Effect of Gelatin Hydrolysate on Properties of Fish Myofibrillar Protein Film

Sunisa Nuanmano

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Author Miss Sunisa Nuanmano

Major Program Packaging Technology

Major Advisor

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

Co-advisor

……
(Prof. Dr. Soottawat Benjakul)

Examining Committee

…..……..… Chairperson
(Dr. Ponusa Jitphuthi)

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

……..……Committee
(Prof. Dr. Soottawat Benjakul)

……..Committee
(Asst. Prof. Dr. Sureurg Khongtong)

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

……...…….
(Assoc. Prof. Dr. Teerapol Srichana)
Dean of Graduate School

This is to certify that the work here submitted is the result of the candidate‘s own investigations. Due acknowledgement has been made of any assistance received.

……Signature
(Asst. Prof. Dr. Thummanoon Prodpran)
Major Advisor

……...……Signature
(Miss Sunisa Nuanmano)
Candidate

I hereby certify that this work has not already been accepted in substance for any degree, and is not being concurrently submitted in candidature for any degree.

……..……Signature

(Miss Sunisa Nuanmano)

Candidate

ชื่อวิทยานิพนธ์ ผลของเจลาตินไฮโดรไลเสตต่อสมบัติของฟิล์มโปรตีนไมโอไฟบริลจากกล้ามเนื้อปลา

ผู้เขียน นางสาวสุนิสา นวลมะโน

สาขาวิชา เทคโนโลยีบรรจุภัณฑ์

ปีการศึกษา 2557

บทคัดย่อ

จากการศึกษาผลของการใช้เจลาตินไฮโดรไลเสตจากปลาที่มีระดับการไฮโดรไลซิสต่างๆ (DH = 23, 61 และ 95%) เป็นพลาสติไซเซอร์ในปริมาณต่างๆ (30 – 60%) ในฟิล์มโปรตีนไมโอไฟบริลจากปลาทับทิมน้ำจืด (*Oreochromis niloticus*) (FMP) เปรียบเทียบกับการใช้กลีเซอรอล พบว่า ฟิล์มทุกชุดการทดลองมีความอ่อนตัวและยืดหยุ่นเพิ่มขึ้นเมื่อเปรียบเทียบกับฟิล์ม FMP ควบคุมที่ไม่เติมพลาสติไซเซอร์ ซึ่งมีความแข็งเปราะ ฟิล์มที่เติมเจลาตินไฮโดรไลเสตที่ระดับ 50 และ 60% ของ FMP มีค่าอิลาสติกมอดุลัส (E) และการต้านทานแรงดึง (TS) สูงกว่า แต่มีค่าการซึมผ่านไอน้ำ (WVP) ต่ำกว่าฟิล์มที่เติมกลีเซอรอลในปริมาณเดียวกัน ($p<0.05$) และที่ระดับการไฮโดรไลซิสเดียวกัน ค่า E และ TS ของฟิล์มมีค่าลดลง ในขณะที่ค่าการยืดตัวเมื่อขาด (EAB) และค่า WVP มีค่าสูงขึ้น เมื่อปริมาณเจลาตินไฮโดรไลเสตที่เติมในฟิล์มสูงขึ้น ($p<0.05$) และพบว่าเมื่อใช้เจลาตินไฮโดรไลเสตในปริมาณเดียวกัน ฟิล์มที่เติมเจลาตินไฮโดรไลเสตที่มีระดับการไฮโดรไลซิส (DH) สูงกว่าจะมีค่า TS และ E ต่ำกว่า แต่มีค่า WVP สูงกว่า อย่างไรก็ตามฟิล์ม FMP ที่เติมเจลาตินไฮโดรไลเสตมีค่า b^* (สีเหลือง) สูงกว่า และการส่องผ่านแสงต่ำกว่า เมื่อเปรียบเทียบกับฟิล์มที่เติมกลีเซอรอล ($p<0.05$)

นอกจากนี้ได้ทำการศึกษาการใช้ของผสมระหว่างกลีเซอรอล (GLY) และเจลาตินไฮโดรไลเสต (61% DH) (GH) ที่อัตราส่วนผสมต่างๆ (GLY/GH = 100/0, 75/25, 50/50, 25/75 และ 0/100) เพื่อเป็นพลาสติไซเซอร์ในฟิล์ม FMP ที่ปริมาณ 50% ของ FMP พบว่า ค่า TS และ E ของฟิล์มมีค่าสูงขึ้น ในขณะที่ค่า EAB ลดลง เมื่อปริมาณเจลาตินไฮโดรไลเสตที่ใช้ในของผสม GLY/GH เพิ่มขึ้น ($p<0.05$) นอกจากนี้เมื่อปริมาณเจลาตินไฮโดรไลเสตในของผสม GLY/GH เพิ่มขึ้น ส่งผลให้ค่า WVP และการส่องผ่านแสงของฟิล์มลดลง แต่ฟิล์มที่ได้มีสีเหลืองมากขึ้น

