	83.72	85.32	86.12	86.65
		15	0.00	1.01	67.64	77.13	83.37	85.15	86.01	86.59
		20	0.01	0.78	66.11	76.04	82.67	84.58	85.53	86.17
	11	0	0.00	1.15	67.51	74.48	81.13	83.82	85.49	86.62
		5	0.00	0.92	65.62	73.65	80.48	83.31	85.15	86.48
		10	0.00	1.36	66.95	74.87	81.63	84.14	85.71	86.76
		15	0.00	2.07	67.12	74.68	81.30	83.86	85.48	86.59
		20	0.00	1.18	65.36	73.36	80.49	83.28	85.08	86.30
FPI	3	0	0.00	1.37	81.92	85.45	87.96	88.96	89.58	90.96
		5	0.01	1.30	80.49	85.15	88.24	89.31	89.91	90.27
		10	0.00	1.09	78.99	84.35	87.87	89.04	89.68	90.25
		15	0.00	1.23	78.54	84.15	87.82	89.02	89.67	90.09
		20	0.01	1.24	79.46	85.09	88.52	89.56	90.06	90.35
	11	0	0.01	1.00	67.59	73.69	80.20	83.13	85.01	86.30
		5	0.01	0.82	67.40	73.19	79.77	83.02	85.12	86.54
		10	0.00	1.04	66.96	74.42	81.12	83.82	85.52	86.67
		15	0.00	1.00	66.72	72.56	79.19	82.46	84.61	86.13
		20	0.00	0.80	68.47	74.39	80.42	82.84	85.28	86.58

4.4.2.8 Film morphology

SEM micrographs of the surface and cross-section of films from washed mince and FPI prepared at pH 3 and 11 are shown in Figure 26. Film from washed mince prepared at pH 3 had the smoother surface than that prepared at pH 11. Film from FPI prepared at pH 3 had the most smooth surface and cross-section, when compared with other film samples. This indicated that those films had the homogeneous structure on both surface and cross-section. This result suggested the alkaline solubilization process could remove unwanted components (sarcoplasmic protein, insoluble protein and lipid) and isolated the myofibrillar protein, rendering the more protein interactions with homogeneous structure. This resulted in the improved mechanical and physical properties of film at both pH (pH 3 and 11). Nevertheless, films from washed mince prepared at pH 3 and 11 and film from FPI prepared at pH 11 were rougher with some discontinuous zone.

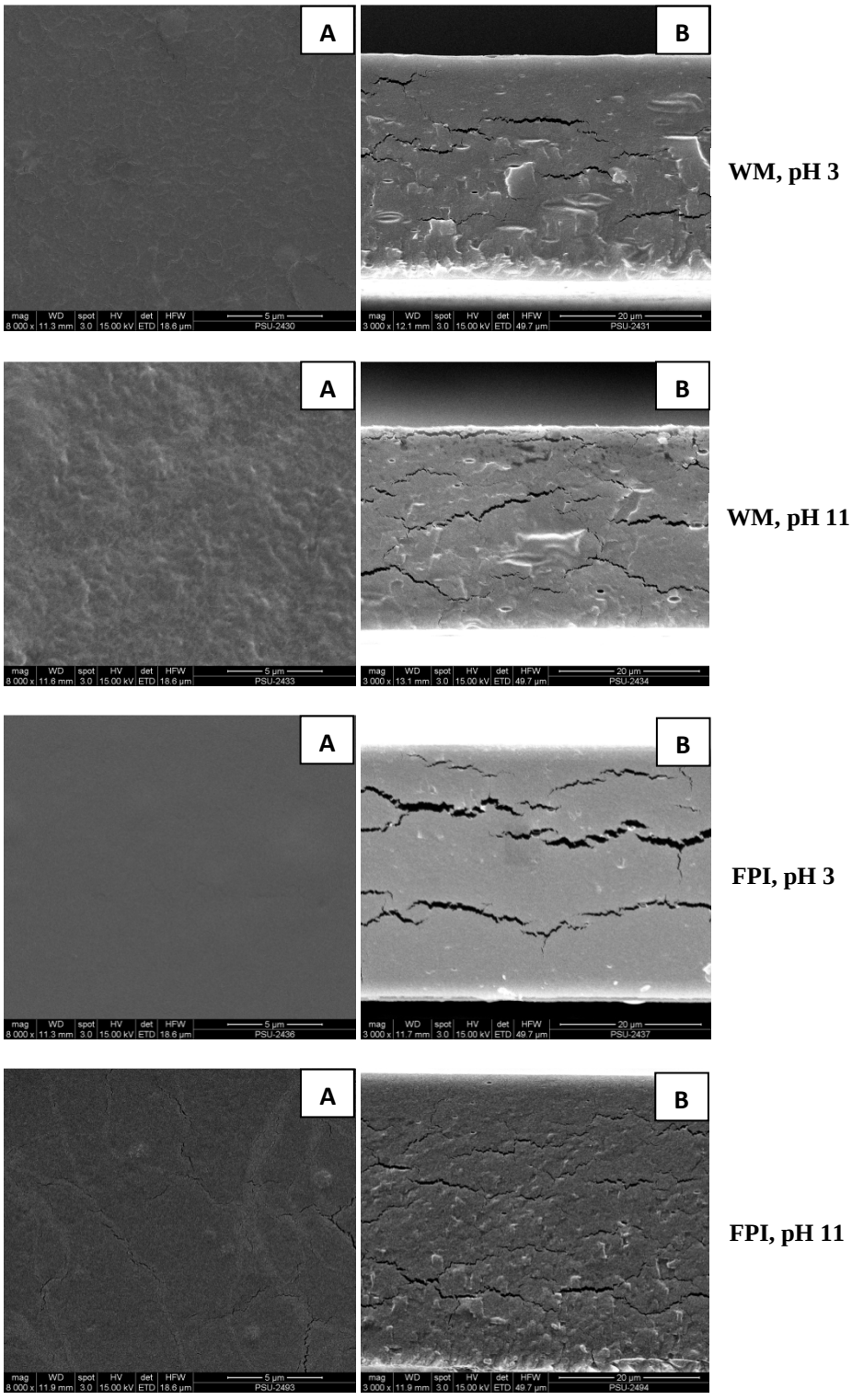


Figure 26. SEM micrographs of films from washed mince (WM) and FPI of red tilapia prepared at pH 3 and 11. Surface (A) and freeze-fractured cross-section (B).

4.5 Conclusion

The use of alkaline solubilization process after conventional washing process could successfully improve the properties of film. This process could lower heme protein and lipid contents from washed mince, leading to the improved mechanical and physical properties of film. Moreover, the lower content of lipid and prooxidants was found, thereby preventing the yellow discoloration caused by lipid oxidation products via Maillard reaction. Wider uses of this film can be achieved without discoloration problem.

CHAPTER 5

PROPERTIES AND STABILITY OF PROTEIN-BASED FILMS FROM RED TILAPIA PROTEIN ISOLATED INCORPORATED WITH ANTIOXIDANT DURING STORAGE

5.1 Abstract

Film from fish protein isolate (FPI) from red tilapia muscle prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT film) was prepared and characterized in comparison with film prepared from unwashed mince film (UWM film) during storage of 40 days. FPIT film had higher tensile strength (TS) and elongation at break (EAB) but lower water vapor permeability (WVP) than UWM film ($P < 0.05$). During the storage, FPIT film had much lower TBARS value than UWM film. Furthermore, FPIT was more transparent and had no yellow discoloration, as evidenced by no change in b^* and ΔE^*-values, during the storage of 40 days. Both UWM and FPIT films were stabilized mainly by hydrogen bond, followed by hydrophobic interaction, disulfide bond and non-disulfide covalent bond. FTIR spectra indicated that FPIT film contained the lower amount of lipids with the lower amplitude of amide B band, compared with UWM film. Higher degradation temperature (Td) was observed in FPIT film, indicating a greater protein-protein interaction in the film matrix. FPIT film had smoother surface and cross-section than UWM film. After 40 days of storage, both films had the increase in TS and Td but lower EAB, WVP and protein solubility. This was more pronounced in UWM film and was associated with the formation of non-disulfide covalent bond in the film network, most likely mediated by the interaction between protein and lipid oxidation products via Maillard reaction. Thus, film from FPI incorporated with antioxidant had the improved mechanical and physical properties without yellow discoloration.

5.2 Introduction

Development of biodegradable packaging materials is breakthrough alternative to synthetic material packaging from petrochemical products, which are non-biodegradable and have the negative impact on environment. Moreover, the biodegradable materials are eco-friendly, non-toxic and have been used to prepare biodegradable films and coating for food preservation and protection. The biodegradable film can be made from neutral biopolymers, including polysaccharides, proteins and lipids or the combination of these materials (Huber *et al*., 2009; Tharanathan, 2003). Among these biopolymers, proteins have been impressively used for the development of biodegradable films due to their relative abundance and film-forming ability. Proteins are heteropolymers containing a variety of amino acids, which can undergo a wide range of interactions and chemical reaction (Stevens, 1999). The protein-based films have excellent oxygen, carbon dioxide and volatile compounds barrier properties, in comparison with synthetic film under low relative humidity condition (Limpan *et al*., 2010). However, protein-based films had poor water vapor barrier properties because their hydrophilicity of amino acids in protein molecules (Gennadios *et al*., 1993; Krochta, 2002).

Fish proteins including myofibrillar and sarcoplasmic proteins have been used for film-forming materials (Artharn *et al.*, 2007; Benjakul *et al.*, 2008; Cuq *et al.*, 1996a; Prodpran *et al.*, 2007; Shiku *et al.*, 2004). The one of major limitations of fish muscle protein-based film are their yellow discoloration during the extended storage (Artharn *et al*., 2009; Tongnuanchan *et al*., 2011). Yellow discoloration of fish muscle protein-based film was mainly caused by non-enzymatic browning reaction or Maillard reaction (Artharn *et al*., 2009; Limpan *et al*., 2010; Tongnuanchan *et al*., 2011). Recently, Tongnuanchan *et al*. (2011) reported that lipid oxidation played an essential role in yellow discoloration of fish muscle protein film, by providing the carbonyl groups involved in Maillard reaction. To tackle the discoloration problem in fish protein-based film, the prevention of lipid oxidation by lowering lipids as well as prooxidants would be a potential approach. The pH-shift process, used for fish protein isolate preparation, might overcome this problem by removal of lipids in fish muscle (Kristinsson *et al.*, 2005; Kristinsson and Liang,

2006; Undeland *et al.*, 2002). Prewashing to eliminate sarcoplasmic proteins including heme proteins with subsequent pH-shift process can lower some prooxidants and simultaneously concentrate myofibrillar proteins, which mainly contribute to film formation. Additionally, the incorporation of appropriate antioxidant into the film could effectively retard the oxidation in film, especially during the extended storage. The objective of this study was to study the properties of protein-based film from red tilapia protein isolate incorporated with antioxidant during extended storage in comparison with film from unwashed mince.

5.3 Materials and method

5.3.1 Chemicals

Glycerol, urea, sodium chloride (NaCl), sodium dodecylsulfate (SDS), thiobarbituric acid, malondialdehyde bis (dimethyl acetal) and β-mercaptoethanol (βME) were purchased from Sigma (St. Louis, MO, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was obtained from Fluka (Buchs, Switzerland).

5.3.2 Fish sample

Fresh red tilapia (*Oreochromis niloticus*) (400–500 g/fish) were purchased from a local market in Hat Yai, Songkhla province, Thailand. Fish were kept in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 30 min. Upon the arrival, fish were immediately washed, filleted and minced to uniformity using a mincer with a hole diameter of 0.5 cm.

5.3.3 Preparation of fish protein isolate from mince

Prior to the isolation of fish protein, mince was subjected to washing as per the method of Toyohara *et al*. (1990) with slight modifications. Fish mince was homogenized with 5 volumes of cold 0.05 M NaCl (2–4 °C) at a speed of 13,000 rpm for 2 min, using an IKA Labortechnik homogenizer (Selangor, Malaysia). The washed mince was filtered through two layers of cheesecloth. The washing process was repeated twice. Unwashed mince and washed mince obtained were stored on ice until used.

To prepare fish protein isolate (FPI), alkaline solubilization process was used as described by Hultin and Kelleher (2000) with slight modifications. Washed mince was added with cold distilled water at the ratio of 1:9 (w/v), followed by homogenization for 1 min at the speed of 13,000 rpm. The pH of the homogenate was then adjusted to 11 using 2 M NaOH. The resulting mixture was centrifuged at 10,000xg for 20 min at 4 °C. The supernatant was collected and the pH was adjusted to 5.5 using 2 M HCl. The precipitate was then filtered through 4 layers of cheesecloth. The retentate was dewatered by centrifugation at 12,000xg for 20 min at 4 °C. The final pH of the sample was adjusted to pH 7.0 using 2 M NaOH. The sample was referred to as ‘fish protein isolate; FPI’. FPI was subjected to analyses and was used for film preparation.

