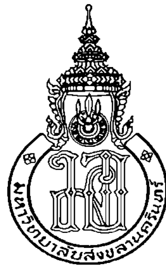

Properties of Biodegradable Film Based on Fish Myofibrillar Protein and Poly(vinyl alcohol) Blend

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A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science in Packaging Technology

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ชื่อวิทยานิพนธ์ สมบัติของฟิล์มย่อยสลายได้จากพอลิเมอร์ผสมระหว่างโปรตีนไมโอไฟบริลจากกล้ามเนื้อปลาและพอลิไวนิลแอลกอฮอล์

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บทคัดย่อ

จากการศึกษาผลของอัตราส่วนระหว่างโปรตีนกล้ามเนื้อปลา (FMP) จากปลาตาหวานหนังหนา (*Priacanthus tayenus*) ต่อพอลิไวนิลแอลกอฮอล์ (PVA) (FMP:PVA; 10:0, 8:2, 6:4, 5:5, 4:6, 2:8, 0:10) และระดับพีเอช (3 และ 11) ต่อสมบัติของฟิล์มผสมระหว่างโปรตีนไมโอไฟบริลจากกล้ามเนื้อปลาและพอลิไวนิลแอลกอฮอล์ พบว่า ฟิล์มผสมมีค่าการต้านทานแรงดึงสูงสุด (TS) และค่าระยะยืดเมื่อขาด (EAB) เพิ่มขึ้นเมื่อเติมพอลิไวนิลแอลกอฮอล์ในปริมาณที่มากขึ้น ($p<0.05$) ฟิล์มผสมที่เตรียมในสภาวะด่างมีค่า TS มากกว่าฟิล์มผสมที่เตรียมในสภาวะกรดเมื่อผสมพอลิไวนิลแอลกอฮอล์ไม่เกินร้อยละ 40 ($p<0.05$) แต่อย่างไรก็ตามเมื่อเติมพอลิไวนิลแอลกอฮอล์มากกว่าร้อยละ 40 พบว่าฟิล์มผสมที่เตรียมในสภาวะกรดมีค่า TS มากกว่าฟิล์มที่เตรียมในสภาวะด่าง ($p<0.05$) ค่าการซึมผ่านไอน้ำ (WVP) ของฟิล์มผสมมีค่าสูงขึ้นตามปริมาณพอลิไวนิลแอลกอฮอล์ที่เพิ่มขึ้นจนกระทั่งพอลิไวนิลแอลกอฮอล์มีปริมาณร้อยละ 40 และเมื่อเติมพอลิไวนิลแอลกอฮอล์ในปริมาณสูงขึ้น กลับมีผลให้ฟิล์มผสมมีค่า WVP ลดลง ($p<0.05$) ฟิล์มพอลิไวนิลแอลกอฮอล์มีค่า TS, EAB และ WVP สูงกว่าฟิล์มโปรตีนและฟิล์มผสม ($p<0.05$) สำหรับค่าสีของแผ่นฟิล์มพบว่า ฟิล์มผสมมีค่า L* และ a* เพิ่มสูงขึ้นแต่ b* มีค่าลดลงเมื่อเติมพอลิไวนิลแอลกอฮอล์มากขึ้น ($p<0.05$) นอกจากนี้พบว่าค่า b* ของฟิล์มผสมที่เตรียมในสภาวะด่างมีค่าสูงกว่าฟิล์มที่เตรียมในสภาวะกรดเมื่อเติมพอลิไวนิลแอลกอฮอล์ในปริมาณที่มากกว่าร้อยละ 40 ($p<0.05$) ฟิล์มโปรตีนและฟิล์มผสมสามารถป้องกันการส่องผ่านของรังสียูวีได้ โดยที่ปริมาณแสงที่ส่องผ่านฟิล์มผสมซึ่งเตรียมในสภาวะกรดเพิ่มมากขึ้นเมื่อปริมาณพอลิไวนิลแอลกอฮอล์ในแผ่นฟิล์มเพิ่มมากขึ้น ทั้งนี้แผ่นฟิล์มผสมทุกอัตราส่วนที่เตรียมในสภาวะด่างมีความใสน้อยกว่าฟิล์มที่เตรียมในสภาวะกรด ($p<0.05$)

เมื่อศึกษาผลของพอลิไวนิลแอลกอฮอล์ (PVA) ที่มีระดับการไฮโดรไลซิส (DH) และน้ำหนักโมเลกุล (MW) ที่แตกต่างกันต่อสมบัติของฟิล์มผสมระหว่างโปรตีนไมโอไฟบริลจากกล้ามเนื้อปลาและพอลิไวนิลแอลกอฮอล์ (อัตราส่วนเท่ากับ 5:5 โดยน้ำหนัก) พบว่าฟิล์มผสมที่เตรียมจากพอลิไวนิลแอลกอฮอล์ซึ่งมีน้ำหนักโมเลกุลสูงมีค่า TS และ EAB สูงกว่าฟิล์มผสมที่

เตรียมจากพอลิไวนิลแอลกอฮอล์ที่มีน้ำหนักโมเลกุลต่ำ ($p<0.05$) การใช้พอลิไวนิลแอลกอฮอล์ที่มีระดับการไฮโดรไลซิสสูงให้ฟิล์มผสมที่มีค่า TS และค่ามอดูลัสยืดหยุ่น (E) สูงกว่าการใช้พอลิไวนิลแอลกอฮอล์ที่มีระดับการไฮโดรไลซิสต่ำ ในขณะที่ฟิล์มผสมที่เติมพอลิไวนิลแอลกอฮอล์ที่ระดับการไฮโดรไลซิสต่ำมีความยืดหยุ่นมากกว่า ($p<0.05$) การเติมพอลิไวนิลแอลกอฮอล์มีผลให้ค่า WVP ของฟิล์มผสมมีค่าลดลงเล็กน้อย โดยฟิล์มผสมที่ใช้พอลิไวนิลแอลกอฮอล์ชนิด BP26 (ระดับการไฮโดรไลซิสต่ำและน้ำหนักโมเลกุลสูง) มีค่า WVP ต่ำที่สุดและสามารถต้านทานแรงดึงได้สูงสุด ($p<0.05$) แต่อย่างไรก็ตามชนิดของพอลิไวนิลแอลกอฮอล์ที่ใช้ไม่มีผลต่อค่าสี การส่องผ่านแสง และความใสของฟิล์มผสม ($p>0.05$)

จากการศึกษาผลของการใช้สารเคมีชนิดต่าง ๆ (มาเลอิกแอนไฮดรายด์ พทาลิกแอนไฮดรายด์ ไกลออกซอล และอิพิคลอโรไฮดริน) ที่ระดับแตกต่างกัน (ร้อยละ 1 3 และ 5โดยน้ำหนักพอลิเมอร์) ต่อสมบัติของฟิล์มผสมระหว่างโปรตีนไมโอไฟบริลและพอลิไวนิลแอลกอฮอล์ (อัตราส่วนเท่ากับ 5:5 โดยน้ำหนัก) พบว่าชนิดและปริมาณสารเคมีที่ใช้มีผลต่อสมบัติเชิงกลของฟิล์มผสม โดยฟิล์มผสมที่ใช้อิพิคลอโรไฮดรินร้อยละ 5 มีค่า TS และ EAB สูงที่สุด ($p<0.05$) ส่วนการใช้มาเลอิกแอนไฮดรายด์ร้อยละ 1 ให้ฟิล์มผสมที่มีค่า WVP ต่ำสุด ($p<0.05$) นอกจากนี้การเติมสารเคมีในฟิล์มผสมยังช่วยให้ฟิล์มที่ได้มีค่าการละลายน้ำลดลง โดยเฉพาะการใช้ไกลออกซอลซึ่งมีผลให้การละลายน้ำของฟิล์มลดลงตามปริมาณไกลออกซอลที่เติม ($p<0.05$) ปริมาณการส่องผ่านแสงของฟิล์มผสมมีค่าลดลงเมื่อมีการเติมสารเคมี โดยฟิล์มผสมที่ใช้มาเลอิกแอนไฮไดรด์ปริมาณร้อยละ 5 ให้ค่าการส่องผ่านแสงน้อยที่สุดและฟิล์มมีความใสน้อยที่สุด ($p<0.05$) การใช้ไกลออกซอลมีผลต่อค่าสีของฟิล์มผสม โดยพบว่าฟิล์มมีสีเหลืองเพิ่มมากขึ้นตามปริมาณการใช้ไกลออกซอลที่เพิ่มขึ้น ($p<0.05$)

จากการวิเคราะห์เปรียบเทียบคุณลักษณะของฟิล์มโปรตีนไมโอไฟบริล ฟิล์มพอลิไวนิลแอลกอฮอล์ และฟิล์มผสมระหว่างโปรตีนไมโอไฟบริลและพอลิไวนิลแอลกอฮอล์ทั้งที่เติมและไม่เติมอิพิคลอโรไฮดรินในปริมาณร้อยละ 5 (โดยน้ำหนักพอลิเมอร์) พบว่าจากผลการทดสอบการละลายของโปรตีนของฟิล์มในตัวทำละลายต่าง ๆ บ่งชี้ว่ามีอันตรกิริยาภายในและระหว่างสายโซ่โปรตีนด้วยพันธะไฮโดรเจน อันตรกิริยาไฮโดรไฟบิค พันธะไดซัลไฟด์ และพันธะโควาเลนท์ที่เกี่ยวข้องกับการเกิดโครงข่ายของฟิล์ม จากผลการวิเคราะห์ด้วยเทคนิค X-ray diffraction (XRD) และเทคนิค differential scanning calorimetry (DSC) พบว่า ฟิล์มโปรตีนมีโครงสร้างแบบอสัณฐาน ในขณะที่ฟิล์มผสมที่เติมและไม่เติมอิพิคลอโรไฮดรินมีโครงสร้างแบบกึ่งผลึก จากผลการศึกษาสมบัติทางความร้อนของฟิล์มพบว่าฟิล์มผสมที่เติมและไม่เติมอิพิคลอโรไฮดรินมีความคงตัวทางความร้อนสูงกว่าฟิล์มโปรตีนโดยมีค่าอุณหภูมิการเปลี่ยนสถานะคล้ายแก้ว (T_g) และอุณหภูมิ

หลอมเหลว (T_m) และอุณหภูมิการสลายตัวทางความร้อน (T_d) สูงกว่าฟิล์มโปรตีน จากผลการวิเคราะห์ด้วยเทคนิค FTIR ยืนยันความเข้ากันได้ของโปรตีนไมโอไฟบริลจากกล้ามเนื้อปลาและพอลิไวนิลแอลกอฮอล์ในแผ่นฟิล์มผสมที่เติมอิพิคลอโรไฮดรินร้อยละ 5 โดยอาศัยพันธะไฮโดรเจนและพันธะโควาเลนท์

จากการเก็บรักษาฟิล์มที่อุณหภูมิห้อง (28-30 องศาเซลเซียส) และความชื้นสัมพัทธ์ร้อยละ 65 พบว่าสมบัติเชิงกล (TS, EAB และ E) ของฟิล์มผสมระหว่างโปรตีนไมโอไฟบริลและพอลิไวนิลแอลกอฮอล์ที่เติมอิพิคลอโรไฮดรินร้อยละ 5 ไม่มีการเปลี่ยนแปลงตลอดการเก็บรักษาเป็นเวลา 8 สัปดาห์ ($p>0.05$) ส่วนสมบัติการซึมผ่านไอน้ำของฟิล์มโปรตีนไม่มีการเปลี่ยนแปลงตลอดระยะเวลาการเก็บรักษา ($p>0.05$) โดยที่ฟิล์มพอลิไวนิลแอลกอฮอล์และฟิล์มผสมที่เติมอิพิคลอโรไฮดรินร้อยละ 5 มีค่า WVP ลดลงในช่วงสองสัปดาห์แรกของการเก็บรักษา ($p<0.05$) หลังจากนั้นมีค่าคงที่ตลอดจนกระทั่งครบเวลาแปดสัปดาห์ เมื่อระยะเวลาการเก็บรักษาเพิ่มขึ้นฟิล์มโปรตีนและฟิล์มผสมที่เติมอิพิคลอโรไฮดรินร้อยละ 5 มีสีเข้มขึ้นและความใสลดลง โดยมีค่า L* และ a* ลดลง และค่า b* เพิ่มขึ้น ($p<0.05$)

Thesis Title	Properties of biodegradable film based on fish myofibrillar protein and poly(vinyl alcohol) blend
Author	Miss Natthaporn Limpan
Major program	Packaging Technology
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ABSTRACT

Effects of the ratios of fish myofibrillar protein (FMP) from bigeye snapper (*Priacanthus tayenus*) to polyvinyl alcohol (PVA) (FMP:PVA; 10:0, 8:2, 6:4, 5:5, 4:6, 2:8, 0:10) and pH levels (3 and 11) on the properties of FMP/PVA blend films were investigated. Both tensile strength (TS) and elongation at break (EAB) of films increased with increasing PVA content ($p<0.05$). When PVA was incorporated up to 40%, films prepared at pH 11 had the higher TS than did those prepared at pH 3 ($p<0.05$). However, as PVA content was greater than 40%, films prepared at pH 3 exhibited the higher TS than did those prepared at pH 11 ($p<0.05$). Water vapor permeability (WVP) of the films prepared at pH 3 increased when PVA content increased up to 40% and decreased with further increases in PVA content ($p<0.05$). PVA films had the higher TS, EAB and WVP than did FMP film and FMP/PVA blend films prepared at both pHs. Films exhibited the increased L* and a*-values but decreased b*-value with increasing PVA content at both pHs. Films prepared at pH 11 showed higher b*-value than did those prepared at pH 3 when PVA content was greater than 40% ($p<0.05$). FMP/PVA blend films exhibited the negligible transmission to the UV light. At pH 3, light transmission of films increased as PVA content increased ($p<0.05$). At all FMP/PVA ratios, films prepared at pH 11 were less transparent than those prepared at pH 3 ($p<0.05$). Therefore, blend composition and pH level influenced the properties of FMP/PVA blend films.

Impacts of various PVA having different degrees of hydrolysis (DH) and molecular weights (MW) on properties of FMP/PVA (5:5, w/w) blend film were investigated. At the same DH of PVA, blend films containing PVA with higher MW exhibited the greater TS and EAB, compared with those incorporated with PVA having the lower MW ($p<0.05$). Blend films containing PVA with higher DH (fully

hydrolyzed type) had the higher TS and elastic modulus (E), while the films incorporated with PVA having the lower DH (partially hydrolyzed type) were more flexible. PVA incorporation slightly decreased WVP of blend films. Blend film added with PVA-BP26 (partial hydrolysis and high MW) exhibited the lowest WVP and the greatest tensile performance. However, PVA types had no effect on color, light transmittance and transparency value of blend films.

Effects of chemicals (maleic anhydride: MA, phthalic anhydride: PA, glyoxal: GLX and epichlorohydrin: ECH) at different levels (1, 3 and 5% w/w based on total polymer) on properties of FMP/PVA (5:5) blend film were studied. Mechanical properties of blend film varied, depending on the type and concentration of chemical added. FMP/PVA blend film incorporated with 5% ECH exhibited the highest TS and EAB ($p<0.05$). However, blend film with 1% MA incorporation had the lowest WVP ($p<0.05$). Incorporation of chemicals generally decreased the solubility in water of FMP/PVA blend films, especially for GLX added films. Solubility was drastically decreased with increasing GLX content ($p<0.05$). Blend film had the lower light transmission when the higher levels of chemicals were used. Blend film containing 5% MA exhibited the lowest film transparency ($p<0.05$). The addition of GLX resulted in the increased b*-value, particularly when GLX content increased ($p<0.05$).

FMP film, PVA film and FMP/PVA blend film without and with 5% ECH were comparatively characterized. Based on protein solubility in various denaturing solutions, different inter- and intra-interactions between protein chains including hydrogen bond, hydrophobic interaction, disulfide bond and non-disulfide covalent bond involved in film network stabilization. Based on x-ray diffraction and differential scanning calorimetry (DSC) results, FMP film was amorphous structure while FMP/PVA blend film exhibited partially crystalline structure, irrespective of ECH incorporation. Greater thermal stability with an increase in the glass transition temperature, melting temperature and degradation temperature was observed in blend film without and with 5% ECH, compared with those of FMP film. Blend of FMP and PVA was partially miscible. FTIR analysis indicated that the interactions between FMP and PVA via hydrogen bond and covalent bond were involved in blend film with

5% ECH addition, reconfirming the compatibility of the blend system.

During the storage under 65±5% RH at room temperature (28-30°C), the mechanical properties (TS, EAB and E) of FMP/PVA blend film incorporated with 5% ECH remained constant up to 8 weeks of storage ($p>0.05$). No changes in WVP were obtained in FMP film during the storage ($p>0.05$). PVA film and FMP/PVA blend film with 5% ECH had the lowered WVP as storage time increased up to 2 weeks, but remained unchanged thereafter. FMP film and FMP/PVA blend film with 5% ECH became darker and less transparent as evidenced by the decrease in L* and a*-values and the increase in b*-value as the storage time increased ($p<0.05$).

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CHAPTER 1

INTRODUCTION

In recent years, there has been an increasing concern of the environmental problem caused by the massive use of synthetic non-biodegradable materials especially for packaging. As a result, much effort has been made to develop biodegradable or edible film from biopolymers to produce environmentally friendly packaging alternative to synthetic plastic packaging films. Among agricultural macromolecules, protein has been empirically used as packaging materials due to its abundance, biodegradability and nutritive value. In addition, agro-packaging based on proteins are generally characterized by remarkable functional properties because of their heterogeneous specific structure (Gerrard, 2002; Cuq *et al*., 1995). Protein-based films have impressive oxygen and carbon dioxide barrier properties in low relative humidity condition, compared to synthetic films. Properties of protein-based films depend on various factors such as the source of protein, pH of protein solution, plasticizers, the preparation conditions and substances incorporated into film-forming solutions (Gerrard, 2002; Cuq *et al*., 1995; Prodpran *et al*., 2007).

Among various proteins, myofibrillar proteins of fish muscles can be used to prepare film-forming solution (Prodpran *et al*., 2007; Pascholick *et al*., 2003). To prepare myofibrillar protein film, pH of the film-forming solution need to be adjusted to higher or lower than the isoelectric point (pI) of the protein to complete solubilization (Iwata *et al*., 2000). As a result, these proteins are fully stretched and closely associated with each other in parallel structures and capable to form a continuous matrix during drying of the solution (Shiku *et al.*, 2003). Like other protein-based films, fish myofibrillar protein films have inferior functional properties to synthetic counterparts. They possess high water absorptivity and water vapor permeability, owing to hydrophilicity of amino acids in protein molecules and to the significant amounts of hydrophilic plasticizers, such as glycerol and sorbitol, incorporated into the films to impart adequate film flexibility (Prodpran *et al*., 2007; McHugh *et al*., 1994). Moreover, as compared to the synthetic films, myofibrillar protein films have relatively poor mechanical properties. Various attempts have been

carried out to improve protein film properties including chemical treatment (Hernandez-Munoz *et al.*, 2004a), enzymetic treatment (Jiang *et al*., 2007), thermal treatment (Lei *et al.*, 2007) and ultraviolet and gamma irradiation (Jo *et al*., 2005). Another effective and widely used approach to improve the properties of protein-based films is polymer blend technique (Perez-Mateos *et al.,* 2009). Polymer blending is a well-used technique whenever modification of properties is required because it has an easy and straightforward procedure and it is low cost (Wang *et al*., 2009).

Polyvinyl alcohol (PVA), a hydrolysis product of polyvinyl acetate, is the polymer of choice being used to blend with various biopolymers and hydrophilic synthetic polymers, due to their great compatibility and ability to be manipulated in water solution (Mansur *et al*., 2008). PVA is a biodegradable and synthetic water soluble crystalline polymer possessing good film forming property (Skeist, 1990). PVA film itself offers good tensile strength (TS), excellent flexibility and toughness as well as good gas and aroma barrier properties (Park *et al*., 2001). Several studies on the development and characterization of films based on PVA and protein blends have been published, for example PVA/gelatin (Maria *et al.*, 2008; Mendieta-Taboada *et al*., 2008), PVA/wheat protein (Zhang *et al*., 2004) and PVA/collagen hydrolysate (Alexy *et al*., 2003; Hoffmann *et al*., 2003). However, use of PVA to modify the properties of fish myofibrillar protein (FMP) film is rare. The properties of FMP/PVA blend films was depended on the compatibility between FMP and PVA molecules which might be affected by blend composition, pH levels, PVA types and chemical reagents incorporated into the films.

Review of Literature

1. Fish muscle protein

Protein, the most important functional components in muscle, confers many desirable physicochemical and sensory attributes of muscle foods. Muscle proteins comprise 15-22% of the total muscle weight (about 60-88% of mass) and can be divided into three major groups on the basis of their solubility: sarcoplasmic proteins (water-soluble), myofibrillar proteins (salt-soluble) and stroma proteins (insoluble) (Ziegler and Action, 1984; Xiong, 1997).

1.1 Sarcoplasmic proteins

Sarcoplasmic proteins are located inside the sarcoplasm and are soluble in water or low salt concentrations (ionic strength < 0.15) (Xiong, 1997). Generally, the sarcoplasmic proteins comprise about 20-30% of the total amount of proteins in fish muscles (Suzuki, 1981; Sikorski, 1990). The contents of sarcoplasmic proteins are higher in pelagic fish muscle than in dermersal fish muscle (Sikorski, 1990). Sarcoplasmic proteins consist of heme protein such as myoglobin and hemoglobin as well as enzymes involving in glycolysis, citric and electron transfer cycles (Shahidi, 1994; Xiong, 1997; Sikorski, 1990).

1.2 Myofibrillar proteins

Myofibrillar proteins are the major structural proteins in fish muscle, which accounts for 55 to 60% of total protein muscle. These proteins can be extracted with neutral salt solutions of ionic strength above 0.15. Myofibrillar proteins can be further divided into three subgroups (Xiong, 1997; Sikorski, 1990) as follows:

1.2.1 Contractile proteins

Contractile proteins, including myosin and actin, are directly responsible for muscle contraction. Myosin makes up 50 to 58% of the myofibrillar fraction. It consists of six polypeptide subunits, two heavy chains and four light chains (Figure 1). The two globular heads with ATPase activity are relatively hydrophobic and are able to bind actin (McCormick, 1994; Xiong, 1997). When myosin is digested by trypsin or chymotrypsin for a short period, it is divided into two heavy meromysin chains with a size of 220,000 Daltons, depending on species and dfiber types (Suzuki, 1981; Xiong, 1997). Actin is the second most abundant myofibrillar protein, comprising about 22% of the myofibrillar protein (Suzuki, 1981). Each actin molecule contains five sulfhydryl groups and is free of disulfide bond. It also contains a myosin binding site, which allows myosin to form temporary complexes via non-disulfide bonds, which can be split by high-energy compounds such as ATP at high ionic strengths (Xiong and Brekke, 1989).

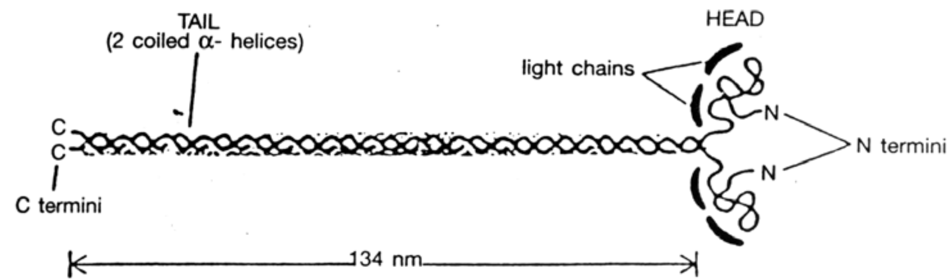


Figure 1. Structure of myosin heavy chain.
Source: McCormick (1994)

1.2.2 Regulartory proteins

The major regulartory proteins are tropomyosin and troponin, located on the thin filaments. Tropomyosin represents approximately 8-10% of the total myofibrillar proteins. Molecule of tropomyosin consists of acidic and basidic amino acids (isoelectric point = 5.1) (Suzuki, 1981; Xiong and Brekke, 1989). Troponin is a globular protein found in thin filament with a molecular weight of 37,000 Daltons.

1.2.3 Cytoskeletal proteins

The proteins in this group include titin, connectin, nebulin, desmin and other proteins. Cytoskeletal proteins are functioned to support and stabilize the contractile proteins of the muscle. The contractile proteins vary in susceptibility to postmortem proteolytic degradation, contributing to the varying meat tenderness (McCromick, 1994).

1.3 Stroma proteins

Stroma protein is the residue after extraction of the sarcoplasmic and myofibrillar proteins. The stroma protein is insoluble in dilute salt solution. It can be extracted by water, acid or alkaline solution and neutral salt solution (Suzuki, 1981). It constitutes about 3% of total muscle proteins. The stroma protein is composed of the main connective tissue proteins such as collagen and elastin (Xiong, 1997).

2. Biodegradable/edible films

Biodegradable films and/or edible films which are able to extend food product shelf-life and preserve food quality have been considered to provide the advantages in the food industry. Films are used in the confectionary, fruits and vegetables, meat, and pharmaceutical industries (Kester and Fennema, 1986; Herald *et al.*, 1995; Krochta and Mulder-Johnston, 1997). Films can prevent the food from interaction with its environment, gains or losses moisture or aroma, taking up oxygen, or contamination with microorganisms (Kester and Fennema, 1986). Furthermore, biodegradable films and edible films can be used to incorporate various food additives such as flavoring, antimicrobial agents and antioxidant agents, into foods at specific locations. This approach can be used to impart a strong localized functional effect, without elevating excessively the overall concentration of the additive in the food (Kester and Fennema, 1986; Herald *et al.,* 1995, Krochta and Mulder-Johnson, 1997).

Biopolymers, including protein, polysaccharides, lipids or their combination have been used to produce biodegradable films and edible films (Arthan *et al*., 2009; Bergo and Sobral, 2007; Jayasekara *et al.*, 2004; McHugh *et al.,* 1994). Polysaccharide films and protein films are good oxygen- and carbon dioxide-barrier properties but show the poor water-vapor barrier property (Jiang *et al.,* 2007; Stuchell and Krochta, 1995).

3. Protein-based films

Proteins cover a broad range of polymeric compounds that provide structure or biological activity in plants or animals. Various proteins can be used as film-forming materials (Alexy *et al,* 2003) such as soy protein isolate (Hang Wan *et al.,* 2005; Tang *et al.,* 2003; Rhim *et al.*, 1999), whey protein isolate (Stuchell and Krochta, 1995), wheat gluten (Zhang *et al.,* 2004), egg white (Gennadios *et al.,* 1996) and fish myofibrillar protein (Cuq *et al.,* 1997a; Shiku *et al.,* 2003; Chinnabhark *et al.,* 2007). Protein-based films generally have the superior mechanical and barrier properties to polysaccharide-based films. Proteins consisting of about 20 amino acids have a specific structure which confers a wider variety of functional properties, compared with polysaccharides which are mostly homopolymers. Furthermore, inter-

and intra-interaction between protein molecules, such as hydrogen bonds, ionic-ionic interactions, hydrophobic interactions and covalent bonds, could be formed during drying condition (Chinnabhark *et al.,* 2007; Iwata *et al*., 2000). Properties of protein-based films are most likely dependent on the protein sources which are different in sequential order of the amino acids, protein structure and the degree of structure extension (Iwata *et al*., 2000).

3.1 Approaches for protein film formation

Several approaches can be used to form protein films (Stuchell and Krochta, 1995) as follows:

3.1.1 Simple coacervation

A Single hydrocolloids is driven from aqueous suspension or caused to undergo a phase change by evaporation of solvent, addition of a water-miscible nonelectrolyte in which the hydrocolloids is not soluble (e.g., alcohol), addition of an electrolyte to cause salting out or crosslink, or alteration of pH.

3.1.2 Complex coacervation

Two solutions of oppositely charged hydrocolloids are combined, causing interaction and precipitation of the polymer complex.

3.1.3 Thermal gelation or precipitation

A sol-gel transformation can occur by heating of a protein to cause denaturation followed by gelation (e.g., egg albumin) or precipitation, or simple cooling of a warm hydrocolloid suspension.

3.2 Mechanism of protein film formation

Protein-based films can be formed in three steps (Figure 2.) (Marquie and Guilbert, 2002):

3.2.1 Break intra- and inter-molecular bonds (non-covalent and covalent bonds) that stabilize polymers in their native forms by using chemical or physical rupturing agents (by solubilization or thermal treatment). As a result, polymer chains became mobile.

3.2.2 Arrange and orient mobile polymer chains in the desired shape.

3.2.3 Allow the formation of new intermolecular bonds and interactions to stabilize the three-dimensional network. The shape obtained in step 2 is maintained by eliminating agents used in step 1 (e.g., solvent removal or cooling).

Based on these three steps, solvent process is based on dispersing and solubilizing the proteins in various solvents and then casting, spraying, or dipping, followed by drying. This process has been extensively studied and applied to produce films from various proteins, particularly from myofibrillar proteins (Cuq *et al*., 1995).

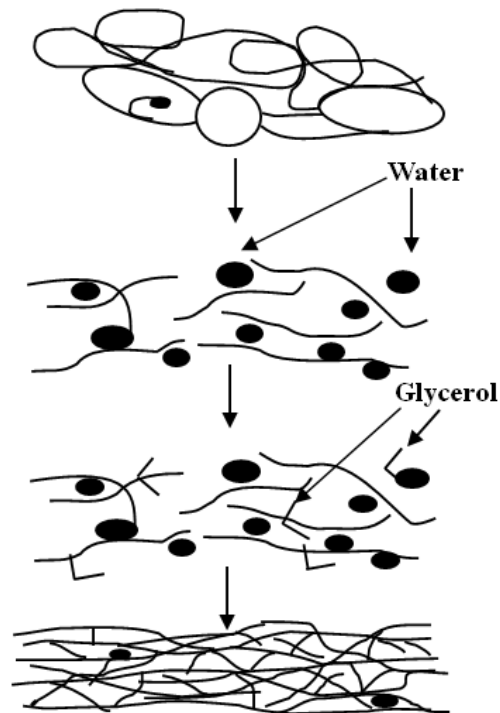


Figure 2. Mechanism of film formation.
Source: Adapted from Marquie and Guilbert (2002)

3.3 Basic properties of protein-based films

Protein films possess different properties depending upon the sources of protein, protein concentration, extrinsic factors, etc.

3.3.1 Barrier properties

Protein films provide the advantage of being excellent oxygen and carbon dioxide barriers (Gennadios *et al*., 1993), but their hydrophilic nature makes them rather ineffective moisture barrier (McHugh and Krochta, 1994; Roy *et al.*, 2000). Park and Chinnan (1995) investigated the effected of film thickness on the properties of protein films from corn-zein and wheat gluten. Barrier property of protein films decrease with increasing film thickness. However, barrier property can

be varied with the source of protein, which can be associated with amino acid composition (Table 1) (Cuq *et al*., 1995).

Table 1. Water vapor permeability of various protein films.

Film	Water vapor permeability ($x10^{-12}$ mol.m/m^2.s.Pa)	Temp (°C)	RH (%) conditions	Thickness ($x10^{-6}$m)
Sodium caseinate film	24.7	25	100 – 0	-
Soy protein film (pH 3)	23.0	25	100 – 50	83
Corn zein film	6.45	21	85 – 0	200
Wheat gluten film	5.08	30	100 – 0	50
Myofibrillar protein film	3.91	25	100 – 0	60

Source: Adapted from Cuq *et al.* (1995)

3.3.2 Mechanical properties

Mechanical properties of protein film are generally poorer than synthetic films (Cuq, 2002; Gennadios *et al.,* 1994). Several factors, including surface charges, hydrophobicity, polymer chain length, etc., may significantly affect the mechanical properties of protein films (Kester and Fennema, 1986). Hydrogen bonds are considered important in contributing to the tensile strength (TS) of protein films (Krochta, 2002). Type and level of plasticizer have a dramatic effect on film properties (Shellhammer and Krochta, 1997; Cuq, 2002). Lim *et al*. (1998) reported that egg white films with higher glycerol contents had greater elongation at break (EAB) values. Myofibrillar protein based-films had greater TS and lower EAB values when compared with other films (Table 2). The distribution and concentration of inter- and intra-molecular interactions allowed by primary and spatial structure most likely affect the mechanical properties of myofibrillar protein-based films.

3.3.3 Solubility property

Film solubility is an important property that relates to intended use. High molecular weight proteins are insoluble or slightly soluble in water and thus have potential for forming water-resistant films (Cuq, 2002). Low molecular weight protein chains such as monomers and small peptides, formed during the film-forming solution and immobilized in the film network, could thus constitute the water-soluble

proteinic component of the films (Cuq *et al.,* 1995). Regardless of plasticizer type (glycerol, sorbitol or sucrose), the increase in plasticizer content in the film normally increased the water-soluble dry matter content. In general, hydrophilic plasticizers enhance water solubility of the protein film (Cuq, 2002; Shiku *et al.,* 2004). Cuq *et al.* (1996a) reported that the thickness variation of myofibrillar protein-based films seemed to have no influence on percent solubility in water. Shiku *et al*. (2004) reported that the film solubility of surimi films was not significantly affected by the quality of surimi.