จากการวิเคราะห์เปรียบเทียบลักษณะเฉพาะของฟิล์ม FMP ที่เติมกลีเซอรอล เจลาตินไฮโดรไลเสต (60% DH) และของผสมระหว่างกลีเซอรอล/เจลาตินไฮโดรไลเสต (GLY/GH = 25/75) เป็นพลาสติไซเซอร์ พบว่า ฟิล์ม FMP ที่เติมกลีเซอรอลมีค่าปริมาณความชื้นและการละลายน้ำของฟิล์มสูงที่สุด เมื่อเปรียบเทียบกับฟิล์มอื่นๆ ($p<0.05$) โดยที่ค่าการละลายน้ำของฟิล์มที่เติม GH และที่เติมของผสมระหว่าง GLY/GH ไม่มีความแตกต่างกันอย่างมีนัยสำคัญ ($p>0.05$) นอกจากนี้ฟิล์มทั้งหมดมีค่าการละลายของโปรตีนในน้ำและการละลายของโปรตีนในตัวทำละลายชนิดต่างๆ ไม่แตกต่างกัน ($p>0.05$) จากผลการวิเคราะห์ค่าการละลายของโปรตีนในตัวทำละลายชนิดต่างๆ และรูปแบบโปรตีน แสดงให้เห็นว่าโครงข่ายของฟิล์ม FMP เกิดจากพันธะไฮโดรเจนเป็นส่วนใหญ่ รวมทั้งอันตรกิริยาไฮโดรโฟบิกและพันธะไดซัลไฟด์ โดยไม่ขึ้นกับชนิดของพลาสติไซเซอร์ที่ใช้ และจากการผลการวิเคราะห์โดยเทคนิค FTIR พบว่า แถบการดูดกลืน Amide-A ของฟิล์มที่เติม GH หรือ GLY/GH มีลักษณะที่กว้างกว่า เมื่อเปรียบเทียบกับของฟิล์มที่เติมกลีเซอรอล ซึ่งแสดงถึงการเกิดอันตรกิริยาโดยพันธะไฮโดรเจนระหว่างสายโซ่ของ FMP และเจลาตินไฮโดรไลเสตที่เกิดได้มากกว่า นอกจากนี้จากผลการวิเคราะห์ด้วยเทคนิค SEM และ TGA พบว่าฟิล์มที่เติมเจลาตินไฮโดรไลเสตมีลักษณะโครงสร้างที่แน่นกว่า และฟิล์มที่ได้มีความคงตัวทางความร้อนสูงกว่า (มีค่าอุณหภูมิการสลายตัวทางความร้อนสูงกว่า) เมื่อเปรียบเทียบกับฟิล์มที่เติมกลีเซอรอลเป็นพลาสติไซเซอร์

จากการศึกษาการเก็บรักษาตัวอย่างฟิล์มที่เลือกที่อุณหภูมิห้อง (28 – 30 องศาเซลเซียส) เป็นเวลา 8 สัปดาห์ พบว่า สมบัติเชิงกล (ค่า E, TS และ EAB) ของฟิล์มที่เติมกลีเซอรอลในปริมาณ 50% มีค่าค่อนข้างคงที่ ($p<0.05$) อย่างไรก็ตามฟิล์มที่เติม GH และ GLY/GH มีค่า E และ TS สูงขึ้น แต่ค่า EAB ต่ำลง ระหว่างการเก็บรักษาเป็นเวลา 8 สัปดาห์ ($p<0.05$) นอกจากนี้พบว่า ฟิล์มทุกชนิดมีค่า WVP และการส่องผ่านแสงต่ำลง แต่มีค่า b^* (สีเหลือง) สูงขึ้นเมื่อเพิ่มระยะเวลาการเก็บรักษา ($p<0.05$) รวมทั้งพบว่า ค่าการละลายของฟิล์ม และการละลายของโปรตีนในน้ำและในตัวทำละลายต่างๆ เพิ่มสูงขึ้นอย่างมาก เมื่อระยะเวลาการเก็บรักษานานขึ้น ทั้งนี้น่าจะเกี่ยวข้องกับการเกิดอันตรกิริยาโดยพันธะโควาเลนต์และที่ไม่ใช่พันธะโควาเลนต์ขององค์ประกอบของฟิล์มมากขึ้นระหว่างการเก็บรักษาที่นานขึ้น

นอกจากนี้ได้ทำการศึกษาความสามารถในการปิดผนึกฟิล์ม FMP ที่เติมเจลาติน ไฮโดรไลเสต โดยการใช้สารยึดติดธรรมชาติ (แป้งมันสำปะหลัง เจลาติน และโปรตีนไอโซ-เลตถั่วเหลือง) ร่วมกับการปิดผนึกด้วยความร้อนแบบอิมพัลส์ที่ระยะเวลาการปิดผนึกต่างๆ (1.5 2.5 และ 3.5 วินาที) จากการตรวจสอบความต้านทานต่อการลอกของรอยปิดผนึก พบว่ารอยปิดผนึกฟิล์มที่ผ่านการปิดผนึกโดยใช้เจลาตินหรือโปรตีนไอโซเลตถั่วเหลืองเป็นสารยึดติด มีค่าความแข็งแรงของรอยปิดผนึกที่สูงขึ้นเมื่อระยะเวลาการปิดผนึกด้วยความร้อนที่ใช้มีค่าสูงขึ้น ($p<0.05$) การใช้เจลาตินเป็นสารยึดติดให้รอยปิดผนึกฟิล์มที่มีความแข็งแรงสูงที่สุด ($p<0.05$) รองลงมาคือการใช้โปรตีนไอโซเลตถั่วเหลืองและแป้งมันสำปะหลังตามลำดับ ทั้งนี้สภาวะการปิดผนึกที่ทำการศึกษาที่ให้รอยปิดผนึกที่มีความแข็งแรงสูงที่สุด คือสภาวะที่มีการใช้เจลาตินเป็นสารยึดติดร่วมกับการปิดผนึกด้วยความร้อนเป็นระยะเวลา 3.5 วินาที