5.3.4 Preparation of film from unwashed mince and FPI

The film-forming solutions (FFS) from unwashed mince and FPI (protein content 17.69 ± 0.58% and 14.63 ± 0.77% wet basis weight, respectively) were prepared according to the method of Chinabhark *et al*. (2007). The samples were added with 3 volumes of distilled water and homogenized at 13,000 rpm for 1 min. The protein concentration of the mixture as determined by Kjeldahl method (AOAC, 2000) was adjusted to 2% (w/v). Glycerol, used as a plasticizer, was added at 50% (w/w) of protein. The mixtures were stirred gently for 30 min at room temperature. The pH of mixture was adjusted to 3, using 1 M HCl, to solubilize the protein. FFS obtained was filtered through two layers of cheesecloth to remove undissolved debris.

For FFS from FPI, Trolox, used as a antioxidant, was incorporated at the level of 100 ppm. Then, both FFS were then stirred gently at room temperature for 10 min.

To prepare the films, FFS (4 g) was cast onto a rimmed silicone resin plate (50 × 50 mm^2) and air-blown for 12 h at room temperature prior to further drying at 25 °C and 50±5% RH for 24 h in an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttlingen, Germany). The resulting films were manually peeled off and used for analyses. Film from unwashed mince and those from FPI containing Trolox were referred to as 'UWM' and 'FPIT' films, respectively. Those films were stored at room temperature (28-30 °C) and subjected to analyses at the designated time.

5.3.5 Characterization of films during extended storage

Both UWM and FPIT films were stored at room temperature (28-32 °C). Samples were taken for determination of thiobarbituric acid reactive substrances (TBARS), color, light transmittance and transparency value every 5 days for totally 40 days. Mechanical properties, WVP and protein solubility of films were tested at day 0 and 40 of storage. FTIR, TGA and SEM analyses were also performed at day 0 and 40 of storage.

5.3.5.1 Film thickness

The thickness of film was measured using a micrometer (Gotech, Model GT-313-A, Gotech Testing Machines Inc, Tawai). Five random locations around each film of ten film samples were used for average thickness determination.

5.3.5.2 Mechanical properties

Prior to testing the mechanical properties, films were conditioned for 48 h at 25 °C and 50 ± 5% RH. 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, Hampshire, UK) equipped with tensile load cell of 100 N. Ten samples (2 × 5 cm^2) with initial grip length of 3 cm were used for testing. Cross-head speed was set at 30 mm/min.

5.3.5.3 Water vapor permeability (WVP)

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

$$WVP\ (\mathrm{gm^{-1}s^{-1}Pa^{-1}}) = wlA^{-1}t^{-1}(P_2 - P_1)^{-1}$$

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).

5.3.5.4 Film and protein solubilities

Film solubility was determined according to the method of Gennadios *et al*. (1998). The conditioned film samples ($2 \times 5\ cm^2$) were weighed and placed in a 50 ml centrifuge tube containing 10 ml of distilled water with 0.1% (w/v) sodium azide, used as antimicrobial agent. The mixture was shaken at a speed of 250 rpm using a shaker (Heidolth Inkubator 10000, Schwabach, Germany) at 30 °C for 24 h. Undissolved debris was removed by centrifugation at 3000xg 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 a percentage of the 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.

5.3.5.5 Color

Color of the film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., VA, USA). D_{65} (day light) and a measure cell with opening of 30 mm were used. The color of the films was expressed as b^*-value (yellowness) and total difference of color (ΔE^*) was calculated as follows (Gennadios *et al.*, 1996):

$$\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

where ΔL^*, Δa^* and Δb^* are the differentials between the color parameter of the samples and the color parameter of the white standard (L^* = 93.27, a^* = - 0.79, b^* = 0.28).

5.3.5.6 Light transmittance and transparency value

The light transmittance of films was measured at the ultraviolet and visible range (200–800 nm) using UV–vis spectrophotometer (Shimadzu UV-1800, Tokyo, Japan) as described by Shiku *et al*. (2004). The transparency value of film was calculated using 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 transparency value represents the lower transparency of film.

5.3.5.7 Thiobarbituric acid reactive substrances (TBARS)

TBARS value of film was determined according to the method of Buege and Aust (1978). Film samples (0.05 g) were mixed with 2.5 ml of TBA solution (0.375 g of thiobarbituric acid, 15 g of trichloroacetic acid, and 0.875 ml of HCl in 100 ml of distilled water). The mixtures were heated for 10 min in a boiling water bath (95–100 °C) to develop pink color, cooled with tap water and centrifuged at 7500xg for 10 min. Absorbance of the supernatant was measured at 532 nm. A

standard curve was prepared using malonaldehyde bis (dimethyl acetal) (MDA) at concentrations ranging from 0 to 10 mM. TBARS value in each sample was expressed as mg MDA/kg dried sample.

5.3.5.8 Protein solubility in various soution

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 (0.2 g) were homogenized in various solvents at a speed of 13,000 rpm for 1 min using a homogenizer. The homogenate was heated in boiling water (100ºC) for 2 min and stirred at room temperature for 4 h. The resulting homogenate was centrifuged at 7500xg for 30 min using a centrifuge (Allegra 25R, Beckman, California, USA). The supernatant (10 ml) was added with 2.5 ml of cold 50% (w/v) trichloroacetic acid to precipitate the proteins. The mixture was kept at 4 ºC for 18 h and centrifuged at 7500xg for 30 min. The precipitate was washed with 10% TCA and solubilized in 10 ml of 0.5M NaOH. The protein content was determined using the Biuret test (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.

5.3.5.9 Protein pattern

Protein patterns of films were determined by SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). 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 homogenised at 13,000 rpm for 1 min. The homogenate was stirred continuously for 24 h at room temperature (28–30 °C). Then, the sample was centrifuged at 7500xg for 10 min at room temperature. The supernatant was subjected to SDS-PAGE as described previously.

5.3.5.10 Attenuated total reflectance-Fourier transforms infrared (ATR-FTIR) spectroscopy

Prior to analysis, films were conditioned in a desiccator containing dried silica gel for 3 weeks at room temperature to obtain the most dehydrated films (Sobral *et al*., 2001). Films were scaned with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45 º ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at room temperature as described by Nuthong *et al*. (2009b). Films were placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra in the range of 700-4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 ºC.

5.3.5.11 Thermo-gravimetric analysis (TGA)

Prior to testing, films were conditioned in a desiccator containing dried silica gel for 3 weeks at room temperature. Conditioned films were scanned using a thermo-gravimetric analyzer (TGA7, PerkinElmer, Norwalk, CT, USA) from 50 to 600 ºC at a rate of 10 ºC/min (Nuthong *et al.*, 2009b). Nitrogen was used as the purge gas at a flow rate of 20 ml/min.

5.3.5.12 Scanning electron microscopy (SEM)

Morphology of surface and cross-section of the film samples were visualized using a scanning electron microscope (SEM) (Quanta 400, FEI, Eindhoven, Netherlands). For cross-section, samples were fractured under liquid nitrogen prior to morphology visualization. Then, the samples were mounted on bronze stub and sputtered with gold (Sputter coater SPI-Module, PA, USA) in order to make the sample conductive. The photographs were taken at an acceleration voltage of 15 kV.

5.3.6 Use of red tilapia protein based-films to extend the shelf life of dried fish powder

5.3.6.1 Preparation of longtail tuna powder

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

5.3.6.2 Shelf life study of dried fish powder covered with round scad protein-based films

Fish powder (15 g) was transferred to a cylindrical glass cup with a diameter of 25 mm. The cup containing fish powder was covered with protein-based films from red tilapia protein isolated incorporated with antioxidant, PP (polypropylene) film and PE (polyethylene) film with a thickness of 0.035 ± 0.003 mm and subsequently sealed with an O-ring. The samples were stored at 28–30 °C. Sample without film covering was used as the control. Samples were taken every 5 days for 30 days for analyses of moisture content (AOAC, 2000), color, TBARS and peroxide value.

5.3.6.3 Peroxide value

Peroxide value was determined as per the method of Richards and Hultin (2002) with a slight modification. Fish powder (1 g) was homogenised at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1, v/v). Homogenate was then filtered using Whatman No. 1 filter paper. Two millilitre of 0.5% NaCl was then added to 7 ml of the filtrate. The mixture was vortexed at a moderate speed for 30 s and then centrifuged at 3000*g* for 3 min to separate the sample into two phases. Two millilitre of cold chloroform/methanol (2:1) were added to 3 ml of the lower phase. Twenty-five microlitre of ammonium thiocyanate and 25 µl of iron (II) chloride were added to the mixture (Shantha and Decker, 1994). Reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. A standard curve was prepared using cumene hydroperoxide at a concentration range of 0.5–2 ppm.

5.3.6.4 TBARS

Thiobarbituric acid-reactive substances (TBARS) were determined as described by Buege and Aust (1978). Fish powder (0.2 g) was mixed with 2.5 ml of a TBA solution containing 0.375% thiobarbituric acid, 15% trichloroacetic acid and 0.25 N HCl. The mixture was heated in a boiling water bath (95–100 °C) for 10 min to develop a pink color, cooled with running tap water and then sonicated for 30 min, followed by centrifugation at 5000*g* at 25 °C for 10 min. The absorbance of the supernatant was measured at 532 nm. A standard curve was prepared using 1,1,3,3-tetramethoxypropane (MDA) at the concentration ranging from 0 to 10 ppm and TBARS were expressed as mg of MDA equivalents/kg of sample.

5.3.7 Statistical analysis

All experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test. For pair comparison, T-test was used Steel and Torrie (1980). Analysis was performed using the SPSS package (SPSS for windows, SPSS Inc., Chicago, IL, USA).

5.4 Results and discussion

5.4.1 Properties and stability of protein-based films from red tilapia during storage

5.4.1.1 Thickness and mechanical properties

Thickness and mechanical properties of film from FPI of red tilapia muscle prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT film) and film from unwashed mince (UWM film) prepared at pH 3 at day 0 and 40 of storage are shown in Table 14. UWM film and FPIT film had similar thickness ($P > 0.05$). FPIT film exhibited the higher TS than UWM film ($P < 0.05$). This result suggested FPI might have the higher content of film forming material, especially myofibrillar proteins, and lower content of undesirable materials such as fat than unwashed mince.

Thus, washing followed by alkaline solubilization, a process used for FPI preparation, effectively improved TS of film from fish mince. The washing process removes sarcoplasmic proteins, such as myoglobin, hemoglobin, enzyme and low-molecular weight proteins and some phospholipids (Morioka *et al*., 1997; Park *et al*., 1997). Artharn *et al*. (2007) and Tongnuanchan *et al*. (2011) reported that washing process could improve the mechanical properties of fish mince-based film, regardless of muscle types and pH used. Alkaline solubilization process was able to separate undesirable constituents such as connective tissues, cellular membrane, neutral storage lipid and membrane lipid (Hultin and Kelleher, 2000; Kristinsson and Liang, 2006). The reduction of lipid was one of factors contributing to the improved TS of FPI-based film. Lipid might interfere protein-protein interaction for film network formation.

For EAB, FPIT film had much higher EAB than UWM film ($P < 0.05$). High EAB of film reflected the extensibility/elasticity of film. The unfolding and stretching of proteins more likely took place during alkaline solubilization process. Those proteins could be more extended and the reactive groups were more exposed. As a consequence, higher interaction of proteins in film matrix occurred, particularly in the way which the longer chains were possibly formed. This led to the enhanced extensibility of film, as evidenced by the increased EAB.

After 40 days of storage, both FPIT and UWM films generally had the increases in TS with the concomitant decreases in EAB ($P < 0.05$). This result was in accordance with Tongnuanchan *et al*. (2011) who reported that protein-based film from unwashed and washed mince of red tilapia muscle prepared at pH 3 had the increases in TS after storage ($P < 0.05$). The increase in rigidity of film might be probably due to the enhanced cross-linking between proteins via non-enzymatic browning reaction. The cross-linking of low and high molecular weight protein could be enhanced via the Maillard reaction (Lertittikul *et al*., 2007). Additionally, the interaction between protein molecules took place to a higher extent with increasing storage time.

Table 14. Properties of films from unwashed mince prepared at pH 3 (UWM) and films from FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) at day 0 and 40 of storage.