Table 2. Tensile strength and elongation at break of various protein-based films.

Film	Tensile strength (MPa)	Elongation at break (%)	Thickness ($x10^{-6}$m)
Fish myofibrillar protein	17	23	34
Whey protein isolate	14	31	110
Soy protein (pH 9)	3.6	160	83
Wheat gluten (pH 11)	3.3	192	150
Corn zein	3.9	213	67

Source: Adapted from Cuq (2002)

3.4 Protein-based films from different sources

3.4.1 Wheat gluten films

Wheat gluten is defined as the water-insoluble protein of wheat flour. Wheat gluten contains the prolamine and glutelin fractions of wheat flour protein, typically referred to as gliadin and glutenin, respectively (Krochta, 2002). Gliadin is soluble in 70% ethanol, but glutenin is not. Both gliadin and glutenin fractions of wheat gluten contain intramolecular disulfide bonds. Intermolecular disulfide bonds, which link individual glutenin protein chains, result in the larger polymers with high molecular weight. The extensive intermolecular interactions in wheat gluten result in quite brittle films with poor water-vapor barrier properties (Gennadios and Weller, 1990). Herald *et al.* (1995) reported that films prepared from spray-dried (SD) and flash-dried (FD) wheat gluten had differences in properties. Films from wheat gluten are comparable to plastic wrap for most properties except water vapor permeability.

SD wheat gluten film exhibited a higher tensile strength (TS) than did the FD wheat gluten films and plastic wrap.

3.4.2 Casein films

Casein, which comprises 80% of milk protein, precipitates when skim milk is acidified to the isoelectric pH, approximately of 4.6 (McHugh and Krochta, 1994). Film formation of aqueous casein solution without heat treatment was due to their random-coil nature. Interactions in the film matrix likely include hydrophobic, ionic and hydrogen bonding (Avena-Bustillos and Krochta, 1993).

3.4.3 Whey protein films

Whey protein comprising 20% of milk protein is the protein that remains soluble after casein is precipitated at pH 4.6. Whey protein consists of several proteins, which are globular and heat labile in nature (McHugh *et al*., 1994). Because of the globular nature of whey proteins, the formation of films requires heat denaturation to open the globular structure, break existing disulfide bonds, and form new intermolecular disulfide and hydrophobic interactions (McHugh *et al*., 1994). McHugh *et al*. (1994) suggested that the best film formation conditions were 10% (w/w) protein solutions with neutral pH and heated for 30 min at 90ºC.

3.4.4 Corn zein fillms

The zein, which is prolamine, is soluble in 70% ethanol. In term of the amino acid composition, zein has a high content of nonpolar hydrophobic amino acids such as leucine, alanine and praline. Zein also contains a high level of glutamic acid (about 20-22%), which exists mostly as glutamine. Glutamine contributes to the insolubility of zein in water (Gennadios and Weller, 1990). Therefore, zein films are generally cast from alcohol solutions (Gennadios *et al*., 1993). The interactions formed in the film matrix likely include hydrophobic interaction, hydrogen bonding and disulfide bond (Ghanbarzadeh *et al.,* 2007).

3.4.5 Myofibrillar protein fillms

Fish muscle proteins consist of sarcoplasmic proteins, myofibrillar proteins and stroma proteins. These proteins are capable of forming a continuous films matrix (Garcia and Sobral, 2005; Sobral *et al.*, 2005). The edible films or biodegradable films based on fish myofibrillar protein have been developed by solution casting process (Sobral *et al.*, 2005; Cuq *et al*., 1995). Various factors

affecting film formation and film properties included protein concentration, pH, temperature and storage time before film casting (Cuq *et al.*, 1995). Cuq *et al.* (1995) found that the pH and protein concentration had strong interactive effects on viscosity of FFS from Atlantic sardines myofibrillar protein. During FFS storage before casting, partial degradation of high molecular weight protein components led to decreased viscosity allowing thin layer casting. They also reported that the optimum film forming condition was at pH 3, 2.0 g protein/100g FFS, 25°C and 6 hr storage. The functional properties of the resulting film were slightly better than other protein-based films (such as whey protein, soy protein, wheat protein and corn zein films), with tensile strength close to those of low density polyethylene films.

3.4.6 Other protein-based films

Various other proteins can be used to prepare biodegradable films or edible films such as gelatin (Simon-Lukasik and Ludescher, 2003), collagen and sarcoplasmic protein (Iwata *et al.*, 2000; Tanaka *et al.*, 2001). Fish sarcoplasmic protein film from blue marlin meat had better flexibility and lower water vapor permeability compared with most of the other protein films. Properties of protein-based films are most likely dependent on the sequential order of the amino acids and protein structure.

4. Fundamental factors affecting properties of protein-based films

4.1 pH

Protein solubility depends on the pH. The net charge of the protein molecule at any given pH is a consequence of the ionization status of all acids. At pH values above or below isoelectric point (pI), all protein molecules have a net charge of the same sign. At pI, protein molecules having large dipoles attract themselves through the countercharged domains and tend to precipitate. This is a general rule that proteins are least soluble at the pI. The isoelectric point of myofibrillar protein is pH = 5 and increasing or decreasing pH from this value results in a more negative or positive charge of the myofibrillar protein (Bertram *et al.*, 2004). Protein film formation is pH dependent. Shiku *et al.* (2003) reported that pH of film-forming solutions had an effect on film formation of edible films based on fish myofibrillar proteins. Myofibrillar protein-based films were formed between pH range of 2-3 and

7-12, whereas films were not formed between pH 4 and 6 because of the poor protein dispersion around the isoelectric point. TS of the films was higher whereas EAB was almost constant irrespective of pH. WVP of myofibrillar protein films was slightly lower than that of other protein-based films and was higher than that of synthetic films. The myofibrillar protein films prepared at acidic and basidic conditions had strong protein networks and their transparency was similar to that of synthetic films. McHugh *et al*. (1994) studied the effect of pH on water vapor permeability of whey protein films. The best film formation was neutral pH, aqueous 10% (w/w) protein solution and heated for 30 min at 90ºC. Differences between WVP values of the films obtained at pHs 7.0, 8.0 and 9.0 were not significant. Cuq *et al.* (1995) examined the effects of protein concentration, pH, temperature and storage time before casting film. The optimal condition for preparing film-forming solution based on myofibrillar protein was at pH of 3, 2 g protein/100g protein solution, 25ºC and 6 h storage time. The TS of the film was close to that of low density polyethylene films. Herald *et al.* (1995) reported that wheat gluten protein film prepared at pH of 3.3 had better TS than that prepared at pH of 10.

4.2 Protein concentration

Protein concentration in filmogonic solution can also influence the formation of protein matrix. The production of films with whey protein isolate, for example, requires a relatively high protein concentration (>8%) in film forming solution (FFS) so that the formation of S-S bridges occurs (Sothornvit and Krochta, 2001). The fish muscle proteins, when made soluble by decreasing pH, provide extremely viscous colloidal solutions (Cuq *et al*., 1995). This way, it is necessary to work with lower protein concentrations in FFS. Cuq *et al.* (1995) developed the condition for film preparation of fish myofibrillar proteins of Atlantic Sardine. The conditions leading to low viscosity FFS, that were to be used to form film, involved pH value between 2.75 and 3.5, and protein concentration between 0.5 and 2.5 g/100g FFS. Iwata *et al*. (2000) prepared FFS with protein concentration between 2-4% of sarcoplasmic proteins of Blue Marlin at pH 10 and heated at 70ºC for 15 min. No significant difference was observed in the tensile strength of films formed from different protein concentrations. In contrast, elongation at break of films increased with increasing protein content. When the protein concentration of FFS was 1.5%, the

film formed was too thin to be peeled off. On the contrary, the formation of films from FFS with more than 4.5% protein was inhibited due to high viscosity. Sobral *et al.* (2005) reported that the effect of protein concentration was observed mainly upon the mechanical properties of Thai Tilapia muscle protein films. The films prepared with 2 g of protein/100g of FFS were more force resistance than those with 1 g of protein/100g of FFS. Chinabhark *et al.* (2007) reported that protein concentration and pH affected the mechanical properties and color of myofibrillar protein film from bigeye snapper surimi. Protein content influenced the mechanical properties and color of films. Film with grater protein content (2%) prepared at acidic condition exhibited higher tensile strength. But similar elongation at break was found between film with 1% and 2% protein content at the same pH used. The film with 2% protein content had more yellowness but lower lightness than those having 1% protein content. And the film was more transparent when the lower protein was used.

4.3 Plasticizers

In addition to the film-forming biopolymer, a major component of edible films is the plasticizer. The addition of a plasticizing agent to edible films is required to overcome film brittleness caused by intensive intermolecular forces. The increase in mobility of polymer chains can improve the flexibility and extensibility of the films (Gontard *et al*., 1993). A variety of common plasticizers used in edible films include glycerol, polyethylene glycol (PEG), sorbitol, propylene glycol (PG) and ethylene glycol (EG), monosaccharide, disaccharide or oligosaccharide, lipids and their derivatives (Yang and Paulson, 2000; Irissin-Mangata *et al*., 2001; Gontard *et al.,* 1993). In general, addition of plasticizer, especially polyols, decreased the mechanical resistance and increased the flexibility and water vapor permeability of the films (Pascholick *et al*., 2003; Irissin-Mangata *et al*., 2001). Jangchud and Chinan (1999) studied the preparation of films from peanut protein. Glycerol was found to be the most suitable plasticizer in peanut protein films, showing the highest mechanical properties when compared with sorbitol, polyethylene glycol and propylene glycol but poor WVP. McHugh *et al*. (1994) studied the effects of various plasticizers on the WVP of whey protein films. Glycerol plasticized whey protein films exhibited lower WVP than films plasticized with PEG 200 and PEG 400 or sorbitol. Orliac *et al.* (2003) reported that types of plasticizers had the effect on homogeneity of films.

Shaw *et al.* (2002) investigated the effects of glycerol, sorbitol and xylitol on the properties of whey protein isolate (WPI) films. Increasing glycerol or sorbitol content led to increase in WVP, EAB and the decrease in TS. However, increasing xylitol had no effect on mechanical properties of WPI films. Tanaka *et al.* (2001) reported that the type and concentration of plasticizers affected the mechanical properties and WVP of edible films from fish water soluble protein. Glycerol and polyethylene glycol (PEG) gave the flexible films. As the concentration of glycerol increased, TS of the films decreased with concomitant increase of EAB and WVP. In contrast, PEG showed more marked influence on TS than on EAB. Films containing a glycerol:PEG ratio of 2:1 exhibited the maximum EAB, while the increase ratio of glycerol to PEG reduced the water vapor barrier property of the films. Sobral *et al.* (2005) studied the effect of plasticizer concentrations (15-65% based on protein) in FFS (pH 2.7) on the physical properties of edible films based on muscle protein of Thai Tilapia. Properties of the resulting films were affected by the plasticizer concentration. The increase of glycerin content caused reduction in color difference (ΔE^*), opacity, tensile strength, elastic modulus but increase in elongation at break of the films.

4.4 Heat treatment

Temperature is a strong denaturing factor for proteins, although the thermal stability and conformation of each protein depend on the amino acid composition. Globular protein, such as sarcoplasmic protein and whey protein have to be thermally denatured in order to form a continuous matrix (Iwata *et al.*, 2000). Furthermore, during the drying period, when water is progressively eliminated, protein conformation changes and the degree of protein unfolding determines the type and proportion of covalent (S-S bonds) or non-covalent (hydrophobic interaction, ionic and hydrogen bonds) interactions that can be established between protein chains. It is known that protein chains can interact more strongly and easily, especially by disulfide bonds, when proteins are heat denatured. So, the cohesion of the final network would be a function of these bonds and determines the properties of the films obtained (Denavi *et al.*, 2009).

Paschoalick *et al.* (2003) reported the effect of glycerol content and thermal treatment on the functional properties of films based on sarcoplasmic proteins of Nile Tilapia. Sarcoplasmic protein films were prepared by casting technique as

follow: 1 g of protein/100 g of FFS, 15-65 g of glycerol/100 g of protein, pH 2.7 and thermal treatment of 40, 65 and 90ºC/30 min. The increasing in temperature of FFS thermal treatment caused an increase in water vapor permeability of the films, especially that the films prepared from FFS treated at 90ºC/30 min were more permeable. Heat treatment also caused a slight increase in film color, possibly due to the occurrence of reaction among the glycerol molecules and the reactive group of lysine. Moreover, the mechanical properties (puncture force and puncture deformation) of the films were increased with increasing in temperature of FFS, except at 90ºC/30 min.

Iwata *et al*. (2000) investigated the influence of FFS heat treatment (heating temperature and time) on the properties of films from sarcoplasmic protein of blue marlin. Films were prepared by adjusting the protein concentration and pH of FFS at 3% and 10, respectively, and FFS were heated at the heating temperatures ranged between 55 and 90ºC for 15 min. The maximum tensile strength was afforded by heating at 70ºC. Moreover, films prepared from FFS heated at 70ºC for more than 3 min had similar tensile strength and elongation at break.

5. Poly(vinyl alcohol)

Poly(vinyl alcohol) (PVA) is a water-soluble synthetic polymer. It is commercially produced by hydrolysis of poly(vinyl acetate). PVA is typically in a dry solid and available in granular or powder form. A wide range of grades, depending on molecular weight and degree of hydrolysis, is offered by PVA manufacturers. However, two main types of PVA, partially hydrolyzed (Figure 3. (A)) and fully hydrolyzed (Figure 3. (B)), are of industrial importance (Skeist, 1990; DeMerlis and Schoneker, 2003).

PVA is biodegradable and synthetic water soluble crystalline polymer possessing good film forming property. In general, PVA is excellent adhesive and highly resistant to solvents, oil and grease. PVA forms tough and clear film that has high tensile strength and abrasion resistance. Its oxygen-barrier properties are superior to those of any known polymers; however, PVA must be protected from moisture, which greatly increased its gas permeability (Skeist, 1990).

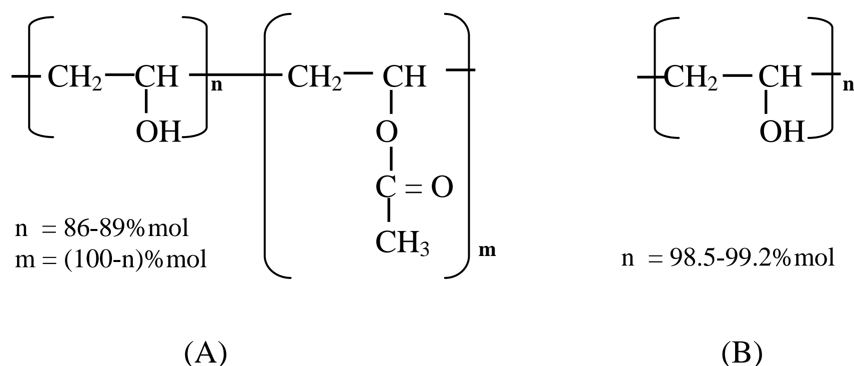


Figure 3. Structural formula of polyvinyl alcohol: (A) partially hydrolyzed; (B): fully hydrolyzed.

Source: DeMerlis and Schoneker (2003)

The physical properties of PVA are controlled by its molecular weight and the degree of hydrolysis. The upper portion of Figure 4 shows the variation in properties with molecular weight at a constant degree of hydrolysis. Also, PVA properties depend on degree of hydrolysis with a constant molecular weight as given in the lower portion of Figure 4 (Skeist, 1990).

The wide range of chemical and physical properties of PVA has led to their broad industrial use. In the USA, the majority of PVA is used in the textile industries as a sizing agent and finishing agent. PVA can also be incorporated into a water-soluble fabric in the manufacture of degradable protective apparel, laundry bags for hospital, rags, sponges, sheets, covers, as well as physiological hygiene products (DeMerlis and Schoneker, 2003; Modern Plastic and Harper, 2000).

PVA is also widely used in the manufacture of paper products. As with textile, PVA is applied as a sizing and coating agent. It provides stiffness to these products making it useful in tube winding, carton sealing and board lamination. PVA is used as a thickening agent for latex paint and common household white glue or in other adhesive mixtures such as remoistenable labels and seals, as well as gypsum-based cement such as is used for ceramic tiles (DeMerlis and Schoneker, 2003). In addition, PVA films are widely used in food packaging. PVA film itself offers good tensile strength (TS), excellent flexibility and toughness as well as good gas and aroma barrier properties (Park *et al*., 2001).

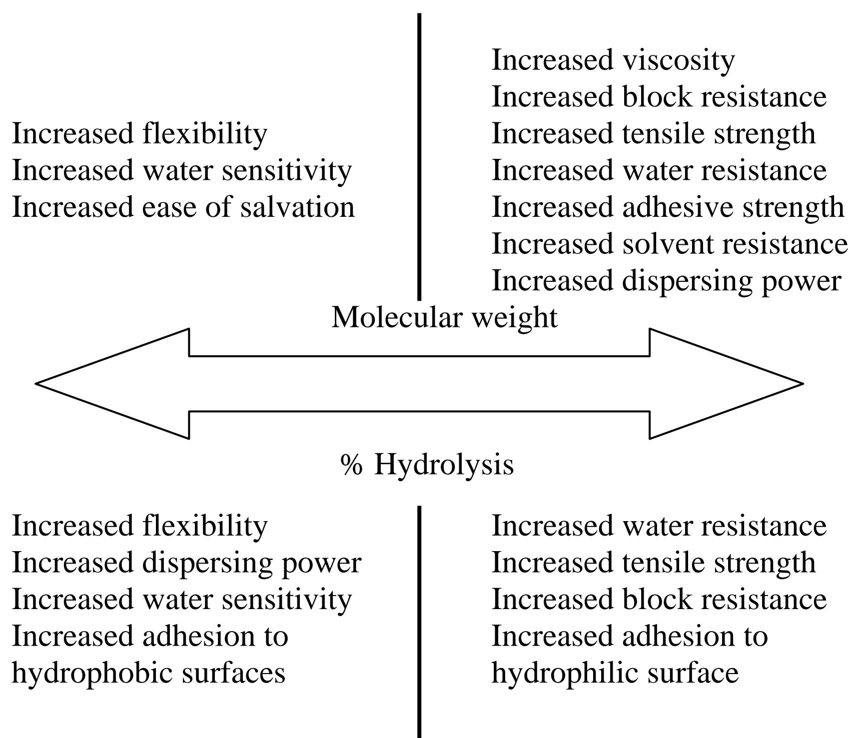


Figure 4. Properties of poly (vinyl alcohol).

Source: Skeist (1990)

6. Some approaches for property improvement of protein films or biodegradable film

6.1 Polymer blend technique

Polymer blend technique is a mixing of two or more polymers together to produce blend, for achieving a specified portfolio of physical properties without the need to synthesize specialized polymer system (Hope and Folkes, 1993). Polymer blend is one of the effective methods for providing new desirable polymeric materials for a variety of applications. Biodegradable blend films, which contain mixture of biopolymer and other biodegradable polymers, either natural or synthetic, have been developed to improve the properties of blend films. Low density polyethylene is the most studied synthetic polymer blended with biopolymers. However, this polymer needs chemical treatment to become biopolymer compatible (Davis, 2003; Kim and Lee, 2002). Another synthetic polymer, poly(vinyl alcohol) (PVA), has been used for the implementation of the mechanical properties of films based on polysaccharides

and proteins, since its hydrophilic and filming character allows for some degree of compatibility with functional natural polymeric materials (Silva *et al*., 2008; Kim *et al*., 2002; Zhang *et al*., 2004).

- Protein/PVA blend films

PVA is the polymer of choice being used to blend with various biopolymers and hydrophilic synthetic polymers, due to their great compatibility (Mansur *et al*., 2008). Silva *et al.* (2008) reported the effect of PVA type and concentration on the properties of biodegradable blend films based on pig skin gelatin and PVA. The blends from five types of PVA with different degree of hydrolysis (DH) (88.0-99.7%), allowed for films with different characteristics but with no direct relationship with the DH of PVA. The increasing PVA content could improve the flexibility of gelatin film in which its tensile strength and puncture resistance decreased with increasing PVA content, but its puncture deformation and elongation at break increased with PVA addition increased.

Zhang *et al*. (2004) studied the properties of wheat proteins (WP)/PVA blend film containing water and glycerol as plasticizers. When blending PVA at 10-25% with WP, the tensile strength and modulus of the blend films were increased by 21-54% and 15-30%, respectively, as compared to those of WP film. However, the elongation of the films was decreased when the PVA component in the blends was increased. Alexy *et al*. (2003) investigated processing parameters and mechanical properties of PVA and collagen hydrolysate (CH) thermoplastic blend film. The addition of CH in the formulations yielded blends with good thermal processability and the resulting films exhibited valuable practical mechanical properties. Tensile strength and elongation at break of the films were not negatively influenced by addition of CH up to 25% in the PVA/CH blends.

6.2 Use of chemicals/ additives

6.2.1 Cross-linking agents/protein modifiers

Properties of protein films can be potentially modified via chemical, physical or enzymatic treatment of protein to enhance the functional properties of films (Gennadios and Weller, 1990; Guilbert *et al.*, 1996). Protein chains possess reactive side groups, which can be modified. Cross-linking agents such as glutaraldehyde, glyoxal or formaldehyde have been widely used to cross-link proteins

(Hernandez-Munoz *et al.,* 2004a; Marquie*,* 2001; Ustunol and Mert, 2004). Cross-linking agents are able to form covalent inter- and/or intra-molecular links between protein chains (Gennadios and Weller, 1990). Incorporation of cross-linking agents could be an alternative means to yield a better structuring of the polymeric matrix. An orderly polymer forming matrix could result in better functional film properties (Carvalho and Grosso, 2004). Carvalho and Grosso (2004) reported that formaldehyde was more efficient in enhancing the mechanical properties of protein films, compared to other cross-linkers. Additionally, the mechanical properties of protein-based films can be improved by transglutaminase (TGase, protein glutamine γ-glutamyltransferase, E.C. 2.3.3.13) (Marinello *et al*., 2003; Carvalho and Grosso, 2004). Transglutaminase catalyzes the formation of strength of films (Faergemand and Qvist, 1997; Mahmoud and Savello, 1993; Marinello *et al.*, 2003). Carvalho and Grosso (2004) examined the cross-linking efficiency of transglutaminase (10 unit/g protein), glyoxal (26.5 mM/100 ml) and formaldehyde (8.8 mM/100 ml) in gelatin films. Film cross-linked by transglutaminase and glyoxal exhibited the lowest TS. In contrast, transglutaminase treated gelatin film yielded the highest water vapor barrier property. Protein cross-linking by glyoxal involves a key reaction with arginine guanidyl groups. This reagent is preferentially active at alkaline pH (Marquie, 2001). Marquie (2001) reported the cross-linking reaction of protein by glyoxal under alkaline conditions during the preparation of cottonseed protein-based film as present in Figure 5. In addition, protein cross-linking by biepoxy compound was reported by Tomihata *et al.* (1994), who studied on the use of biepoxy compound cross-linking agent for collogen and gelatin films. They reported the cross-linking reaction between amino groups of protein and biepoxy compound as depicted in Figure 6. Cross-linking can improve the tensile properties of these materials to such a level as they are handled with ease.

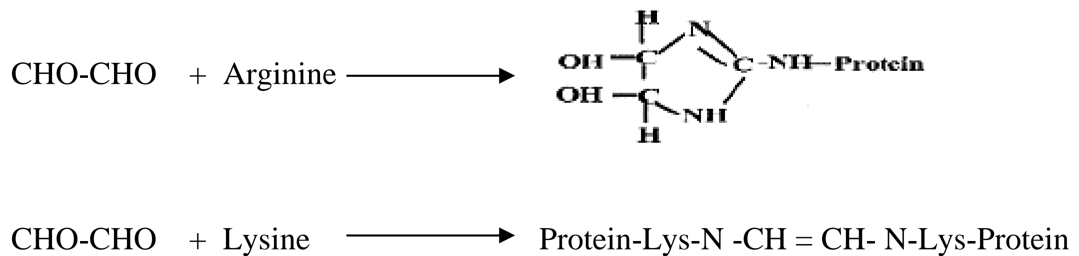


Figure 5. Postulated mechanism of protein cross-linking by glyoxal.
Source: Adapted from Marquie (2001)

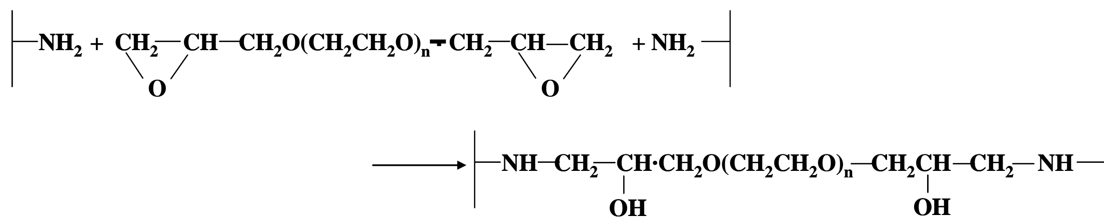


Figure 6. Reaction of protein cross-linking by biepoxy compounds.
Source: Tomihata *et al.* (1994)

Chemical modification of proteins includes the derivatization of the amino acid side chains of proteins as well as hydrolysis of the peptide bonds. The versatile chemical and physical behavior of proteins stems from their complex structure. Thus, the permutations and combinations in the arrangement of high molecular mass chains lead to numerous possibilities for modification. The typical chemical reactions are often classified based on the type of reagent used. Succinic anhydride introduces anionic succinate residues to the ε-amino group (Figure 7). As a result, the net charge on the protein becomes negative and is accompanied by major conformational changes and greater solubility. Although the principal reaction of succinic anhydride and proteins is through the amino groups of lysine, secondary reaction occur via histidine and tyrosine residues and with aliphatic hydroxyl and sulfhydryl group (Nakai and Modler, 1996).

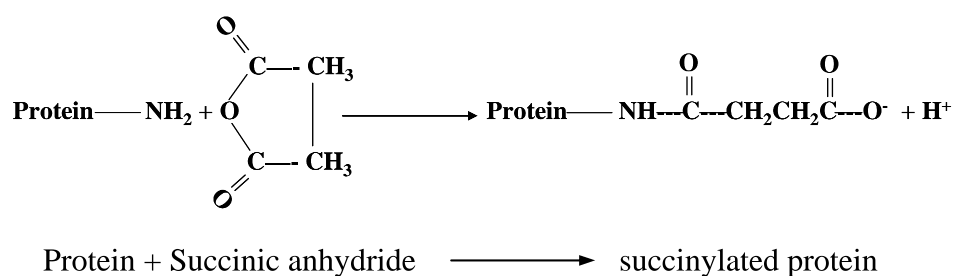


Figure 7. Reaction of succinic anhydride and protein.
Source: Nakai and Modler (1996)

Maleic anhydride reacts with proteins in a similar way to succinic anhydride but results in products that are labile to hydrolysis. It is there for a useful reagent for the reversible modification of amino groups (Nakai and Modler, 1996). Moreover, maleic anhydride was used as a compatibilizer of polymer blend system. Jose *et al.* (2006) reported the properties of polyamide (PA) 12/polypropylene (PP) blends influenced by reactive compatibilizer. The compatibilizer used was maleic anhydride (MA) functionalized polypropylene (PP-g-MA). The amount of compatibilizer was varied from 1 to 20 wt% of compatibilizer was added into the PA12/PP blends. The compatibilization stabilized the morphology of blends by reducing the particle size as well as interparticle distance and enhancing the interfacial area and interface adhesion. The particle size of disperse phase decreased with the compatibilizer concentration increased and the optimum compatibilizer concentration was found at 5% (wt). Moreover, it was found that compatibilization significantly improved the mechanical properties of PA12/PP blends. A good correlation has been observed between the mechanical properties and morphological parameter. The mechanism of the interfacial chemical reactions is based on (a) the amine-anhydride reaction which involves an acid/amide intermediate that cyclices to produce an imide group and a water molecule (Figure 8 (a)), or (b) an amide-anhydride mechanism which involves an acid/imide intermediate which cylclices, leading to a cyclic imide and an acid chain end (Figure 8 (b)).

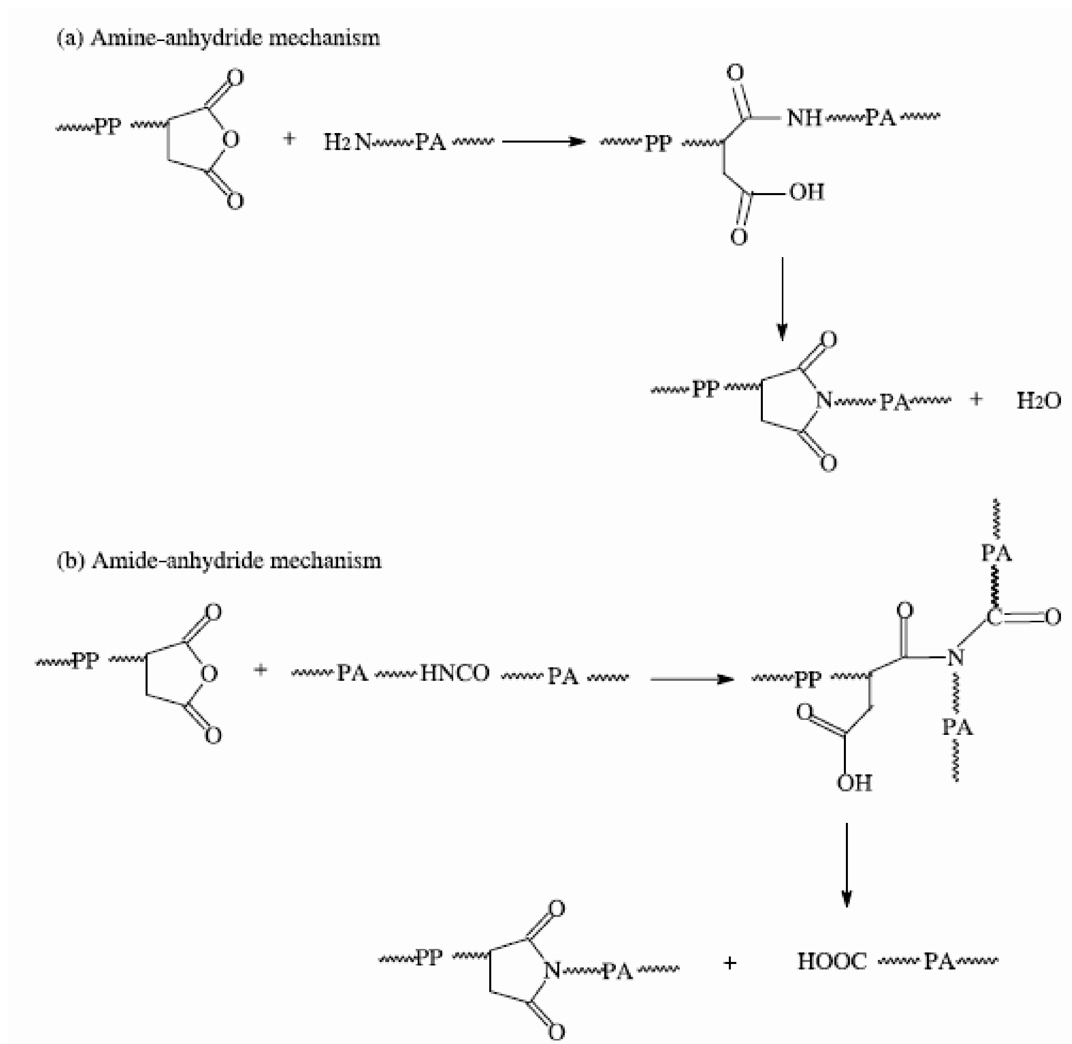


Figure 8. The mechanism of interfacial chemical reaction between PA12 and PP-g-MA: (a) amine-anhydride mechanism, (b) amide-anhydride mechanism.
Source: Jose *et al.* (2006)

Furthermore, the properties of blend films based on biopolymer and poly(vinyl alcohol) could be enhanced by chemical treatment. Kim *et al*. (2002) reported the used of epichlorohydrin (ECH) as a cross-linking agent between hydrolyzed starch-g-poly(acrylonitrile) (HSPAN) and PVA blend film to overcome the phase separation and improve the mechanical properties of blend films. The absorbency of HSPAN/PVA blend films decreased with PVA contents due to the reduction of HSPAN contents and also decreased with the ECH contents due to the cross-linking. The compatibility of HSPAN/PVA blend films was improved by the cross-linking reaction with ECH between hydroxyl group of starch and PVA, resulted

in enhanced mechanical properties and water resistance. Ray *et al.* (2009a) prepared starch/PVA blend film at two different ratios (starch:PVA =60:40 and 50:50) incorporated with glycerol at 30% wt (based on dry weight of polymer) and 20% wt epichlorohydrin (ECH) (based on dry weight of polymer). Tensile property (tensile strength, elastic modulus and energy at break) of the blend at starch/PVA=50:50 was enhanced by cross-linking of ECH between starch and PVA through covalent bonds (Figure 9).