Thesis Title Effect of Gelatin Hydrolysate on Properties of Fish MyofibrillarProtein Film

Author Miss Sunisa Nuanmano

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ABSTRACT

The effect of using fish gelatin hydrolysates having different degree of hydrolysis (DH: 23, 61 and 95%) as plasticizer at various levels (30 – 60%) in film of fish myofibrillar protein (FMP) from red tilapia (*Oreochromis niloticus*) was investigated, in comparison with glycerol. All films exhibited increased film flexibility as compared to the control FMP film without plasticizer which was brittle. Regardless of DH, FMP films incorporated with gelatin hydrolysates at 50 and 60% of FMP generally had higher elastic modulus (E) and tensile strength (TS) but lower water vapor permeability (WVP), compared with those added with glycerol at the same level ($p<0.05$). At the same DH, both E and TS of film decreased, while elongation at break (EAB) and WVP increased with increasing levels of gelatin hydrolysate ($p<0.05$). When hydrolysate at the same level was used, the decrease in TS and E but the increase in WVP was found as DH increased. Nevertheless, FMP film added with gelatin hydrolysate exhibited the higher *b**-value (yellowness) and lower transparent, compared with the film containing glycerol ($p<0.05$).

Moreover, use of the blend of glycerol (GLY) and 61% DH gelatin hydrolysate (GH) of various GLY/GH ratios (100/0, 75/25, 50/50, 25/75 and 0/100) at 50% of FMP as plasticizer in the FMP film was also studied. Both TS and E of films increased while EAB of the films decreased as GH level in the blend increased ($p<0.05$). The presence of GH at higher level in the GLY/GH blend resulted in decreased WVP and transparency but increased yellowness of the FMP film.

FMP films plasticized with glycerol, 60% DH fish gelatin hydrolysate (GH) and glycerol/gelatin hydrolysate blend (GLY/GH = 25/75) were comparatively characterized. In general, the FMP film plasticized with glycerol possessed the highest moisture content and film solubility in water as compared to the others ($p<0.05$). Film solubility of the films added with GH and GLY/GH was not

significantly different ($p>0.05$). Moreover, all films exhibited similar protein solubilities in water and different denaturing solvents ($p>0.05$). As revealed by protein solubility in various solvents and SDS-PAGE protein patterns, FMP films were stabilized by mainly hydrogen bond as well as hydrophobic interaction and disulfide bond, regardless of type of plasticizer used. Based on FTIR analysis, the Amide-A peak became broader for films added with GH or GLY/GH blend as compared to those added with only glycerol, suggesting the greater interaction via hydrogen bond between FMP chains and gelatin hydrolysate. Moreover, SEM and TGA results respectively indicated that the films added with gelatin hydrolysate had more compact structure and thermal stability (higher degradation temperature), compared to that added with glycerol as plasticizer.

During the storage at room temperature (28 – 30 °C) for 8 weeks of the selected film samples, mechanical properties (E, TS and EAB) of the film added with 50% glycerol remained constant ($p>0.05$). However, films added with GH and GLY/GH blend had increased E and TS but decreased EAB upon 8 weeks of storage ($p<0.05$). In addition, WVP and transparency of all films seemed to decrease but *b**-value (yellowness) increased with increasing storage time ($p<0.05$). Dramatic decreases in film solubility as well as protein solubility in water and various solvents were noticed as storage time increased ($p<0.05$), more likely associated with the formation of various interactions via non-covalent and covalent bonds of the components in the film during the extended storage.

Moreover, sealing ability of FMP film incorporated with gelatin hydrolysate, sealed by means of applying different natural adhesives (tapioca flour, gelatin and soy protein isolate) in combination with impulse heat sealing at various times (1.5, 2.5 and 3.5 s) was investigated. As evaluated by peel strength test, films sealed with gelatin or soy protein isolate had increased seal strength with increasing heat sealing time used ($p<0.05$). Irrespective of heat sealing time, use of gelatin as adhesive rendered the seal with the highest seal strength ($p<0.05$), followed by soy protein isolate and tapioca starch, respectively. Among sealing conditions studied, the highest seal strength was observed when gelatin was used as adhesive and heat sealed for 3.5 s.

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CHAPTER 1

INTRODUCTION

Biodegradable packaging from biopolymers have been received the increasing attention to produce environmentally friendly packaging alternative to synthetic plastic packaging films which generally made from non-biodegradable synthetic polymers (Kester and Fennema, 1986; Krochta and De Mulder-Johnston, 1997). Among all biopolymers, protein has been empirically used as packaging materials because of their relative abundance, good film formation ability, biodegradability and nutritive value (Krochta, 2002). In addition, protein films exhibit better oxygen barrier, carbon dioxide barrier and mechanical properties when compare with polysaccharide films (Baldwin and Baker, 2002).

Among various proteins, myofibrillar proteins of fish muscles can be utilized to prepare film with good strength. These proteins are capable of forming a continuous matrix during drying of the protein solution (Krochta, 2002; Prodpran and Benjakul, 2005; Prodpran *et al*., 2007). However, the film from fish myofibrillar protein (FMP) alone is too brittle due to extensive protein-protein interactions involving disulfide covalent bond, hydrogen bond, electrostatic force and hydrophobic interactions (Krochta and De Mulder-Johnston, 1997; Prodpran and Benjakul, 2005). To improve flexibility of protein films including FMP films, addition of plasticizer such as glycerol, sorbitol, polyethylene glycol and sucrose are required. In particular for FMP-based films which contain disulfide covalent bond, a high amount of plasticizer in the range of 40-60% of protein is generally added to render the films with sufficient flexibility (Prodpran and Benjakul, 2005). However, those common synthetic plasticizers used in protein-based films are highly hydrophilic which result in dramatically increased water-vapor adsorption and permeability of the film. The differences in composition, size, structure and shape of plasticizers directly affect their ability to function in the film matrix (Orliac *et al.*, 2003).