Storage time (Days)	Samples	TS (MPa)	EAB (%)	WVP (x 10^{-11} $gm^{-1}s^{-1}Pa^{-1}$)	Thickness (mm)
0	UWM	2.85 ± 0.50^{bB}	63.74 ± 9.57^{bA}	5.14 ± 0.27^{aA}	$0.030 \pm 0.005^{aA*}$
	FPIT	8.40 ± 0.65^{aB}	123.95 ± 7.38^{aA}	4.17 ± 0.15^{bA}	0.029 ± 0.002^{aA}
40	UWM	3.58 ± 0.42^{bA}	44.24 ± 6.38^{bB}	4.73 ± 0.18^{aB}	0.031 ± 0.003^{aA}
	FPIT	10.37 ± 1.37^{aA}	84.60 ± 8.51^{aB}	3.98 ± 0.11^{bA}	0.030 ± 0.004^{aA}

*Mean $\pm$ SD (n=3).

Different lowercase superscripts in the same column under the same storage time indicate significant differences ($P < 0.05$). Different uppercase superscripts in the same column under the same material and pH levels used indicate significant differences ($P < 0.05$).

5.4.1.2 Water vapor permeability (WVP)

WVP of film from FPIT and UWM films at day 0 and 40 of storage is shown in Table 14. Fish protein-based film is known to have poor water vapor barrier properties, due to its hydrophilic nature (Hamaguchi *et al*., 2007). At beginning of storage (0 day), WVP of UWM film was higher than FPIT film ($P < 0.05$). It was suggested that polar or hydrophillic amino acids and proteins, including the sarcoplasmic proteins, were leached out by washing process (Artharn *et al*., 2007; Tongnuanchan *et al*., 2011). In general, the high content of polar amino acids was found in myofibrillar protein of blue marlin (Shiku *et al*., 2003) and Nile tilapia (Paschoalick *et al*., 2003). Furthermore, alkaline solubilization process could remove the undesirable components and concentrated the film forming materials. As a result, the strong interaction in film matrix as evidenced by the highest TS might be able to prevent the migration of water vapor more effectively (Table 14). The higher interaction of protein molecules in film matrix decreased the free volume and mobility of polymeric structure, thereby lowering the diffusion of water as indicated by the

lower WVP. The higher aggregation of myofibrillar proteins rendered the denser and stronger film matrix, which could retard the migration of water (Artharn *et al*., 2008).

Generally, the decrease in WVP of both films was observed as storage time increased ($P < 0.05$). This result suggested that the film matrix became denser and more rigid due to the arrangement and cross-linking between protein molecules during storage. Such a film matrix might retard diffusion of water from surrounding atmosphere through the films.

5.4.1.3 Moisture content, film solubility and protein solubility

Moisture content, film solubility and protein solubility of film from FPIT film and UWM film at day 0 and 40 of storage are shown in Table 15. At day 0, UWM film had the higher moisture content than FPIT based film ($P < 0.05$). For film and protein solubility, FPIT film had the lower values than UWM film ($P < 0.05$). The removal of the water soluble components such as sarcoplasmic proteins resulted in the less adsorption of water of film. The stronger and denser film network with the high compactness might lower film and protein solubility of FPIT film. Fish protein-based films prepared at acidic condition had much higher in both of film and protein solubility than those prepared with alkaline condition ($P > 0.05$) (Tongnuanchan *et al*., 2011). Alkaline condition favored the formation of protein cross-linking via disulfide bonds in fish protein based film (Shiku *et al*., 2003). After 40 days of storage, film and protein solubility of all film samples decreased markedly ($P < 0.05$). This observation suggested that protein molecules in film might undergo higher cross-linking, in which larger molecular weight cross-links were formed. Aldehydes or carbonyl compounds produced from lipid oxidation can interact with protein amino groups via Maillard reactions (Chaijan *et al.,* 2007). Secondary products from lipid oxidation, especially aldehydes, can induce myofibrillar protein cross-linking, resulting in structural and functional changes in these proteins (Li and King, 1999; Tironi *et al*., 2002). The cross-linked proteins in film polymer were insoluble, resulting in the decreased film and protein solubility during storage. Lower solubility of films was coincidental with the increased TS and decreased of EAB with increasing storage time (Table 14). Artharn *et al.* (2009) also found the decrease in film and protein solubility of round scad protein-based film as the storage time increased.

Table 15. Moisture content, film solubility and protein solubility of films from unwashed mince prepared at pH 3 (UWM) and films from FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) at day 0 and 40 of storage.

Storage time (Days)	Samples	Moisture Content (%)	Film solubility (%)	Protein solubility (%)
0	UWM	37.16 ± 2.25^{aA}	89.29 ± 5.31^{aA}	87.35 ± 1.86^{aA}
	FPIT	31.97 ± 2.14^{bA}	75.00 ± 3.84^{bA}	74.45 ± 1.83^{bA}
40	UWM	34.83 ± 0.43^{aA}	29.34 ± 1.96^{aB}	25.37 ± 2.90^{aB}
	FPIT	29.23 ± 0.99^{bA}	16.80 ± 2.67^{bB}	12.69 ± 5.04^{bB}

*Mean $\pm$ SD (n=3).

Different lowercase superscripts in the same column under the same storage time indicate significant differences ($P < 0.05$). Different uppercase superscripts in the same column under the same material and pH levels used indicate significant differences ($P < 0.05$).

5.4.1.4 Color

The color expressed as b^*-value (yellowness) and ΔE^*-value (total color difference) of FPIT and UWM films during storage of 40 days is shown in Figure 27. FPIT film showed the lower b^* and ΔE^*-values than UWM film ($P < 0.05$). This result indicated the lower yellowness in FPIT film in comparison with UWM film. Washing could remove pigments such as hemoglobin or myoglobin, leading to less amount of coloring compounds in the washed mince. Additionally, during casting and drying, UWM film might undergo the high level of lipid oxidation, which produced the carbonyl compound (aldehyde and ketone). Those compounds could react with amino groups of proteins, which were the main components of film via non-enzymatic browning reaction. This resulted in higher b^*-value (yellowness) as well as ΔE^*-value, compared with FPIT film.

During storage of 40 days, UWM film showed the continuous increase in b^* and ΔE^*-values ($P < 0.05$), while FPIT film had no changes in both of b^* and ΔE^*-values ($P > 0.05$). Both b^* and ΔE^*-values of UWM film increased at a higher rate within the first 10 days of storage. FPIT film incorporated with the antioxidant could prevent the yellow discoloration effectively (Figure 28). The removal of lipids from fish muscle using alkaline solubilization process also contributed to the retarded lipid oxidation, which was associated with the yellow discoloration of film. In general, the yellow discoloration was observed in fish muscle protein-based film (Artharn *et al.*, 2009; Cuq *et al.*, 1996a). Thus, the use of FPI in conjunction with antioxidant was a potential means to prevent yellow discoloration of film during the extended storage.

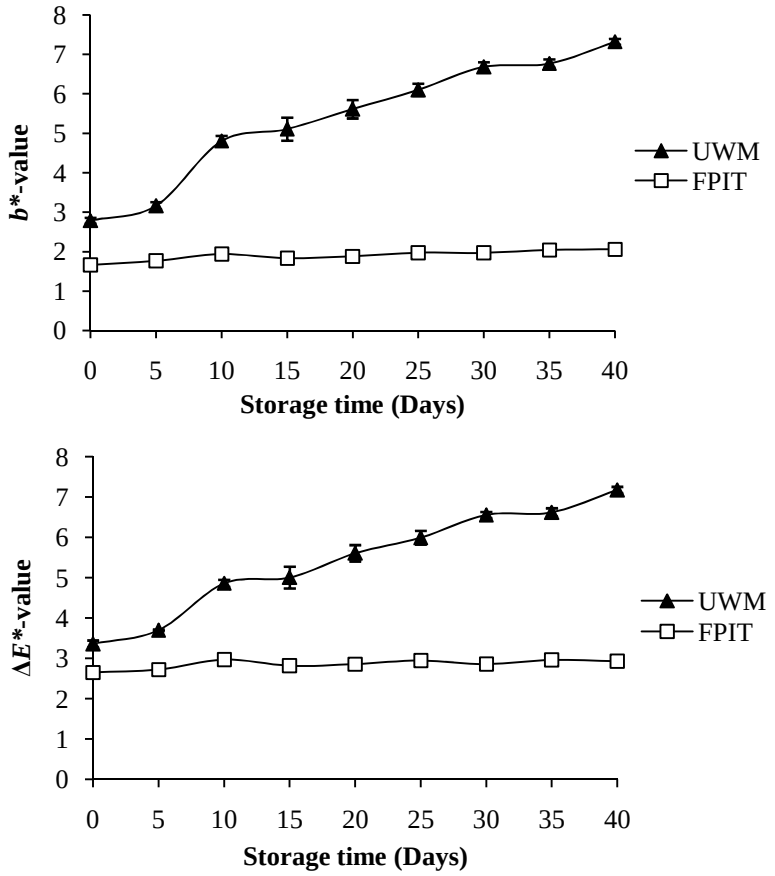


Figure 27. Changes in b^* and ΔE^*-values of films from unwashed mince prepared at pH 3 (UWM) and films from FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) during the storage of 40 days. Bars represent the standard deviation (n=3).

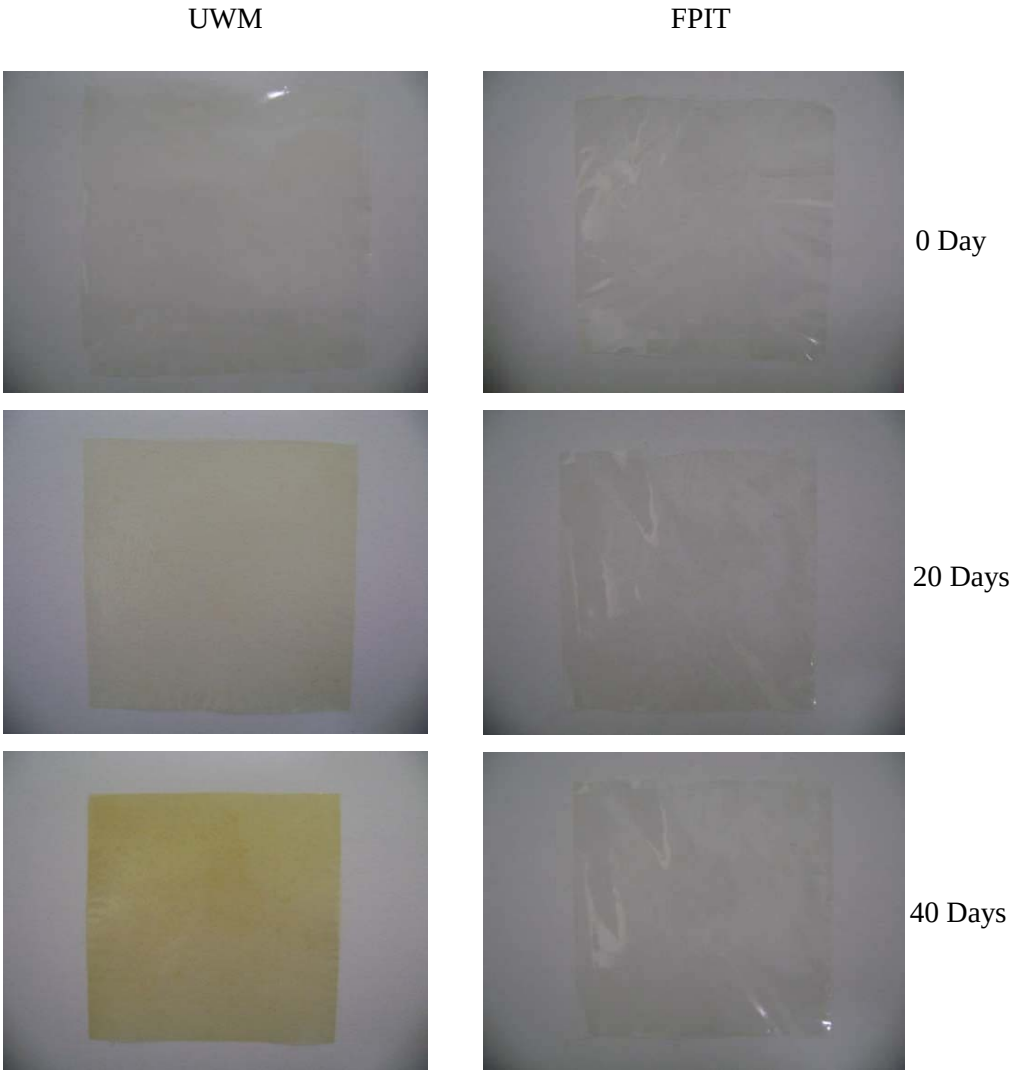


Figure 28. Photograph of films from unwashed mince prepared at pH 3 (UWM) and films from FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) during the storage of 40 days.