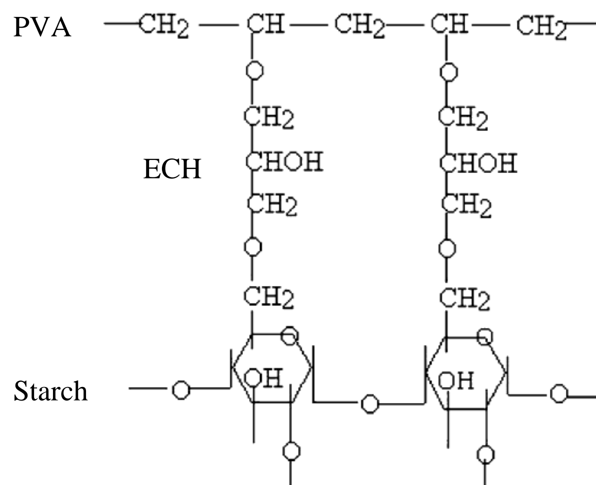


Figure 9. The cross-linking structure of starch, poly(vinyl alcohol) and epichlorohydrin.
Source: Ray *et al.* (2009a)

6.2.2 Other additives

Besides the use of protein modifier, incorporation of other additives such as oil/lipid and nano fillers has been used to modify the properties of protein and other biopolymer films. Oil and lipids are non-polar hydrophobic substances wildly used as a barrier against moisture migration (Morillon *et al.,* 2002). Sunflower oil was added to cod skin gelatin at various concentrations (0%, 0.3%, 0.6%, and 1%) to improve the hydrophobic properties and decrease water vapor permeability and soluble matter content of gelatin films. Added oil increased film thickness, whiteness, optical absorbance and decreased film transparency. Water vapor permeability of the film did not decrease when oil was added, though oil added yielded more insoluble films due to oil-protein interactions. Furthermore, Fourier-transform infrared (FTIR)

spectra revealed some lipid-protein interaction (hydrogen bonds, ester formation) and early oil oxidation (Perez-Mateos *et al*., 2009). Prodpran *et al*. (2007) reported the effect of palm oil and chitosan on the properties of film from round scad muscle protein. Films added with 25% palm oil (as glycerol substitution) had the slight decrease in water vapor permeability (WVP) and elongation at break (EAB) of films. WVP and tensile strength (TS) of films increased but EAB decreased when 10-40% chitosan (as protein substitution) was incorporated. Hydrophobic interactions and hydrogen bonds, together with disulfide and non-disulfide covalent bonds played an important role in stabilizing the film metrix. The a* and b*-value increased with increasing chitosan levels. Films added with chitosan were less transparent and had the lowered transmission in the visible range. The incorporation of 25% palm oil and 45% chitosan yielded the films with the improved TS but decreased water vapor barrier property.

Recently, polymer nonocomposites have received a great interest due to the ability of nanosized material fillers to improve polymer properties when compared with single polymer or micro-scale composites. The potential improvements include enhanced mechanical strength, weight reduction, increased heat resistance and improved barrier properties (Ray and Okamoto, 2003). Biodegradable nanocomposites were successfully fabricated from corn starch and montmorillonite (MMT) nano-clay by melt extrusion processing (Tang *et al*., 2008). Sothornvit *et al*. (2009) reported the effect of nano-clay types (Cloisite Na^+, Cloisite 20A and Cloisite 30B) on the properties of whey protein isolate (WPI)/clay composite films. The nanocomposite films exhibited an opaque appearance and haze, and degree of this effect depended on type of nano-clays added. The type of nano-clay used significantly influenced the tensile and water vapor barrier properties of the composite films with the exception of Cloisite 30B, which had no negative effect. Tensile properties (tensile strength, tensile modulus and elongation at break) of WPI/Cloisite Na^+ or 30B composite films did not significantly decrease as compared with those of WPI pure film. On the other hand, all tensile properties of WPI/Cloisite 20A film were lower than those of WPI pure film. This may also be due to the incomplete dispersion of the nano-clay (Cloisite 20A) into the polymer metrix, which is caused by the incompatibility of hydrophobic nanoclay with hydrophilic biopolymer. The

WVP of WPI/clay composite films changed with type of nano-clays used. The WVP of WPI/Cloisite Na^+ decreased the most followed by WPI/Cloisite 30B and WPI/Cloisite 20A. In addition, the WPI/Cloisite 30B composite films exhibited remarkably significant bacteriostatic effect against Gram-positive bacteria, *L. monocytogenese.* Bae *et al.* (2009) investigated the influences of clay content, homogenization rpm, pH and ultrasonication on the mechanical and barrier properties of fish gelatin/montmorillonite nanocomposite films. The addition of 5% nano-clay (w/w) increased the film tensile strength and oxygen and water barrier properties. The ultrasonically treated nanocomposite films exhibited an exfoliated type structure with improved tensile strength and barrier properties, and the films produced were uniform in thickness and relatively transparent.

7. Stability of films during storage

Properties of edible/biodegradable films generally vary at the same degree with storage time, especially if compared with those of synthetic films, due to the intrinsic instability of their raw materials. These variations could affect their functionality on foods, and, therefore, a high degree of stability of film properties for a long time is generally desired.

When the films are exposed during storage time to certain environmental conditions, it is possible to observe both physical and chemical changes in their nature. Chemical changes, such as oxidation of the protein sulfhydryl groups could cause degradation of the polymeric chains (Micard *et al.*, 2000). Physical changes include polymeric recrystallization (as the retrogradation procuced in starch films) and those due to the migration of low molecular weight components, such as plasticizers, used in film formulation (Anker *et al.*, 2001). This migration of additives can be considered the most important cause of physical instability of films. Park *et al.* (1994) reported that glycerol could migrate slowly from the film bulk to the surface of gluten-based films during storage at 25ºC and 50% RH, even when glycerol was initially well dispersed in the film-forming solution. Sommanathan *et al.* (1992) found the changes in mechanical property of casein film treated with triethanolamine during storage at 25ºC and 65% RH for 1 year. Film became yellowish and considerably less resistant. Cuq *et al.* (1996b) studied the stability of

myofibrillar protein-based films during storage for 8 weeks at 20ºC and 58.7% RH. The solubility in water, WVP and mechanical properties of film remained constant for 8 weeks of storage. However, those films turned yellowish and discolored due to non-enzymatic browning.

Objectives

1. To prepare and investigate the properties of fish myofibrillar protein (FMP) and poly(vinyl alcohol) (PVA) blend films.

2. To study the effects of some factors (pH, blend composition, PVA types with different degree of hydrolysis and molecular weight) on properties of FMP/PVA blend films.

3. To investigate the effect of some chemicals on properties of FMP/PVA blend films.

4. To monitor the changes in properties of films from FMP/PVA blend during storage.

CHAPTER 2

RESEARCH METHODOLOGY

1. Material

1.1 Raw material

Fresh bigeye snapper (*Priacanthus tayenus*) (Figure 10) with an average weight of 200-250 g were obtained from the dock in Songkhla within 48 h after capture. The fish were washed with tap water and stored in ice until used.

Figure 10. Photograph of bigeye snapper (*Priacanthus tayenus*).

1.2 Chemicals

Sodium chloride, magnesium chloride, sodium nitrite, potassium chloride, urea and sodium dodecylsulfate (SDS) were purchased from Univar (New South Wales, Australia). Lithium chloride, potassium acetate, potassium carbonate, magnesium nitrate and phthalic anhydride were purchased from Unilab (New South Wales, Australia). Glycerol, Coomassie Brilliant Blue G250 and chloromethyloxirane (epichlorohydrin) were purchased from Wako Pure Chemical Industry, Ltd. (Tokyo, Japan). Methanol, trichloroacetic acid (TCA), sodium hydroxide and hydrochloric acid were obtained from Merk (Darmstadt, Germany). β-mercaptoethanol (β-ME) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acrylamide, *N,N,N'N'*-tetramethylethylenediamine (TEMED), bis-acrylamide, potassium persulfate, glyoxal (40% in water) and maleic anhydride were procured from Fluka Chemical Co. (Buchs, Swizerland). Polyvinyl alcohol (PVA) was purchased from Dusit Chemical Co., Ltd. (Bangkok, Thailand). Figures 11-13 show chemical structures of glyoxal,

maleic anhydride, phthalic anhydride and epichlorohydrin (ECH) used in this study.

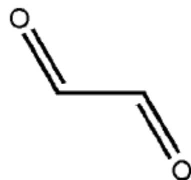
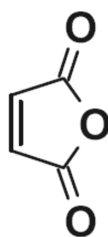
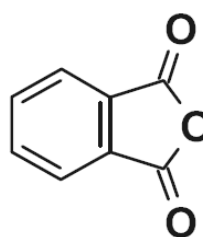


Figure 11. Chemical structure of glyoxal.

Source: Shangari *et al.* (2006)





(A) (B)

Figure 12. Chemical structures of maleic anhydride (A) and phthalic anhydride (B).

Source: Kshirsagar and Argade (2009)

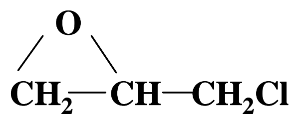


Figure 13. Chemical structure of epichlorohydrin.

Source: Kim *et al.* (2002)

2. Equipment

The equipments used in this experiment are shown in Table 3.

Table 3. List of equipments used in this work.

Equipments	Model	Company/Country
Refrigerated centrifuge	RC-5B plus	Sorvall, California, USA
pH meter	pH/Ion 510	Eutech Instruments Pte Ltd., Singapore
Homogenizer	WIGGEN HAUSER D-500	TE Scientific Sdn. Bhd of Lot 8, Selangor, Malaysia
Magnetic stirrer	Ro 15 power	IKA labortechnik, Stanfen, Germany
Vortex mixer	G-560E	Scientific Indrustries Inc., NY, USA
Shaker	Heidolth Inkubator 10000	Schwabach, Germany
Microcentrifuge	MIKRO20	ZENTRIFUGEN, Hettich, Germany
Electrophoresis apparatus	Mini-Protean II	Bio-Rad Laboratory Int., California, USA
Universal testing machine	LR 30 K	LLOYD Instruments Ltd., Hampshire, UK
Environmental chamber	KBF 115	WTB Binder, Tuttlingen, Germany
Double-beam spectrophotometer	UV-16001	Shimadzu, Kyoto, Japan
CIE colorimeter	Color Flex	HunterLab Reston, Virginia, USA
Fourier transform infrared spectrometer	Bruker Model Equinox 55	Bruker Co., Ettlingen, Germany
Scanning electron microscope	JSM-5800 LV	JEOL, Tokyo, Japan
Differential scanning calorimeter	DSC 7	Perkin Elmer, Norwalk, CT, USA
Thermo-gravimetric analyzer	TGA 7	Perkin Elmer, Norwalk, CT, USA

3. Method

3.1 Preparation of fish myofibrillar protein and compositional analysis

3.1.1 Preparation of fish myofibrillar protein (FMP)

FMP was prepared according to the method of Benjakul *et al*. (2003). The fish were filleted and manually chopped. The fish mince was mixed with 3 volumes of cold distilled water and homogenized at 13,000 rpm for 2 min, followed by filtering through a layer of nylon cloth. The mince was mixed with 5 volumes of 50 mM NaCl for 5 min and filtrated through a layer of nylon cloth. The washing process was repeated twice. Then, washed mince (refered as "FMP") obtained was stored on ice until used for analysis and film preparation.

3.1.2 Compositional analysis of fish myofibrillar protein (FMP)

The washed mince or FMP obtained was subjected to compositional analysis for protein, moisture, fat and ash contents according to AOAC (2000). Protein pattern was determined by SDS-PAGE (using 4% stacking gel and 10% running gel) according to the method of Laemmli (1970). Muscles (3 g) were solubilized in 27 ml of 5% SDS. The mixture was homogenized for 1 min at a speed of 13,000 rpm using a homogenizer (WIGGEN HAUSER D-500, Selangor, Malaysia) and incubated at 85°C for 1 h to dissolve total proteins. The sample was centrifuged at 8,500 xg for 10 min at room temperature using a microcentrifuge (MIKRO20, Hettich Zentrifugan, Germany). Protein (15 μg) determined by the Biuret method (Robinson and Hodgen, 1940) was load onto the gel and subjected to electrophoresis at a constant of 15 mA per gel using a Mini-Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 15 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h.

3.2 Study on effect of pH level and blend composition on properties of fish myofibrillar protein (FMP)/poly(vinyl alcohol) (PVA) blend films

3.2.1 Preparation of film-forming solutions (FFS)

To prepare FMP-FFS, washed mince (or FMP) was added with distilled water to obtain the final protein concentration of 2% (w/v). The mixture was

homogenized at 13,000 rpm for 1 min. Glycerol was then added at 50% (w/w) of protein content. The mixture was stirred gently for 30 min at room temperature. The pH of the mixture was adjusted to 3 or 11 using 1 N HCl and 1 N NaOH, respectively, to solubilize the protein. The solution was filtered through a layer of nylon cloth to remove undissolved debris. PVA-FFS was prepared by adding PVA powder (PVA-BP17: hydrolysis degree of 86-89 %mol and molecular weight of 84,000 – 89,000 g/mol) in distilled water to obtain PVA concentration of 2% (w/v). The mixture was stirred gently at 90°C for 30 min to completely dissolve PVA. Glycerol was then added at 50% (w/w) of PVA content. The pH of PVA solution was adjusted to 3 or 11 using 1 N HCl and 1 N NaOH, respectively. FFSs of the blend were prepared by mixing the designed amount of FMP-FFS and PVA-FFS to obtain the different FMP:PVA ratios (10:0, 8:2, 6:4, 5:5, 4:6, 2:8 and 0:10). Then, FFS was stirred gently at room temperature for 10 min.

3.2.2 Film casting and drying

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

3.2.3 Determination of film properties

3.2.3.1 Film thickness

The thickness of film was measured using a micrometer (Mitutoyo Absolute, Tokyo, Japan). Five random positions of each film of five films were used for thickness determination.

3.2.3.2 Mechanical properties

Prior to the measurement of mechanical properties, the films were conditioned for 48 h in a ventilated oven at 25°C and 50±5% RH. Elastic modulus (E), tensile strength (TS) and elongation at break (EAB) of films were determined as described by Iwata *et al.* (2000) with a slight modification using a Universal Testing Machine (Lloyd Instruments, Hampshire, UK) equipped with tensile load cell of 100 N. Ten samples (2x5 cm^2) with initial grip length of 3 cm were used for testing. The samples were clamped and deformed under tensile load with the cross-head speed of

30 mm/min until the samples were broken. The maximum load and the final extension at break were used for calculation of TS and EAB, respectively. The elastic modulus (E) was calculated as the initial slope of the linear portion of stress-strain curve.

3.2.3.3 Water vapor permeability (WVP)

WVP of films was determined using a modified ASTM D-882 method (1989) as described by Shiku *et al.* (2004). The film was sealed on an aluminum cup containing silica gel (0% RH) with silicone vacuum grease and rubber gasket. The cup was placed at 30°C in a desiccator containing the distilled water. The cup was weighed at 1 h intervals over a 10 h period. WVP of the film was calculated as follows:

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

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

3.2.3.4 Color, light transmittance and transparency value

Color of film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., Reston, Virginia, USA), working with D_{65} (day light). The color parameters were expressed as L* (lightness), a* (redness/greenness) and b* (yellowness/blueness) values. The light transmittance of films was measured at the ultraviolet and visible range (200 – 800 nm) using UV-Vis spectrophotometer (Jasco V530, Tokyo, Japan) as described by Shiku *et al.* (2004). The transparency value of film was calculated by the following equation (Han and Floros, 1997):

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

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

3.2.3.5 Film solubility

Film solubility was determined according to the method of Gennadios *et al.* (1998). A portion of the film (2x4 cm^2) was weighed and immersed in 10 mL of distilled water containing sodium azide (0.1% w/v) to prevent microbial growth. The mixture was shaken at a speed of 250 rpm using a shaker (Heidolth Inkubator 10000, Schwabach, Germany) at 30ºC for 24 h. Undissolved debris was removed by centrifugation at 3000xg for 20 min. The pellet was dried at 105ºC for 24 h using hot air oven (Binder FED115, Tuttlingen, Germany). Film solubility was calculated by subtracting the weight of unsolubilized dry matter from initial weight of dry matter and expressed as a percentage of the total weight.

3.2.3.6 FTIR spectroscopy

Prior to analysis, films were conditioned in a desiccator containing dried silica gel for 7 days at room temperature to obtain the most dehydrated films. The films were scanned with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany). The samples were measured in a horizontal ATR Trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI). The spectra were performed in the 4000 - 650 cm^{-1} regions to resolve overlapping bands.

3.2.3.7 Film morphology

Morphology of surface and freeze-fractured cross section of the film samples were visualized using a scanning electron microscope (SEM) (JSM-5800LV, JEOL, Tokyo, Japan) at an acceleration voltage of 10 kV. 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, and photographs were taken at selected magnification.

The condition yielding film sample which had the highest mechanical properties was chosen for further study.

3.3 Study on effect of PVA types on the properties of FMP/PVA blend films

Six types of poly(vinyl alcohol) (PVA), with different degree of hydrolysis (DH) and molecular weight (MW) (Table 4), were used for film

preparation. Film forming solution of the blend was prepared in the same way as mentioned in section 3.2.1 with selected condition (pH and blend composition).

Table 4. Properties of PVA used in this work.

PVA types	Characteristics		
	DH (%mol)	DP*	MW (g/mol)
PVA-BP05	86- 89	550-650	27,000 – 32,000
PVA-BP17	86- 89	1,700-1,800	84,000 – 89,000
PVA-BP26	86- 89	2,500-2,650	124,000 – 130,000
PVA-BF05	98.5-99.2	500-600	22,000 – 27,000
PVA-BF17	98.5-99.2	1,700-1,800	75,000 – 80,000
PVA-BF26	98.5-99.2	2,500-2,600	112,000 – 120,000

* DP = degree of polymerization.

The films obtained were determined as described in section 3.2.3. PVA type used in FMP/PVA blend which rendered the film with highest mechanical properties was chosen for next study.

3.4 Study on effect of some chemicals on the properties of FMP/PVA blend films

Maleic anhydride, phthalic anhydride, epichlorohydrin and glyoxal at different concentrations (1, 3 and 5% (w/w) of polymeric content) were added into film-forming solution (FFS) after glycerol addition. FFSs were prepared as described in section 3.2.1 and the resulting films were determined as mentioned in section 3.2.3.

The FMP/PVA blend film which had the highest mechanical properties was chosen for further study.

3.5 Film characterization

The film samples obtained from section 3.3, 3.4 and control films (FMP film and PVA film) were subjected to the following analyses:

3.5.1 Film solubility and protein solubility

Film solubility was determined according to the method of Gennadios *et al.* (1998) as mentioned in section 3.2.3.5. To examine the protein solubility, the samples were prepared in the same manner with film solubility test. Protein in supernatant (10 ml) was precipitated by adding 50% (w/v) cold TCA to a final concentration of 10%. The mixture was kept at 4°C for 18 h and centrifuged at 7,500xg for 30 min. The precipitate was washed with 10% TCA and solubilized in 0.5 M NaOH. The protein content was measured using the Biuret method (Robison and Hodgen, 1940). To obtain the total amount of protein, films were solubilized in 0.5 M NaOH. Protein solubility was expressed as percentage of the total protein in film.

3.5.2 Protein solubility in various solvents

To ascertain different kinds of bond/interactions formed in the film matrix, protein solubility of the selected films in various solvents was determined as described by Chawla *et al.* (1996). The solvents used included

S1: 20 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS

S2: 20 mM Tris-HCl (pH 8.0) containing 1 % (w/v) SDS and 8 M Urea

S3: 20 mM Tris-HCl (pH 8.0) containing 1% (w/v) SDS, 8 M Urea and 2 % (v/v) β-ME

The film samples (0.5 g) were homogenized in various solvents for 1 min using a homogenizer (IKA Labortechnik, Malaysia). The homogenate with S3 was heated in boiled water (100°C) for 2 min. All homogenates were stirred at room temperature for 4 h. The resulting homogenates were centrifuged at 7,500xg for 30 min using a microcentrifuge (MIKRO 20, Hettich Zentrifugan, Germany). Protein in supernatant (10 ml) was precipitated by adding 50% (w/v) cold TCA to give a final concentration of 10% (w/v) TCA. The mixture was then kept at 4°C for 18 h and centrifuged at 7,500xg for 30 min. The precipitate was washed with 10% TCA and solubilized in 0.5 M NaOH. The protein content was measured using the Biuret method (Robinson and Hodgen, 1940). To obtain the total amount of protein, films were solubilized in 0.5 M NaOH. The solubility was reported as percentage of the total protein in film.

3.5.3 Protein pattern

Protein patterns of FFSs and their films were determined by SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). FFS (at pH 3) was neutralized using 1 N NaOH. Then, the neutralized solution was mixed with 5% SDS at a ratio of 1:1 (v/v). The mixture was incubated at 85ºC for 15 min. To solubilize the films prior to SDS-PAGE analysis, films were mixed with 20 mM Tris HCl (pH 8.0) containing 2% SDS and 8 M urea in the presence and the absence of 2% β-ME. The mixture was homogenized at 13,000 rpm for 1 min. The homogenate was stirred continuously for 24 h at room temperature (28-30ºC). Undissolved debris was removed by centrifuge at 8,500xg for 10 min at room temperature using a microcentrifuge (MIKRO20, Hettich Zentrifugan, Germany). Protein (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-Protein II unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After separation, the proteins were stained with 0.02% (w/v) Coomassie Brilliant Blue and 7.5% (v/v) acetic acid and destained with 50% (v/v) methanol and 7.5% (v/v) acetic acid for 15 min, followed by 5% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h.

3.5.4 FTIR spectroscopy (as described in section 3.2.3.6)

3.5.5 X-ray diffractometry (XRD)

XRD measurements were carried out by using a wide angle X-ray diffractometer (Philips X'Pert MPD, Almelo, Netherland), with Cu source, operating at room temperature, 40 kV and 30 mA current. The samples were cut into the circular shape of 30 mm diameter and placed in a sample holder. Then, the set was placed inside the chamber of the apparatus, in order to perform the measurements. The measurement angles (2θ) were varied from 5º to 30º. The time of each scanning was about 32 min.

3.5.6 Thermal properties

Thermal transitions of the films were measured by means of differential scaning calorimetry (DSC7, Perkin Elmer, Norwalk, CT, USA) as described by Langmaier *et al*. (2008). The instrument was calibrated with Indium as a standard. Films were conditioned over silica gel at 25°C for 3 weeks before testing to

obtain the most dehydrated films. The conditioned samples (2-5 mg) were placed in an aluminium pan. The samples were heated at 5°C/min from -40 to 150°C and followed by quench cooling with dried ice to -40°C. This step was done in order to dry the sample. Next, the second-step heating scan was performed by heating the sample at 5°C/min from -40 to 250°C. Samples were also determined for thermal degradation using thermogravimetric analyzer (TGA7, Perkin Elmer, Norwalk, CT, USA). Films were scanned from room temperature to 800ºC at a rate of 10ºC/min. Nitrogen was used as the purge gas with flow rate of 20 ml/min.

3.5.6 Film morphology (as described in section 3.2.3.7)

3.6 Study on moisture sorption isotherms

Moisture sorption isotherms of the selected films from section 3.4 and control films (FMP film and PVA film) were determined as described by Srinivasa *et al.* (2003). Prior to analysis, all films were conditioned at room temperature (28-30°C) over dry silica gel for 3 weeks. Moisture sorption isotherms of the films were determined at room temperature under different relative humidity (RH) conditions (18±0.5%, 23±0.5%, 34±0.5%, 46±0.5%, 54±0.5%, 64±0.5%, 73±0.5% and 90±0.5%) prepared using different saturated salt solutions of $LiCl.H_2O$, $KC_2H_3O_2$, $MgCl_2$, $K_2CO_3.2H_2O$, $Mg(NO_3)_2.6H_2O$, $NaNO_2$, NaCl and KCl, respectively. The water activity (a_w) of each salt solution was calculated as %RH/100. Equilibrium moisture content (EMC) of the films at each RH was calculated as followed (Labuza, 1982):

$$EMC = \left[\frac{We(Mi+1)}{Wi} - 1\right] x100$$

where *EMC* is equilibrium moisture content (EMC) (g moisture/100 g dry mass); *We* is final weight of film sample at equilibrium state; *Mi* is initial moisture content (fractional dry basis weight); *Wi* is initial weight of film sample. Three films were used for measurement.

The moisture sorption isotherm of the samples was constructed by plotting the EMC against a_w.

3.7 Study on changes of FMP/PVA blend films during storage

Films obtained from section 3.4 and control films (FMP film and PVA film) were stored in a dessicator containing a saturated salt solution of $NaNO_2$ (65±0.5% RH) at room temperature (28-30ºC). Film samples were taken at week 0, 1, 2, 4, 6 and 8 of storage for the following analyses:

3.7.1 Mechanical properties (as described in section 3.2.3.2)

3.7.2 Water vapor permeability (as described in section 3.2.3.3)

3.7.3 Moisture content (AOAC, 2000)

3.7.4 Color and film transparency (as described in section 3.2.3.4)

4. Statistical analysis

Experiments were run in duplicate. Data were subjected to Analysis of Variance (ANOVA) and the differences between means were evaluated by Duncan's Multiple Range Test (Steel and Torrie, 1980). SPSS statistic program (SPSS 11.0 for window, SPSS Inc., Chicago, IL, USA.) was used for data analysis.

CHAPTER 3

RESULTS AND DISCUSSION

1. Composition and protein pattern of fish myofibrillar protein

Proximate composition of fish myofibrillar protein (FMP) from bigeye snapper (*Priacanthus tayenus*) is shown in Table 5. Protein content was found as a major constituent at 14.40 % (wet weight) with the negligible fat and ash contents. The flesh of fish normally contains 11-24% crude protein, depending on the species, the type of muscle, etc. (Sikorski *et al.,* 1990). During washing process, some lipids, minerals as well as water soluble proteins were removed. As a consequence, the myofibrillar proteins became more concentrated.

Protein pattern of mince (M) and washed mince (referred as "FMP") are revealed in Figure 14. From the result, it indicated that muscle protein consist of several protein bands corresponding to myosin heavy chain (MHC), actin, troponin, tropomyosin as well as myosin light chain and sarcoplasmic protein. After washing, sarcoplasmic protein band intensity was lowered, while the band intensity of myofibrillar proteins (MHC and actin) was increased. Myofibrillar protein conventionally prepared by water washing in which most of sarcoplasmic proteins are removed (Lanier, 2000). Myosin is the most dominant protein, which constitutes about 50-60% of total myofibrillar protein (Suzuki, 1981). Actin is another protein associated with myosin as actomyosin, which plays an essential role in contraction-relaxation (Trinick, 1991). Due to filamental nature of myofibrillar proteins, the strong film matrix could be formed (Cuq, 2002).

Table 5. Proximate composition of fish myofibrillar protein (FMP) from big eye snapper.

Composition	Quantity (%)	
	Wet wt.	Dry wt.
Moisture	83.87 ± 0.27*	-
Protein	14.40 ± 0.22	89.27 ± 1.34
Fat	0.17 ± 0.02	1.07 ± 0.12
Ash	0.30 ± 0.01	1.83 ± 0.07

*Mean ± SD from triplicate determinations.

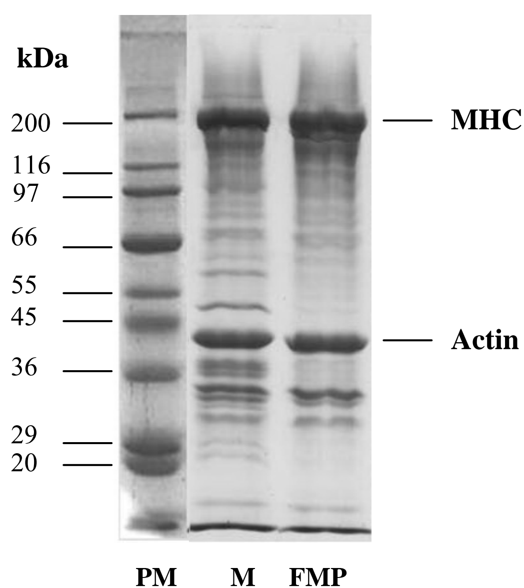


Figure 14. Protein patterns of mince (M) and washed mince (FMP) of bigeye snapper under reducing condition. PM: high molecular weight protein marker, MHC: myosin heavy chain.

2. Effect of pH level and blend composition on properties of fish myofibrillar protein (FMP)/poly(vinyl alcohol) (PVA) blend films

2.1 Visualized appearance of films

Figure 15 shows photograph of the selected film samples (FMP, FMP/PVA blend and PVA films) prepared at pH 3 and pH 11. The films could be easily separated from the casting plates and easy to handling. All of the resulting films were homogeneous, rather transparent and flexible. Their surfaces were smooth without visible pores and crack. In general, PVA film was clearer and more transparent than FMP/PVA blend and FMP films, respectively. FMP-based films prepared at pH 3 were more transparent than those prepared at pH 11.

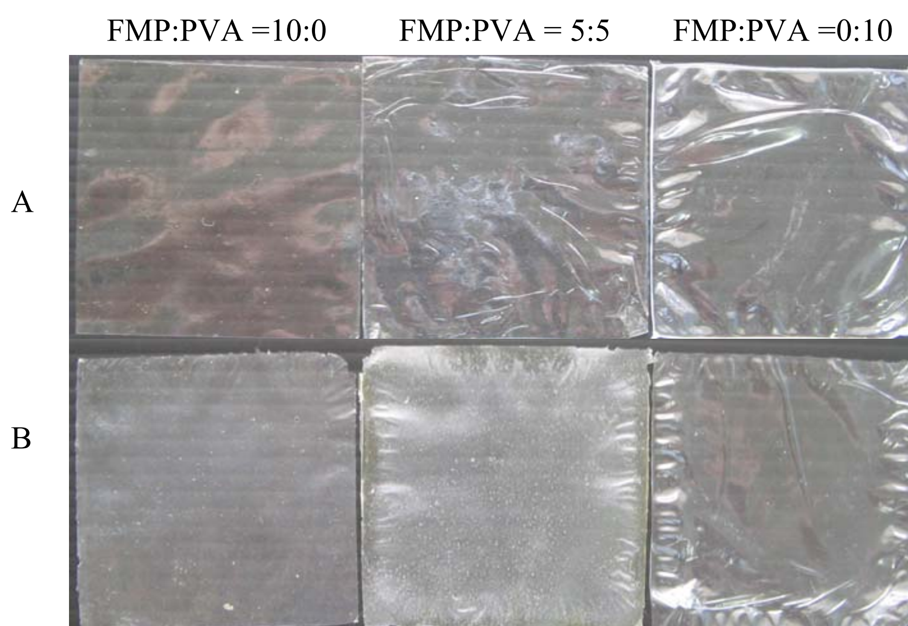


Figure 15. Photograph of FMP films, FMP/PVA blend films (FMP:PVA = 5:5) and PVA films prepared from film-forming solutions at pH 3 (A) and pH 11 (B).

2.2 Thickness and mechanical properties

Thickness of fish myofibrillar protein (FMP) film, PVA film and FMP/PVA blend films was in the range of 0.026 – 0.030 mm (data not shown). Figure 16 illustrates tensile stress-strain curves of representative film samples. As compared

to PVA and FMP/PVA blend films, FMP films at both pHs were stiffer and more resistant to tensile deformation, which exhibited lower deformation (Figure 16 (A, B) vs. Figure 16 (E, F)). Moreover, stress-strain diagram of FMP/PVA blend films (Figure 16 (C, D)) showed ductile behavior in which highly plastic deformation was observed, which was most likely due to PVA incorporation.

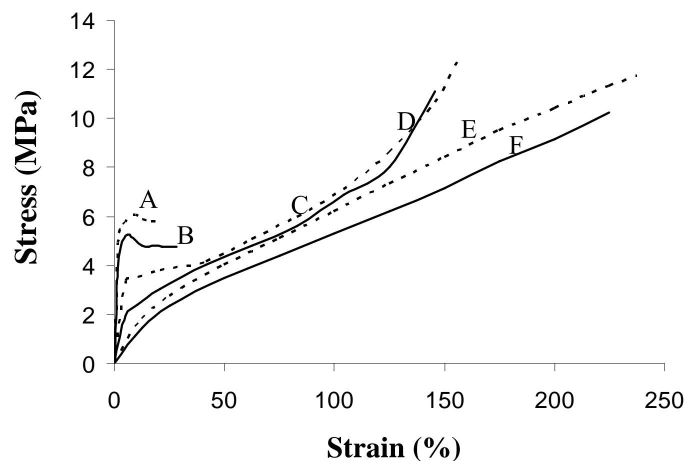


Figure 16. Representative tensile stress-strain diagrams of selected film samples: FMP films prepared at pH 3 (A) and pH 11 (B), FMP/PVA blend films (FMP:PVA = 5:5) prepared at pH 3 (C) and pH 11 (D) and PVA films prepared at pH 3 (E) and pH 11 (F).