Gelatin is produced from by-products such as bone, skin, fin and scale. It is derived from thermal degradation of collagen by acidic or alkaline process

(Arvanitoyannis, 2002; McHugh *et al*., 1994). Gelatin can be used to prepared film with high transparency. Gelatin films are generally more flexible than the FMP film since it contains no disulfide and covalent bonds (Jongjareonrak *et al.*, 2006). Hoque *et al.* (2011) reported that degree of hydrolysis of gelatin had an impact on the mechanical properties of the film. At higher degree of hydrolysis, short gelatin molecules with the high mobility of chain and high number of chain ends exhibited plasticizing effect by preventing protein-protein interaction. Therefore, gelatin hydrolysate, the short chain peptides with high mobility, might act as plasticizer in FMP film. The gelatin hydrolysate incorporated may reduce interaction among long chains of FMP and increase the free volume in the protein network of film, resulting in plasticizing effect. Moreover, gelatin hydrolysate added as plasticizer in FMP film may be more compatible to the FMP molecules which can lower the water vapor permeability of the film with less migration to the film surface, compared to a common hydrophilic and hygroscopic plasticizer like glycerol.

In addition, sealing ability of protein film based on FMP has not been reported. Sealing efficiency of protein film is one of important properties for being used as food packaging material. Therefore, the feasibility of sealing of FMP film by the aid of bio-based adhesive in combination with applied heat was also investigated in this study.

Review of Literature

1. Fish muscle protein

Composition of fish muscle varies with species. Different fish have varying amount of dark and ordinary muscle. 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 muscles than in dermersal fish muscles (Sikorski, 1990). Sarcoplasmic proteins consist of heme proteins such as myoglobin and hemoglobin as well as enzymes involving in glycolysis, citric and electrontransfer cycles (Shahidi, 1994; Xiong, 1997; Sikorski, 1990). Sarcoplasmic proteins can have an adverse effect on the strength and deformability of myofibrillar protein gels (Hultin and Kelleher, 2000). These proteins may interact with myosin upon heating, which can prevent the cross-linking during gel matrix formation, resulting in gels with poorer water holding capacity (Sikorski, 1994).

1.2 Myofibrillar proteins

Myofibrillar proteins are the major structural proteins in fish muscle, which accounts for 55-60% of total proteins in 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 meromyosin chains with a size of 220,000 daltons and four light meromyosin chains ranging in size from 16,000 to 25,000 daltons, depending on species and fiber 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 (Figure 2) contains five sulfhydryl groups and is free of disulfide bond. It also contains a myosin binding site, which allows myosin to form temperatory complexes via non-disulfide bonds, which can be split by high-energy compounds such as ATP or at high ionic strengths.

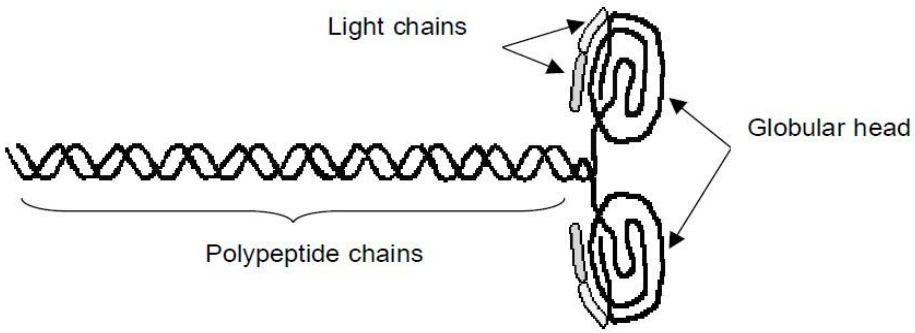


Figure 1. Molecules of myosin.
Source: Tamarkin (2004)

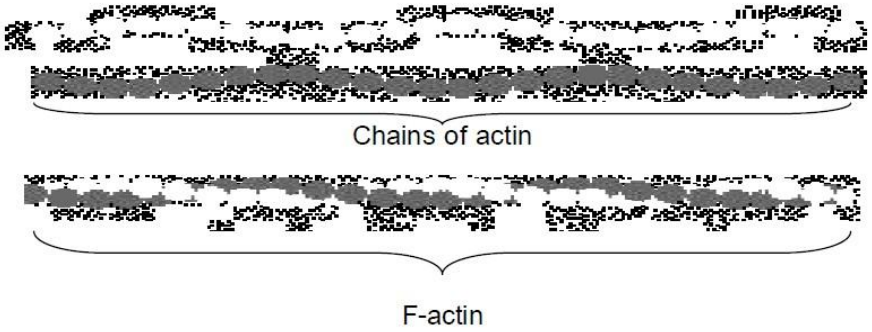


Figure 2. Molecules of actin.
Source: Tamarkin (2004)

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 (Figure 3).