5.4.1.5 Light transmittance and film transparency

Table 16 presents the light transmission, at selected wavelengths of 200 - 800 nm, of FPIT and UWM films during storage of 40 days. Both films had the excellent barrier properties against UV light in the range of 200-280 nm. Protein-based films had good UV barrier properties, owing to their high content of aromatic amino acids that absorb UV light (Hamaguchi *et al*., 2007). Light transmission of visible range (350-800 nm) of UWM film ranged from 69.83% to 83.34%, whereas

higher values were found for FPIT film (80.34-88.87%). FPIT film was clearer and more transparent when compared with UWM film. Thus, light transmission of FPIT film was considerably increased by the removal of unwanted components during washing and alkaline solubilization process. Light transmission of film was most likely governed by the arrangement or alignment of polymer in film network (Limpan *et al*., 2010). With increasing storage time, the decrease in light transmission was found, particularly in the wavelength of 280-400 nm. The higher cross-linking with increasing storage time might result in denser network, in which light could not transmit through the film easily.

The transparency value of all film samples during storage is shown in Figure 29. The transparency value of FPIT film was lower than that of UWM film ($P < 0.05$). The lower transparency value indicated that the film was more transparent. UWM film was generally more turbid than FPIT film. The lower amount of undesirable components such as sarcoplasmic proteins, pigments, insoluble proteins and lipids, etc of FPI used for film preparation might contribute to the higher transparency of films. Prodpran *et al.* (2007) reported that round scad protein-based film decreased in opacity when incorporated with palm oil. The differences in opacity of film was determined by the optical properties of lipid incorporated (Yang and Paulson, 2000). Thus, FPI with lower lipid more likely rendered the clearer film.

During storage of 40 days, no marked change in transparency value was observed in both films. However, Artharn *et al*. (2009) reported a slight increase in transparency value of round scad protein-based film during storage at 28-30 ºC for 8 weeks.

Table 16. Light transmittance (%) of films from unwashed mince prepared at pH 3 (UWM) and films from FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) during 40 days of storage.

Samples	Storage time	Light transmission (%) at different wavelength (nm)							
	(Days)	200	280	350	400	500	600	700	800
UWM	0	0.01	0.66	69.83	74.54	79.48	81.35	82.51	83.34
	5	0.01	0.66	69.83	74.54	79.48	81.35	82.51	83.34
	10	0.01	0.54	65.77	72.65	78.78	81.08	82.46	83.45
	15	0.01	0.39	64.04	72.79	79.81	81.92	83.07	83.88
	20	0.00	0.11	60.41	70.60	79.04	81.43	82.67	83.53
	25	0.01	0.22	59.24	69.49	78.15	80.81	82.27	83.32
	30	0.00	0.44	62.94	71.33	78.66	81.08	82.44	83.42
	35	0.00	0.29	61.74	71.31	79.22	81.48	82.68	83.53
	40	0.01	0.29	58.34	69.28	78.55	81.17	82.54	83.51
FPIT	0	0.01	0.58	80.34	83.04	86.03	87.35	88.25	88.87
	5	0.01	0.53	80.53	83.32	86.21	87.43	88.23	88.85
	10	0.01	0.48	79.82	82.80	85.79	87.02	87.81	88.32
	15	0.01	0.46	79.31	82.48	85.56	86.87	87.72	88.34
	20	0.00	0.45	79.77	83.05	86.09	87.30	88.06	88.55
	25	0.01	0.46	78.18	81.87	85.29	86.75	87.68	88.36
	30	0.01	0.42	77.23	81.07	84.71	86.25	87.24	87.97
	35	0.01	0.36	77.93	81.84	85.45	86.90	87.80	88.36
	40	0.01	0.37	77.32	81.46	85.28	86.81	87.78	88.44

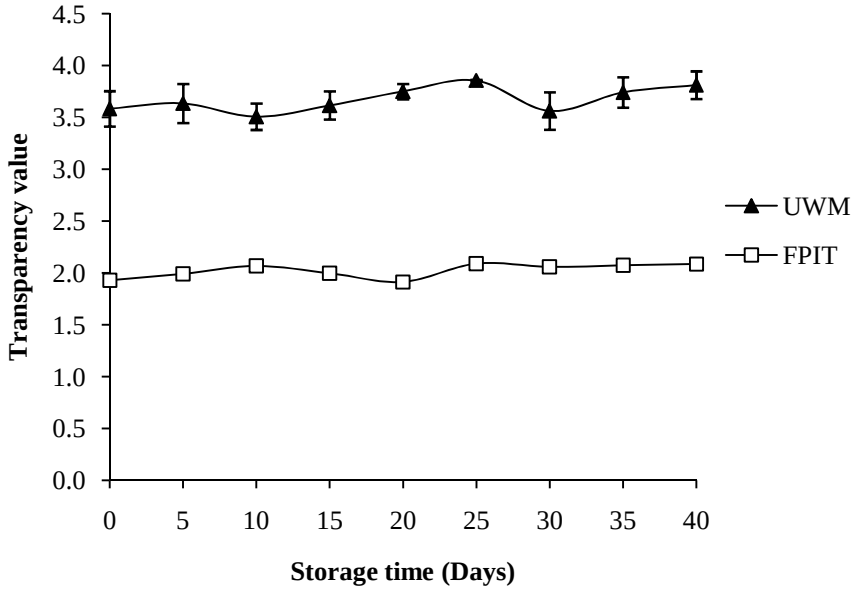


Figure 29. Changes in transparency value of films from unwashed mince prepared at pH 3 (UWM) and films from FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) during the storage of 40 days. Bars represent the standard deviation (n=3).

5.4.1.6 TBARS

Lipid oxidation of film from FPIT and UWM films during storage of 40 days was measured by monitoring TBARS value (Figure 30). The increase in TBARS value in UWM film was found within the first 5-10 days of storage ($P < 0.05$). Thereafter, the decrease in TBARS was noticeable at day 15, followed by the constant value up to the end of storage (day 40). The decrease in TBARS might be caused by a loss of low molecular weight decomposition products during the advancement of oxidation (Nawar, 1996). For FPIT film, slight increase in TBARS was found after 20 days of storage and the value remained constant until the end of storage. FPIT film had the much lower TBARS value than UWM film ($P < 0.05$). The increase in TBARS of UWM film during storage was related with the increase in yellowness (*b**-value) (Figure 27). It was noted that the oxidation occurred to some degree in films during storage. This result suggested that the washing in combination with alkaline solubilization processes more likely decreased the lipids and prooxidant

in resulting film. Apart from myoglobin, hemoglobin in fish muscle was able to be an effective catalyst of lipid oxidation (Apte and Morrissey, 1987). Heme dissociation, heme destruction and iron release play a role in the acceleration of lipid oxidation (Grunwald and Richards, 2006). The membrane phospholipids are known to be the main substrate for oxidative reactions in fish muscle (Gandemer, 1999; Hultin, 1994). Their removal was expected to greatly enhance the stability of film during storage. Even though the large amount of lipids could be removed, the lipid oxidation of FPIT film containing Trolox, an antioxidant, still took place to a small extent. This was probably because some membrane lipids were still retained in FPI. Some portion of storage lipids could be co-precipitated with the proteins during the isoelectric precipitation step of pH-shift process (Kristinsson *et al.*, 2005). Thus, the incorporated antioxidant, Trolox at 100 ppm, could retard the lipid oxidation of FPIT film during the storage to some extent. Under acidic condition, prooxidative potential of heme proteins, which were denatured at low pH, increased (Kristinsson and Liang, 2006). The increased prooxidant by acid condition enhanced the lipid oxidation (Hultin and Kelleher, 2000; Undeland *et al.*, 2002). Thus, the removal of heme proteins (hemoglobin and myoglobin) and lipids from fish muscle by washing in combination with alkaline solubilization process could prevent the lipid oxidation of fish muscle protein-based film by lowering lipid precursor as well as prooxidants. Additionally, the incorporation of Trolox was an effective means to prevent oxidation of film.

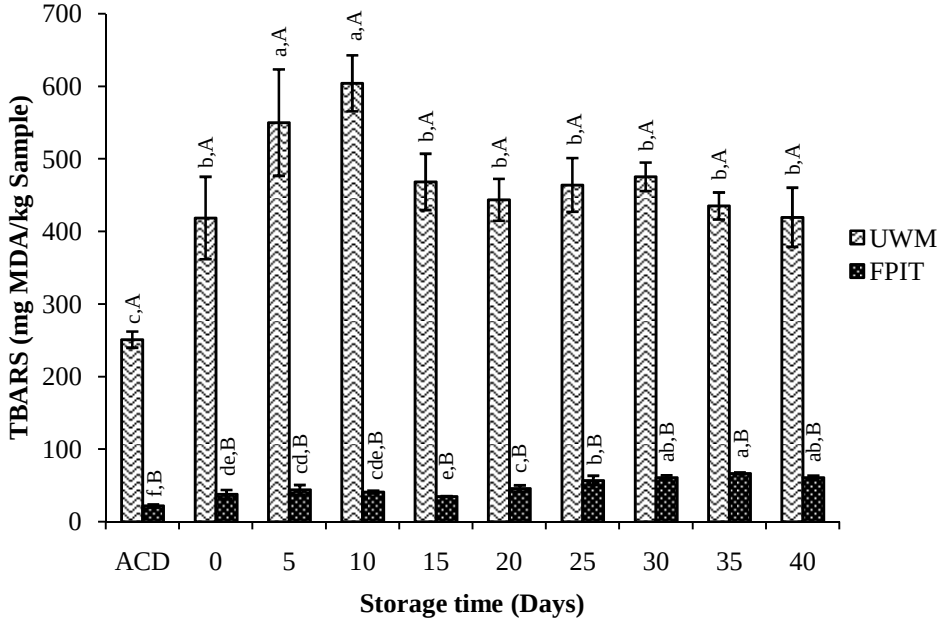


Figure 30. Changes in TBARS of films from unwashed mince prepared at pH 3 (UWM) and films from FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) during the storage of 40 days. ACD: after casting and drying. Bars represent the standard deviation (n=3). Different lowercase letters on the bars within the same material indicate the significant differences ($P < 0.05$). Different uppercase letters on the bars within the same storage time indicate significant differences ($P < 0.05$).

5.4.1.7 Protein solubility in various solvents

Protein solubility of FPIT and UWM films at day 0 and 40 of storage is shown in Table 17. Protein solubility of FPIT film was lower than that of UWM film for all solvents used. When S1 (20 mM Tris, pH 8 containing 1% (w/v) SDS) was used, protein solubility of FPIT film was lower than that of UWM film ($P < 0.05$). S1 containing SDS might increase repulsive force between protein molecules via charge modification, leading to enhanced the dissolvation of film matrix. SDS has been known to destroy hydrogen bond (Prodpran *et al.*, 2007). The result suggested that the matrix of both films was mainly stabilized by hydrogen bonds, as evidenced by the high protein solubility in S1. When S2 (20 mM Tris, pH 8 containing 1% (w/v) SDS

and 8 M urea) was used, a slight increase in protein solubility was observed in both films. Urea has been known to disrupt the hydrophobic interaction (Prodpran *et al*., 2007; Shiku et al., 2004). The increase in solubility caused by urea suggested that hydrophobic interaction was involved in the matrix of both films. S3 contained 2% β-mercapthoethanol, which was able to destroy disulfide bond, along with SDS and urea. The slightly increased protein solubility was observed in both films, compared with those of S1 and S2. This result indicated the presence of disulfide bond in film matrix. However, Shiku *et al*. (2004) reported that the addition of β-mercaptoethanol did not increase the protein solubility of Alaska pollack surimi films, indicating that disulfide bonds were not involved in the formation of surimi films. It has been known that MHC molecules contain about 40 sulfhydryl groups (Roussel and Cheftel, 1990). Therefore, inter-molecular disulfide bonds could be formed during casting and drying of the FFS in both films. Iwata *et al.* (2000) also reported that disulfide bond formation plays an important role in the development of the fish protein film. It was noted that the lower protein solubility was found in FPIT film than UWM film ($P < 0.05$) for all solvents used. It was noted that both film samples were not completely solubilized even when S3 was used. This result indicated the presence of non-disulfide covalent bonds, which stabilized the film matrix. A similar result was observed in Alaska pollack surimi film (Shiku *et al*., 2004), porcine-plasma protein film containing different cross-linking agents (Nuthong *et al.*, 2009b) and fish skin gelatin film incorporated with seaweed extract (Rattaya *et al*., 2009). The lower solubility in S3 of FPIT films suggested the higher content of non-disulfide covalent bond, which was in agreement with the higher TS of FPIT film, in comparison with UWM film (Table 14).