TS, EAB and E of the films prepared from film forming solutions (FFS) at pHs of 3 and 11 are shown in Figure 17(A), 17(B) and 17(C), respectively. PVA film exhibited the higher TS and EAB but lower E than did FMP film ($p<0.05$), regardless of pH used. FMP film prepared at pH 3 had the lower TS, but higher EAB and E, compared with that prepared at pH 11 ($p<0.05$). The result was in agreement with Chinabhark *et al.* (2007) who reported that EAB of surimi film prepared at pH 3 was higher than that prepared at pH 11. However, no difference in TS was found for surimi film prepared at pH 3 and 11. Acidic and alkaline solubilizing processes are the prerequisite for myofibrillar protein-based film preparation and have the impact on the properties of the resulting films (Iwata *et al.,* 2000; Hamaguchi *et al.,* 2007). During drying, solubilized myofibrillar proteins underwent interaction via various

bonds including hydrogen bond, hydrophobic and ionic interactions as well as disulfide covalent bond, resulting in film formation (Shiku *et al.,* 2003). For PVA film, that prepared at pH 3 showed the higher TS and E than did that prepared at pH 11 ($p<0.05$). Nevertheless, no difference in EAB was observed between PVA films prepared at both pHs used ($p>0.05$).

For FMP/PVA blend films prepared at both pHs, TS and EAB increased with increasing PVA content up to 60% ($p<0.05$). However, no difference in TS was noticeable when PVA at levels of 60-100% was incorporated ($p>0.05$). Blend films prepared using 60-80% PVA had similar EAB ($p>0.05$). All FMP/PVA blend films had the lower EAB than did PVA film ($p<0.05$). When E value of all films was determined, blend films had the decrease in E as PVA levels increased up to 60% ($p<0.05$). When PVA levels ranging from 60 to 80% were incorporated, the resulting films possessed similar E value to that found for PVA film ($p>0.05$). For blend films incorporated with PVA at levels up to 40%, films prepared at pH 11 had the higher TS than those prepared at pH 3 ($p<0.05$). Conversely, blend films prepared at pH 3 showed the higher TS than did pH 11 counterpart when PVA level of 50 to 80% was used ($p<0.05$). The similar result was obtained for EAB. For E, blend films prepared at pH 3 showed the higher value than did those prepared at pH 11, irrespective of PVA level used.

PVA could improve the flexibility and decrease the stiffness of FMP-based film as evidenced by the increased EAB and the decreased E, respectively. This was most likely due to the decrease in intermolecular interaction between protein molecules caused by the dispersed PVA molecules in the film matrix. Furthermore, PVA most likely interacted with protein molecules via H-bond, leading to the lowered rigidity governed by covalent bonds between protein molecules. Hydrogen bonds between the reactive groups of protein, acting as hydrogen acceptor, and –OH groups of PVA were most likely formed in blend films, leading to the enhanced mechanical properties of resulting films, especially as PVA levels incorporated increased. The improved mechanical properties of film based on biopolymers was also reported for hydrolyzed starch-g-PAN (HSPAN) when PVA was incorporated, in which the superior TS was found at HSPAN/PVA ratio = 80:20 (Kim *et al.,* 2002). Methylated corn starch and PVA blend film had the increases in TS and EAB with PVA

incorporated from 20 to 100% when compared with film without PVA (Gouhua *et al.,* 2006). This tensile behavior is typical for homogeneous and thermodynamically miscible systems. Zhang *et al*. (2004) investigated mechanical properties of wheat protein/PVA blend films and found that TS and E of films were significantly improved as compared to those of wheat protein film. Moreover, Srinivasa *et al.* (2003) reported that PVA incorporation could decrease rigidity of chitosan film.

Thus, the incorporation of PVA at a particular level could maneuver the strength and flexibility of resulting FMP/PVA blend films. Furthermore, pH of film-forming solution for FMP/PVA blend film preparation had the influence on the mechanical properties of resulting blend film.

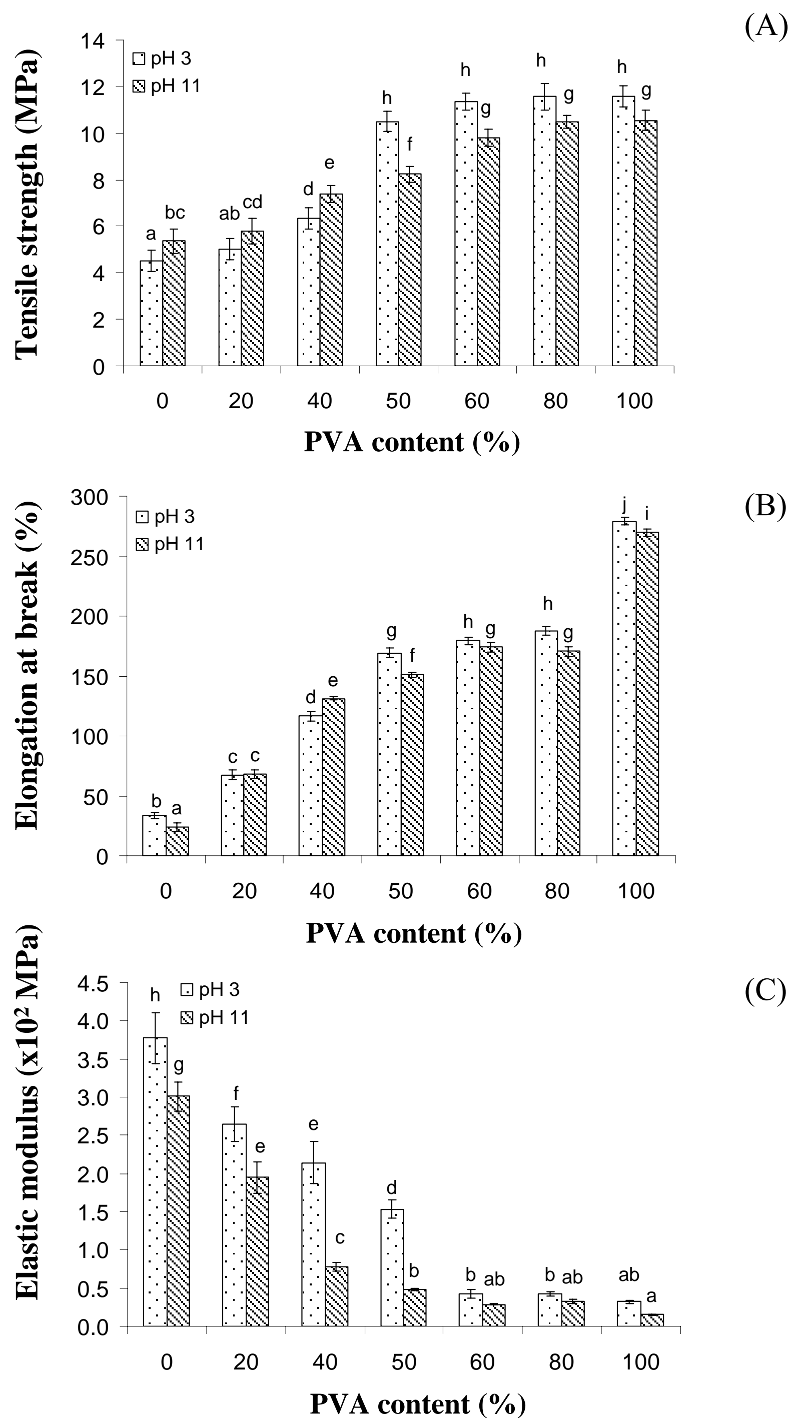


Figure 17. Mechanical properties of FMP film, PVA film and FMP/PVA blend films prepared at pH 3 and 11: (A) tensile strength, (B) elongation at break and (C) elastic modulus. Bars represent the standard deviation from ten determinations. Different letters indicate the significant differences ($p<0.05$).

2.3 Water vapor permeability

Water vapor permeability (WVP) of FMP film, PVA film and FMP/PVA blend films is shown in Figure 18. No differences in WVP of both FMP and PVA films were found at different pHs ($p>0.05$). Bigeye snapper surimi film and blue marlin edible film prepared from FFS with acidic and alkaline pH had similar WVP (Chinnabhark *et al*., 2007; Hamaguchi *et al.,* 2007). In general, WVP of PVA film was higher than that of FMP and FMP/PVA blend films, except for the blend with 40% PVA, which showed the highest WVP. FMP film might have the denser protein network with the lower polarity than PVA film. As a result, it could be resistant to water molecule transfer through the film. For PVA film, it contained a large number of hydroxyl group (-OH), resulting in the increased hydrophilic property of the film material, limiting its ability to exhibit the moisture barrier property (Skeist, 1990). As PVA at levels of 20-40% was incorporated, WVP of resulting blend films increased. This was most likely associated with the increase in the hydrophilicity of film governed by PVA. However, when PVA content higher than 40% was incorporated, WVP value decreased. High amount of –OH group possibly interacted with protein chain, resulting in the lower content of free OH groups. WVP of FMP/PVA blend films was higher than that of glutenin-rich films (Hernandez-Munoz *et al*., 2004b), but lower than that of surimi film (Chinnabhark *et al*., 2007), blue marlin edible film (Shiku *et al.,* 2003; Hamaguchi *et al.,* 2007), porcine plasma protein film (Nuthong *et al*., 2008), whey protein isolate and pullulan blend film (Gounga *et al.,* 2007) and cod gelatin and sunflower oil blend film (Perez-Mateos *et al*., 2009). For FMP/PVA blend film, higher WVP was obtained in films prepared at pH 11, compared with those prepared at pH 3 except for film with 60% PVA, which had no difference in WVP ($p>0.05$). The result suggested that film material especially myofibrillar protein became more charged at pH 11. It was presumed that myofibrillar protein might have acidic amino acids as the dominant amino acids. At pH 11, the carboxyl groups were deprotonated and the negative charge could be predominant. At pH 3, no much changes in protonation or deprotonation took place. Therefore, pH had an impact on WVP of resulting FMP/PVA films.

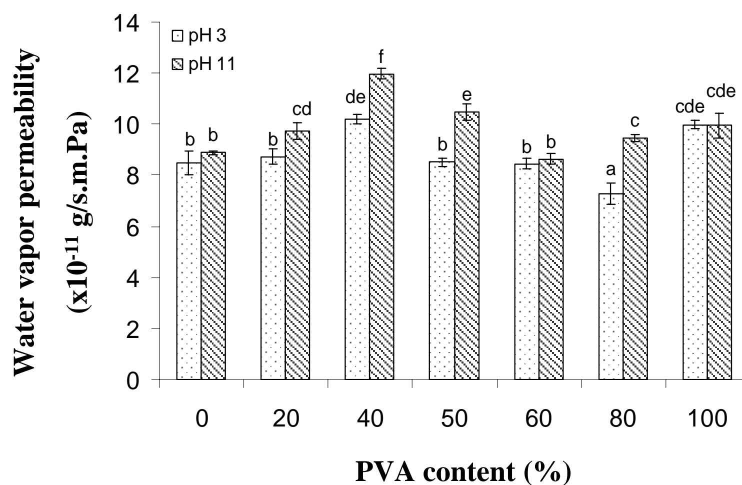


Figure 18. Water vapor permeability (WVP) of FMP film, PVA film and FMP/PVA blend films. Bars represent the standard deviation from four determinations. Different letters indicate the significant differences ($p<0.05$).

2.4 Optical properties

2.4.1 Light transmittance and film transparency

Transmittance (%T) in UV- Visible range and transparency value of FMP film, PVA film and FMP/PVA blend films prepared at pH 3 and pH 11 are presented in Table 6. FMP film had the excellent barrier for light transmission in UV range, while PVA film showed the poorer barrier property. Similar results were obtained for films prepared at both pHs. %T in visible range (350 – 800 nm) of FMP/PVA blend films at pH 3 ranged from 68.9 to 87.0%, but the much lower values were found for blend films prepared at pH 11 (10.2 – 65.19%). At pH 3, %T of film slightly increased with increasing PVA content. PVA film was clear and transparent as shown by the highest %T value. Thus, %T of blend films increased as the level of PVA increased. %T value of FMP/PVA blend films prepared at pH 11 decreased as PVA levels incorporated increased up to 60%. However, the increase in %T of blend film was noticeable when PVA at 80% was incorporated. %T of film was most likely governed by the arrangement or alignment of polymer molecules in film network. The result suggested that FMP film and FMP/PVA blend films could retard lipid oxidation induced by UV light in food system (Fang *et al*., 2002). Surimi films (Shiku *et al.*,

2004), blue marlin myofibrillar protein (Shiku *et al.,* 2003; Hamaguchi *et al.,* 2007) and whey protein isolate (Gounga *et al.,* 2007) had the excellent barrier property for UV light owing to their high content of aromatic amino acids that absorb UV light (Hamaguchi *et al.,* 2007).

The transparency value of FMP film prepared at pH 3 (3.26) was lower than that prepared at pH 11 (6.59), indicating that the former was more transparent than the latter ($p<0.05$). Shiku *et al.* (2003) also observed similar results for myofibrillar protein-based films from blue marlin meat. However, no differences in transparency value were observed between surimi film from bigeye snapper prepared at pH 3 and 11 as reported by Prodpran and Benjakul (2005). For all FMP/PVA blend films, films prepared at pH 3 were more transparent (lower transparency value) than those prepared at pH 11 ($p<0.05$). In general, no differences were found in blend film prepared at pH 3 with different PVA levels ($p>0.05$). At pH 11, blend film had the higher transparency value than those of FMP film and PVA film ($p<0.05$). Transparency value of blend film increased continuously as PVA levels increased up to 60% ($p<0.05$). Nevertheless, a decrease in transparency value was observed when PVA at 80% was incorporated in the film ($p<0.05$). The decrease in transparency value was coincidental with the increase in %T of blend film containing 20% FMP and 80% PVA prepared at pH 11. It could be suggested that the pHs of film forming solution had the impact on film transparency. Therefore, blend films were more transparent and clear enough for packaging the products, in comparison with FMP film with less transparency.

Table 6. Light transmittance (%T) and transparency value of FMP film, PVA film and FMP/PVA blend films prepared at pH 3 and 11.

pH levels	FMP:PVA ratios	%T at particular wavelength (nm) 200	280	350	400	500	600	700	800	Transparency value*
3	10: 0	2.30	1.94	72.12	78.70	81.52	82.53	82.78	82.28	3.26±0.17[bc**]
	8: 2	2.33	2.28	68.09	74.34	78.51	79.92	80.34	80.10	3.61±0.09[c]
	6: 4	2.89	4.86	80.10	76.59	80.02	81.31	81.82	81.61	3.40±0.06[bc]
	5: 5	2.83	8.95	81.00	78.62	81.12	81.89	82.42	82.00	3.34±0.24[bc]
	4: 6	2.99	12.89	81.55	80.16	81.78	82.42	82.52	81.93	3.11±0.09[bc]
	2: 8	3.33	30.05	87.60	81.12	81.76	82.21	82.33	81.85	3.30±0.20[bc]
	0: 10	28.33	77.15	89.68	85.56	85.77	86.42	86.75	86.52	2.27±0.19[a]
11	10: 0	2.20	1.69	59.56	60.46	63.44	64.80	65.34	65.19	6.59±0.03[d]
	8: 2	1.35	1.71	34.00	38.27	42.99	45.30	46.63	47.16	12.80±0.29[e]
	6: 4	0.45	0.84	10.88	15.40	18.69	20.90	22.66	24.03	20.90±0.97[g]
	5: 5	0.14	1.39	10.32	15.52	18.61	20.53	21.96	23.10	24.97±0.13[i]
	4: 6	0.20	1.65	10.74	13.53	15.65	16.97	17.65	18.24	24.16±0.72[h]
	2: 8	0.73	7.67	21.94	28.44	33.92	37.10	39.12	40.41	15.99±0.51[f]
	0: 10	19.26	71.73	92.68	82.43	83.25	84.24	84.86	84.87	2.80±0.44[ab]

*Mean ± SD from three determinations.

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

2.4.2 Color of films

L*, a* and b* values of FMP film, PVA film and FMP/PVA blend films prepared at pH 3 and 11 are shown in Table 7. For FMP film, higher a* and b* values but lower L* value were observed for film prepared under acidic condition, in comparison with that prepared under alkaline condition ($p<0.05$). This result suggested that an acidic condition could induce the formation of yellowish pigment, especially via Maillard reaction. Acidic condition induced the degradation of myofibrillar proteins, leading to the availability of free amino group for browning reaction (Chinnabhark *et al.,* 2007; Prodpran and Benjakul, 2005). As the temperature increased, the reaction between the glycerine molecule and the reactive group of lysine took place (Pascholick *et al*., 2003).

FMP/PVA blend films exhibited the increased L* and a* values but decreased b* value with increasing PVA content at both pHs. The decrease in b* value of blend film with higher level of PVA incorporated was mainly associated with the lower content of myofibrillar proteins. As a result, the amount of amino groups was lowered and Maillard reaction became lower. However, the dilution of FMP by increasing PVA had the less effect on Maillard reaction occurred at pH 11. It was found that no difference in b* value was found in blend film containing PVA at level of 20-60%. Srinivasa *et al.* (2003) prepared chitosan and PVA blend film with various PVA contents (0-100%) and found that films with higher concentration of PVA had the lighter color as indicated by the increased L* value. The result indicated that PVA as well as pH of FFS had the influence on the color of FMP/PVA blend film.

Table 7. L*, a* and b* -values of FMP film, PVA film and FMP/PVA blend films prepared at pH 3 and 11.

pH levels	FMP:PVA ratios	L*#	a*#	b*#
3	10:0	88.69 ± 0.08 [a¥]	-1.37 ± 0.01 [bc]	2.91 ± 0.07 [g]
	8:2	89.06 ± 0.13 [b]	-1.30 ± 0.04 [de]	2.99 ± 0.03 [g]
	6:4	89.38 ± 0.11 [cd]	-1.33 ± 0.07 [cde]	2.75 ± 0.04 [f]
	5:5	89.27 ± 0.04 [c]	-1.28 ± 0.10 [de]	2.04 ± 0.05 [d]
	4:6	89.33 ± 0.04 [cd]	-1.08 ± 0.01 [f]	1.66 ± 0.08 [c]
	2:8	89.44 ± 0.11 [d]	-1.09 ± 0.04 [f]	0.92 ± 0.06 [b]
	0:10	89.63 ± 0.14 [e]	-0.93 ± 0.08 [g]	0.63 ± 0.03 [a]
11	10:0	89.89 ± 0.06 [g]	-1.51 ± 0.06 [a]	2.79 ± 0.04 [f]
	8:2	90.31 ± 0.02 [g]	-1.43 ± 0.03 [abc]	2.16 ± 0.05 [e]
	6:4	90.23 ± 0.06 [g]	-1.42 ± 0.08 [abc]	2.12 ± 0.09 [de]
	5:5	90.61 ± 0.07 [h]	-1.46 ± 0.03 [ab]	2.19 ± 0.06 [e]
	4:6	90.02 ± 0.04 [f]	-1.30 ± 0.02 [de]	2.19 ± 0.04 [e]
	2:8	90.65 ± 0.13 [h]	-1.23 ± 0.10 [e]	1.60 ± 0.07 [c]
	0:10	90.29 ± 0.02 [g]	-1.01 ± 0.1 [fg]	0.63 ± 0.03 [a]

[#] Mean ± SD from three determinations.

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

2.5 Film solubility

Film solubility in water of FMP film, PVA film and FMP/PVA blend films prepared at pH 3 and 11 is presented in Figure 19. Among all films, FMP film had the lowest solubility and FMP film prepared at pH 3 had the lower solubility than did that prepared at pH 11 ($p<0.05$). FMP films were mostly stabilized by various bonds, including intermolecular disulfide covalent bonds (Chinnabhark *et al.,* 2007). This resulted in the decreased solubility. Due to higher degraded protein molecules at pH 3, Maillard reaction was more favorable, leading to the formation of strong protein cross-links stabilized by covalent bond. However, glycerol used as a plasticizer could be leached out. Glycerol is hydrophilic plasticizer added into film forming solution and could enhance film solubility in water. Cuq *et al.* (1997a) studied the effect of plasticizer concentration on the properties of films from fish myofibrillar protein. The solubility of these films was increased with increasing plasticizer content. A linear relationship between water soluble dry matter content and hydrophilic plasticizer content in the film was observed. The similar result was found for glutenin-rich film (Hernandez-Munoz *et al.*, 2004a). For PVA film, the highest solubility was observed, regardless of pH used. High solubility of PVA film was associated with the weak bond, particularly hydrogen bond. This film could be hydrated in the presence of water. This led to the ease of solubilization.

Film solubility of FMP/PVA blend films prepared at both pHs increased with increasing levels of PVA (Figure 19). The increase in solubility was probably due to the increase in proportion of hydrophilic compound, PVA. Kim *et al.* (2002) found the higher solubility of hydrolyzed starch-g-polyacrylonitrile (HSPAN)/PVA blend film with increasing PVA content. However, the solubility of the blend system between *Amaranthus cruentus* flour and PVA was considerably reduced for film blended with more than 10% PVA (Elizondo *et al.,* 2009). The result suggested that solubility of FMP/PVA blend film was affected by FMP/PVA ratio as well as pH of FFS.

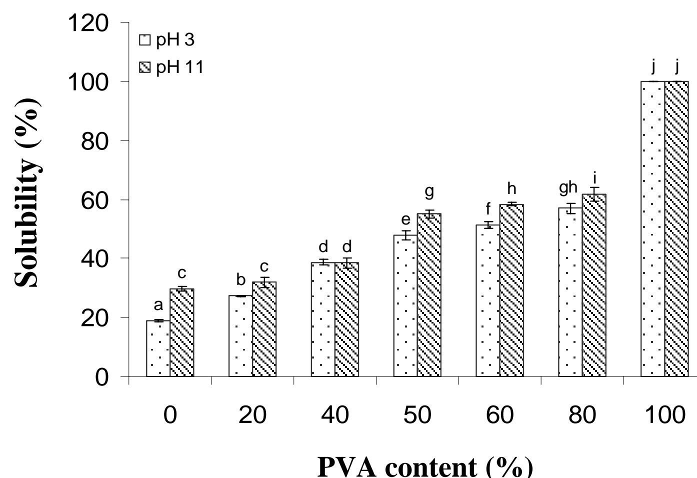


Figure 19. Film solubility (% based on dry basis weight) of FMP film, PVA film and FMP/PVA blend films. Bars represent the standard deviation from four determinations. Different letters indicate the significant differences ($p<0.05$).

2.6 Fourier-transform infrared (FTIR) spectroscopy

The FTIR spectra of FMP film, PVA film and FMP/PVA blend films prepared at pH 3 and 11 are presented in Figure 20 (A) and (B), respectively. Similar pattern of FTIR spectra were found in blend films with the same FMP/PVA ratio, prepared at alkaline and acidic condition. The peak situated around 1033 cm^{-1} in all spectra might be related to the glycerol added as a plasticizer (Bergo and Sobral, 2007). For FMP film and FMP/PVA blend film, peaks representing N-H stretching vibration at ~3271 cm^{-1} (Amide III). The bending vibration of N-H group and stretching vibration of C-N group at ~1540 cm^{-1} (Amide II) (Bergo and Sobral, 2007; Pawlak and Mucha, 2003) were observed in FMP film and FMP/PVA blend film, regardless of FMP/PVA ratio. FMP film and FMP/PVA blend film also showed the peak at ~1643 cm^{-1} representing carbonyl group (Amide I). The intensity of Amide I and Amide-II peaks decreased with PVA addition. For PVA film, high intensity of O-H stretching (~3273 cm^{-1}), C-H stretching (~2938 cm^{-1}) and C-H bending (~848 cm^{-1}) peaks were found, reflecting PVA structure. The peak at 1712 cm^{-1} was attributed to the stretching C=O and C-O from residual acetate groups remaining in

PVA molecule. Those amplitudes of peak in FMP/PVA blend film decreased as the level of PVA incorporated decreased.

The shift of Amide-I and Amide-II peaks was noticeable for FMP-based films incorporated with PVA, as compared to FMP film without PVA. Moreover, the peak corresponding to N-H stretching (3273 cm^{-1}) of FMP film incorporated with PVA was broader than that of FMP film without PVA. The spectra changes suggested the presence of protein-protein and protein-PVA interactions via hydrogen bond. Hydrogen bonding was involved in interaction between PVA and *A. cruentus* flour, as indicated by a broad band at 3293 cm^{-1}. This was due to hydrogen-bonded hydroxyl groups (O-H) in the polymer. The shift to lower vibrational frequencies observed in *A. cruentus* flour/PVA film as PVA concentration increased (3272-3278 cm^{-1}) indicated an increase in hydrogen bonding between two components (Elizondo *et al.,* 2009). FTIR spectra result reconfirmed the interaction between myofibrillar protein and PVA, which was governed by FMP/PVA ratio.

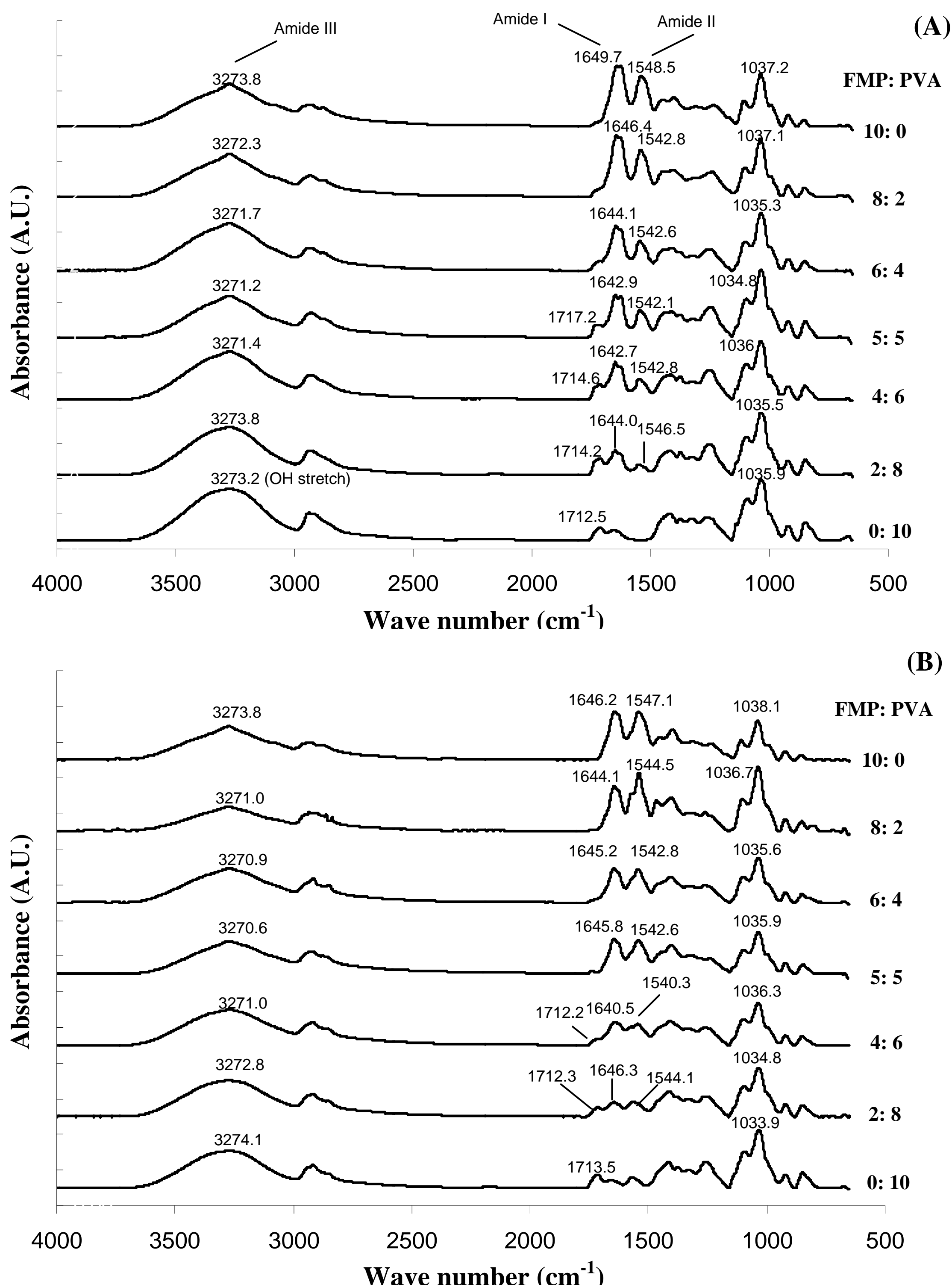


Figure 20. FTIR spectra of FMP film, PVA film and FMP/PVA blend films prepared at pH 3 (A) and pH 11 (B).

2.7 Microstructure of films

Surface and freeze-fractured cross-sectional images of FMP film, PVA film and FMP/PVA (FMP:PVA = 5:5) blend film prepared at pH 3 are shown in Figure 21. Surface of FMP film was smoother than that of PVA film and FMP/PVA blend film. FMP film image revealed a homogeneous structure, where myofibrillar proteins aggregated to form the dense and continuous network. This network was associated with the relative low solubility (Figure 19) and water vapor permeability (Figure 18). However, a rough fracture surface was found in cross-section image of FMP film. From SEM images, no distinct separation of the matrix or void in the film was observed in FMP/PVA blend film, indicating the good compatibility of the blend between FMP and PVA. The compatibility of FMP and PVA arises from the presence of their molecular interaction in the film matrix, thereby resulting in the improved mechanical and physical properties of FMP/PVA blend film. Elizondo *et al*. (2009) found that upper surface of *Amaranthus cruentus* flour/PVA blended film was dense and showed some roughness distributed along the surface, but without the cracks. PVA incorporation into starch provoked the changes in the biopolymer structure at both molecular and morphological levels, reducing its rigidity. Jayasekara *et al*. (2004) reported that the surface of wheat starch/PVA blend film did not show any sign of cracking.

From the result, FMP/PVA blend film at FMP/PVA ratio of 5:5 prepared at pH 3 had the highest mechanical properties, compared with other FMP/PVA blend films. Therefore, FMP/PVA (5:5) blend at pH 3 was chosen and used for further study.

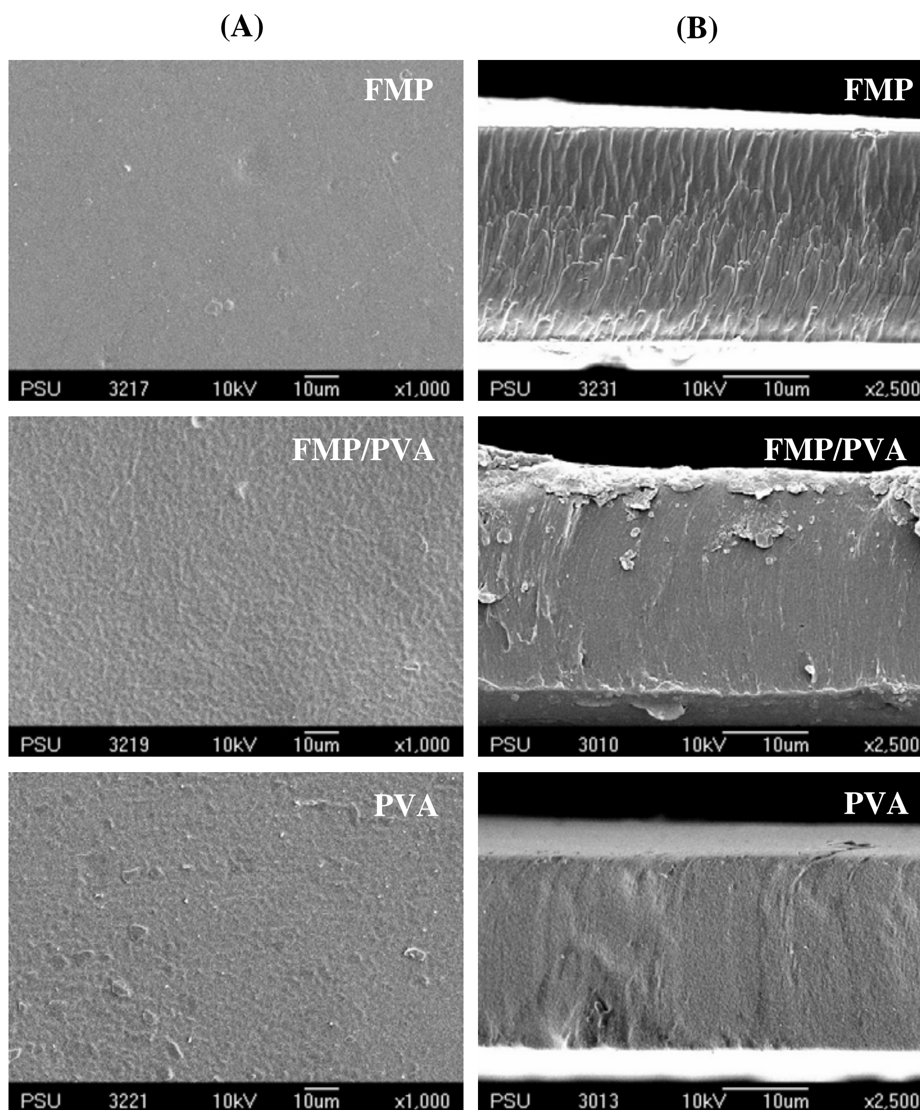


Figure 21. Surface (A) and freeze-fractured cross-sectional (B) images of FMP film, PVA film and FMP/PVA (FMP:PVA = 5:5) blend film prepared at pH 3.