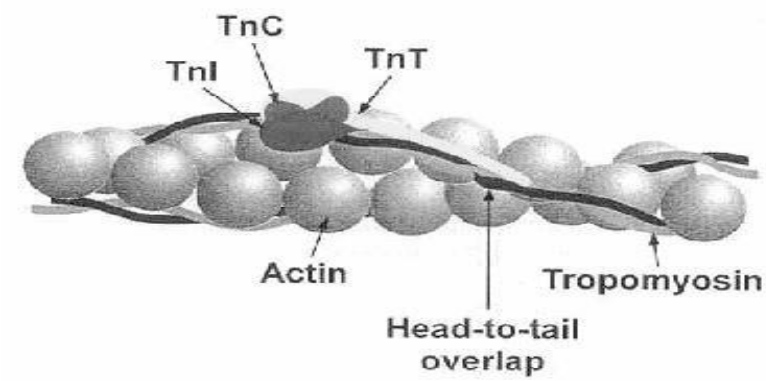


Figure 3. Molecules of Tropomyosin (TnC), Troponin I (TnI) and Troponin T (TnT).
Source: Cohen (1975)

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 of the muscle. The contractile proteins vary in susceptibility to postmortem proteolytic degradation, contributing to the varying meat tenderness (McCormick, 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 solutions. 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 films

The environmental pollution caused by using non-biodegradable materials has led to the research and development of biodegradable materials owing to their environmentally friendly, biocompatibility and being alternative packaging to synthetic polymers or plastics.

Biodegradable films or edible films which are able to protect food product, extend food product shel-life and prevent quality loss of foods due to mass transfer such as moisture, gases and flavours (Skurtys *et al*, 2010). Indeed, 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).

Biopolymer materials used for biodegradable or edible film can be divided into 4 groups: biopolymer hydrocoilloids (proteins and polysaccharides), lipids, resins and composites (Krochta *et al.,* 1994). Physical and chemical characteristics of the biopolymer greatly influence the properties of resulting films and coatings (Sothornvit and Krochta, 2000). 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). The use of natural-based polymers films depends on cost, availability, functional attributes, mechanical properties (strength and flexibility), optical properties (gloss and opacity), barrier requisites (water vapor, O_2 and CO_2 permeabilities), structural resistance to water and sensorial acceptances. These characteristics are considerably influenced by several parameters such as the type of material used as structural matrix, film manufacturing conditions and the type and concentration of additives (Debeaufort *et al*., 1998; Guilbert *et al*., 1996).

3. Protein-based films

Proteins are thermoplastic heteropolymers of both polar and nonpolar amino acids. They are macromolecules with specific amino acid sequences and there are able to form numerous intermolecular linkages, and undergo different interactions, yielding a wide range of potential functional properties (Song and Zheng, 2008). Proteins used to form films can be divided into two categories: animal and plant proteins. Animal proteins include whey protein, casein, gelatin, collagen, fish myofibrillar protein, egg-white protein, and keratin. Plant proteins include wheat gluten, corn zein, soy protein, peanut protein, and cottonseed protein (Sothornvit and Krochta, 2005). Protein-based films generally have the superior mechanical and barrier properties to polysaccharide-based films (Cuq *et al.*, 1995). 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). In addition, properties of protein based films are most likely dependent on various factors such as the source of protein, pH of protein solution, plasticizer and formation process (Sobral *et al.*, 2005).

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 Protein film formation mechanism

Protein-based films can be formed in three steps (Figure 4) (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.

3.2.2 Allow the formation of new inter-molecular 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).

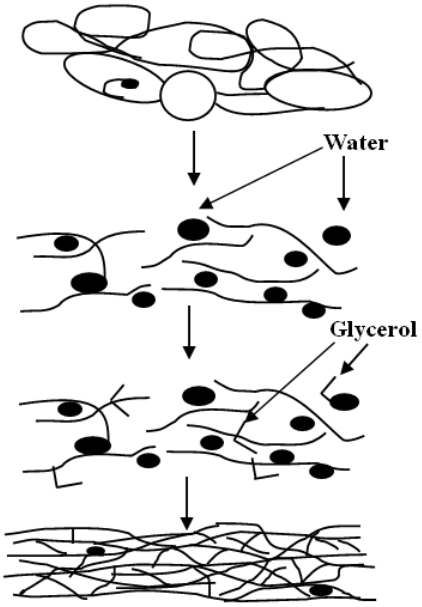


Figure 4. Mechanism of film formation.
Source: Adapted from Marquie and Guilbert (2002)

3.3 Fundamental properties of protein-based films

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

3.3.1 Mechanical properties

Tensile strength (TS) and elongation at break (EAB) are the major mechanical properties of several films. Generally, TS and EAB values for protein film range widely from 0.5 to 17 MPa and from 1 to 260%, respectively (Cuq *et al.,* 1998; Krochta, 2002). However, mechanical properties of protein films are poorer than synthetic films (Table 1) (Cuq, 2002; Gennadios *et al*., 1994). The mechanical properties of protein vary depending on surface charges, hydrophobicity, polymer chain length, etc., (Kester and Fennema, 1986). Hydrogen bonds are considered important in contributing to the tensile strength (TS) of protein films (Krochta, 2002). Moreover, type and level of plasticizer have a strong effect on film properties (Shellhammer and Krochta, 1997; Cuq, 2002). It is necessary to add optimal plasticizers into protein films to increase flexibility and EAB value. However, increasing concentration of plasticizers resulted in decreased TS. Jongjareonrak *et al.* (2006) reported that decreased TS and increased EAB were obtained in gelatin film

from bigeye snapper skin and brownstripe red snapper skin with increasing glycerol content (25-75%, based on protein). Audic and Chaufer (2005) and Abdorreza *et al*. (2011) reported that the use of plasticizer decreased the interactions between protein chains, thereby decreasing TS value for films derived from protein sources.