After 40 days of storage, UWM film and FPIT film had the decrease in protein solubility for all solvents used ($P < 0.05$). This result indicated that non-disulfide covalent bonds took place during extended storage. This was possibly caused by the formation of cross-links induced by lipid oxidation products generated during storage (Figure 30). The polymerized proteins in film matrix might lose their solubility. The oxidation products of lipids in film matrix could interact with protein via Maillard reaction, resulting in their insolubilization (Damodaran, 1996). The decrease in protein solubility of UWM film was much higher than FPIT film after 40

days of storage. Higher lipids in unwashed mince were more prone to oxidation, thereby yielding lipid oxidation products readily for Maillard reaction. As a consequence, a marked decrease in solubility was obtained in UWM film after 40 days.

Table 17. Protein solubility in various solvents of films from unwashed mince prepared at pH 3 (UWM) and films from FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) at day 0 and 40 of storage.

Storage time (Days)	Samples	Protein solubility (%)		
		S1**	S2	S3
0	UWM	84.30 ± 0.65^{aA}	90.76 ± 2.08^{aA}	93.84 ± 2.75^{aA}*
	FPIT	81.83 ± 1.77^{aA}	84.26 ± 1.37^{bA}	87.50 ± 2.01^{bA}
40	UWM	46.85 ± 0.50^{bB}	47.86 ± 0.50^{bB}	52.29 ± 0.37^{bB}
	FPIT	60.64 ± 0.44^{aB}	65.00 ± 1.73^{aB}	79.53 ± 0.83^{aB}

*Mean $\pm$ SD (n=3).

Different lowercase superscripts in the same column under the same storage time indicate significant differences ($P < 0.05$). Different uppercase superscripts in the same column under the same material and pH levels used indicate significant differences ($P < 0.05$).

** S1: 20 mM Tris- HCl (pH 8.0) + 1% (W/V) SDS

S2: 20 mM Tris- HCl (pH 8.0) + 1% (W/V) SDS + 8.0 M Urea

S3: 20 mM Tris- HCl (pH 8.0) + 1% (W/V) SDS + 8.0 M Urea + 2% βME

5.4.1.8 Protein pattern

Protein patterns of film from FPIT film and UWM film at day 0 and 40 of storage determined under reducing and non-reducing conditions are presented in Figure 31. The low band intensity of MHC and actin was found in both films. The result suggested that the cross-linking via covalent bond was observed in both films. When comparing protein pattern between both films, it was noted that protein with MW of 140-150 kPa was found in UWM film, while actin was dominant in FPIT film. Under reducing condition, band intensity of MHC and actin increased for both films. Both MHC and actin bands were more regained under reducing condition in comparison with non-reducing condition, especially for FPIT film. This result suggested that those proteins were cross-linked preferably by disulfide bond, particularly for FPIT films. Disulfide bond was a strong bond stabilizing film network of FPIT film. This was reflected by the higher TS and lower solubility of this film. The sulfhydryl groups in muscle proteins could form disulfide bonds involved in film matrix upon casting and drying of film (Shiku *et al.*, 2003). Apart from cross-linking, the degradation could take place to some extent. The degradation of protein, particularly MHC, was possibly caused by acid hydrolysis (Chinabhark *et al.*, 2007). After storage for 40 days, no marked changes in protein patterns were observed in comparison with those found at day 0. This result suggested that the polymerization of protein probably took place during storage, mainly via weak bonds. This contributed to the stronger film network as indicated by the increased TS and lowered solubility.

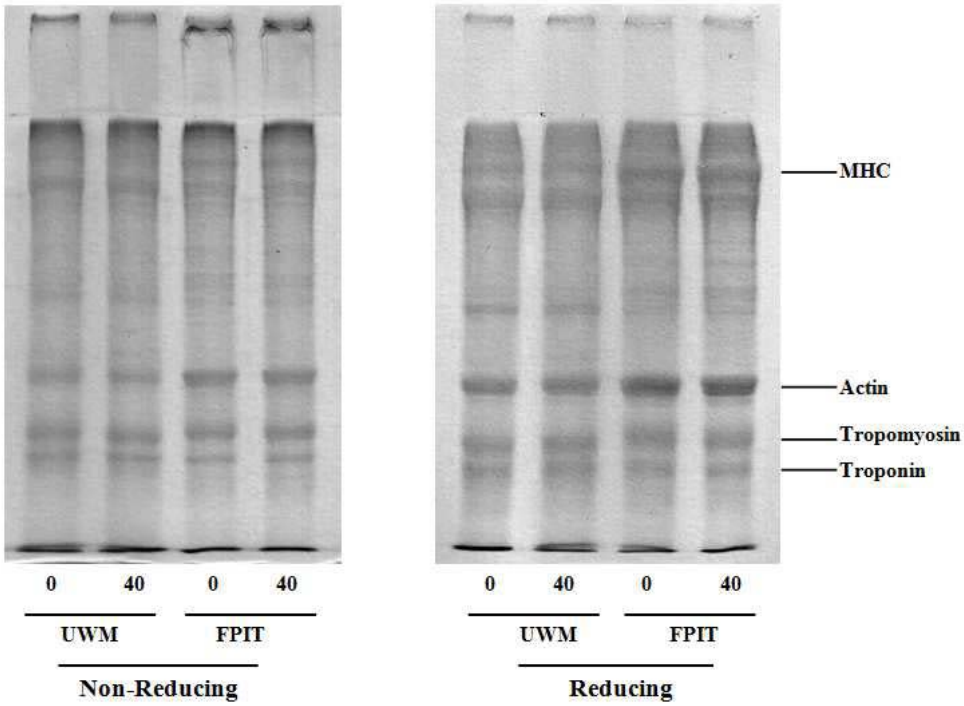


Figure 31. Protein patterns under non-reducing and reducing conditions of films from unwashed mince prepared at pH 3 (UWM) and films from FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) at day 0 and 40 of storage.

5.4.1.9 Fourier-transform infrared (FTIR) spectroscopy

FTIR spectra of FPIT and UWM films at day 0 and 40 of storage are illustrated in Figure 32A and 32B, respectively. Generally, FTIR spectra of FPIT and UWM films showed the similar major peaks but the amplitudes of peaks were different. The band situated at the wavenumber 1037-1038 cm^{-1} was found in both of FPIT and UWM films, corresponding to the glycerol (OH group) added as a plasticizer (Bergo and Sobral, 2007). Both films had the similar spectra in the range of 1700-700 cm^{-1}, covering with amide-I, II and III. Both films had the major bands at 1645 cm^{-1} (amide-I, illustrating C=O stretching/hydrogen bonding coupled with COO), 1537 cm^{-1}(amide-II, presenting the bending vibrations of N-H groups and stretching vibrations of C-N groups) and 1234 cm^{-1} (amide-III, illustrating the vibrations in-plane of C-N and N-H groups of bound amide or vibrations of CH_2 groups of glycine) (Aewsiri *et al.*, 2009; Muyonga *et al.*, 2004a). Limpan *et al.* (2010)

also found the similar result for bigeye snapper (*Priacanthus tayenus*) myofibrillar prtein film, where amide-I, II and III bands were found at the wavenumber 1649, 1548 and 1235 cm^{-1}, respectively. It was noted that the amplitude of amide-I, II and III of FPIT film was higher than that of UWM film. This might be due to the the higher content of proteins in FPI, in comparison with UWM. During washing and alkaline solubilization, lipids and other components were removed, leading to the concentrated proteins. Moreover, the greater dissociation into monomer of myofibrillar protein during FPI preparation was presumed. Monomers with free functional groups rendered the higher amplitude, though those dissociated proteins underwent interaction during casting and drying.

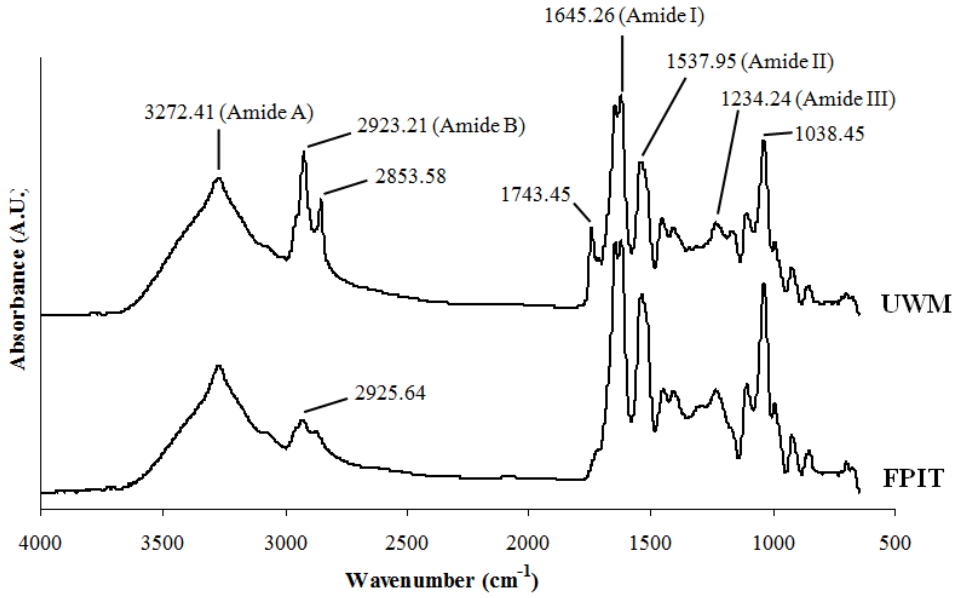
Moreover, amide-A band was observed at the wavenumber of 3272 and 3271 cm^{-1} for UWM and FPIT films, respectively. Amide-B band was also found at 2923 and 2925 cm^{-1} for UWM and FPIT films, respectively. The amide-A band represented the NH-stretching coupled with hydrogen bonding and amide-B band represented the CH stretching and $-NH_3^+$ at wavenumber 2928 cm^{-1} (Muyonga *et al.*, 2004b). The spectrum of FPIT film showed the much lower amplitude of amide-B peak, compared with that of UWM film. The lower amount of $–NH_2$ or NH_3^+ indicated that those amino groups of FPIT film plausibly underwent interaction by hydrogen bond with hydrogen bond acceptor at higher extent than UWM film. Additionally, those amino groups might be involved in Maillard reaction, where non-disulfide bonds were formed. The decrease in amide-B amplitude was also indicative of hydrophobic interaction via –CH between protein chains.

Furthermore, the peaks at wavenumbers 2923.58 $cm^{-1}$and 2853.62 cm^{-1} might represent the methylene asymmetrical and symmetrical stretching vibration of the aliphatic CH_2 group, respectively (Guillén and Cabo, 1997; Guillén and Cabo, 2004). Both the methylene asymmetrical stretching bands at approximately 2924 cm^{-1} and methylene symmetrical stretching band near 2853 cm^{-1} were obviously present in most of lipid samples (Guillén and Cabo, 2004). For UWM film, the amplitude of those peaks was much higher than FPIT film. Moreover, the carbonyl absorption of triglyceride ester linkage was observed at 1746 cm^{-1} (Setiowaty *et al*., 2000). For UWM film, the stretching vibration peak assignable to the C=O group of triglycerides was found at wavenumber 1746 cm^{-1}, but there was no peak at 1746 cm^{-1} was found

in FPIT film. This result confirmed that UWM film had higher lipid content than FPIT film. These results suggested that the washing process could remove lipids to some extent. Nevertheless, lipoproteins associated with muscle proteins might not be leached out easily. When alkaline-aided process was implemented, proteins were more likely dissociated and the lipid or membrane phospholipids were separated into the upper phase during the centrifugation effectively. The removal of lipid by washing in combination with alkaline solubilization process was confirmed by the disappearance of peak at wavenumber 1746 cm^{-1}.

After storage for 40 days, slight changes in FTIR spectra were found in comparison with those found at day 0. However, slight decrease in amplitude of amide A was found in UWM film, suggesting the greater interaction of amino groups via Maillard reaction and the greater cross-linking of protein molecules. This was confirmed by lower solubility, the increase in TS and increase in b^*-value after 40 days of storage. Additionally, the increase in amplitude of peak with wavemunber of 1743 cm^{-1} was obtained with UWM film after 40 days of storage. This more likely represented the formation of carbonyl compounds, especially aldehyde, ketone, etc, the lipid oxidation produces, during the extended storage.