3. Effect of PVA type on the properties of FMP/PVA blend films

FMP/PVA (5:5) blend films with different types of PVA (BP05, BP17, BP26, BF05, BF17, BF26) were prepared and subjected to characterization, in comparison with the corresponding PVA films.

3.1 Thickness and mechanical properties

All films with the thickness of 0.024-0.028 mm had the varying mechanical properties. Films prepared from PVA with different degree of hydrolysis (DH) and molecular weight (MW) and the corresponding blend films showed the different TS, EAB and E values ($p<0.05$) (Table 8). For PVA control films, at the same MW, films made from higher DH PVA (BF type) showed the higher TS and EAB than those prepared from lower DH PVA (BP type) ($p<0.05$). Moreover, E value of PVA films was slightly increased with increasing DH. At the same DH value, PVA films with higher MW exhibited higher tensile performance than the films produced from lower MW PVA as shown by the higher TS and EAB values ($p<0.05$). Therefore, both DH and MW of PVA had the impact on the mechanical properties of resulting films. When PVA was incorporated into FMP film, the resulting blend films had the altered mechanical property, compared with either FMP or PVA films (Table 8). For FMP/PVA blend films, PVA added markedly improved the flexibility and decreased the stiffness of FMP-based film as evidenced by increased EAB and decreased E, respectively. The properties of blend films were influenced by DH and MW of PVA incorporated. Similar trends were observed in blend films, in comparison with those found in PVA films with different DH and MW. Generally, the decrease in intermolecular interaction between protein molecules resulted from the dispersed PVA molecules in the film matrix. At the same DH of PVA used, TS and EAB of the FMP/PVA blend film increased with the increase in molecular weight of PVA due to greater intermolecular interaction along the chains of PVA and protein. At the same MW of PVA, FMP/PVA blend film incorporated with PVA of higher DH (BF type PVA) had the greater E and TS but lower EAB ($p<0.05$). This result was in agreement with the tensile properties of *A. Cruentus* flour/PVA blend film with different DH and MW of PVA used. High DH PVA yielded the film with the greater TS and puncture force than those with low DH PVA (Elizondo *et al*., 2009). Maria *et*

al. (2008) reported the effect of DH and MW of PVA on the properties of pig skin gelatin/PVA (1:1) blend film. DH and MW of PVA did not affect TS, EAB and E of gelatin/PVA blend film except for the lowest DH and MW PVA used (MW 31-50 KDa and 88%DH), which rendered the film with the lowest tensile properties. Silva *et al.* (2008) also reported the properties of pig skin gelatin/PVA blend film with various DH of PVA. No significant difference of EAB of the films with different DH of PVA. The higher degree of hydrolysis increases the amount of hydroxyl groups present in the PVA molecule, allowing for the formation of hydrogen interactions between –OH groups of PVA and polar groups (amino, carbonyl and carboxylic groups) of proteins. Among all FMP/PVA blend films tested, PVA-BP26 (partial hydrolysis and high MW) produced film with higher TS (11.20 MPa) and EAB (179.44%).

Table 8. Mechanical properties of fish myofibrillar protein (FMP), different poly(vinyl alcohol) (PVA) and FMP/PVA (5:5) blend films.

Films	**TS* (MPa)**	**EAB* (%)**	**E* ($x10^2$ MPa)**
PVA-BP05	6.51 ± 0.27^{b}**	175.64 ± 2.55^{e}	0.09 ± 0.03^{a}
PVA-BP17	11.72 ± 0.53^{e}	374.75 ± 3.98^{h}	0.13 ± 0.02^{a}
PVA-BP26	14.60 ± 0.27^{g}	359.08 ± 6.84^{g}	0.10 ± 0.01^{a}
PVA-BF05	12.77 ± 0.39^{f}	282.76 ± 4.97^{f}	0.72 ± 0.14^{bc}
PVA-BF17	19.55 ± 0.43^{h}	410.33 ± 11.45^{i}	0.33 ± 0.05^{a}
PVA-BF26	24.71 ± 0.67^{i}	445.44 ± 6.58^{j}	0.43 ± 0.06^{ab}
FMP	6.97 ± 0.82^{b}	32.95 ± 3.24^{a}	4.18 ± 0.60^{f}
FMP/PVA-BP05	5.15 ± 0.60^{a}	87.19 ± 5.41^{b}	0.89 ± 0.15^{cd}
FMP/PVA-BP17	8.17 ± 0.20^{c}	154.92 ± 3.47^{d}	0.78 ± 0.18^{bcd}
FMP/PVA-BP26	11.20 ± 0.47^{de}	179.44 ± 3.65^{e}	0.93 ± 0.12^{cd}
FMP/PVA-BF05	6.52 ± 0.31^{b}	95.33 ± 3.70^{b}	1.36 ± 0.17^{e}
FMP/PVA-BF17	10.70 ± 0.27^{d}	125.07 ± 2.44^{c}	1.11 ± 0.17^{de}
FMP/PVA-BF26	10.65 ± 0.47^{d}	150.21 ± 6.89^{d}	1.34 ± 0.11^{e}

* Mean ± SD from ten determinations.

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

3.2 Water vapor permeability

Water vapor permeability (WVP) of PVA films and FMP/PVA (5:5) blend films is shown in Table 9. PVA films exhibited the varying WVP, depending on DH and MW of PVA used. With the same DH, PVA film with the higher MW showed the higher WVP ($p<0.05$). Increasing MW of PVA was possibly associated with increasing degree of disorder of amorphous region, leading to increased free volume and thus higher WVP. For the same MW, films with the higher DH possessed the higher WVP than those having lower DH ($p<0.05$). FMP/PVA blend films, except FMP/PVA-BF26, had the lower WVP than the control (FMP) film ($p<0.05$). The decrease in WVP of blend film might result from inter- and intra-molecular interactions between protein and PVA molecules, plausibly via hydrogen bonding. As a result, the overall amount of free hydrophilic functional groups (NH_2 and COOH groups in protein and OH group in PVA) available for binding with water molecules in the film matrix decreased, thereby decreasing WVP of the resulting blend film. However, no differences of WVP of all FMP/PVA blend films containing PVA of different MW and DH was found ($p>0.05$). Among all FMP/PVA blend films, FMP/PVA-BP26 exhibited the lowest WVP value ($8.70x10^{-11}$ $g.s^{-1}.m^{-1}.Pa^{-1}$). WVP of FMP/PVA blend films was higher than that of gelatin and gellan or *K*-carragenan blend films (Pranoto, *et al*., 2007), glutenin-rich films (Hernandez-Munoz *et al*., 2004a), blue marlin edible film (Shiku *et al.,* 2003; Hamaguchi *et al.,* 2007) and was similar to that of FMP film from round scad (Arthan *et al*., 2007). Nevertheless, WVP of blend film in this study was lower than that of surimi film (Chinnabhark *et al*., 2007), porcine plasma protein film (Nuthong *et al*., 2008), whey protein isolate and pullulan blend film (Gounga *et al.,* 2007), and cod gelatin and sunflower oil blend film (Perez-Mateos *et al*., 2009). Su *et al.* (2007) reported the WVP value of soy protein isolate (SPI)/PVA blend films depended on PVA (0-50%) and glycerol content (0-4%). SPI/PVA blend film at 20% PVA content without plasticizer expressed the lowest WVP value and then increased from 7.2 to 9.5 g mm/m^2 h kPa when glycerol used increased from 0% to 4%. Therefore, PVA with the appropriate DH and MW was incorporated into FMP film, the decreased WVP could be obtained in the resulting blend film.

Table 9. Water-vapor permeability and film solubility of fish myofibrillar protein (FMP), different poly(vinyl alcohol) (PVA) and FMP/PVA (5:5) blend films.

Films	**WVP*** ($\mathbf{x10^{-11}\ g.s^{-1}.m^{-1}.Pa^{-1}}$)	**Film solubility*,** † **(%)**
PVA-BP05	6.83 ± 0.66^{a}**	100.00 ± 0.00^{g}
PVA-BP17	7.61 ± 0.16^{b}	100.00 ± 0.00^{g}
PVA-BP26	8.60 ± 0.26^{c}	100.00 ± 0.00^{g}
PVA-BF05	9.48 ± 0.88^{de}	39.03 ± 0.77^{d}
PVA-BF17	10.08 ± 0.14^{e}	33.43 ± 1.92^{c}
PVA-BF26	11.88 ± 0.37^{f}	23.63 ± 1.90^{a}
FMP	9.95 ± 0.13^{e}	24.03 ± 1.72^{a}
FMP/PVA-BP05	9.13 ± 0.20^{cd}	58.07 ± 1.16^{f}
FMP/PVA-BP17	9.20 ± 0.26^{cd}	54.52 ± 1.83^{e}
FMP/PVA-BP26	8.70 ± 0.16^{c}	54.82 ± 1.70^{e}
FMP/PVA-BF05	9.17 ± 0.23^{cd}	33.38 ± 1.16^{c}
FMP/PVA-BF17	9.33 ± 0.37^{d}	31.22 ± 0.80^{b}
FMP/PVA-BF26	9.57 ± 0.22^{de}	25.69 ± 0.81^{a}

* Mean ± SD from four determinations.

† Based on dry basis weight.

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

3.3 Film solubility

Solubility in water of PVA films and FMP films with and without incorporation of different types of PVA having various DH and MW was presented in Table 9. PVA film with low DH was completely soluble in water, regardless of MW. On the other hand, the much lower solubility was obtained in PVA film with the high DH and those with high MW showed the lower solubility ($p<0.05$). PVA molecules with high DH might interact each other via hydrogen bonding and the larger complexes could be formed. The hydrolysis degree increases water resistance of PVA (Carvalho *et al.*, 2009). Fully hydrolyzed (BF type) PVA film is typically more water resistant than partially hydrolyzed (BP type) PVA film (Skeist, 1990) as evidenced by

lower solubility value. FMP film had the lower solubility than PVA films, irrespective of PVA types. Nevertheless, FMP/PVA-BF26 had no difference in solubility, compared with FMP or corresponding PVA-BF26 films ($p>0.05$). Since protein network was not likely to solubilize or disperse in water caused by high interaction density and certainly the presence of intermolecular covalent bonds in the film matrix. The solubility of FMP film was most likely reflected the leaching of glycerol, a hydrophilic plasticizer, from the film matrix (Cuq *et al*., 1997a; Prodpran and Benjakul, 2005; Shiku *et al*., 2003). Due to the hydrophilic nature of PVA, the solubility in water of FMP/PVA blend films was considerably higher than that of the FMP film ($p<0.05$). However, the solubility of PVA is directly related to the DH and/or MW. FMP/PVA-BF blend films had lower film solubility than FMP/PVA-BP blend films ($p<0.05$). The decreasing film solubility of FMP/PVA blend films was obtained when PVA with higher DH was incorporated. The result was in accordance with that of pig skin gelatin/PVA blend film (Carvalho *et al*., 2009; Elizondo *et al*., 2009). Silva *et al*. (2008) found that film solubility in water of gelatin/PVA (ratio 1:1) blend films reduced from 35.3% to 15.5% when DH of PVA increased from 88% to 99.7%. Considering the MW of PVA used, the solubility in water of FMP/PVA blend film was decreased with increasing MW of PVA used. The decrease in solubility of blend film might be because long chain PVA molecules could form the greater inter-molecular interactions with protein molecules. The solubility of FMP/PVA-BF 26 blend film was lower than that of *A. cruentus* flour/PVA blended films (Elizondo *et al*., 2009), pigskin gelatin/PVA blend films (Carvlho *et al*., 2009) and whey protein isolate/pullulan blends films (Gounga *et al*., 2007). Low water solubility is important when films are in contact with water during processing and storage. Thus, FMP/PVA blend films could be used for further application as packaging material.

3.4 Light transmittance and film transparency

Light transmittance (%T) in UV-Visible range and transparency value of PVA films and FMP films with and without the incorporation of various PVAs with different DH and MW are shown in Table 10. For the UV range, PVA film showed the decreased %T at 200 nm as the MW increased. Conversely, the increase in %T at 280 nm was noticeable with increasing MW. However, PVA film had the

much lower barrier property against UV light, in comparison with FMP film. FMP/PVA blend films had the excellent barrier property against UV light in the wavelength of 200-280 nm. PVA types had the impact on %T in UV range of blend film, most likely related with those found in the corresponding PVA films. In visible rage (350-800 nm), PVA films showed the poorer barrier property against visible light. PVA with higher MW rendered the film with higher %T, indicating the poorer barrier properties. In general, the %T of FMP/PVA blend films were range from 77% to 87%. This result indicated that the resulting films were quite clear. Therefore, FMP film and FMP/PVA blend films with different PVAs could retard lipid oxidation induced by UV light in food system (Fang *et al*., 2002). Surimi films (Shiku *et al.,* 2004), blue marlin myofibrillar protein (Shiku *et al.,* 2003; Hamaguchi *et al.,* 2007) and whey protein isolate (Gounga *et al.,* 2007) had the excellent barrier property for UV light owing to their high content of aromatic amino acids that absorb UV light (Hamaguchi *et al.,* 2007).

All PVA films showed the similar transparency value ($p>0.05$). Transparency values, indicating lower transparent of the films, tended to increased in the blend film, compared with PVA films. However, FMP film had the highest transparency value ($p<0.05$), which was similar to that of FMP/PVA-BP05 blend film. Regardless of PVA type used, FMP/PVA blend films were more transparent (lower transparency value) than FMP film ($p<0.05$). No differences in transparency value of those films with different DH and MW of PVA were observed ($p>0.05$). In addition, the transparency value of FMP/PVA blend films observed in this study was lower than that of FMP film from big eye snapper (4.3–5.9) (Chinabhark *et al*., 2007), FMP based film from blue marlin (6.65-36.9) (Shiku *et al*., 2003), film from whole fish meat of blue marlin (5.1-13) (Hamaguchi *et al*., 2007) and whey protein isolated-based film (3.41-7.42) (Gounga *et al*., 2007) but was similar to that of Alaska Pollack surimi film which ranging from 2.19 to 3.47 (Shiku *et al*., 2004). The result suggested that PVA incorporated contributed to the increase in transparency of the resulting films.

Table 10. Light transmittance (%T) and transparency value of fish myofibrillar protein (FMP), different poly(vinyl alcohol) (PVA) and FMP/PVA (5:5) blend films.

Films	**%T at particular wavelength (nm)**								**Transparency value***
	200	**280**	**350**	**400**	**500**	**600**	**700**	**800**	
PVA-BP05	70.90	70.20	83.46	85.10	86.47	87.58	87.76	88.54	2.02 ± 0.24[a]**
PVA-BP17	70.01	73.87	80.68	83.13	85.15	85.97	86.49	87.28	2.20 ± 0.21[ab]
PVA-BP26	50.20	78.98	80.40	85.54	87.45	88.16	88.58	89.32	2.30 ± 0.23[ab]
PVA-BF05	79.94	70.92	77.57	81.56	83.53	85.19	85.75	86.37	2.09 ± 0.22[a]
PVA-BF17	74.26	79.30	84.58	85.60	86.78	87.52	87.93	88.65	2.05 ± 0.24[a]
PVA-BF26	55.94	80.07	86.95	85.72	86.87	87.56	87.96	88.64	2.18 ± 0.26[ab]
FMP	6.19	2.42	80.36	81.53	84.02	85.24	85.71	84.93	3.18 ± 0.08[d]
FMP/PVA-BP05	6.01	6.43	77.06	77.31	80.35	81.77	82.25	82.83	2.77 ± 0.05[cd]
FMP/PVA-BP17	5.53	6.89	81.64	81.47	84.55	85.53	85.84	86.30	2.23 ± 0.09[ab]
FMP/PVA-BP26	2.83	8.95	81.00	78.62	81.12	81.89	82.42	82.00	2.56 ± 0.09[bc]
FMP/PVA-BF05	3.44	7.62	80.02	80.53	83.72	84.70	85.00	85.43	2.52 ± 0.12[bc]
FMP/PVA-BF17	4.18	7.93	82.91	81.43	84.54	85.56	85.95	86.13	2.20 ± 0.10[ab]
FMP/PVA-BF26	3.76	10.43	82.99	81.99	84.87	85.83	86.15	86.63	2.34 ± .20[ab]

[*] Mean ± SD from three determinations.

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

3.5 Color of films

Color expressed as L*, a* and b*-values of films are shown in Table 11. PVA films had L*-value of 91.16-92.26, a*-value of -1.34- (-1.20) and b*-value of 0.40 – 0.47. In general, PVA film had the higher L*-value but lower b*-value than FMP and FMP/PVA blend film. Different PVA used did not affect overall color of FMP/PVA blend films. However, PVA incorporation could decrease the yellowness of the FMP-based film as evidenced by the lower b*-value of FMP/PVA blend films, compared to the control (FMP) film. It was mainly associated with the lower content of myofibrillar proteins in the film system. As a result, the amount of amino groups was lowered and Maillard reaction became lower. Maria *et al.* (2008) reported that the properties of gelatin/PVA (ratio 1:1) blend film depended on PVA types and glycerol concentration. PVA incorporated gelatin films could decrease the overall difference in

color (ΔE^*) of gelatin film. Moreover, the gelatin/PVA blended film with 45% glycerol had the lower ΔE^* than those without plasticizer and with 25% of glycerol. L* and a*-values of gelatin/PVA blend film did not change with different DH of PVA used, but b*-value was slightly decreased from 3.1 to 2.7 as DH value increased from 88% to 99.7% (Silva *et al.*, 2008). PVA incorporation could lower yellowness of blend film and showed negligible impact on L* and a*-values.

Table 11. L*, a*, b*-values of fish myofibrillar protein (FMP), different poly(vinyl alcohol) (PVA) and FMP/PVA (5:5) blend films.

Films	**L*#**	**a*#**	**b*#**
PVA-BP05	91.17 ± 0.26^{c**}	-1.27 ± 0.00^{fg}	0.41 ± 0.00^{ab}
PVA-BP17	92.26 ± 0.20^{e}	-1.21 ± 0.04^{g}	0.47 ± 0.07^{b}
PVA-BP26	92.07 ± 0.32^{e}	-1.32 ± 0.02^{def}	0.42 ± 0.03^{ab}
PVA-BF05	91.15 ± 0.21^{c}	-1.20 ± 0.03^{g}	0.44 ± 0.02^{ab}
PVA-BF17	91.16 ± 0.10^{c}	-1.29 ± 0.02^{ef}	0.44 ± 0.02^{ab}
PVA-BF26	91.53 ± 0.23^{d}	-1.34 ± 0.04^{def}	0.40 ± 0.01^{a}
FMP	90.72 ± 0.05^{b}	-1.45 ± 0.05^{b}	1.63 ± 0.05^{g}
FMP/PVA-BP05	90.49 ± 0.05^{ab}	-1.42 ± 0.06^{bc}	1.15 ± 0.04^{c}
FMP/PVA-BP17	90.41 ± 0.07^{a}	-1.39 ± 0.05^{bcd}	1.29 ± 0.06^{d}
FMP/PVA-BP26	90.46 ± 0.45^{ab}	-1.57 ± 0.07^{a}	1.39 ± 0.04^{e}
FMP/PVA-BF05	90.40 ± 0.03^{a}	-1.35 ± 0.05^{cde}	1.37 ± 0.03^{e}
FMP/PVA-BF17	90.36 ± 0.04^{a}	-1.35 ± 0.02^{cdef}	1.49 ± 0.03^{f}
FMP/PVA-BF26	90.40 ± 0.05^{a}	-1.57 ± 0.07^{a}	1.39 ± 0.04^{e}

Mean ± SD from three determinations.

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

3.6 Fourier-transform infrared (FTIR) spectroscopy

FTIR spectra of PVA films and FMP films with and without incorporation of different PVA having various DH and MW are shown in Figure 22. For all films tested, peaks around 2927- 2938, 1031-1038, and 847- 853 cm^{-1} were observed, representing C-H stretching, C-O stretching and C-H bending, respectively (Jayasekara *et al.,* 2004). The peak situated around 1031-1038 cm^{-1} in all spectra might be related to the glycerol (Bergo and Sobral, 2007). For FMP-based film, their spectra revealed additional N-H stretching at 3273 cm^{-1}, N-H bending at 1538 cm^{-1} (Amide-II) and high intensity of the peak of C=O stretching of amide in protein at 1644 cm^{-1} (Amide-I). For PVA film, high intensity of O-H stretching from the inter-molecular and intra-molecular hydrogen bonds between 3550 and 3200 cm^{-1}, C-H stretching (in the range of 2840 to 3000 cm^{-1}), C-O-H stretching (673-677 cm^{-1}) and C-H bending (847-853 cm^{-1}) peaks were found due to the PVA structure. The peaks around 1714 cm^{-1} was attributed to the stretching of C=O and C-O from acetate groups remaining in partially hydrolyzed PVA (BP type) (Costa-Junior *et al*., 2009; Mansur *et al*., 2008). This peak was also found in FMP/PVA-BP blend films. On the other hand, the C-O stretching band at around 1141 cm^{-1} due to crystalline portion of fully hydrolyzed PVA was appeared in the spectra of the PVA-BF film (Mansur *et al*., 2008; Park *et al*., 2001). This peak was also observed in the spectra of FMP/PVA-BF blend films. From the results, the shift of wave numbers of Amide-I and Amide-II peaks was noticeable for FMP-based films incorporated with PVA as compared to the control (FMP) film. The intensity of Amide-I and Amide-II peaks decreased with PVA addition. In addition, the peak related to N-H stretching (3273 cm^{-1}) of protein of the films incorporated with PVA was broader than that of the control film. These changes observed in the spectra suggested the presence of protein-protein and protein-PVA interactions plausibly via hydrogen bonding.

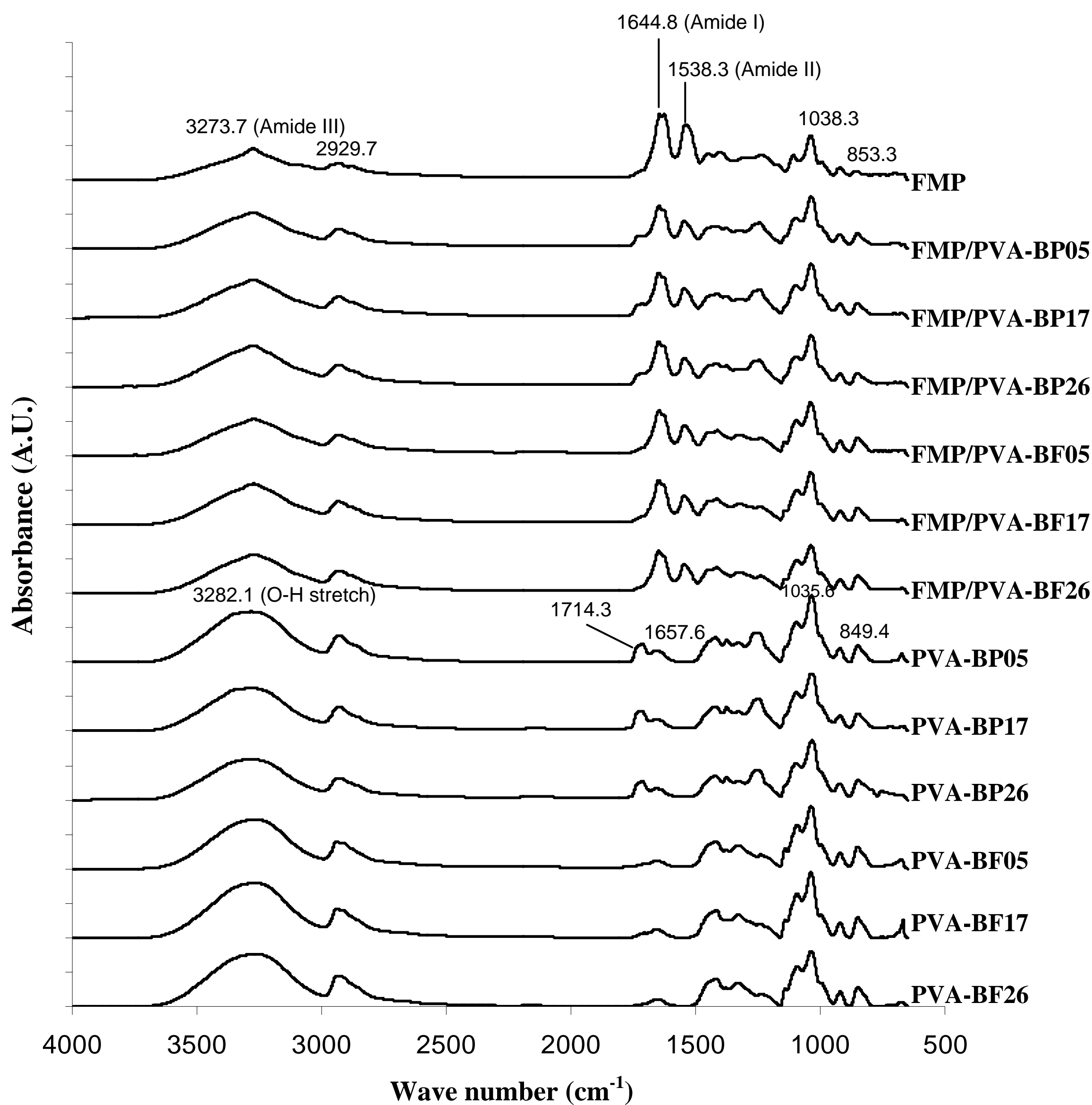


Figure 22. FTIR spectra of FMP film, FMP/PVA (5:5) blended films and PVA films with different PVA types.

3.7 Microstructure of films

SEM micrographs of the surface and freeze-fractured cross-section of the selected films (FMP, FMP/PVA-BP26 and PVA-BP26) are presented in Figure 23. PVA film had rougher surface than FMP and FMP/PVA blend films. FMP film image revealed a homogeneous structure, where myofibrillar proteins aggregated to form the dense and continuous network. This network was associated with the relative low solubility in water (Table 9). No difference in surface morphology of FMP and FMP/PVA blend films was observed. The FMP film exhibited rougher cross-section than PVA and FMP/PVA blend films. From the SEM images, no distinct separation of film matrix was observed in FMP/PVA blend film, which indicated the compatibility of the blend between FMP and PVA. The compatibility of FMP and PVA was most likely arising from the presence of their molecular interaction in the film matrix, thereby yielding the improved mechanical and physical properties of the FMP/PVA blend film. Elizondo *et al*. (2009) found that upper surface of *Amaranthus cruentus* flour/PVA-325 (DH 98%-98.8% and MW 85,000-124,000 Da) blend film was dense and showed some roughness distributed along the surface, but without the cracks. PVA incorporation into starch provoked the changes in the biopolymer structure at both molecular and morphological levels, reducing its rigidity. These observations were confirmed by the results of the mechanical properties of FMP/PVA blend film as shown in Table 8. Su *et al*. (2007) reported that the film surface of soy protein isolate/PVA blend film was relatively smooth except for a few scratches produced as cast. SEM micrographs of the fracture surfaces of starch/poly(lactic acid) (PLA) (starch:PLA = 50:50 w/w) and PVA (DH 80% and MW 6,000 Da) blend at various PVA concentration (10%, 20%, 30%, 40% and 50%) indicated that fewer starch granules and gaps were observed as the concentration of PVA increased (Ke and Sun, 2003).

From the result, blend film containing PVA-BP26 (partial hydrolysis and high molecular weight) had the highest mechanical properties, compared with other blend films. Therefore, FMP/PVA-BP26 (5:5) blend at pH 3 was chosen and used for further study.

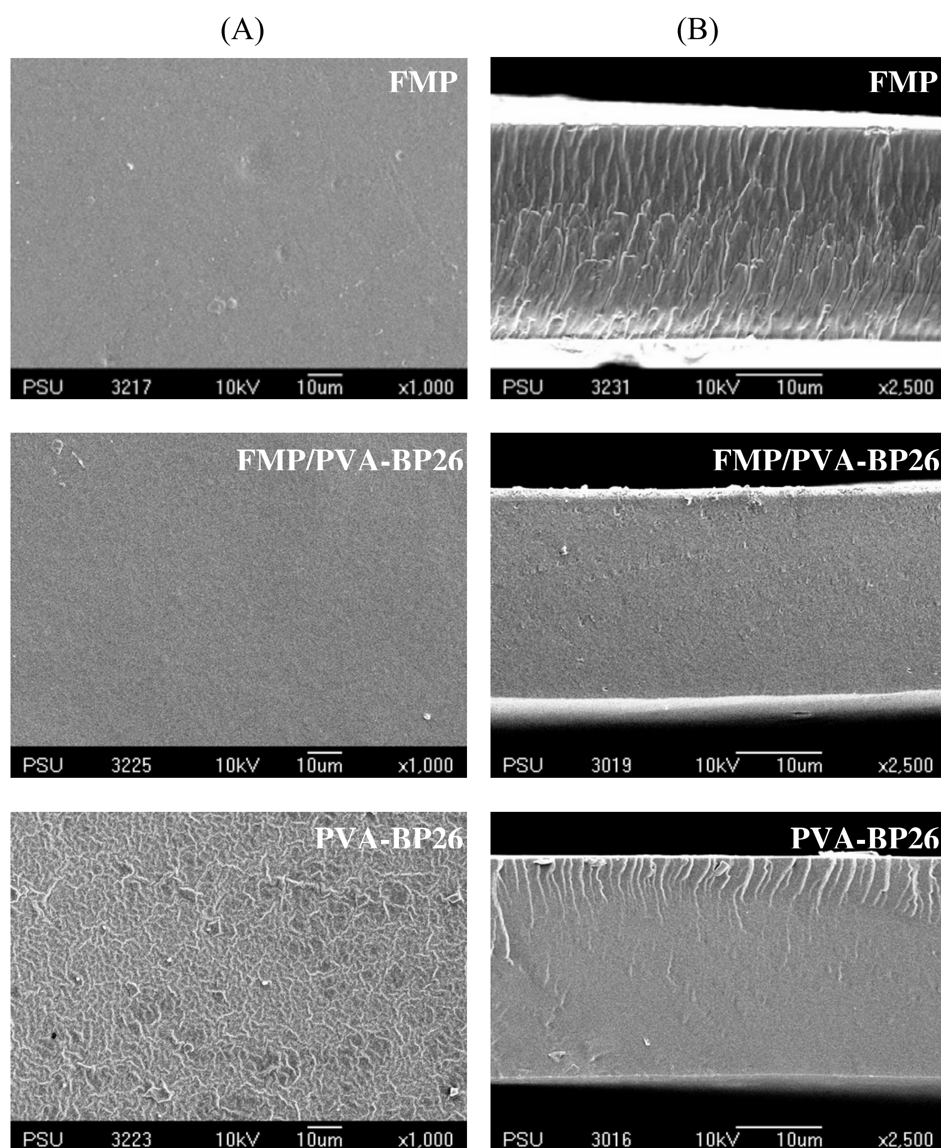


Figure 23. SEM micrographs of the surface (A) and cross-section (B) of the FMP, FMP/PVA-BP26 and PVA-BP26 films.

4. Effect of some chemicals on the properties of FMP/PVA blend films

4.1 Thickness and mechanical properties

Mechanical properties of FMP/PVA blend films containing different chemicals at different levels were compared with those of FMP film, PVA film and FMP/PVA blend film without the addition of chemicals (Figure 24). All films had the thickness ranging from 0.028-0.033 mm. TS, EAB and E values of FMP/PVA blend film varied, depending on the type and concentration of chemical added. TS of FMP/PVA blend film added with epichlorohydrin (ECH) increased with increasing ECH ($p<0.05$). For blend film added with maleic anhydride (MA) or glyoxal (GLX), TS was decreased with MA or GLX at levels of above 1% was used ($p<0.05$). Nevertheless, no differences in TS was observed in films added with 3 and 5% MA or GLX ($p>0.05$). Film added with phthalic anhydride (PA) had similar TS when PA at levels of 1 or 3% was added, while TS decreased at 5% PA was used ($p<0.05$). Among all sample tested, blend film added with 5% ECH showed the highest TS ($p<0.05$). For EAB, blend film added with 5% ECH showed higher value than those added with 1 or 3% ECH ($p<0.05$). For those added with MA or PA, no differences in EAB were found between films added with chemical at level of 1 and 5% ($p>0.05$). On the other hand, blend film added with GLX had the decrease in EAB when 5% GLX was used, in comparison with 1% GLX ($p<0.05$). When considering EAB of all films, those added with 5% ECH or 5% MA exhibited the highest EAB ($p<0.05$). When the same chemical was incorporated, E of films added with ECH or PA increased ($p<0.05$), compared with the control blend film, regardless of concentrations used. However, the addition of MA and GLX had no effect on E-value of resulting films ($p>0.05$), irrespective of concentration used. The result indicated that MA and GLX did not have the impact on film stiffness. From the result, it could be suggested that the selected chemical used yielded the stronger film with improved flexibility, as shown by higher TS and EAB, as compared to those of control films (FMP/PVA blend film). Also, ECH and PA could increase E value of resulting film, indicating the improved stiffness of the blend film. In general, ECH and PA might enhance the interaction or formation of cross-linking between FMP and PVA. ECH could form the covalent bond between amino group of protein and epoxide ring of

ECH, while hydroxyl group of PVA might react with chlorine (Cl) moiety of ECH (Tomihata *et al.,* 1994; Ray *et al.*, 2009a). For PA and MA incorporation, anhydride group might form the covalent bond with amino group of protein and hydroxyl group of PVA (Jose *et al.*, 2006). This result was in agreement with the tensile properties of starch/PVA blend film added with 20% ECH as reported by Ray *et al*. (2009a). The presence of cross-linking with ECH was found to have considerable effect on the properties of the blends. Kim *et al.* (2002) reported that the mechanical properties, the strength and the strain at break, of the hydrolyzed starch-g-poly(acrylonitrile) (HSPAN)/PVA blend films incorporated with ECH were improved, compared with HSPAN film, mainly via cross-linking reaction. Costa-Junior *et al.* (2009) reported the properties of chitosan/PVA blend film added with glutaraldehyde (1, 3 and 5%) were significantly altered by changing the blend composition and chemical cross-linking. Polymer chains are covalently linked, consequently becoming more rigid and brittle as shown by less flexibility. Zhang and Sun (2004) reported the mechanical properties of poly(lactic acid) (PLA)/starch composites compatibilized by maleic anhydride. Mechanical properties increased markedly compared to the control PLA/starch composites. Vaz *et al.* (2003) studied the effect of cross-linking by glyoxal on the mechanical properties of several natural protein films (gelatin, soy, casein and sodium-caseinate). Glyoxal cross-linking of proteins increased the mechanical strength and reduced the ductility for all proteins studied, except gelatin. It should be mainly attributed to the cross-linking reaction between aldehyde groups of the glyoxal and the free ε-amino groups of lysine (or hydroxylysine) residues of the studied proteins. From the result, ECH at a level of 5% was shown to be the most effective for enhancing mechanical properties of protein/PVA blend, possibly due to its cross-linking reaction. ECH might also lengthen the chain of polymers. As a result, the increase in EAB, especially in the presence of PVA, was observed. As governed by enhanced interaction between FMP and PVA molecules within the film matrix, the stiffer and tougher blend film could be obtained.