3.3.2 Water vapor barrier properties

The proteins-based films possess polar hydrophilic characteristics; consequently, they have poor water-vapor barrier properties. The aqueous alcohol-soluble cereal protein, gluten and zein seem to produce films that have a low water-vapor permeability (WVP) when compared with other protein films, except for fish myofibrillar proteins which also form films with a low WVP (Krochta, 2002). Zein and gluten consisting of prolamine are relatively hydrophobic proteins thus resulting in lower WVP (Esen, 1987). Moreover, temperature, RH gradients applied across films and film thickness affect WVP values of hydrophilic protein films (McHugh *et al.,* 1993; Gennadios *et al.,* 1994)

3.3.3 Oxygen and carbon dioxide barrier properties

Protein films provide the advantage of being excellent oxygen and carbon dioxide barriers (Gennadios *et al*., 1993). Protein films also have low carbon dioxide permeability (Mujica-Paz and Gontard, 1997). In general, at low to intermediate RH, protein films have lower oxygen permeability than LDPE and HDPE, which are not good oxygen barriers (Krochta, 2002). At higher RH conditions, protein films are plasticized by absorbed moisture and their oxygen permeability increases drastically (Mujica-Paz and Gontard, 1997). Sucrose and sorbital plasticized β-lactoglobulin films were the best oxygen barriers (Sothornvit and Krochta, 2000). However, barrier property can be varied with the source of protein (Table 1), which can be associated with amino acid composition (Cuq *et al*., 1995).

Table 1. The properties of selected synthetic and biopolymer films.

Film	Tensile Strength (MPa)	Elongation (%)	WVP[a]	OP[b]
Polyester	178	85	0.02	12
Polyvinyl chloride	93	30	1.2	23
Low-density polyethylene	13	500	0.04-0.05	1003
High-density polyethylene	26	300	0.014	224
Hydroxypropylcellulose	15	33	6.2	300
Wheat gluten	3.3	192	5.1	1290
Soy protein	3.6	160	194	14
Corn zein	3.9	213	6.5	35
Fish myofibrillar protein	17	23	3.9-3.8	1-873[c]

[a]Water-vapor permeability (WVP) ($\times 10^{-12}$ mol.m/m^2.s.Pa)

[b]Oxygen permeability (OP) ($\times 10^{-18}$ mol.m/m^2.s.Pa)

[c]1 measured under dry conditions, 873 measured under high relatively humidity.

Source: Data from Cuq (2002), Gontard *et al*. (1996) and Krochta *et al*. (1994)

3.3.4 Solubility properties

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.* (1996) 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.

4. Protein-based films from different sources

4.1 Myofibrillar protein film

Fish muscle proteins consist of sarcoplasmic proteins, myofibrillar proteins and stroma proteins. Myofibrillar protein and sarcoplasmic protein are capable of forming a continuous films matrix (Chinabhark *et al*., 2007; Garcia and Sobral, 2005; Sobral *et al.*, 2005). Myofibrillar proteins are salt soluble protein, which accounts for 55 to 60% of total muscle protein (Xiong, 1997; Sikorski, 1990). Various factors affecting film formation and film properties included protein concentration, pH, temperature and storage time before film casting (Cuq *et al*., 1995). Film-forming ability of protein can be influenced by amino acid composition, distribution and polarity, ionic cross-links between amino and carboxyl groups, hydrogen bonding and intra- and inter-molecular disulfide bonds (Gennadios and Weller, 1991). Tongnuanchan *et al.* (2011) found that films from both unwashed and washed mince of red tilapia (*Oreochromis niloticus*) prepared at pH 3 had higher tensile strength (TS) than those films prepared at pH 11. Shiku *et al.* (2004) reported that transparent and flexible protein films were successfully made from frozen Alaska Pollack surimi. 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. Myofibrillar protein-based films have interesting functional properties such as mechanical or water vapor barrier properties, compared to other protein-based films (Cuq *et al*., 1995; Paschoalick *et al*., 2003; Shiku *et al*., 2003).

4.2 Sarcoplasmic protein films

Sacoplasmic proteins comprise about 20-30% of the total amount of proteins in fish muscle (Suzuki, 1981; Sikorski, 1990). Sarcoplasmic protein can be used for film preparation (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 (Hamaguchi and Tanaka, 2007). The functional properties of sarcoplasmic protein-based film are sensitive to heating because their globular proteins must be thermally denatured to form a continuous matrix (Iwata *et al*., 2000).

4.3 Gelatin films

Gelatin is a thermal denatured protein obtained from collagen, isolated from animal skin, bones and fish skins (Gómez-Guillén *et al*., 2002). Gelatin has been widely employed as an ingredient to improve the elasticity, consistency and stability of foods. In addition, it can be used as a material for preparing biodegradable films (Giménez *et al*., 2009). The physical and structural properties of gelatins are related not only to the molecular weight distribution but also to the amino acid composition that plays a vital role in the rheological and barrier properties of the resulting films (Gómez-Guillén *et al*., 2009). The properties of film from gelatin depend on the source of gelatin, plasticizer and other factors. Jongjareonrak *et al*. (2006) observed the lower TS of the films prepared from the bigeye snapper-skin gelatin than that of the brownstripe red snapper-skin gelatin films, where bigeye snapper-skin gelatin possessed the lower concentrations of high-molecular weight fractions with a concomitant increase in degradation peptides, compared with brownstripe red snapper-skin gelatin. Decreased TS and increased EAB were obtained in gelatin film from bigeye snapper skin and brownstripe red snapper skin with increasing glycerol content (25-75%, based on protein) (Jongjareonrak *et al.*, 2006). Gómez-Guillén *et al.* (2009) also reported that gelatin containing higher amount of lower molecular weight fractions yielded the film with higher percent elongation and lower tensile strength.