(A)



(B)

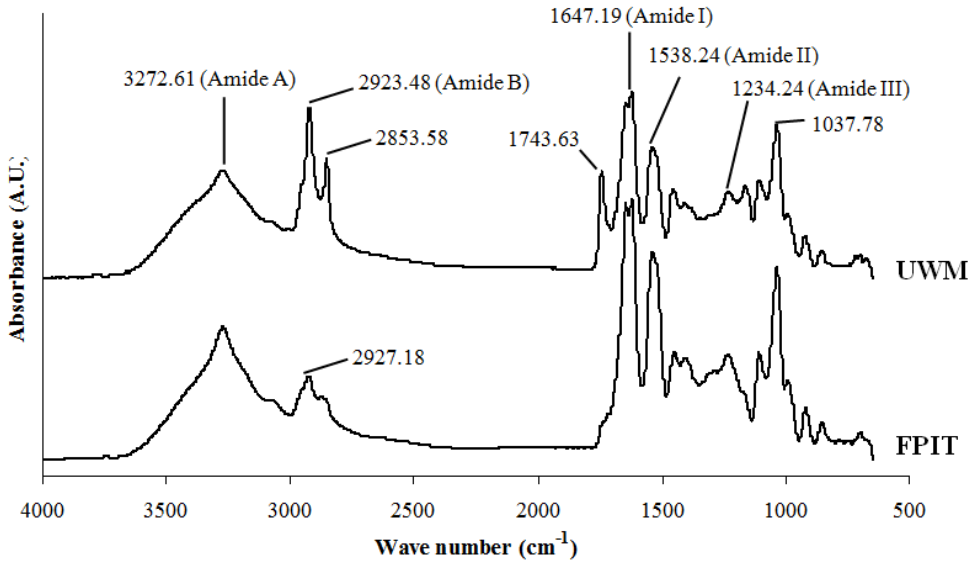


Figure 32. FTIR spectra of films from unwashed mince prepared at pH 3 (UWM) and films from FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) at day 0 (A) and 40 (B) of storage.

5.4.1.10 Thermal-gravimetric analysis (TGA)

TGA thermograms presenting the thermal degradation behavior of FPIT film and UWM film at day 0 and 40 of storage are illustrated in Figure 33A and 33B, respectively. The degradation temperatures (Td), weight loss (Δw) and residue (%) of both film samples are presented in Table 18. Both FPIT and UWM films generally exhibited three main stages of weight loss. At day 0 of storage, UWM and FPIT films had the first stage of weight loss at 36.99 ºC and 42.47 ºC, respectively. The higher weight loss was found in UWM film (7.34 %), compared with FPIT film (3.57%). Additionally, the weight loss observed at 46.62-61.31 ºC was possibly due to the loss of free and bound water absorbed on the film. The similar result was observed in cuttlefish skin gelatin film (Hoque *et al.*, 2011a) and porcine-plasma protein film (Nuthong *et al.*, 2009b). The second stage of weight loss was observed at 170.25 ºC and 171.61 ºC for UWM and FPIT films, respectively with Δw_2 of 30.33% and 28.13%, respectively. The degradation temperature in the range 196.30-216.71 ºC of protein film was mostly associated with the loss of glycerol compound (plasticizer) and smaller size protein fraction as well as structurally bound water (Hoque *et al.*, 2011a). For the third stage of weight loss, Δw_3 were 43.02 and 51.09 and Td_3 of 304.52 and 311.56 ºC were obtained for UWM and FPIT films, respectively, mostly associated with the degradation of the larger-size or associated protein fraction. The result indicated that the degradation of UWM and FPIT films began at ≈170 ºC. This result was in agreement with Nuthong *et al*. (2009b) who reported that the initial temperature degradation of porcine plasma protein-based film was observed at ≈170 ºC. Higher Td of all three stages were found in FPIT film, compared with UWM film. This result indicated the higher thermal stability of FPIT film was plausibly due to the higher protein interaction in film matrix than UWM film. This indicated that FPIT film had the higher cross-links, which were in accordance with the higher TS and lower solubility, in comparison with UWM film. Apparently, thermally stable structures formed were due to the occurrence of cross-linking reaction (Schmidt *et al*., 2005).

The stronger matrix was found in FPIT film, probably owing to less undesirable components especially lipids and heme proteins, which exhibited interfering effect on the protein-protein interaction in the film network. It was noted

that the higher residue was found in UWM film, suggesting the presence of very thermal stable substance or cross-links.

After 40 days of storage, FPIT film still showed the higher heat resistance than UWM film as evidenced by the higher Td_1, Td_2 and Td_3 and lower of weight loss for Δw_1 and Δw_2. However, FPIT film showed the higher Δw_3, compared with UWM film. Thus, both UWM and FPIT films generally had the increase in thermal degradation temperatures after 40 days of storage, possibly caused by the higher protein interaction. This was coincidental with the increased TS and lowered solubility of film after storage for 40 days. In general, higher residue from thermal degradation was found in both UWM and FPIT films, compared with those observed at day 0. This confirmed that both films had the stronger interaction between protein molecules in the film network after the extended storage. Normally, the presence of residue or char after thermal degradation is typically found in thermosetting materials which associated with the presence of highly covalent cross-linked structure. From the result, the increase residue or char at 40 days of storage found in UWM film was more pronounced compared to that of FPIT film. Therefore, this result suggested that protein cross-linking via non-disulfide covalent bonds possibly from Maillard reaction dramatically took place in UWM film during extended storage.

Table 18. Thermal degradation temperatures (Td, ºC) and weight loss (Δw, %) of film from unwashed mince prepared at pH 3 (UWM) and FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) at day 0 and 40 of storage.

Storage time (Days)	Samples	Δ_1		Δ_2		Δ_3		Residue (%)
		$Td_{1,onset}$	Δw_1	$Td_{2,onset}$	Δw_2	$Td_{3,onset}$	Δw_3	
0	UWM	36.99	7.34	170.25	30.93	304.52	43.02	18.53
	FPIT	42.47	3.57	171.61	28.13	311.56	51.90	16.40
40	UWM	38.44	8.17	174.00	30.92	309.35	32.09	28.82
	FPIT	48.69	4.96	184.96	28.79	318.12	49.16	17.09

Δ_1, Δ_2 and Δ_3 denote the first, second and third stage weight loss, respectively, of film during heating scan.

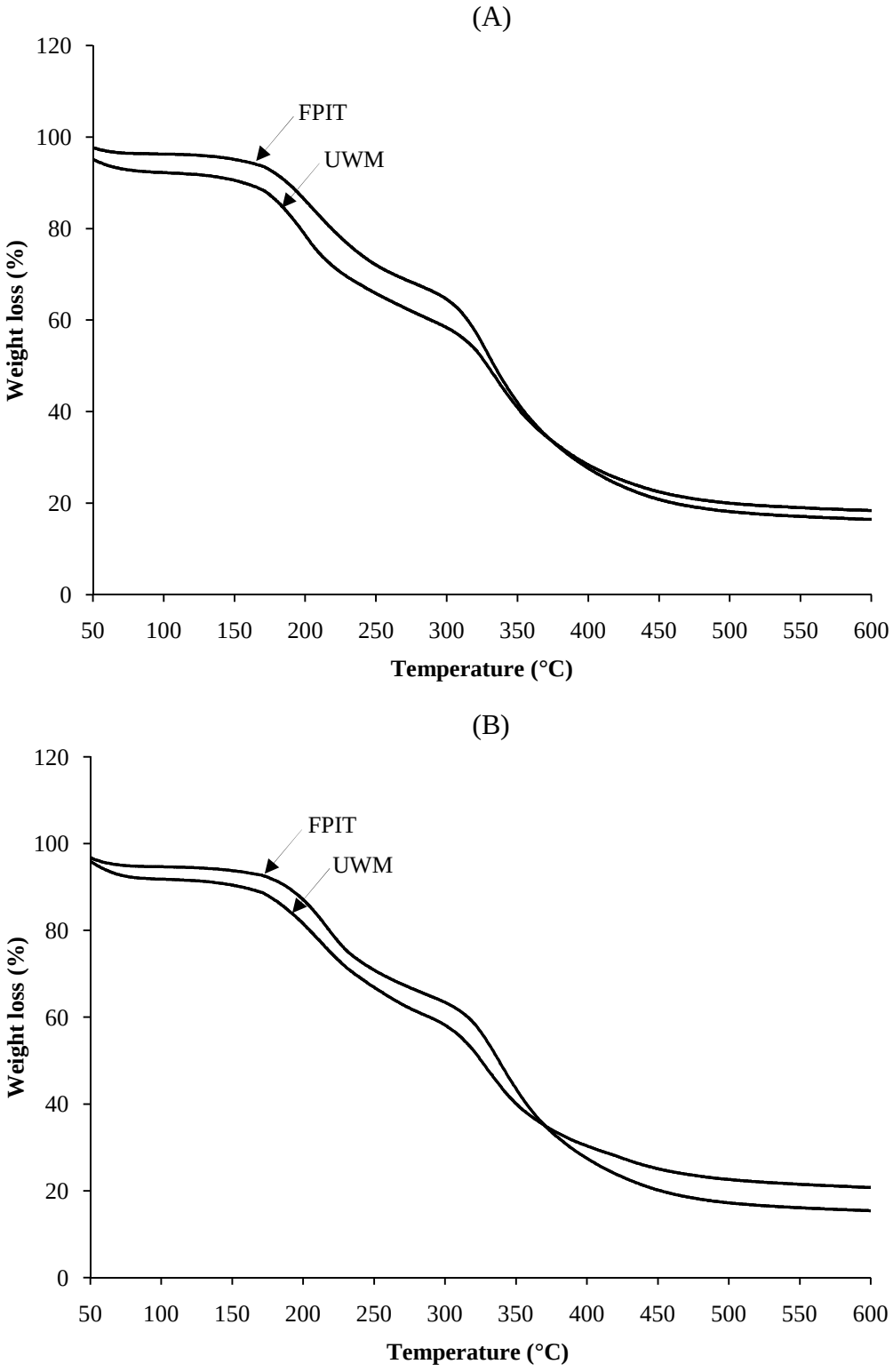


Figure 33. Thermo-gravimetric curves of films from unwashed mince prepared at pH 3 (UWM) and films from FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) at day 0 (A) and 40 (B) of storage.

5.4.1.11 Film morphology

Surface and freeze-fractured cross-sectional images of FPIT and UWM films at day 0 and 40 of storage are shown in Figure 34. The surface and cross-section of FPIT film were smoother than those of UWM film. This revealed that FPIT film had more homogeneous structure than UWM film. Myofibrillar proteins with less amounts of undesirable components more likely underwent higher interaction to form a continuous film matrix. This result suggested that washing in combination with alkaline solubilization process could isolate the myofibrillar proteins by dissociating the proteins to monomers. The dissociated proteins could align themselves more orderly and uniformly. This resulted in the improved mechanical and physical properties of FPIT film. Nevertheless, both films showed some cracks in the cross-section.

After 40 days of storage, FPIT and UWM films exhibited rougher surface, compared with those observed at day 0. Moreover, the increase in crack with higher gap in cross-section was found in both films. The significant decrease in moisture content and intensive cross-linking between protein molecules possibly led to the presence of non-uniform shrinkage of the internal structure network, resulted in formation of micro-crack in the film matrix. This network was associated with the relative increase in TS and decrease in solubility of film stored for an extended time.