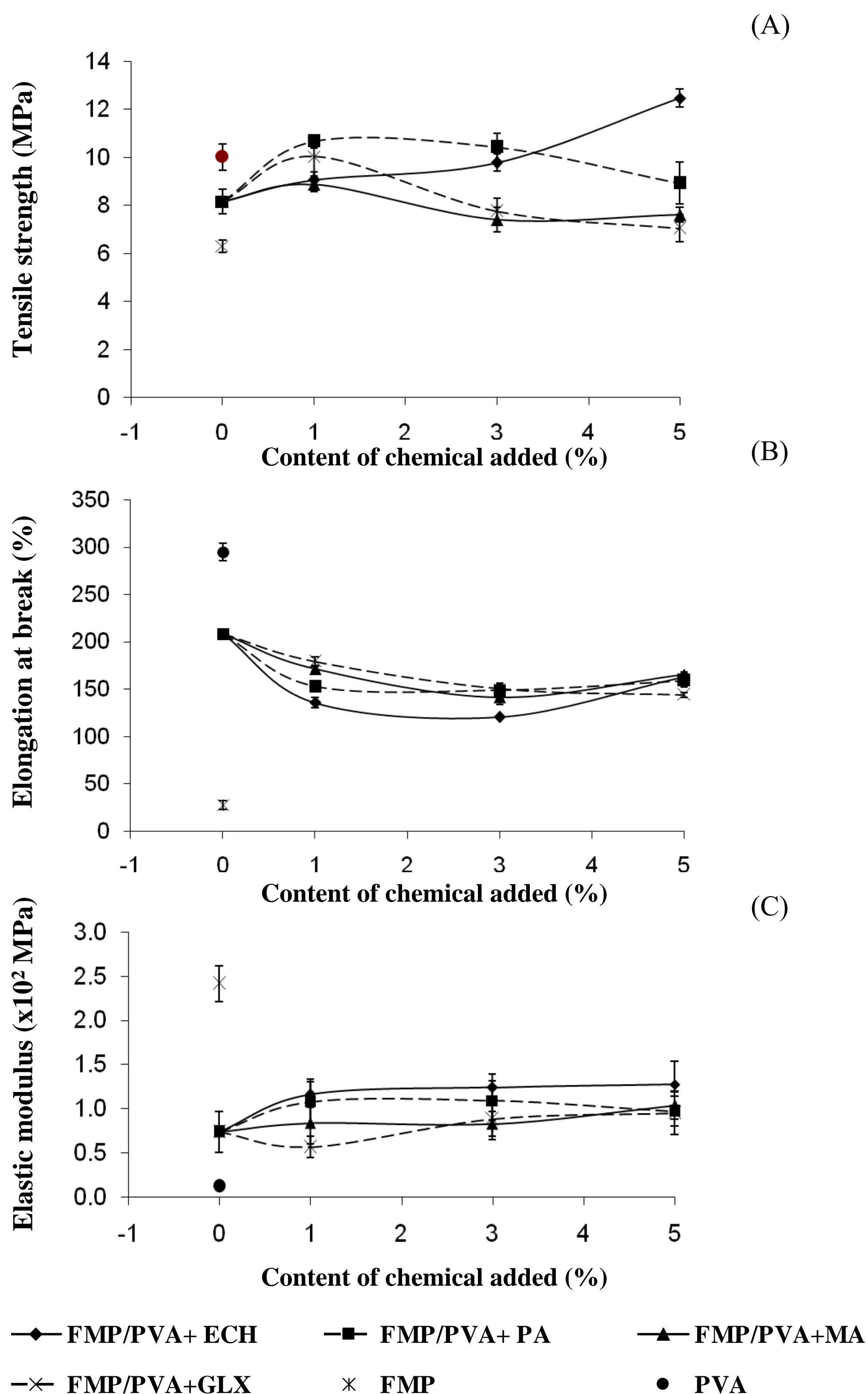


Figure 24. Mechanical properties of FMP/PVA blend films added with various chemicals at different levels: (A) tensile strength, (B) elongation at break and (C) elastic modulus. Bars represent the standard deviation from ten determinations.

4.2 Water vapor permeability

Table 12 presents the water vapor permeability (WVP) of FMP film, PVA film and FMP/PVA blend film without and with different chemicals (MA, PA, ECH and GLX) at various concentrations (1-5%). FMP films exhibited the highest water barrier properties and its WVP was increased when PVA was incorporated. As the chemicals were added, WVP of resulting films varied, depending on type and level used. When ECH or PA at a level of 1% was added, the film had increased WVP ($p<0.05$). For film added with ECH, the further increase had no effect on WVP ($p>0.05$). Conversely, slight decrease in WVP was noticeable when film was added with PA at level of 3 and 5% ($p<0.05$). Among films added with different levels of GLX, that added with 3% GLX showed the lowest WVP ($p<0.05$). For film added with MA, that with 1% MA had the lowest WVP, while the addition of MA at level of 3 and 5% resulted in the increased WVP ($p<0.05$). Different chemicals incorporated affected on WVP of FMP/PVA blend films at different degrees. In general, water vapor permeation through a hydrophilic film is closely related to the solubility and diffusivity of water molecules in polymer matrix. In the presence of cross-linker, the decrease of WVP is due to the formation of some densely cross-linked region. It decreases the free volume in the films, thereby decreasing the absorption site for water molecules as well as limiting molecular mobility (Hernandez-Munoz *et al.,* 2004a; Carvalho and Grosso, 2004). In addition, anhydride group of MA might form the complex structure between amino group of FMP and hydroxyl group of PVA (Jose *et al.*, 2006; Nakai and Modler, 1996) and yielded the film with higher water vapor barrier property, in compared with FMP/PVA control film. Interestingly, some differences in WVP values can also be observed for the films added with different chemicals. FMP/PVA blend films incorporated with 5% ECH, which yielded the film with the most improved mechanical properties, had no differences in WVP, compared with FMP/PVA blend film without chemicals ($p<0.05$). This result demonstrated that the intrinsic nature of chemicals used had some ability to affect WVP of the films as a result of the formation of different cross-linked/supra-structures and the kinds of interactions formed. Thus, some particular chemicals at appropriate amount could improve the barrier properties of FMP/PVA blend film. Hernandez-Munoz *et al.* (2004a) reported that cross-linked glutenin-rich film with glutaraldehyde, glyoxal and

formaldehyde showed better water vapor barrier property than that of films without treatment. WVP values decreased by around 30% when cross-linking agents were incorporated. The increasing reticulation of the network with the low molecular weight aldehydes could decrease the free volume of the polymeric matrix and increase the tortuosity of the pathway of the water molecules through the network, thus decreasing diffusion rate of water molecules through the films. Carvalho and Grosso (2004) also found the similar result for WVP of gelatin-based films modified with transglutaminase, glyoxal and formaldehyde. The greatest reduction in WVP was observed for the enzymatic modified films (~35%) as compared to native film, followed by chemical treated films. On the other hand, Rhim *et al.* (1998) reported that soy protein isolate (SPI)/dialdehyde starch films had slightly greater WVP than control SPI films. It might have resulted from the bulky dialdehyde starch molecules widening the interstitial spaces in the protein matrix, thus allowing for increased diffusion rate of water molecules through the films.

Table 12. Water vapor permeability of FMP/PVA blend films with various chemicals at different levels.

Films	Water vapor permeability[*] ($x10^{-11}$ $g.s^{-1}.m^{-1}.Pa^{-1}$)
FMP	$6.74 \pm 0.17^{b**}$
PVA	9.72 ± 0.19^{g}
FMP/PVA	8.31 ± 0.72^{ef}
FMP/PVA-1% ECH	9.90 ± 0.35^{g}
FMP/PVA-3% ECH	8.34 ± 0.43^{ef}
FMP/PVA-5% ECH	8.86 ± 0.43^{f}
FMP/PVA-1% MA	5.60 ± 0.10^{a}
FMP/PVA-3% MA	7.42 ± 0.12^{cd}
FMP/PVA-5% MA	7.57 ± 0.33^{cd}
FMP/PVA-1% PA	9.54 ± 0.33^{g}
FMP/PVA-3% PA	7.55 ± 0.30^{cd}
FMP/PVA-5% PA	7.86 ± 0.42^{cde}
FMP/PVA-1% GLX	8.05 ± 0.56^{de}
FMP/PVA-3% GLX	7.29 ± 0.23^{bc}
FMP/PVA-5% GLX	8.40 ± 0.35^{ef}

[*] Mean ± SD from four determinations.

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

4.3 Film solubility

Film solubility in water of different FMP/PVA blend films incorporated with MA, PA, ECH or GLX at various levels is presented in Figure 25. FMP film showed the much lower solubility, in comparison with FMP/PVA blend film ($p<0.05$). In contrast, PVA films were completely dissolved in water. This may be associated to the hydrophilic and hygroscopic character of PVA, which depend on hydrolysis degree, ranged from 86% to 99.2%. Since PVA-BP26 used in this part had low hydrolysis degree (~86%) and thus exhibited high water absorption and low water resistance. Incorporation of all chemicals decreased the solubility of the resulting

FMP/PVA blend films ($p<0.05$), depending on type of chemicals used. Among all blend films added with different chemicals, that with GLX exhibited the lowest solubility in water ($p<0.05$), especially when the higher levels were used. It might be associated with highly cross-linking reaction between protein chains in the film matrix, possibly associated with predominant covalent bonds. Inter- and intra-interactions between protein chains, such as covalent bond, hydrophobic interaction, hydrogen bond and ionic interaction, stabilized film network determine the solubility of protein films (Shiku *et al.,* 2003; Cuq *et al.,* 1997a; Nuthong *et al.,* 2009). Cross-linking using glyoxal involves the reaction between the aldehyde goups of glyoxal and amino groups of arginine and lysine (or hydroxylysine) residues of the polypeptide chains. Treatment with glyoxal decreased the solubility of protein film (Vaz *et al.,* 2003). For the blend film added with ECH, MA or PA, no significant differences in solubility of FMP/PVA blend films were found at all levels used ($p>0.05$). Kim *et al.* (2002) reported that the use of epichlorohydrin as a cross-linking agent in the range of 0-20% of polymer content could reduce the solubility in water of HSPAN/PVA blend films because of the cross-linking reaction between hydroxyl groups of HSPAN and hydroxyl groups of PVA by ECH. Carvalho and Grosso (2004) reported that the increase in the degree of cross-linking as a result of enzyme and chemical treatments could have led to a reduction in the low molecular weight fractions, thus decreasing the solubility of gelatin films. Hernandez-Munoz *et al.* (2004a) studied the effect of cross-linking agents, glutaraldehyde, glyoxal and formaldehyde, on properties of glutenin-rich films. Due to the high insolubility of glutenin matrix, cross-linking with glyoxal and glutaraldehyde did not change solubility of the films but formaldehyde treated films slightly decreased the solubility, probably due to the reinforcement of protein network. Gennadios *et al.* (1998) reported the properties of egg white-dialdehyde starch (DAS) films. The solubility in water of egg white films decreased significantly by ~15%, when DAS was added at 2.5 or 5%. Therefore, solubility of FMP/PVA blend film was governed by chemicals used as the cross-linker or modifier in the film matrix. The degree of modification varied most likely depending on nature of chemicals used as well as types, amount and distribution of interactions formed in the film matrix.

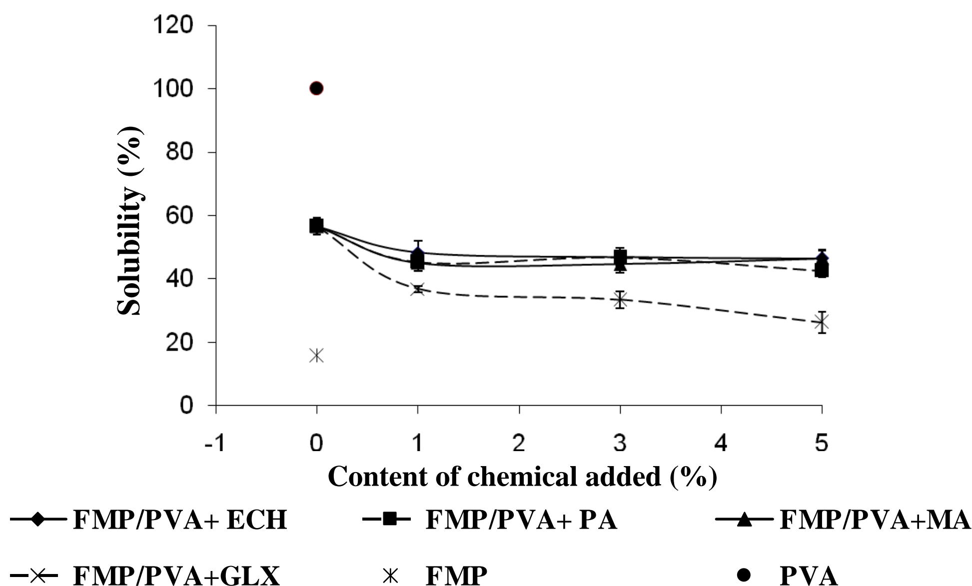


Figure 25. Film solubility (% based on dry basis weight) of FMP/PVA blend films added with various chemicals at different levels. Bars represent the standard deviation from four determinations.

4.4 Light transmittance and film transparency

Light transmittance (%T) in UV (200-400 nm) and visible (350-800 nm) ranges and transparency value of FMP/PVA blend films in the absence and presence of different chemicals at various levels are shown in Table 13. Visually, FMP/PVA blend film was more transparent than FMP film. At the wavelength of 280 nm, the FMP films showed the higher transmission, while blend films had the lower transmission especially at higher levels of chemicals. The results showed that FMP film and FMP/PVA blend films had the excellent barrier properties in UV light. It was suggested that FMP and FMP/PVA blend films can potentially retard lipid oxidation induced by UV light in food system. This is in agreement with other studies done on Blue marlin muscle protein-based films (Hamaguchi *et al.,* 2007), round scad muscle protein-based films (Arthan *et al.,* 2007), surimi film from Alaska Pollack (Shiku *et al.,* 2004) and whey protein isolate-based film (Gounga *et al.,* 2007). In addition, the transmission in the visible range of FMP and FMP/PVA blend films ranged from 58.80 to 88.30%. When ECH, PA or GLX were added, the resulting films had a slightly decrease in transmittance. Nevertheless, the marked decreasing in

transmittance were noticeable when MA was used ($p<0.05$). The lowest transmittance was found when 5% MA was incorporated ($p<0.05$), compared with those observed in other films. Visually, 5% MA treated films possessed white spot distributed along the film surface and might prevent the light transmission at selected wavelength.

Addition of various chemicals in blend films showed the lower transparency value than that of the control film ($p<0.05$). Higher transparency values indicated the lower transparency of the films. Similar transparency value was noticeable among all blend films added with different chemicals at all levels used, except for film added the 5% MA, which showed the marked increase in transparency value (4.85). The increase in transparency value was in agreement with the decrease in transmittance of light in the visible range.

Table 13. Light transmittance (%T) and transparency value of FMP/PVA blend films with various chemicals at different levels.

Films	**%T at particular wavelength (nm)***								**Transparency value***
	200	**280**	**350**	**400**	**500**	**600**	**700**	**800**	
FMP	0.00	2.23	75.03	77.60	80.87	82.53	83.07	83.47	2.65 ± 0.04^{f}**
PVA	28.93	78.40	83.63	85.40	86.93	87.97	88.27	88.40	2.02 ± 0.09^{abcd}
FMP/PVA	0.00	11.57	79.03	83.10	85.80	87.03	87.50	87.83	2.58 ± 0.25^{f}
FMP/PVA-1%ECH	0.00	8.10	78.23	81.90	84.50	85.53	85.93	85.80	2.21 ± 0.18^{cde}
FMP/PVA-3%ECH	0.00	5.13	77.47	82.43	85.93	87.50	87.97	88.30	1.85 ± 0.20^{a}
FMP/PVA-5%ECH	0.00	6.93	77.03	81.87	85.10	86.43	87.03	87.63	2.18 ± 0.12^{cde}
FMP/PVA-1%MA	0.00	6.13	76.37	80.73	83.67	85.03	85.63	86.03	2.29 ± 0.11^{e}
FMP/PVA-3%MA	0.00	3.18	75.85	80.95	84.35	85.90	86.60	87.03	2.25 ± 0.01^{de}
FMP/PVA-5%MA	0.00	3.20	58.80	63.10	66.43	68.23	69.33	69.77	4.85 ± 0.17^{g}
FMP/PVA-1%PA	0.00	4.20	78.10	82.93	86.00	87.40	87.80	88.10	1.82 ± 0.05^{a}
FMP/PVA-3%PA	0.00	1.20	75.77	81.03	84.43	85.87	86.23	86.53	2.17 ± 0.12^{cde}
FMP/PVA-5%PA	0.00	0.47	75.10	80.63	84.37	85.87	86.40	86.83	1.96 ± 0.19^{abc}
FMP/PVA-1%GLX	0.00	5.40	77.00	82.03	85.33	86.77	87.33	87.73	1.90 ± 0.10^{ab}
FMP/PVA-3%GLX	0.00	4.53	72.30	78.90	83.20	84.93	85.67	86.17	2.17 ± 0.12^{cde}
FMP/PVA-5%GLX	0.00	5.13	71.57	79.23	84.03	85.80	86.53	86.93	2.14 ± 0.09^{bcde}

* Mean ± SD from three determinations.

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

4.5 Color of films

Table 14 shows L*, a* and b*-values of FMP/PVA blend films in the absence and presence of different chemicals at various levels. When chemicals were added, no marked changes in L* and a*-values were found. However, the addition of those chemicals affected b*-value differently. As ECH, MA and PA incorporated increased, the increasing in b*-value was found ($p<0.05$). At the same level of chemicals added, films added with GLX had the much higher b*-value ($p<0.05$). This was mainly due to the enhanced browning reaction, Maillard reaction. Carbonyl group of GLX underwent Maillard reaction with free amino group of myofibrillar protein. Therefore, FMP/PVA blend film became yellowish with GLX treated films. In general, the yellow/brown coloration associated with protein-aldehyde interactions is due to the various intermediate or final products of the Maillard reaction (Cheftel *et al.,* 1985). The similar result was found for the color of glutenin-rich films cross-linked with aldehyde (glutaraldehyde, glyoxal and formaldehyde) (Hernandez-Munoz *et al*., 2004a). Hernandez-Munoz *et al*. (2004a) found that proteins treated with glutaraldehyde and glyoxal rendered the darker and yellowish films. Reaction of glyoxal with proteins produced a brown discoloration owing to the formation of Schiff base with arginine (Marquie, 2001). The incorporation of dialdehyde starch increased film yellowness as evidenced by greater b*-value. Moreover, dialdehyde starch containing protein films were slightly darker (Rhim *et al.*, 1998, Gennadios *et al.,* 1998).

From the result, blend film incorporated with ECH at 5% had the highest mechanical properties, compared with other blend films. Therefore, FMP/PVA blend film added with 5% ECH (FMP/PVA+5% ECH) was chosen and used for further study.

Table 14. L*, a*, b*-values of FMP/PVA blend films with various chemicals at different levels.

Films	L*#	a*#	b*#
FMP	90.39 ± 0.12[def]**	-1.23 ± 0.05[a]	1.47 ± 0.06[d]
PVA	91.80 ± 0.39[h]	-1.13 ± 0.03[bc]	0.55 ± 0.01[a]
FMP/PVA	90.71 ± 0.04[g]	-1.08 ± 0.07[cd]	1.34 ± 0.07[c]
FMP/PVA-1% ECH	90.34 ± 0.15[def]	-1.13 ± 0.05[bc]	1.18 ± 0.03[b]
FMP/PVA-3% ECH	90.00 ± 0.07[ab]	-0.91 ± 0.01[e]	1.44 ± 0.14[d]
FMP/PVA-5% ECH	90.43 ± 0.04[ef]	-1.00 ± 0.04[d]	1.62 ± 0.04[e]
FMP/PVA-1% MA	90.12 ± 0.23[abcd]	-1.16 ± 0.02[abc]	1.61 ± 0.03[e]
FMP/PVA-3% MA	89.92 ± 0.03[a]	-1.20 ± 0.01[ab]	1.33 ± 0.02[c]
FMP/PVA-5% MA	90.05 ± 0.10[abc]	-1.08 ± 0.04[cd]	1.79 ± 0.04[g]
FMP/PVA-1% PA	90.60 ± 0.11[fg]	-1.08 ± 0.04[cd]	1.71 ± 0.06[efg]
FMP/PVA-3% PA	90.31 ± 0.04[cde]	-1.02 ± 0.04[d]	1.75 ± 0.03[fg]
FMP/PVA-5% PA	90.35 ± 0.15[def]	-1.16 ± 0.01[abc]	1.95 ± 0.08[h]
FMP/PVA-1% GLX	90.34 ± 0.17[def]	-1.02 ± 0.02[d]	1.66 ± 0.06[ef]
FMP/PVA-3% GLX	90.05 ± 0.01[abc]	-1.17 ± 0.09[ab]	2.22 ± 0.01[i]
FMP/PVA-5% GLX	90.22 ± 0.14[bcde]	-1.19 ± 0.03[ab]	2.44 ± 0.01[j]

Mean ± SD from three determinations.

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

5. Film characterization

5.1 Moisture content, film solubility and protein solubility

Moisture content, film solubility and protein solubility in water of the selected blend film (FMP/PVA; 5:5) without and with 5% ECH were determined in comparison with FMP film and PVA film (Table 15). Among all films, FMP films had the lowest moisture content, film and protein solubilities, followed by FMP/PVA blend film and PVA film, respectively ($p<0.05$). PVA film was hydrophilic in nature and more likely absorbed the water from the environment, resulting in the high moisture content. FMP/PVA added with 5% ECH had a lower moisture content,

compared with that without ECH ($p<0.05$). For protein solubility, FMP film exhibited the lowest solubility, while FMP/PVA blend films had the higher protein solubility ($p<0.05$). From the result, it was noted that PVA film was completely dissolved in water, which was associated with the hydrophilic and hygroscopic character of PVA, governed by hydrolysis degree (Skeist, 1990; Pal *et al.,* 2006; Maria *et al.,* 2008). Since PVA-BP26 with partial hydrolysis (DH ≈ 86-89%) was incorporated, high water absorption and low water resistance of resulting film were obtained. Moreover, PVA incorporation in FMP film provoked film solubility and protein solubility in water. It was most likely due to the weak interaction between FMP and PVA, particularly hydrogen bond leading to the ease of solubilization. The used of ECH in FMP/PVA blend film slightly decreased film solubility and protein solubility ($p<0.05$). ECH added might promoted interaction or cross-linking between protein and PVA molecules in the film. In general, almost protein-based films were not dissolved in water and the small amount of material released from the film was mainly from hydrophilic plasticizer and low molecular weight proteinaceous compounds (Cuq *et al*., 1997a; Orliac *et al*., 2003). Strong inter- and intra-interaction between protein chains, such as covalent bond, hydrophobic interaction, hydrogen bond and ionic interaction, has been known to stabilize film network (Shiku *et al*., 2003; Cuq *et al*., 1997a; Nuthong *et al*., 2009).

Biodegradable blend films from waste gelatin and PVA had a strong decline in the water resistance as the concentration of PVA in the polymer matrix increased (Chiellini *et al*., 2001). Moreover, waste gelatin/PVA blend film was easy to disintegrate and dissolve after 1 h when the higher PVA content (80-90%) was used. Furthermore, Ke and Sun (2003) studied on the property of starch, poly(lactic acid) (PLA) and PVA blend film. They also found an increase in the swelling rate of the polymeric matrix with the augmentation of PVA concentration in the blends. The solubility property of the HSPAN/PVA blend films was also increased with increasing PVA content (Kim *et al*., 2002). The use of epichlorohydrin as a cross-linking agent in the range of 0-20% of polymer content could reduce the solubility in water of blend films because of the cross-linking reaction between HSPAN and PVA by ECH.

Table 15. Moisture content, film solubility and protein solubility of FMP film, PVA film and FMP/PVA (5:5) blend films without and with 5% epichlorohydrin.

Films	Moisture content* (%)	Film solubility*, † (%)	Protein solubility*, † (%)
FMP	28.92 ± 0.24[a**]	14.27 ± 2.75[a]	1.23 ± 0.09[a]
FMP/PVA	36.10 ± 0.38[c]	52.39 ± 3.56[b]	13.68 ± 0.01[c]
FMP/PVA+5%ECH	34.03 ± 0.50[b]	49.17 ± 0.93[b]	12.35 ± 0.02[b]
PVA	42.17 ± 0.38[d]	100.00 ± 0.00[c]	-

* Mean ± SD from four determinations.

† Based on dry basis weight.

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

5.2 Protein solubility in various solvents

Protein solubility of FMP film, PVA film, FMP/PVA (5:5) blend films without and with 5% epichlorohydrin (ECH) in various solvents is shown in Table 16. The distribution and extents of inter- and intra-molecular interactions between proteins, give rise to a three-dimensional network structure of films. The solubility of films in three different denaturing solutions was used to determine the major associative forces involved in the film matrix. S1 which contains SDS is able to disrupt hydrogen bonds. FMP-based film was solubilized at very low extent (approximately 5% of protein in the film), while FMP/PVA blend film showed the higher protein solubility (about 20% of protein in the film), regardless of ECH used. Thus, the solubility of FMP/PVA film suggested the presence of hydrogen bonds in the films. When S2 was used for film solubilization, the solubility of FMP film and FMP/PVA film increased to 49.27% and 69.69-70.21%, respectively. With the addition of 8.0 M Urea (S2), hydrophobic interactions can be destroyed. The result suggested that the main forces involved in the formation of film were hydrogen bonds and hydrophobic interactions. In addition, the higher solubility of films in denaturing solvent containing 2% βME (S3), compared to those of S1 and S2, indicated the contribution of disulfide bond in the film network. Myosin heavy chain contains about 40 sulfhydryl groups and might undergo oxidation, in which inter-molecular disulfide

bonds can be formed during the drying the protein solution (Shiku *et al*., 2004). From the results, it was elucidated that hydrogen bonds, hydrophobic interactions, together with disulfide bonds play an important role in the formation of FMP film and FMP/PVA blend films. This result was agreed with Shiku *et al.* (2004) who found that the hydrogen bonds, hydrophobic interactions, as well as disulfide bonds stabilized the film network of surimi films from Allaska Pollack. For FMP film, the non-disulfide covalent bond was formed to a higher extent compared with FMP/PVA blend films as evidenced by the lower solubility in S3. For FMP/PVA blend films, hydrogen bond was more involved and played a role in film matrix stabilization and non-disulfide bonds constituted at a lower level. ECH addition resulted in more non-disulfide covalent bonds, possibly between protein and PVA molecules, in the blend film as compared to that without ECH. Therefore, the differences in bonding involved in the film formation directly affect the mechanical and molecular related properties of resulting films.

Table 16. Protein solubility in various solvents of FMP film, PVA film and FMP/PVA (5:5) blend films without and with 5% epichlorohydrin in various solvents.

Films	**Protein solubility (%)**[*, †]		
	S1[***]	**S2**	**S3**
FMP	5.10 ± 0.66[a**]	49.27 ± 0.14[a]	52.04 ± 0.48[a]
FMP/PVA	20.52 ± 0.87[b]	70.21 ± 1.76[b]	81.24 ± 1.42[c]
FMP/PVA+5%ECH	20.84 ± 0.96[b]	69.69 ± 1.89[b]	76.73 ± 2.52[b]

[*] Mean ± SD from triplicate determinations.

[†] Based on dry basis weight.

[**] The different superscripts in the same column indicate the 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

5.3 Protein pattern

The Figure 26 shows SDS-PAGE patterns of myofibrillar protein (FMP), film forming solution (FFS) of FMP, FMP/PVA, FMP/PVA with 5% epichlorohydrin (ECH) and the corresponding films including FMP-based film, FMP/PVA blend film and FMP/PVA blend film added with 5% ECH. FMP contained myosin heavy chain (MHC) (~200 kDa) and actin (~40 kDa) as the major proteins. When FMP was used for FFS preparation, regardless of PVA blending, it was noted that the lower band intensity of MHC with the coincidental occurrence of proteins with MW of 140 and 68 kDa was found. During the solubilization of myofibrillar protein under acidic condition, the hydrolysis of MHC took place, most likely due to autolysis under acidic condition. No differences in the protein pattern and band intensity between FMP-FFS and FMP/PVA-FFS were visually observed, regardless of ECH used. When protein patterns of different resulting films were determined, similar pattern was obtained between FMP film and FMP/PVA blend films with and without 5% ECH. It was observed that the protein patterns of resulting films were slightly different, compared to those found in corresponding FFSs. The lower band intensity of MHC and protein with 140 kDa as well as actin was noticeable in the resulting films. This might be associated with the pronounced degradation during film casting and drying. Cuq *et al*. (1995) reported that the degradation of MHC in sardine FFS took place, mostly in the acidic pH range, due to cathepsins which are strongly associated with the myofibrillar proteins and are not removed by the washing treatment. In addition, Chinabhark *et al*. (2007) found the similar result for protein patterns of protein-based films from bigeye snapper (*Priacanthus tayenus*) surimi, prepared under acidic condition (pH 3) and alkaline condition (pH 11). The degraded proteins with molecular weight of 140-150 kDa were found in the film with acid solubilizing process, while the proteins with the molecular weight ranging from 60 to 70 kDa were obtained in the film prepared from alkaline FFS. Actin was also degraded into different degradation products. For FMP/PVA films, no differences in protein patterns were noticeable in the absence and presence of ECH. Thus, ECH had no impact on protein pattern or degradation of protein in FMP/PVA blend films. It was noted that slightly lower band intensity of MHC was found in FMP film, compared with FMP/PVA films. This was in agreement with the highest formation of

non-disulfide bond as indicated by the lowest solubility in S3 (Table 16).

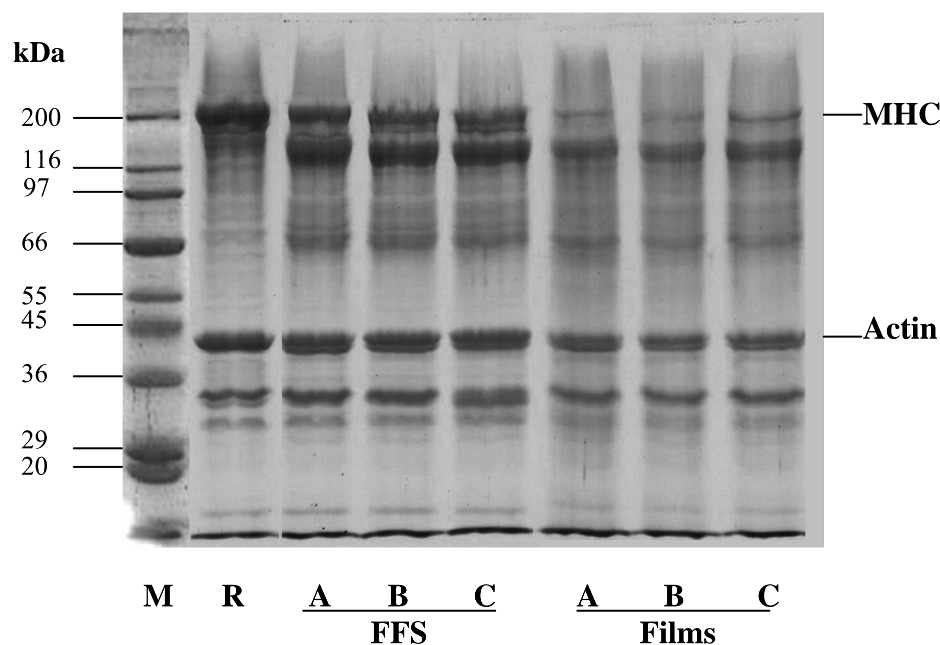


Figure 26. Protein patterns under reducing condition of fish myofibrillar protein (R), film forming solution (FFS) and resulting films. A: FMP; B: FMP/PVA; C: FMP/PVA with 5% ECH; M: protein marker.