4.4 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). 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 90 ^{o}C. Whey proteins films exhibit transparent, flexible, colourless and flavourless films, with a poor moisture barrier (Fairley *et al.,* 1996; McHugh *et al*., 1994). Whey protein films without plasticizers are very brittle. Therefore, to improve flexibility of films, addition of plasticizers is required. Increased concentration of plasticizers in edible whey protein films resulting in films with decreased tensile strength and Young's modulus, but increased elongation at break (Shaw *et al.,* 2002).

4.5 Wheat gluten films

Wheat gluten consists mainly of wheat storage protein (70 to 80%, dry matter basis) with traces of starch and non-starch polysaccharides (10 to 14%), lipid (6 to 8%), and minerals (0.8 to 1.4%) (Guilbert, 2002). Wheat gluten consists of prolamine and glutelin fractions of wheat flour protein, typically referred to as gliadin and glutenin, respectively (Krochta, 2002). Gliadin is soluble in alcohol and the glutenins are soluble in dilute acid or alkali solutions. Both gliadin and glutenin include intra-molecular disulfide bonds. Inter-molecular disulfide bonds, which link individual glutenin protein chains, result in the larger polymers with high molecular weight. The extensive inter-molecular interactions in wheat gluten result in quite brittle films with poor water-vapor barrier properties (Gennadios and Weller, 1990). Wheat gluten films are usually prepared by solution casting method using water and/or ethanol as co-solvent and polyols as plasticizers (Gennadios *et al*., 1994) Herald *et al.* (1995) found 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. To improve the water-vapor barrier and tensile strength properties, covalent crosslinking of gliadin polypeptide chains using dialdehydes and thermal treatment of the casting films can be used (Hernandez-Munoz *et al*., 2004).

4.6 Casein films

Caseinates obtained by adjusting acid-coagulated casein to pH 6.7 using sodium hydroxide. Caseins are importantly phosphoproteins that precipitate at pH 4.6 and 20 ^{o}C (Gennadios *et al*., 1994). The composition of amino acid in casein is characterized by a low level of cysteine. Therefore, disulfide bond cannot form to render water-insoluble films (Chen, 2002; Gennadios *et al*., 1994). The high proline content led to the better emulsifying properties when compared with whey protein film (Khwaldia *et al.,* 2004). Casein films from aqueous solution without heat treatment was due to their random-coil nature and ability to hydrogen bond extensively (Gennadios *et al.,* 1994). Interactions in the film matrix likely include hydrophobic, ionic and hydrogen bonding (Avena-Bustillos and Krochta, 1993).

4.7 Corn zein films

Zein consists of alcohol-soluble proteins found in corn proteins (Padua and Wang, 2002). Zein has a high content of non-polar hydrophobic amino acids, that improve the water vapor permeability of films (Dangaran *et al*., 2009). Zein films can be prepared by dissolving zein in 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). Soliman *et al.* (2009) found that treatment of zein film by gamma irradiation can improve the water barrier properties, color, and appearance of film (Soliman *et al.*, 2009).

4.8 Soy protein film

Soy proteins are composed of a mixture of albumins and globulins, 90% of which are storage proteins with globular structure. Soy protein consists of four major fractions: 2S, 7S, 11S and 15S (Kinsella, 1979). However, soy proteins are mostly globulin, with the 7S and 11S factions representing 37% and 31%, respectively, of the total protein (Sun *et al*., 1999). Globulins are protein fractions in which the subunits are associated via hydrophobic and hydrogen bonding (Thanh and Shibasaki, 1976). Park *et al.* (2002) reported that the interactions of SPI films include disulfide bonds, hydrogen bonds, and hydrophobic interactions. Due to the native hydrophilic of proteins, soy protein films generally possess low moisture resistance and poor water vapour barrier abilities (Lim *et al.*, 1998). However, under low relative humidity environments, SPI films have been shown to possess good oxygen barrier properties (Song *et al.*, 2011). The mechanical properties of films are affected by interactions between proteins and other small molecules including water, plasticizers, lipids, and other additives dispersed in the matrix (Chen, 1995).

5. Plasticizers

Plasticizers are low molecular weight, high boiling point liquids with the average molecular weights of between 300 and 600, and linear or cyclic carbon chains. The low molecular weight of a plasticizer allows it to occupy intermolecular spaces between polymer chains to reduce brittleness, increase flexibility and enhance toughness of films. Since plasticizers reduce intermolecular forces along the polymer

chains, free volume and chain movements of polymer system are increased (Irnrnergut and Mark, 1965).

Plasticizers can aid processing and modify the properties of the final product. As a processing aid, plasticizers decrease the processing temperature, reduce sticking in molds and enhance wetting. As a property modifier of final product, plasticizers increase the temperature range of usage, increase flexibility, elongation and toughness and lower the glass transition temperature (Sears and Darby, 1982).