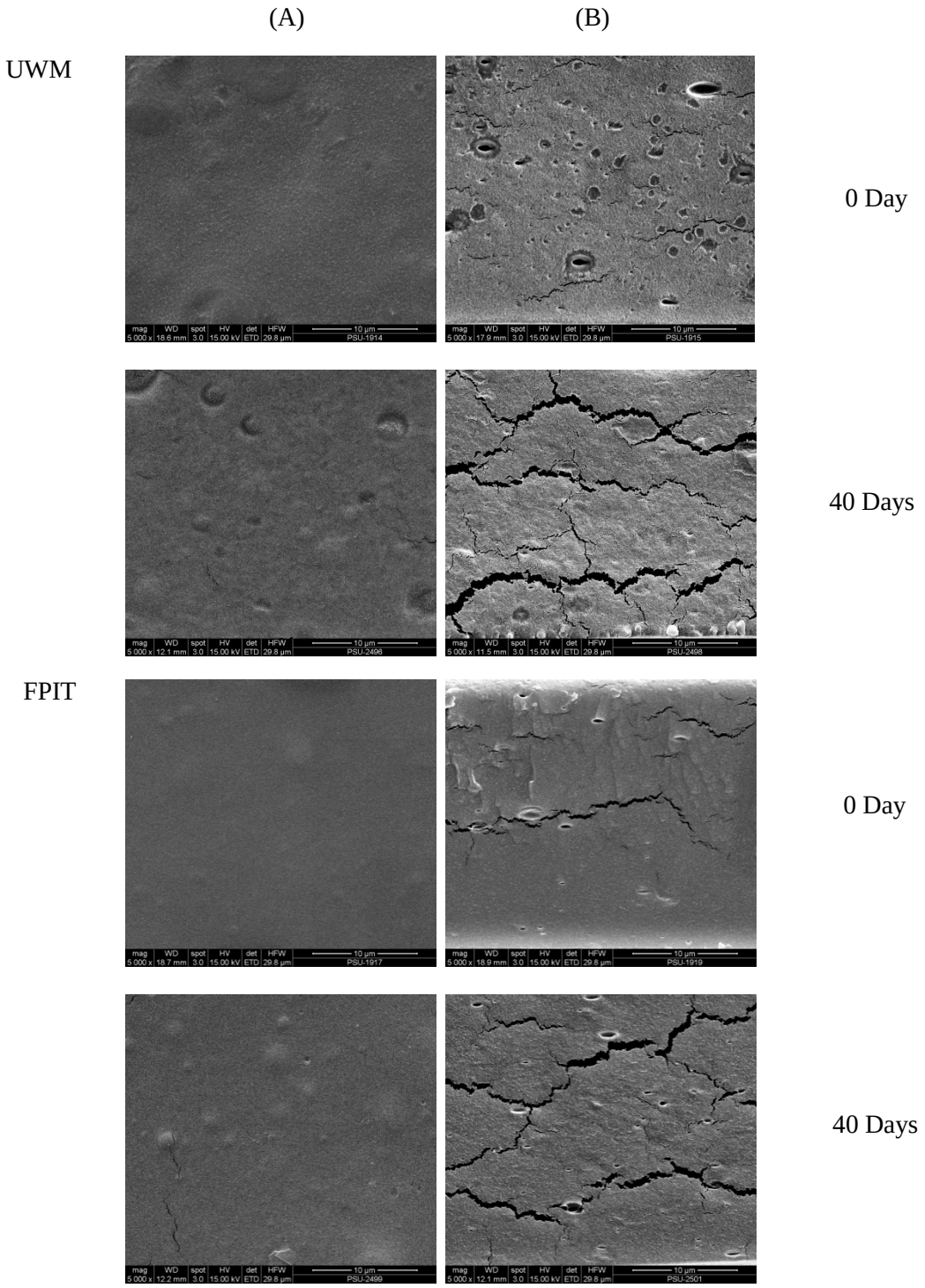


Figure 34. SEM micrographs of films from unwashed mince prepared at pH 3 (UWM) and FPI of red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT) at 0 and 40 days of storage. Surface (A) and freeze-fractured cross-section at 5000 x (B).

5.4.2 Use of red tilapia protein based-films to extend the shelf life of dried fish powder

5.4.2.1 Change in moisture content of dried fish powder

Moisture content of dried fish powder covered with FPIT film (film from of red tilapia muscle prepared at pH 3 and incorporated with 100 ppm Trolox), polyethylene (PE) film and polypropylene (PP) film during storage of 30 days at 28-30 ºC is shown in Figure 35. In general, the moisture content of dried fish powder covered with all films increased, especially within the first 15 days ($P < 0.05$). Thereafter, the increasing rate was much lower for the sample kept with FPIT film. However, a slight decrease in moisture content was found in sample stored with PE and PP films after 15 days. After 30 days of storage, uncovered sample (Control) and samples covered with FPIT film, PE film and PP film had the increase in moisture content by 3.25, 2.95, 1.28 and 1.23-fold, respectively. The moisture content of dried fish powder covered with PE and PP films had the lower increase in moisture content than that of uncovered and covered with FPIT film ($P < 0.05$). No difference in moisture content was found between PE and PP films ($P > 0.05$). The result indicated that the water vapor barrier property of film from FPIT film was lower than that of PE and PP films. Fish muscle is known for its hydrophilic characteristics; water vapor barrier properties of protein films and other hydrocolloid-based films were poorer, compared to those from non-hydrophilic material or synthetic polymer (Hamaguchi *et al*., 2007; Shiku *et al*., 2003). The adsorption of water vapor by dried fish powder is generally mediated by binding of water molecules to specific hydrophilic sites, such as carboxylic, amino and hydroxyl residues of proteins, even at low relative humidity (D'Arcy and Watt, 1981). The higher moisture diffusion from the environment through the packaging material increases the moisture content of packed sample. Artharn *et al*.(2009) reported that moisture content of dried fish powder packed with round scad protein-based film and chitosan film was higher than that of those packed with HDPE film ($P < 0.05$) during storage.

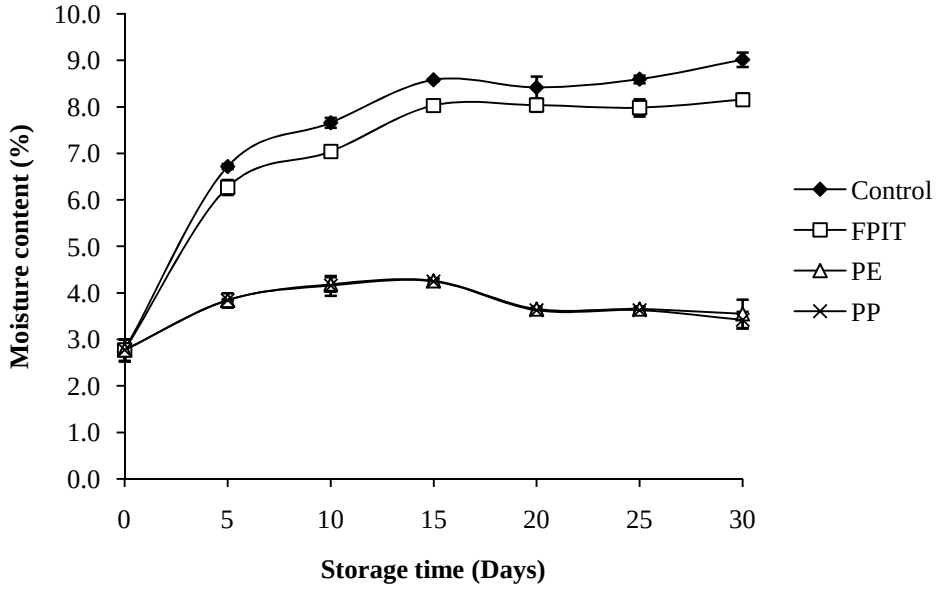


Figure 35. Change in moisture content of uncovered dried fish powder (Control) and fish powder covered with FPIT film, polyethylene film (PE) and polypropylene film (PP) during storage of 30 days at 28-30 ºC. Bars represent the standard deviation (n=3).

5.4.2.2 Changes in lipid oxidation of dried fish powder

Lipid oxidation of dried fish powder covered with FPIT film, polyethylene (PE) film and polypropylene (PP) films was monitored by measuring PV and TBARS values during 30 days of storage at 28-30 ºC (Figure 36 and 37, respectively). PV value of all samples increased after 5 days of storage, followed by the decrease up to 15 days. Thereafter, a continuous increase in PV value was observed up to 30 days of storage. The sample covered with FPIT film, generally showed a lower PV than the other samples during 30 days of storage ($P < 0.05$), except at day 25 of storage.

TBARS values of all samples increased within the first 5 days of storage, followed by slight decrease up to 10 days. Subsequently, the gradual increase in TBARS was observed up to 30 days of storage. The sample covered with FPIT film showed the lower TBARS value than those covered with both PE and PP films during storage. This result indicated that oxygen barrier properties of film from FPI based

film might be higher than HDPE and PP films. The result was in agreement with Artharn *et al*. (2009) who reported that round scad protein based film could retard the lipid oxidation of dried fish powder during storage better than HDPE films. Protein-based films have impressive oxygen and carbon dioxide barrier properties in low relative humidity condition compared to synthetic films (Limpan *et al*., 2010; Shiku *et al*., 2003). Therefore, the protein-based film can be used as the material to prevent rancidity of foods and also can be served as alternative material for chemically synthesized polymeric films. The decrease in TBARS value was probably due to the losses in volatile secondary oxidation products, particularly those with low molecular weights. Alghazeer *et al*. (2008) reported the gradual increase of PV and TBARS in frozen mackerel and instant decrease in both PV and TBARS after 10 weeks of storage.

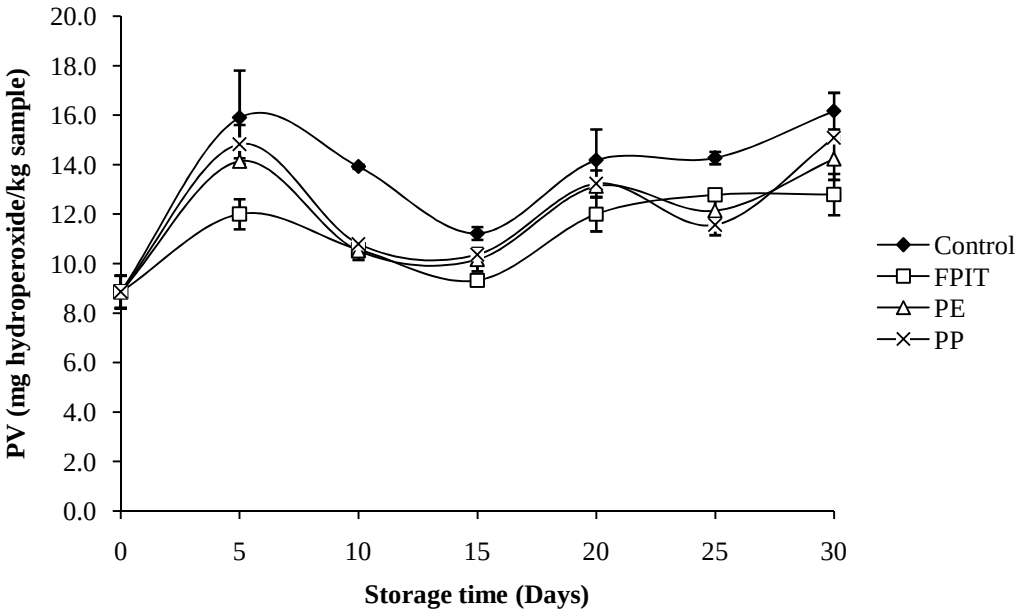


Figure 36. Change in peroxide value of uncovered dried fish powder (Control) and fish powder covered with FPIT film, polyethylene film (PE) and polypropylene film (PP) during storage of 30 days at 28-30 ºC. Bars represent the standard deviation (n=3).

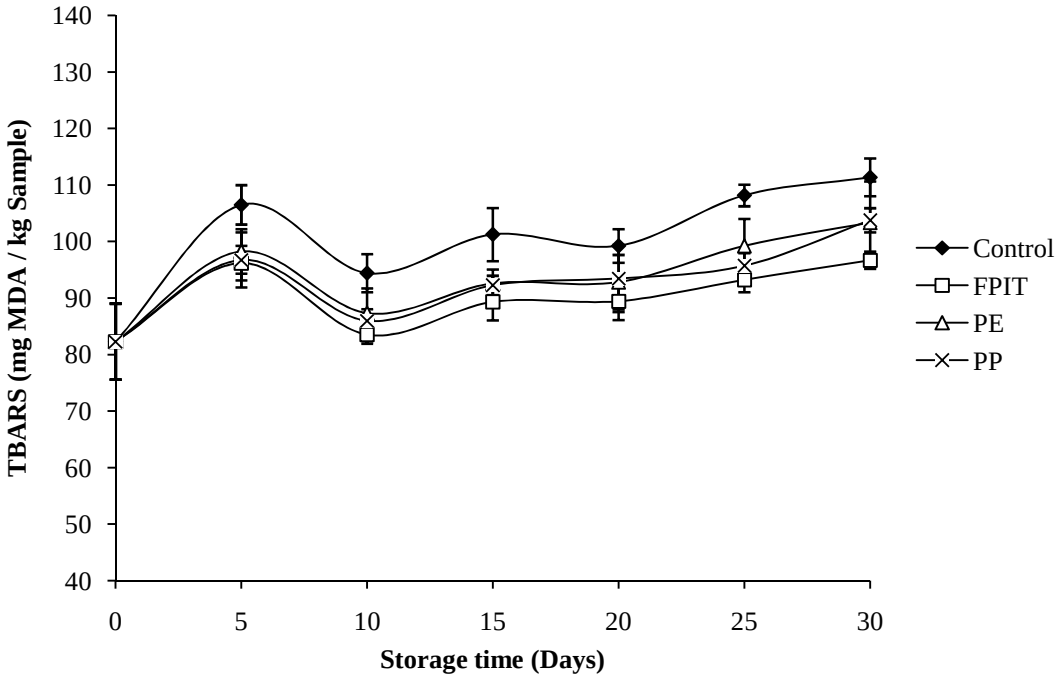


Figure 37. Change TBARS value of uncovered dried fish powder (Control) and fish powder covered with FPIT film, polyethylene film (PE) and polypropylene film (PP) during storage of 30 days at 28-30 ºC. Bars represent the standard deviation (n=3).