5.4 Fourier-transform infrared (FTIR) spectroscopy

Figure 27 illustrates the FTIR spectra of FMP film, PVA film and FMP/PVA (5:5) blend film without and with 5% ECH. For all spectra, peaks around 2928-2931, 1318-1327 and 1035-1038 and 849-853 cm^{-1} were observed which were due to C-H stretching, C-O stretching and C-H bending of hydrocarbon in the film structure (Jayasekara *et al.,* 2004). The four peaks situated at 849-853, 922 cm^{-1} (C-C skeletal vibrations), 1032-1037 cm^{-1} (C-O stretch at C^1 and C^3), and 1107-1108 cm^{-1} (C-O stretch at C^2) in all spectra were associated to the glycerol structure (Lodha and Netravali, 2005). For FMP-based films, the absorption bands at 1645 cm^{-1}, 1544 cm^{-1}, 1449 cm^{-1} and 3272 cm^{-1} were contributed to C=O stretching (Amide-I), N-H bending (Amide-II), C-H deformation and N-H stretching (Amide-III), respectively. Moreover, the peak situated at 1236 cm^{-1} is associated to the N-H bending and C-N stretching vibration (Schmidt *et al*., 2005). For PVA structure, the very strong broad band observed from 3200 to 3500 cm^{-1} may be assigned to O-H stretching due to the

strong hydrogen bond of intramolecular and intermolecular type. The C-H stretching vibration was observed at 2935 cm^{-1}. The peak at 1714 cm^{-1} and 1093 cm^{-1} may be attributed to the non-hydrolyzed vinyl acetate group of PVA. The peak at 1374 cm^{-1} is due to –CH_2- wagging and that at 1328 cm^{-1} is due to –C-H- and –O-H bending (Costa-Junior *et al.*, 2009; Mansur *et al.*, 2008; Gohil *et al.*, 2006). These characteristic peaks due to PVA structure were also found in all spectra of FMP/PVA blend films, regardless of ECH addition. In addition, the shift of wave numbers of Amide-I and Amide-II peaks was noticeable for FMP/PVA blend film as compared to the control (FMP) film. The intensity of Amide-I and Amide-II peaks decreased with PVA addition. Moreover, the peak related to N-H stretching (3273 cm^{-1}) of protein of the films incorporated with PVA was broader than that of the control film. The change in the characteristic shape of the spectrum as well as the peak shift to a lower frequency range, suggested increased hydrogen bonding between –OH of PVA and –OH, -COOH and –NH_2 of protein in the blend. The FTIR spectrum of FMP/PVA blend film added with 5% ECH was rather similar to that of FMP/PVA blend film. However, slight shift of the band positions of Amide-I and Amide-II was noticeable. Also, the decrease in intensity of peak at 3273-3283 cm^{-1} which related to –NH and –OH in FMP/PVA+5%ECH film was observed as compared to that of FMP/PVA film without ECH. The covalent bond might be occurred between FMP and PVA in the presence of ECH. Nuthong *et al.* (2009) reported the characterization of porcine plasma protein-based films as affected by oxygenation pretreatment and cross-linking agent (2% glyoxal, 3% caffeic acid and 3% caffeic acid with oxygenation). The cross-linked films show the shift to lower frequency of amide-I peak and lower peak amplitude as compared to control film (without cross-linking and oxygenation). Film with oxygenated 3% caffeic acid had the higher Amide-I peak amplitude, in comparison with other films, since these conditions might induce the protein cross-linking to a higher extent. Sreedhar *et al.* (2006) reported the FTIR spectra of cross-linked starch/PVA blends, in which a decrease in intensity of the –OH band upon cross-linking of the blends was found with ECH. Moreover, Ray *et al.* (2009a) reported FTIR spectra of starch/PVA (6:4) blend added with 30% glycerol and 20% ECH (based on total polymer). They found that the peak at 1644 cm^{-1} which contributed to moisture in the blend was decreased significantly in the blend

containing ECH. The -OH stretching band was also lowered in starch/PVA blend containing ECH as compared to that of the blend without ECH used.

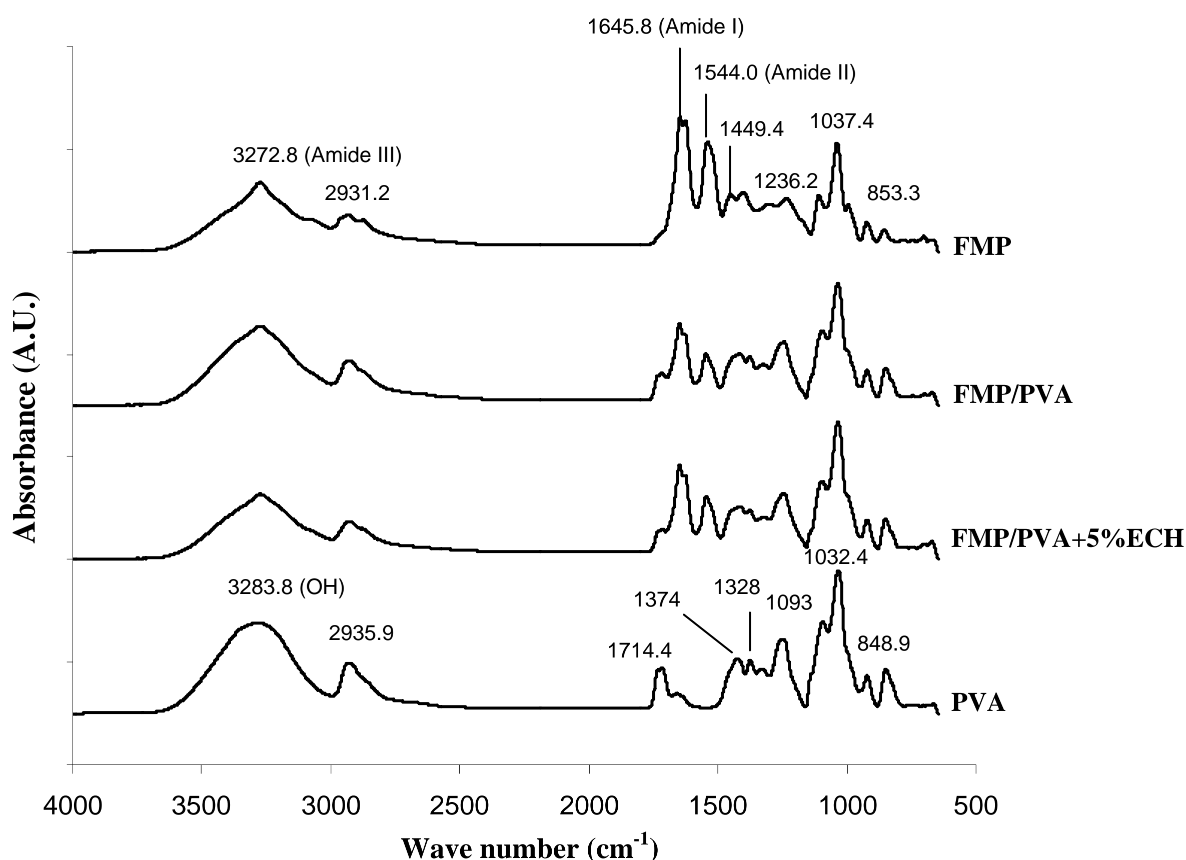


Figure 27. FTIR spectra of FMP film, PVA film and FMP/PVA (5:5) blend films without and with 5% ECH.

5.5 X-ray diffractometry (XRD)

X-ray diffraction patterns of the selected films (FMP film, PVA film and FMP/PVA blend film without and with 5% ECH) are shown in Figure 28. The X-ray diffraction was analyzed in the 2θ ranged from 5° to 30°. FMP film exhibited two broad diffraction peaks at 2θ around 9.29° and 19.97°. Similar XRD characteristic has been reported for films prepared from other proteins such as soy protein isolate (Su *et al.,* 2007) and pig skin gelatin (Maria *et al.,* 2008). This halo diffraction pattern indicated amorphous of structure FMP in the film matrix. On the other hand, X-ray diffractogram of PVA film showed a strong characteristic peak at 2θ around 19°. This result was in agreement with Ricciardi *et al.* (2004) and Shi *et al.* (2008), who

investigated the XRD pattern of PVA, showing the strong maximum reflections at $2\theta = 19.4°$ with a shoulder at $2\theta = 20°$, typical of crystalline structure of PVA. PVA has a flexible structure, which favors close molecular packing and crystallization (Xiao *et al.,* 2000). From the result, PVA films obtained showed partially crystalline structures. For FMP/PVA blend film without and with 5% ECH, their XRD diffractograms showed broad diffraction peak at 2θ around 9° due to amorphous FMP and strong peak at $2\theta \approx 19°$ arised from PVA crystallites. These blend films were thus partially crystalline materials. Maria *et al.* (2008) reported the XRD pattern of pig skin gelatin/PVA blend film. They also found that the blend of gelatin/PVA was a partially crystalline material, with a characteristic peak at 2θ around 20°. From a result, a slight shift of characteristic peak ($2\theta \approx 19°$) was observed in FMP/PVA blend films with and without ECH, compared to that of PVA. This possibly suggested the interaction between FMP and PVA present in the film matrix. Furthermore, the intensity of characteristic peak ($2\theta \approx 19°$) of FMP/PVA and FMP/PVA+5%ECH blend films decreased as compared to that of PVA film. It was most likely because FMP and especially ECH crosslinker inhibited close packing of the PVA molecules by reducing the degree of freedom in the 3-D conformation, limiting or even preventing the formation of crystalline regions (Shi *et al.,* 2008). Similar results have been reported in blend films from soy protein isolate (SPI)/PVA plasticized by glycerol (Su *et al.,* 2007) and PVA/corn starch (75:25) blend with glycerol plasticizer (20% wt based on polymer) and citric acid as crosslinker (Shi *et al.,* 2008).

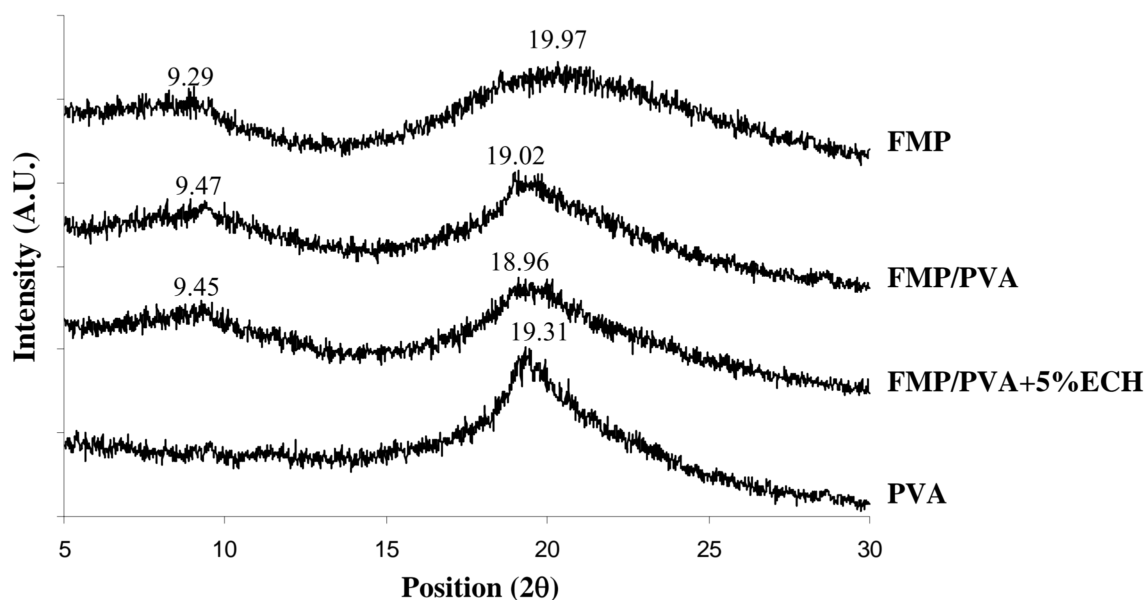


Figure 28. X-ray diffraction patterns of FMP film, PVA film and FMP/PVA (5:5) blend films without and with 5% ECH.

5.6 Thermal properties

5.6.1 DSC measurement

DSC was used to examine the transition temperatures, such as glass transition temperature (T_g) and melting temperature (T_m), of the films. Figure 29 shows typical DSC thermograms of the control films (FMP and PVA films) and FMP/PVA blend films without and with 5% ECH. From the first-step DSC scan (from -40°C to 150°C), it was observed the wide endothermic peaks around 36-48°C and 98-150°C (data not shown), presumably associated with evaporation of free water and bound water, respectively, absorbed in the film samples. This was in agreement with Langmaier *et al.* (2008), who found the wide endothermic peak due to evaporating adsorbed moisture from collagen hydrolysate films in the 30-120°C regions. From the second-step heating scan (Figure 29), transition temperatures associated with endothermic relaxation of the film matrix were observed. The observed transition temperature of the film indicated the temperature causing the disruption of the polymer interaction formed during film preparation. FMP film exhibited glass transition temperature (T_g) at about 144.02°C. The T_g is a very important physical parameter, which serves to explain the physical and chemical behavior of material system, and it is defined as the temperature at which the material changes from the

glassy state to the rubbery state for a given heating rate (Perdomo *et al.,* 2009). This transition was associated with molecular segmental motion of amorphous structure. The high T_g of FMP film most likely resulted from high and strong interaction between FMP molecules. No melting transition was observed in FMP film, which was due to amorphous structure of the film matrix as indicated by X-ray diffractogram (Figure 28). The DSC curve of FMP film also exhibited enthalpy relaxation peak (or aging enthalpy) superimposed on the glass transition change. This relaxation was possibly related with destroying some residual order structures presented in the film matrix. This result was in agreement with that of collagen hydrolysate protein film and gelatin film (Sarti and Scandola, 1995). Cuq *et al*. (1997b) reported T_g of 70°C for Atlantic sardine myofibrillar protein film containing 35% plasticizer (sorbitol and sucrose) and 5% moisture content. For PVA film, its thermogram showed broad T_g around 4.13°C. This broad T_g might be because the commercial PVA used consist of wide distribution of its molecular size. The quite low T_g of PVA film was more likely contributed from plasticization due to plasticizer (glycerol) added together with water absorbed. PVA film possessed melting transition at peak temperature of 182.18°C, due to order and crystalline structures in the film. The presence of T_g and T_m of PVA film reflected its partially crystalline structure. FMP/PVA blend films without and with 5% ECH had generally similar DSC thermograms. FMP/PVA film and FMP/PVA+5%ECH film had T_g of 7.2°C and 12.5°C, respectively, and T_m of 193.93°C and 198.10°C, respectively. Only one broad T_g was observed in blend films suggested partial miscibility between FMP and PVA molecules. Partial miscible blend between PVA and other biopolymers has been reported such as in gelatin/PVA (Mendieta-Taboada *et al.,* 2008; Silva *et al.,* 2008), collagen hydrolysate/PVA (Sarti and Scandola, 1995), gellan/PVA (Sudhamani *et al.,* 2003) and corn starch/PVA (Shi *et al.,* 2008). Su *et al.* (2007) observed the transition temperatures of soy protein isolate (SPI)/PVA blend film containing 2 wt% glycerol plasticized. T_g decreased from 136.5°C to 97.5°C and T_m increased from 157.4°C to 179.8°C as PVA incorporated increased from 0 to 40%. The co-existance of both glycerol and PVA possibly changed the aggregate structure of SPI. Mendieta-Taboada *et al*. (2008) studied thermal properties of gelatin/PVA blend films with 0-40% PVA incorporation

and without plasticizer. They reported that T_g occurred between 43°C and 49°C and T_m ranged from 116 to 134°C as observed in the first DSC scan. From the result, incorporation of ECH slightly increased T_g and T_m of the FMP/PVA blend film. ECH might interact or cross-link FMP and PVA molecules, which resulted in decreased chain mobility in the film matrix. However, Sreedhar *et al.* (2006) found that ECH cross-linking decreased T_g of starch/PVA blend film. They inferred that this lowering was due to the decrease in the regularity of the –OH groups on cross-linking.

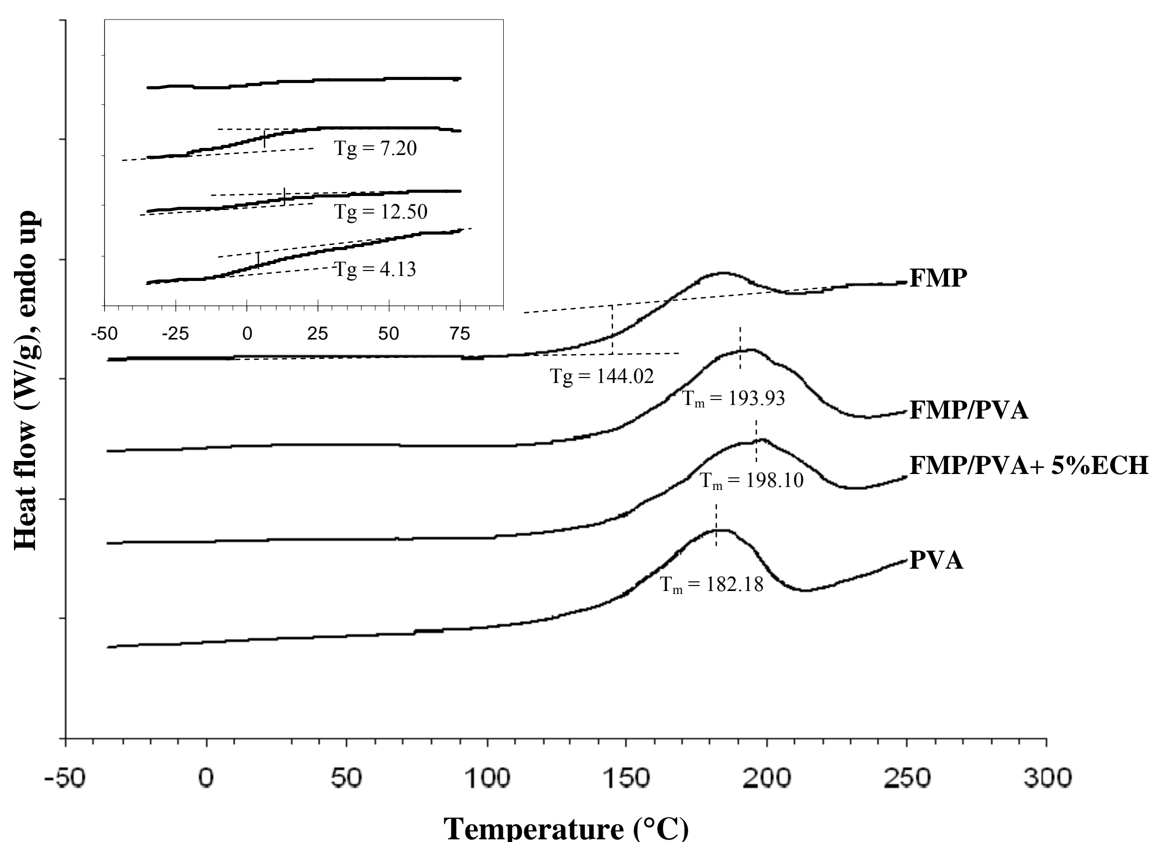


Figure 29. DSC thermograms of FMP film, PVA films and FMP/PVA (5:5) blend films without and with 5% ECH.

5.6.1 TGA measurement

Thermal degradation behavior of the polymeric film can be studied by using thermogravimetric analyzer (TGA). TGA thermograms of the control films (FMP film and PVA film) and FMP/PVA blend films without and with 5% ECH are depicted in Figure 30 and corresponding degradation temperatures (T_d) and weight loss (Δw) are

shown in Table 17. The initial weight loss (Δw_1= 4.4-8.5% wt) at temperature (T_{d1}) about 35.28-37.03°C of all films was related to the loss of free water adsorbed in the films. This was in agreement with Langmaier *et al*. (2008) and Nuthong *et al*. (2009). The second weight loss (Δw_2 = 26.96-31.06%) observed at temperatures (T_{d2}) ranged from 165.65°C to 171.37°C was most likely associated with the loss of bound water. Langmaier *et al*. (2008) also reported the similar result for glycerol plasticized collagen hydrolysate film in which the bound water was removed out at temperature ≈ 163-186°C. These T_{d1} and T_{d2} observed, presumably related to loss of water, were coincidental with transition temperatures (36.92- 48.58°C and 98-150°C) found in the first-step DSC scan which intended to dry the sample in DSC. The FMP film showed the third weight loss (Δw_3 = 50.85%) at 305.77°C, which mainly associated with the degradation of the major protein component as well as plasticizer incorporated in the film matrix. This degradation pattern of FMP film was similar to that of other protein films such as sodium casinate film, whey protein film and gelatin film (Barreto *et al.,* 2003). The initial temperature of degradation in the range 295-300°C of the pure protein films has been reported (Barreto *et al.,* 2003). Barreto *et al.* (2003) and Schmidt *et al.* (2005) investigated FTIR spectra of gas products evolved during the thermal degradation of collagen hydrolysate protein film. The degradation involved the formation of CO_2, CO, NH_3 and other unsaturated compounds, suggesting that the reaction mechanism included at the same time the scission of the C-N, C(O)-NH, C(O)-NH_2, -NH_2 and C(O)-OH bonds of the proteins and the mechanism of reaction occurred by random scission of the protein chains. For PVA film, it showed the third weight loss (Δw_3 = 49.79%) at 353.77°C followed by the forth weight loss (Δw_4 = 12.39%) at 440.23°C, most likely due to the decomposition of PVA molecules in the film matrix. From the result, it was noted that degradation of PVA film occurred at higher temperature than that of FMP film. Pawlak and Mucha (2003) reported that thermal degradation of PVA in the solid state involved predominantly the elimination of water and observed the formation of C=C double bonds in the polymer backbone due to molecular chain scissions. FMP/PVA blend films without and with 5% ECH showed similar degradation behavior in which the third weight loss (Δw_3 = 40.22-40.69%) and the forth weight loss (Δw_4 = 12.39-15.55%) were observed at 326.51-332.06°C and

419.93-422.42°C, respectively. This most likely reflected the characteristic degradation of FMP and PVA as major components in the blend films. From the result, the FMP/PVA blend film with 5% ECH exhibited higher degradation temperature than FMP/PVA blend film without ECH and FMP film, respectively. ECH added might cross-link between FMP and PVA in the film, resulted in enhanced thermal stability. Ray *et al.* (2009b) studied thermal stability of starch/PVA blend films with glycerol plasticized and cross-linked with 20% ECH. Intercomponent H-bonding between starch, PVA and glycerol enhanced the thermal stability of the films. But, incorporation of ECH lowered the thermal stability of the films. From the result, as temperature up to 500-800°C, there was the residual mass (or char) about 3, 7.7, 7.2 and 14% for PVA, FMP/PVA, FMP/PVA+5%ECH and FMP films, respectively. Among all films, FMP film had the highest residue mass, most likely due to the presence of highly cross-linked network via non-covalent and covalent bonds which stabilized film structure and also found in the same way of FMP/PVA blend film with the intermediated residue mass. On the contrary, almost of PVA film (97% wt) could be degraded when heated up to 800°C; this was most likely because PVA film network was stabilized by weak bonds. Therefore, thermal properties of FMP, PVA and blend films were varied depending on the differences in film compositions and molecular interactions which stabilized the film matrix. PVA incorporation together with ECH addition could improve thermal stability of FMP film.

Table 17. Thermal degradation temperature (T_d, °C) and weight loss (Δw, %) of FMP film, PVA film and FMP/PVA (5:5) blend films without and with 5% ECH.

Films	Δ_1*		Δ_2		Δ_3		Δ_4		**Residual mass (%)**
	T_{d1}	Δw_1	T_{d2}	Δw_2	T_{d3}	Δw_3	T_{d4}	Δw_4	
FMP	35.28	6.38	165.65	27.81	305.77	50.85	-	-	14.80
FMP/PVA	37.03	8.46	164.86	26.96	326.51	40.22	419.93	16.70	7.19
FMP/PVA + 5%ECH	36.92	4.56	163.92	31.06	332.06	40.69	422.42	15.55	7.68
PVA	36.07	4.44	171.37	30.38	353.77	49.79	440.23	12.39	2.92

* Δ_1, Δ_2, Δ_3 and Δ_4 referred to the first, second, third and fourth stage weight loss, respectively, as observed in TGA thermogram.

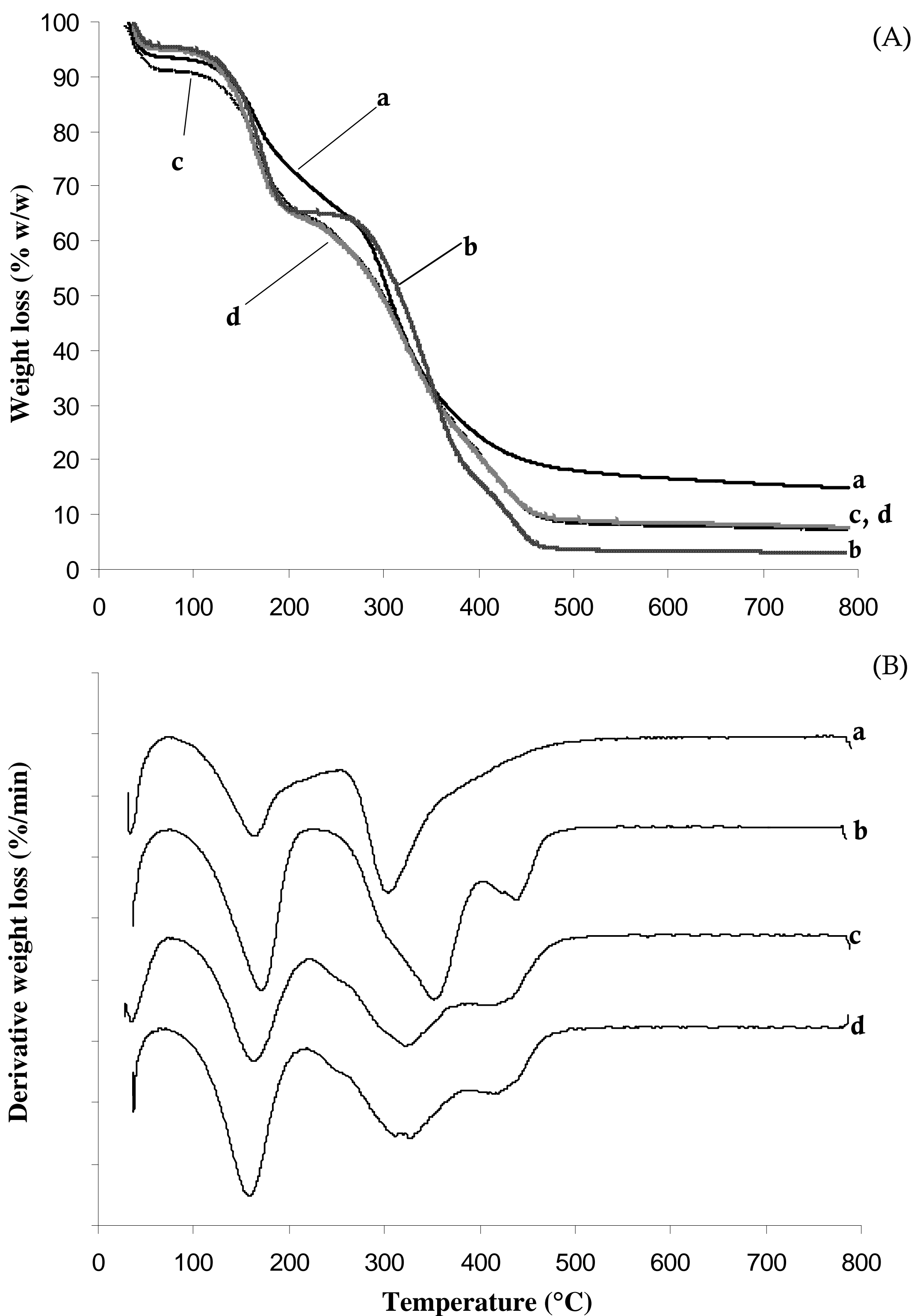


Figure 30. TGA data showing weight loss (A) and derivative weight loss (B) as a function of temperature of FMP film (a), PVA film (b) and FMP/PVA (5:5) blend films without (c) and with (d) 5% ECH.

5.7 Film morphology (SEM technique)

Figure 31 illustrates the SEM micrographs of the surface and freeze-fractured cross-section of the selected films (FMP film, PVA film and FMP/PVA blend films without and with 5% ECH). The surface morphology of FMP/PVA blend film with 5% ECH was similar to that of FMP film and FMP/PVA blend film, showing the formation of uniform and continuous structures without cracks or pores. This indicated that blend films with ordered matrix and homogeneous structure were formed. For PVA film, rough and discontinuous surface morphology was evident. The FMP film exhibited rougher cross-section area than that of the PVA and FMP/PVA blend films. On the contrary, cross-sectional image of FMP/PVA blend film and PVA film show smooth surface. The FMP/PVA blend films with and without ECH did not show any evidence of distinct separation or void in the matrix, which indicated the compatibility of the blend between FMP and PVA. The compatibility of FMP and PVA was most likely arising from the presence of their molecular interaction in the film matrix. From the result, protein molecules and PVA molecules might form highly interaction by both intra- and intermolecular H-bonds. With the addition of 5% ECH for FMP/PVA blend film, the cross-sectional surface of the blend film became slightly rougher. This suggested the inhomogenity of the film matrix, resulted from ECH cross-linking. When ECH was located between the chains, the molecular chains were push apart, increasing free volume in the film matrix. This could explain why the WVP of this film did not much decrease as compared to that of FMP/PVA blend film (Table 12). Costa-Junior *et al.* (2009) reported the morphological structure of chitosan/PVA (1:1) blend film cross-linked with glutaraldehyde. The surface morphology of the blend showed the formation of uniform and continuous film. Nevertheless, some effect of phase segregation was detected with "droplet-like" form onto these chitosan/PVA blends. They suggested that polymers, PVA and chitosan, prior to chemical cross-linking have their chains mostly physically entangled in the hydrogel network, but formed a chemical bonded hydrogel after glutaraldehyde cross-linking has taken place.

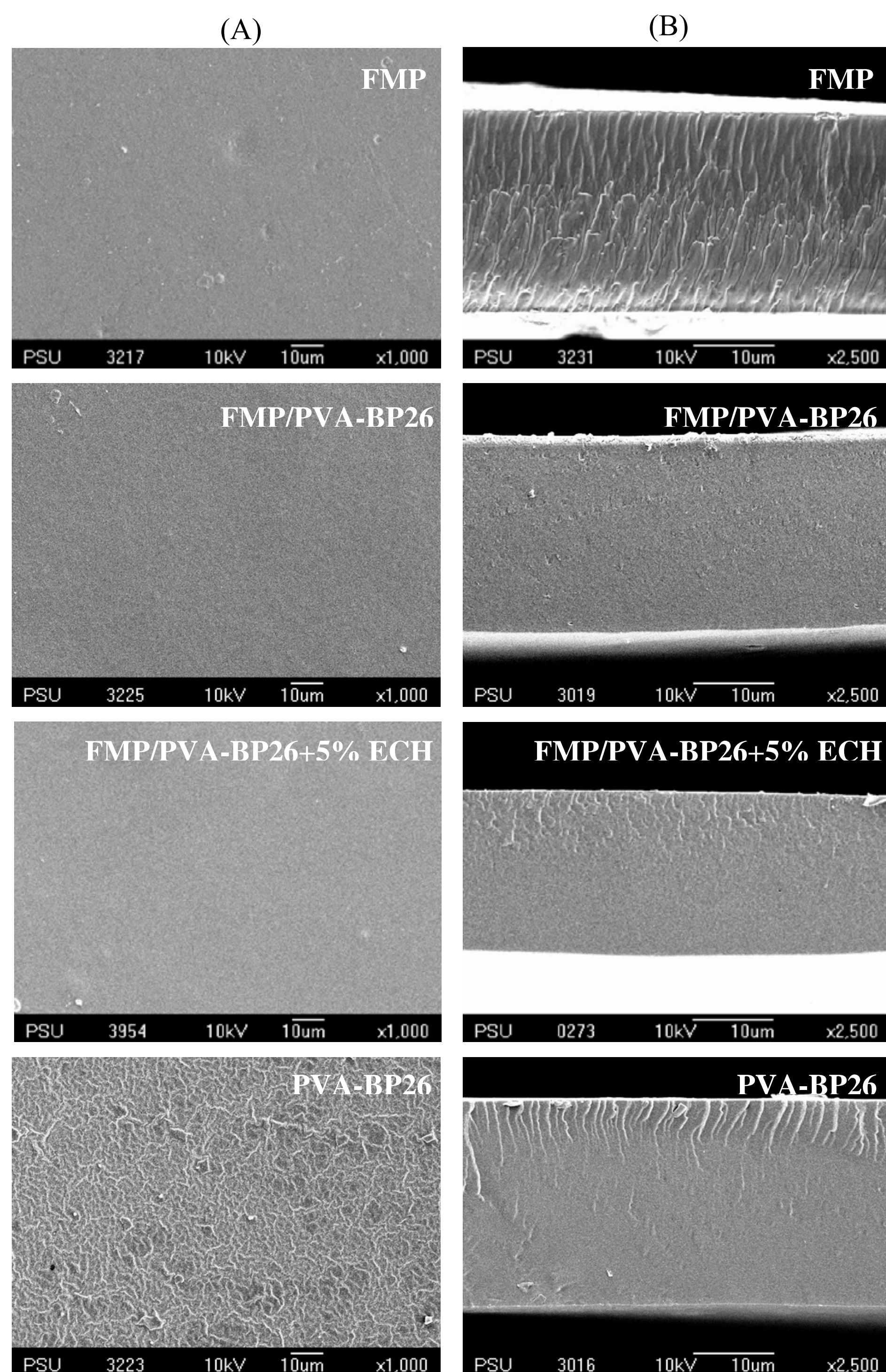


Figure 31. SEM micrographs of the surface (A) and freeze-fractured cross-section (B) of FMP film, PVA film and FMP/PVA blend films without and with 5% ECH.

6. Moisture sorption isotherm

The moisture sorption isotherm is a means to characterize the water absorption of the film, which in turn is transmitted to the product inside. Knowledge of sorption isotherm is also important for predicting stability and quality changes during packaging and storage product (Srinivasa *et al.,* 2003). Figure 32 shows moisture sorption isotherms of FMP film, PVA-BP26 film and FMP/PVA (5:5) blend film incorporated with 5% epichlorohydrin (ECH) (FMP/PVA+5%ECH) determined at room temperature (28-30°C). All films exhibited type-II sorption isotherm in which equilibrium moisture content increases with increasing water activity (A_w) in sigmoidal manner. This characteristic of sorption isotherm was normally found with those of most foods and bio-based films (Perdomo *et al*., 2009; Sudhamani *et al*., 2005; Al-Muhtaseb *et al*., 2002). Arthan (2006) reported that moisture sorption isotherm of round scad protein based-films was sigmoidal. At low water activity (0.18-0.46), moisture content of the films determined at room temperature increased slowly. Moisture content of films increased rapidly at A_w between 0.67-0.90. From the result, PVA film showed higher moisture sorption than FMP/PVA+5%ECH blend film and FMP film, respectively, most likely due to its hydrophilic nature. This result agreed with that of Srinivasa *et al*. (2003), who found the same sorption behavior in chitosan/PVA blend films. The equilibrium moisture content in the chitosan/PVA blend films increased with increase in the PVA concentration.

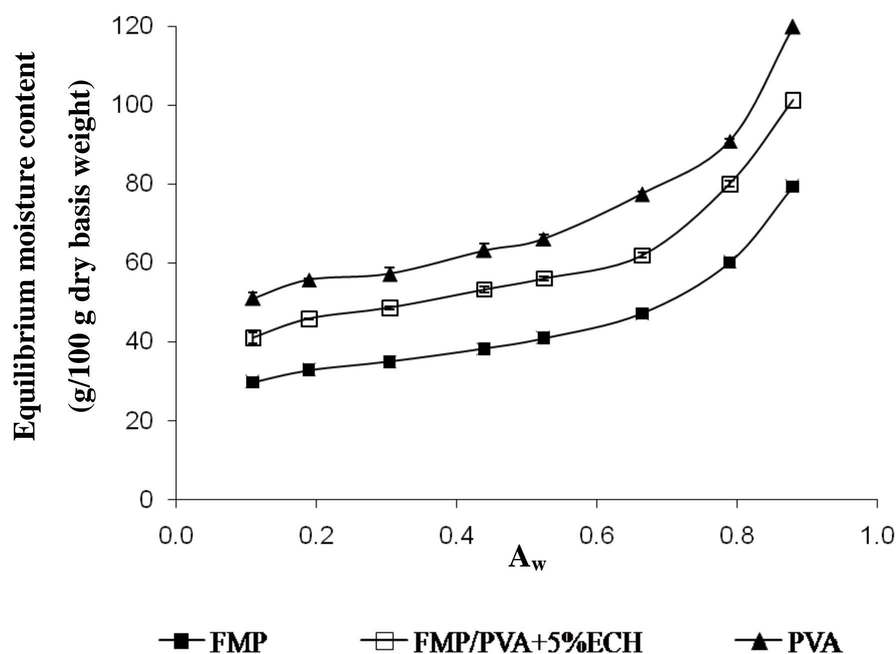


Figure 32. Moisture sorption isotherms (at 28-30°C) of FMP film, PVA-BP26 film and FMP/PVA (5:5) blend film incorporated with 5% epichlorohydrin (ECH). Bar represents the standard deviation from five determinations.

7. Changes in properties of FMP/PVA blend films during storage

7.1 Changes in mechanical properties

Mechanical properties of control films (FMP and PVA films) and FMP/PVA (5:5) blend film incorporated with 5% epichlorohydrin (ECH) stored under 65±5% RH at room temperature (28-30°C) are shown in Figure 33 (A-C). Elongation at break (EAB) of films remained generally constant during the 8 weeks of storage ($p>0.05$).Tensile strength (TS) and elastic modulus (E) of the films were increased at the beginning of the storage time (0-2 weeks) ($p<0.05$) and then seemed to level off. The increased strength and stiffness of the films suggested the more rigid or compact structure which could be resulted from the rearrangement or aggregation of polymer molecules in the film matrix. The aggregated structure possibly associated with the formation of non-covalent intermolecular interactions between the protein-protein, protein-PVA or PVA-PVA molecules. In addition, the increase in TS was postulated to be due to the migration of glycerol from bulk to the surface of film, due to the

limited bonds existing between protein molecules and glycerol. This might lead to the greater formation of cross-links (Anker *et al*., 2001; Park *et al*. 1994). Park *et al.* (1994) reported that the changes in mechanical properties of films made from wheat gluten protein and corn-zein mixtures plasticized with glycerol during 20 days of storage at 25°C and 50% RH were caused by the slowly migration of plasticizers from the bulk film to the surface, even when glycerol was initially well dispersed in the film forming solution. Moreover, the effect of storage time at 23°C, 50% RH for 16 weeks on the functional properties of glutenin-rich films plasticized with glycerol, sorbitol and triethanolamine was studied by Hernandez-Munoz *et al*. (2004b). They concluded that the mechanical properties of films plasticized with glycerol changed drastically with time due to glycerol migration; then, the films became harder and less flexible as the storage time increased, while the properties of films plasticized with sorbitol or triethanolamine remained stable during storage time. Nevertheless, Cuq *et al.* (1996b) reported that mechanical properties of myofibrillar protein-based films plasticized with saccharose did not change during storage for 8 weeks at 20°C and 58% RH. Similar results have been reported for the aging of whey protein films (Anker *et al*., 2001; Oses *et al*., 2009). From the result, it can be observed that TS and EAB of FMP/PVA blend film added with 5% ECH remained basically constant during the 8 weeks of storage. The ECH addition might be an assistance in stabilizing the film network. Thus, the FMP/PVA+5% ECH blend film was stable throughout the storage of 8 weeks at 28-30°C and 65±5% RH.

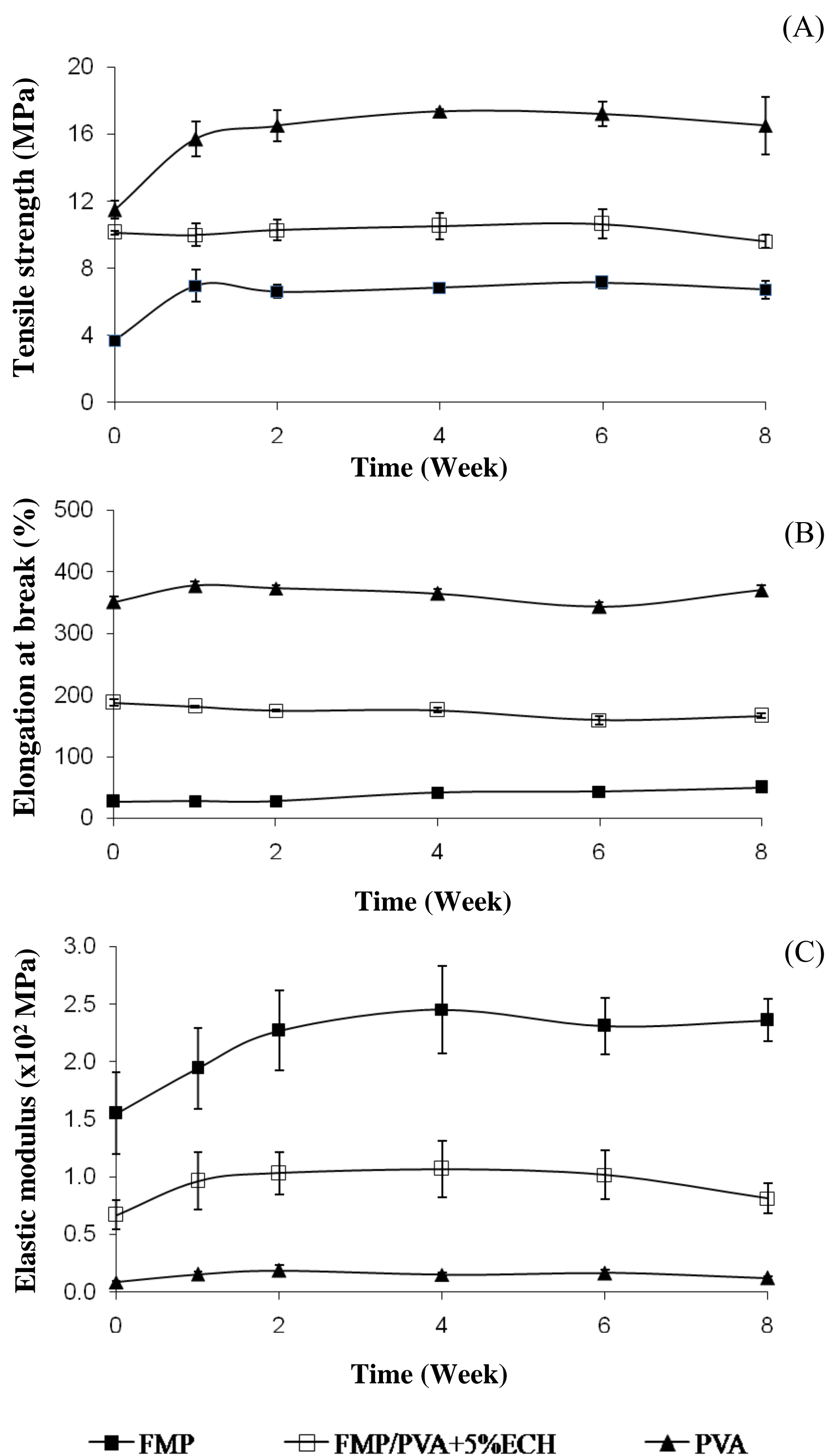


Figure 33. Changes in tensile strength (A), elongation at break (B) and elastic modulus (C) of control films (FMP and PVA) and FMP/PVA (5:5) blend film added with 5% epichlorohydrin (ECH) during the storage at 28-30°C and 65±5% RH. Bar represents the standard deviation from ten determinations.

7.2 Changes in moisture content and water vapor permeability

Moisture content and water vapor permeability (WVP) of control films (FMP and PVA films) and FMP/PVA-BP26 (5:5) blend film added with 5% ECH (FMP/PVA+5%ECH) stored under 65±5% RH at room temperature (28-30°C) are presented in Figure 34 (A and B), respectively. Moisture content of all films was continuously reduced during 0-4 weeks of storage and tended to be constant thereafter. PVA film had higher moisture content than FMP/PVA+5%ECH blend film and FMP film, respectively, throughout the storage time. For WVP, PVA and blend films exhibited decreased WVP as storage time increased up to 2 weeks, after that WVP remained constant. No change in WVP of FMP film was observed over 8 weeks of storage ($p>0.05$). The decrease in moisture content and WVP of the films during the first two weeks of storage was most likely associated with the molecular arrangement leading to the more order film structure. This also possibly caused the concomitant increase in TS and E of films observed at 0-2 weeks of storage (Figure 33A and 33C). Anker *et al.* (2001) and Hernandez-Munoz *et al*. (2004b) also found the decrease in moisture content and WVP with extended storage time of glycerol plasticized whey protein isolate film and glutenin-rich film. They postulated that this was due to the glycerol migration. However, Cuq *et al*. (1996b) reported that WVP of film from Atlantic sardine myofibrillar protein remained unchanged upon 8 weeks of storage at 20°C and 58% RH.

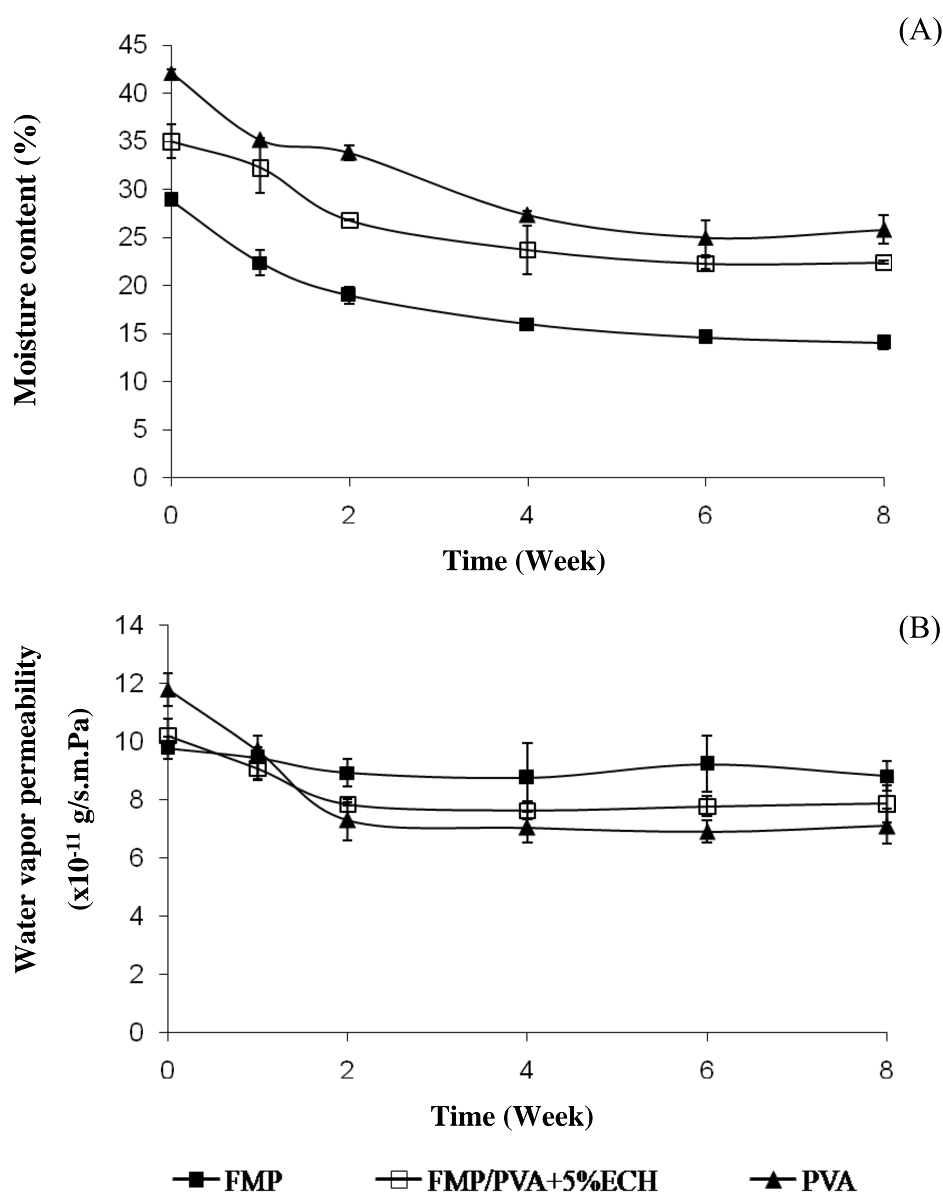


Figure 34. Changes in moisture content (A) and water vapor permeability (WVP) (B) of control films (FMP and PVA film) and FMP/PVA (5:5) blend film added with 5% epichlorohydrin (ECH) during the storage at 28-30°C and 65±5% RH. Bar represents the standard deviation from five determinations.

7.3 Changes in color and film transparency

L*, a* and b*-values of control films (FMP and PVA films) and FMP/PVA-BP26 (5:5) blend film added with 5% ECH (FMP/PVA+5%ECH) during storage under 65±5% RH at room temperature (28-30°C) are presented in Figure 35 (A-C). The color of PVA film did not change during 8 weeks of storage ($p>0.05$). This film remained transparent and clear after conditioning. In contrast, FMP and FMP/PVA+5%ECH films became darker as evidenced by the decrease in L* and a*-values ($p<0.05$). The increased in b*-value was observed for those films stored for the longer time ($p<0.05$). The results suggested that the films were more yellowish with increasing storage time. The increase in b*-value could be a result of non-enzymatic browning reaction. In general, the yellow/brown coloration has been reported to be associated with protein-aldehyde interactions via Maillard reaction, and the reaction rate is strongly dependent on the material composition, temperature, moisture content, relative humidity and pH (Cuq *et al.,* 1996b). After 8 weeks of storage, the FMP film was translucent with a yellowish color as expressed the lowest L* and a*-values and the highest b*-value. During increased storage time, protein might undergo degradation for some extents, which resulted in increased free amino groups available for Maillard reaction. The results were in agreement with the finding of Arthan *et al*. (2009), who observed the increased b*-value but decreased L* and a*-values of round scad protein-based films incorporated without and with oil or oil/chitosan during storage under 54% RH at room temperature (28-30°C) for 8 weeks. The similar results have been reported for aging of fish myofibrillar protein-based film from Atlantic sardines (Cuq *et al.*, 1996b). The yellow hue of fish myofibrillar protein-based film with saccharose plasticized was slightly increased during storage at 58% RH and 20°C for 8 weeks, plausibly due to the result of non-enzymatic browning reactions between protein and reducing sugars produced by partial hydrolysis of the saccharose introduced in the formulation.

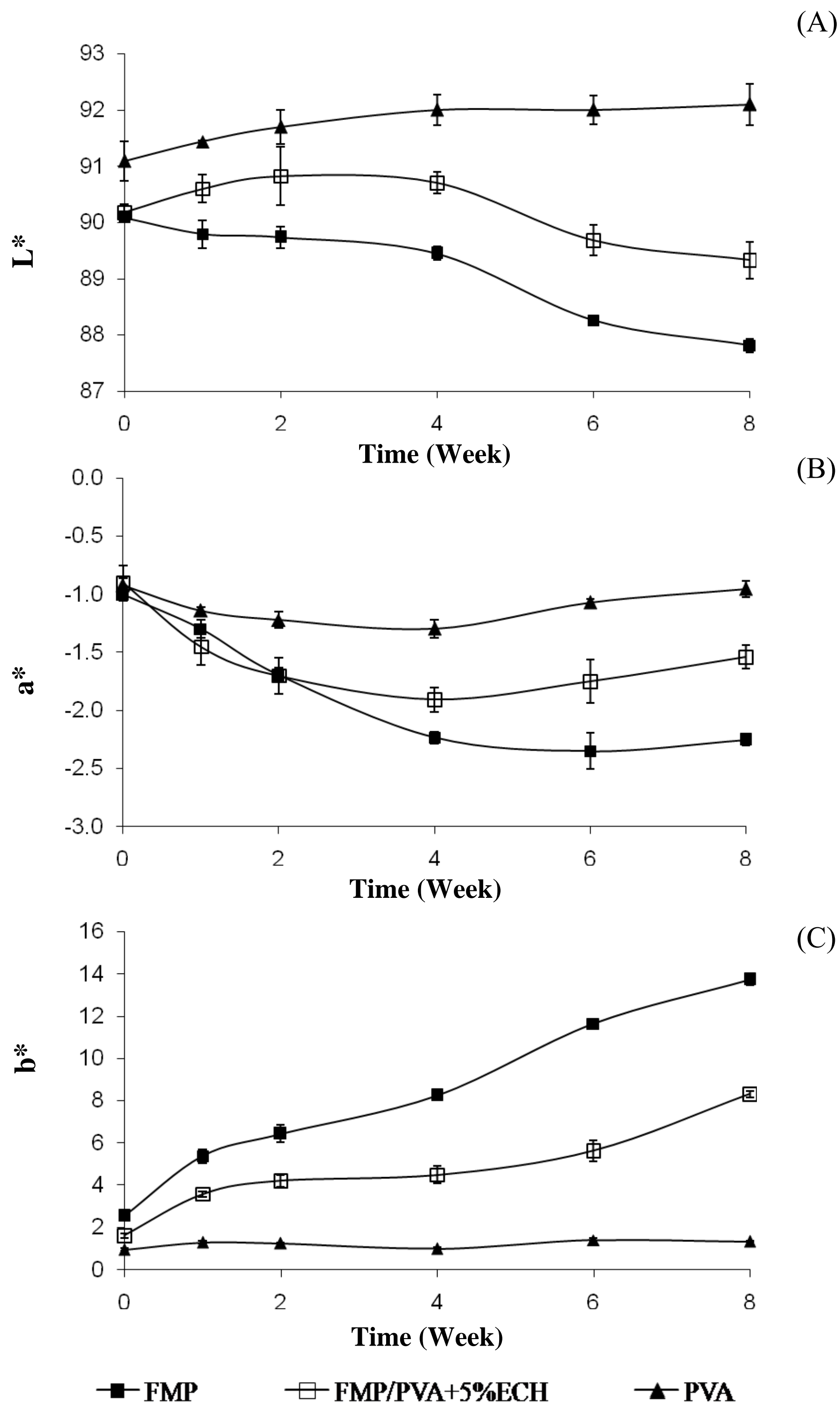


Figure 35. Changes in L*, a* and b* values of control films (FMP and PVA) and FMP/PVA (5:5) blend film added with 5% epichlorohydrin (ECH) during the storage at 28-30°C and 65±5% RH. Bar represents the standard deviation from five determinations.

Transparency value of control films (FMP and PVA films) and FMP/PVA-BP26 (5:5) blend film added with 5% ECH (FMP/PVA+5%ECH) was evaluated as a function of storage time and presented in Figure 36. Among all films, PVA film exhibited the most transparent as evidenced by the lowest transparency value throughout the storage. A slightly increased transparency value (i.e., decreased film transparence) was observed in all films stored for 1 week of storage. This was possibly caused by the reorganization or aggregation of polymer molecules in the film matrix. However, the transparency value remained relatively constant after 1 weeks of storage. The similar results have been reported for aging of cod skin gelatin-based film (Perez-Mateos *et al.*, 2009). Transparency value of this film did not change during storage under 22°C and 58% RH condition for 1 month. Arthan *et al*. (2009) observed the increase in opacity of round scad protein-based films incorporated without and with oil or oil/chitosan during storage under 54% RH at room temperature (28-30°C) for 8 weeks.

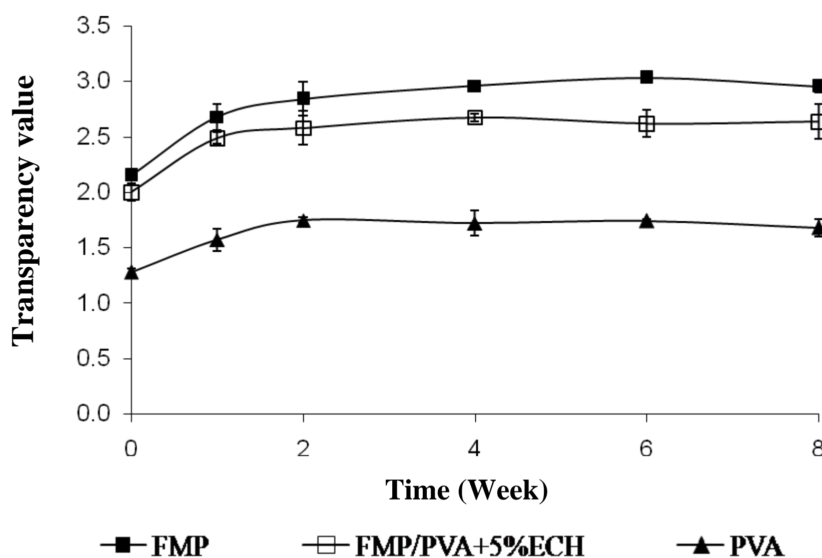


Figure 36. Changes in transparency value of control film (FMP and PVA) and FMP/PVA (5:5) blend film added with 5% epichlorohydrin (ECH) during the storage at 28-30°C and 65±5% RH. Bar represents the standard deviation from triplicate determinations.

CHAPTER 4

CONCLUSION

1. Properties of fish myofibrillar protein (FMP) films could be modified by poly(vinyl alcohol) (PVA) blending. The level of PVA incorporated and pH affected the properties of FMP/PVA blend film. PVA incorporation at an appropriate level could improve the mechanical properties by increasing TS and EAB and lowering the redness and yellowness of FMP films. Those properties were governed by the interaction between myofibrillar protein and PVA. FMP/PVA blend film at a ratio of 5:5 and prepared at acidic condition (pH 3) had relatively higher mechanical properties as well as water-vapor barrier property in comparison with other blend samples.

2. The properties of FMP/PVA blend films varied, depending on the degree of hydrolysis (DH) and molecular weight (MW) of PVA used. In general, PVA of higher MW yielded the blend film with greater TS and EAB. PVA with higher DH resulted in the higher rigidity of the blend film. Incorporation of an appropriate PVA could improve the water-vapor barrier property of the blend film. The compatibility of protein and PVA molecules played an important role in the improved properties of the FMP/PVA blend film. FMP film incorporated with PVA-BP26 (DH = 86-89% mol (partial hydrolysis type) and MW= 124,000 – 130,000 g/mol) exhibited the best mechanical properties with a comparatively low WVP.

3. Various chemicals incorporation had the impact on the properties of FMP/PVA blend film differently, mostly governed by the type and concentration used. Each chemical at an appropriate amount could improve mechanical properties of blend films. FMP/PVA blend films incorporated with ECH at the level of 5% (w/w of total polymer) had the highest mechanical properties and relatively low WVP. Solubility in water and WVP of FMP/PVA blend film were decreased with GLX incorporation. MA incorporation rendered blend film with the lowest WVP and film transparency.

4. FMP and FMP/PVA blend film were stabilized by hydrogen bonds, hydrophobic interactions, as well as disulfide and non-disulfide covalent bonds. The compatibility of FMP and PVA was most likely arisen from the presence of their inter-molecular interaction in the film matrix. PVA and ECH incorporation increased thermal stability as well as transition temperatures of FMP film.

5. FMP/PVA blend film incorporated with 5% ECH displayed high stability during the extended storage under 65±5% RH at room temperature (28-30°C). However, FMP film and the blend film became darker and more yellowish during 8 weeks of storage.

SUGGESTIONS

1. The improvement of water vapor barrier property of FMP/PVA blend film by adding other additives which significantly increase hydrophobicity of resulting film should be further studied.

2. More research on preventing discoloration of film induced by Maillard reaction during drying and storage should be conducted.

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APPENDIX

APPENDIX

ANALYTICAL METHODS

1. Determination of moisture content (AOAC, 2000)

Method

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

Calculation

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

where W1 = weight (g) of sample before drying
W2 = weight (g) of sample after drying

2. Determination of protein content (AOAC, 2000)

Reagents

1. Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) with 1 part of copper sulphate ($CuSO_4$)
2. Sulfuric acid (H_2SO_4)
3. 40% NaOH solution (w/v)
4. 0.2 N 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 Kjedahl catalyst and 20 ml of conc. H_2SO_4
3. Prepare a tube containing the above chemical except sample as blank. Place flasks in inclined position and heat gently unit frothing ceases. Boil briskly until solution clears.
4. Cool and add 60 ml distilled water cautiously.
5. Immediately connect flask to digestion bulb on condenser and with tip of condenser immersed in standard acid and 5-7 indicator in receiver. Rotate flask to mix content thoroughly; then heat until all NH_3 is distilled.
6. Remove receiver, wash tip of condenser and titrate excess standard acid distilled with standard NaOH solution.

Calculation

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

where

A	= volume (ml) of 0.2 N HCl used sample titration
B	= volume (ml) of 0.2 N HCl used in blank titration
N	= normality of HCl
W	= weight (g) of sample
14.007	= atomic weight of nitrogen
6.25	= the protein-nitrogen conversion factor for fish and its by-products

3. Determination of ash content (AOAC, 2000)

Method

1. Place the crucible and lid in the furnace at 550°C overnight to ensure that impurities on the surface of crucible are burned off.
2. Cool the crucible in the desiccator (30 min).
3. Weigh the crucible and lid to 3 decimal places.
4. Weigh about 5 g sample into the crucible. Heat over low Bunsen flame

with lid half covered. When fumes are no longer produced, place crucible and lid in furnace.

5. Heat at 550°C overnight. During heating, do not cover the lid. Place the lid after complete heating to prevent loss of fluffy ash. Cool down in the desiccator.
6. Weigh the ash with crucible and lid when the sample turns to gray. If not, return the crucible and lid to the furnace for the further ashing.

Calculation

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

4. Determination of fat content (AOAC, 2000)

Reagent

1. Petroleum ether

Method

1. Place the bottle and lid in the incubator at 105°C overnight to ensure that weight of bottle is 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 is completely evaporated and bottle is 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

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

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

Reagents

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

Method

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

Table: Experimental set up for the Biuret's assay

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

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

Reagent

1. 30% Arylamide-0.8% bis Acrylamide
2. Sample buffer: Mix 4 ml of 10% of SDS, 10 ml of glycerol, in the present or absence of β-mercaptoethanol 1 ml, 12.5 ml of 50 mM Tris-HCl, pH 6.8, and 0.03 g bromophenol blue. Bring the volumn to 10 ml with distilled water. Divide into 1 ml aliquots, and store at -20 °C.
4. 10% (w/v) Ammonium persulfate
5. 10% (w/v) SDS
6. TEMED (*N,N,N'N'*- tetramethylethylenediamine)
7. 0.5 M Tris-HCl, pH 6.8
8. 1.5 M Tris-HCl, pH 8.8
9. Electrode buffer: Dissolve 3 g of Tris-HCl, 14.4 g of glycine and 1 g of SDS in distilled water. Adjust to pH 8.3. Add distilled water to 1 liter to total volume.
10. Staining solution: Dissolve 0.04 g of Coomassie blue R-250 in 100 ml methanol. Add 15 ml of glacial acetic and 85 ml of distilled water.
11. Destaining solution I: 50% methanol-7.5% glacial acetic acid
12. Destaining solution II: 5% methanol-7.5% glacial acetic acid

Method

Pouring the running gel:

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

Pouring the stacking gel:

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

Table: Experimental set up for running and stacking gel

Reagents	10 % running gel	4% stacking gel
30% Acrylamide-bis	3.333 mL	0.665 mL
1.5 M Tria-HCl buffer, pH 8.8	2.500 mL	-
0.5 M Tris-HCl buffer, pH 6.8	-	1.25 mL
Distilled water	4.012 mL	3.00 mL
10% SDS	100 µL	50 µL
10% Ammonium persulfate	50 µL	25 µL
TEMED	5 µL	3 µL

Sample preparation:

1. Fish muscle 3 g and 27 ml of 5% SDS were mixed and homogenized at 13,000 rpm for 1 min.
2. The sample was incubated at 85ºC for 1 h to dissolve total protein and then centrifuged at 8,500*xg* for 10 min at ambient temperature and collect supernatant.
3. Protein 30 µg was determined by Biuret method.

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°.
2. Remove the comb without tearing the edge of the polyacrylamide wells.
3. Fill the wells with electrode buffer.
4. Place the upper chamber over the sandwich and lock the upper buffer

chamber to the sandwich. Pour electrode buffer into the lower buffer chamber. Place the sandwich attached to the upper buffer chamber into the lower chamber.

5. Fill the upper buffer chamber with electrode buffer so that the sample wells of the stacking gel are filled with buffer.
6. Use a 10-25 µL syringe with a flate-tipped needle; load 15 µg 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 constant current at 30 Am.
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 was and gently pry the two plates apart.
4. Remove the gel from the lower plate. Place the plate with the gel attached into the small plastic box and swishing the plate.

Staining the gel:

1. Cover the gel 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 destaining solution II. Agitate until the gel back ground is clear except for the protein bands.