5.4.2.3 Changes in color

*L**, *a** and *b**-values of dried fish powder covered with FPIT film, polyethylene (PE) film and polypropylene (PP) films during storage of 30 days at 28-30 ºC are shown in Figure 38. Generally, the uncovered dried fish powder and powder covered with all films had the decrease in *L** and the increase in *a** and *b**-values during storage of 30 days ($P < 0.05$). During storage, the uncovered sample had the highest rate of increase in *a** and *b**-values and the decrease in *L**- value, followed by the sample covered with FPIT film. Generally, fish powder covered with both synthetic films (PE and PP) had the lowest increases in *b**-values (yellowness) during 30 days of storage. The increase in *b**-value indicated the formation of yellowish pigment, presumably via the Maillard reaction, which might be associated with increasing moisture content in fish powder (Artharn *et al.*, 2009). The rate of non-enzymatic browning reactions increases as the water activity increases (Jouppila and Roos, 1994). Yeo and Shibamoto (1991) reported that the browning intensity of L-cysteine/D-glucose model system increased with increasing moisture content.

Furthermore, the browning was likely related with the increased lipid oxidation as indicated by the increase in PV and TBARS values. Lipid oxidation products, especially those with carbonyl group such as aldehydes, could undergo glycation with amino group of protein in fish powder (Chaijan *et al.*, 2007). As a consequence, Maillard reaction took place at a higher extent in the presence of lipid oxidation products. Thus, the increase in lipid oxidation enhanced the non-enzymatic browning (Maillard reaction) as evidenced by the increased *b**-value (yellowness) during storage.

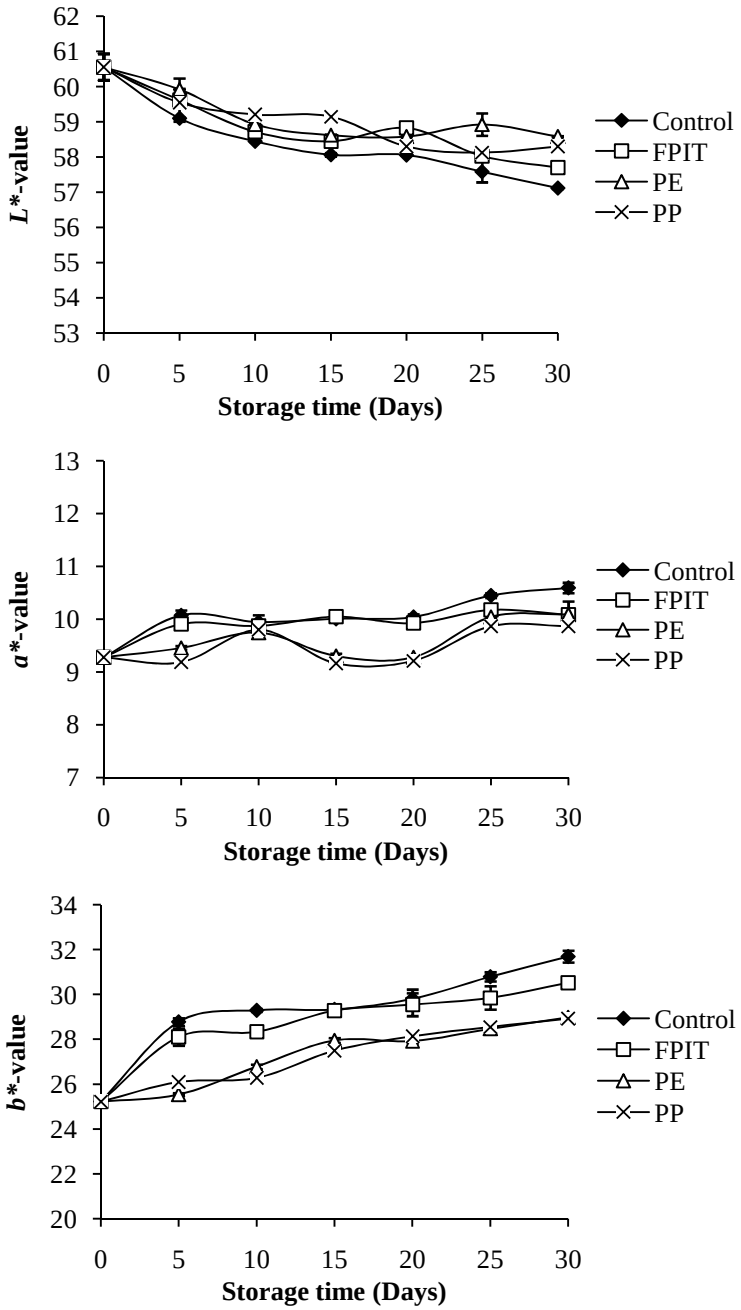


Figure 38. Change in *L**, *a** and *b**-values of uncovered dried fish powder (Control) and fish powder covered with FPI from red tilapia prepared at pH 3 and incorporated with 100 ppm Trolox (FPIT), polyethylene film (PE) and polypropylene film (PP) during storage of 30 days at 28-30 ºC. Bars represent the standard deviation (n=3).

5.5 Conclusion

Film from FPI incorporated with Trolox had superior physical properties to film from unwashed mince. This film was more stable and had the negligible yellow discoloration during extended storage. Therefore, film prepared from FPI incorporated with appropriate antioxidant can be of further potential application. On the other hand, the changes were more pronounced in UWM film, mainly associated with the greater lipid oxidation and the higher formation of cross-links mediated by non-disulfide covalent bond. Therefore, FPIT film can be used as biodegradable film for effective retardation of lipid oxidation of food products without its yellow discoloration.

CHAPTER 6

SUMMARY AND FUTURE WORKS

6.1 Summary

1. Mechanical and physical properties of film from red tilapia muscle were affected by washing process and pH level of FFS. These factors also affected the yellow discoloration of film mediated by Maillard reaction during storage. Lipid oxidation and alkaline pH more likely played a role in yellow discolouration of fish muscle film, mainly by providing the carbonyl groups involved in Maillard reaction, which was enhanced at alkaline pH.
2. Lipid oxidation of fish muscle protein-based film exposed to oxygen was the key factor affecting yellow discoloration during extended storage. The inhibition of lipid oxidation by incorporation of antioxidants (Trolox and catechin) in the range of 100-400 ppm was able to prevent discoloration, mainly via the retardation of lipid oxidation of film during the storage.
3. The use of alkaline solubilization process after conventional washing process could successfully improve the properties of film. This process could lower heme protein and lipid contents from washed mince, leading to the improved mechanical and physical properties of film. Moreover, the lower content of lipid and prooxidants was found, thereby preventing the yellow discoloration caused by lipid oxidation products via Maillard reaction.
4. Film from FPI incorporated with Trolox had superior physical properties to film from unwashed mince. This film was more stable and had the negligible yellow discoloration during extended storage. Therefore, film prepared from FPI incorporated with appropriate antioxidant can be of further potential application.
5. FPI films incorporated with Trolox could be used to prevent lipid oxidation of dried fish powder during storage at room temperature (28-30 °C). Nevertheless, the film had the lower ability to prevent water vapor migration into the sample.

6.2 Future works

1. The improvement of water barrier properties of fish protein film, especially from FPI, should be further studied
2. The sealability of FPI film should be intensively investigated, in which the bag can be prepared and practically used as food packaging.

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APPENDICES

Analytical Methods

1. Determination of moisture content (AOAC, 2000)

Method

1. Dry the empty dish and lid in the oven at 105 °C for 30 min and transfer to desiccator to cool (30 min). 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 or overnight at 105 °C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried content.

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

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

W2 = weight (g) of sample after drying

2. 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 burn 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 on after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.
6. Weigh the ash with crucible and lid until turning to gray. If not, return the crucible and lid to the furnace for further ashing.

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

3. Determination of protein content (AOAC, 2000)

Reagents

1. Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) anhydrus, nitrogen free with 1 part of copper sulphate ($CuSO_4$)
2. Sulfuric acid (H_2SO_4)
3. 40% NaOH solution (w/v)
4. 0.02N HCl solution
5. 4% H_3BO_3 solution (w/v)
6. 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 Kjeldahl 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 until 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 in distillate with standard NaOH solution.

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

where: A = volume (ml) of 0.02N HCl used for sample titration

B = volume (ml) of 0.02N HCl used for 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 byproducts

4. Determination of fat content (AOAC, 2000)

Reagents

- Petroleum ether

Method

1. Place the bottle and lid in the incubator at 105 °C overnight to ensure that weight of bottle was stable.
2. Weigh about 3-5 g of sample to paper filter and wrap.
3. Take the sample into extraction thimble and transfer into soxhlet
4. Fill petroleum ether about 250 ml into the bottle and take it on the heating mantle.
5. Connect the soxhlet apparatus and turn on the water to cool them and then switch on the heating mantle.
6. Heat the sample about 14 h (heat rate of 150 drop/min).
7. Evaporate the solvent by using the vacuum condenser.
8. Incubate the bottle at 80-90 °C until solvent was completely evaporated and bottle was completely dried.
9. After drying, transfer the bottle with partially covered lid to the desiccator to cool. Reweigh the bottle and its dried content.

Calculation

$$\% \text{ Fat} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100$$

5. Biuret method for quantitation of protein (Robison 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 A1.

2. To all tubes containing standards or unknow sample, 500 µl of protein sample were added and mix well by using the vortex mixer.
3. Add 2.0 ml of the biuret reagent to each tube, and mix well.
4. Incubate the mixture at room temperature for 30-45 min, and then read the absorbance of each tube at 540 nm.
5. 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/ 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

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

Reagents

1. Protein molecular weight standards
2. 30% Acrylamide-0.8% bis Acrylamide
3. 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 5-10 mg Bromophenol blue (enough to give dark blue color to the solution). Bring the volume 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’-tetramethylenediamine) - 0.5 M Tris-HCl, pH 6.8
7. 1.5 M Tris-HCl, pH 8.8
8. Electrode buffer: Dissolve 3 g of Tris, 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.
9. Staining solution: Dissolve 0.05 g of Coomassie blue R-250 in 15 ml of methanol. Add 5 ml of glacial acetic acid and 80 ml of distilled water.
10. 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 isobutyl alcohol by gently squirting the isobutyl alcohol 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 A2.
3. Transfer stacking gel solution to tickle into the center of the sandwich along an edge of 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 gel and stacking gel

Reagents	7.5% running gel	4% stacking gel
30% Acrylamide-bis	2.500 ml	0.665 ml
1.5 M Tris-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	100 µl	50 µl
2% Ammonium persulfate	50 µl	25 µl
TEMED	5 µl	2 µl

Sample preparation:

1. Weigh 3 g of sample and homogenize with 5% (w/v) SDS in a final volume of 30 ml.
2. Incubate the mixture at 85 °C for 1 hr.
3. Centrifuge at 3,500xg 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 polyacylamide 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. Insert a spatula between the glass plates at one corner where the spacer is, and gently pry the two plates apart.
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 fresh solution. Repeat until the gel is clear except for the protein bands.

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Bachelor of Science (Food Science and Nutrition)	Prince of Songkla University	2007

List of Publication and Proceedings

Publications

1. Tongnuanchan, P., Benjakul, S. and Prodpran, T. 2011. Roles of lipid oxidation and pH on properties and yellow discolouration during storage of film from red tilapia *(Oreochromis niloticus)* muscle protein. Food Hydrocolloids. 25: 426-433.
2. Tongnuanchan, P., Benjakul, S., Prodpran, T. and Songtipya, P. 2011. Characteristics of film based on protein isolate from red tilapia muscle with negligible yellow discoloration. Int. J. Biol. Macromol. In Press, Uncorrected Proof:
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