5.1 Plasticizer classification

In polymer science, two types of plasticizers are generally defined: internal and external plasticizers (Irnmergut and Mark, 1965)

5.1.1 Internal plasticizers

Internal plasticizers are part of the polymer molecules and become part of the product, which can be either co-polymerized into the polymer structure or reacted with the original polymer (Frados, 1976). Internal plasticizers generally have bulky structures that provide polymers with more space to move around and prevent polymers from coming close together. Therefore, they soften polymers by lowering the glass transition temperature (T_g) and thus reducing the elastic modulus or stiffness.

5.1.2 External plasticizers

External plasticizers are low volatility substances that are added to polymers. They interact with polymer chains and produce swelling, but are not chemically reacted and thus being lost by evaporation, migration or extraction. The benefit of using external plasticizers over internal plasticizers is the opportunity to select from a variety of plasticizers, depending on the film properties desired (Banker, 1966; Wilson, 1995).

The properties of some plasticizers are shown in Table 2 and 3. Most plasticizers used in biopolymers consist of hydroxyl groups which will form hydrogen bonds with biopolymers, and thus increasing the free volume and flexibility of the film matrix. However, moisture sorption of various plasticizers plays an important role in affecting different film properties (Sothornvit and Krochta, 2001; Mathew and Dufresne, 2002; Cao *et al*., 2009).

Table 2. The properties of some plasticizers for biodegradable films.

Plasticizer type	Molecular weight (MW)	Formular	Shape	Oxygen atom
Propylene glycol	76	$C_3H_8O_2$	Straight chain	2
Glycerol	92	$C_3H_8O_3$	Straight chain	3
Sorbital	182	$C_6H_{14}O_6$	Straight chain	6
Polyethylene glycol	200	$H(OCH_2\text{-}CH_2)_4OH$	Straight chain	5
Sucrose	342	$C_{12}H_{22}O_{11}$	Ring structure	11
Polyethylene glycol	400	$H(OCH_2\text{-}CH_2)_8OH$	Straight chain	9

Source: Adapted from Sothornvit and Krochta (2001)

Table 3. Chemical and physical properties of some plasticizers.

	Glycerol	Xylitol	Sorbital	Maltitol	PEG-400
Carbon number	3	5	6	12	16
Molecular weight	92	152	182	344	400
Melting point (^oC)	20	94	100	157	4-8
Heat stability (^oC)	>160	>160	>160	>160	>160
Hygroscopy	High	High	Medium	Medium	Low
T_g (^oC)	-75	-27	0	45	-25

Source: Adapted from Mathew and Dufresne (2002)

5.2 Some synthetic plasticizers used in biopolymers

5.2.1 Glycerol

Glycerol is a hydrophilic polyol (Lindsay, 1996). It is a relatively small molecule when compared with other plasticizers, with 3 carbons per carbon chain and a molecular weight of 92 (Figure 5a). Glycerol action was attributed to it being easily inserted between protein chains due to its small size. It will reduce the protein-protein interactions and form hydrogen bonds with the amide group of the proteins. This reduces the closeness between the protein chains, resulting in the protein structure with less dense. Cuq *et al.* (1997) reported that increasing the plasticizer led to the protein network less dense as it modifies the molecular 3-dimensional organization, decrease in attractive protein-protein intermolecular forces and increase in free volume and chain mobility and consequently more permeable. However, the addition

of glycerol should be limited since it has detrimental effect on the strength and the water-vapour barrier properties of films. Cuq *et al*. (1997) also suggested that WVP and water content in films increased with increasing glycerol content due to higher amount of polar groups of glycerol (hydrophilic plasticizer being compatible with hydrophilic film-forming materials like gelatin, thereby enhancing sorption of polar molecules like water).

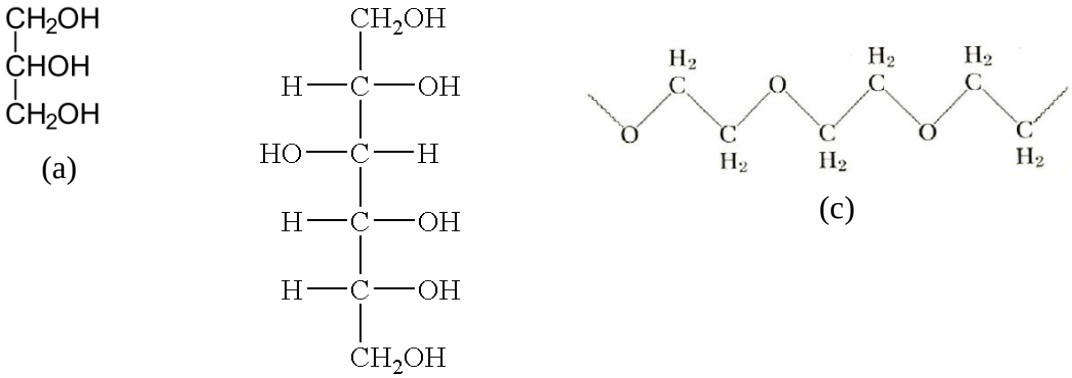


Figure 5. The chemical structures of glycerol (a), sorbitol (b) and polyethylene glycol (c).

Source: Crosby (1992); BeMiller and Whistler (1996); Lee *et al.* (1967)

5.2.2 Sorbitol

Sorbitol is also a hydrophilic polyol with a low molecula weight (182) (Table 2). Its molecules can easily insert between protein chains to form hydrogen bonds with the reactive groups of the proteins (Cuq *et al.*, 1997). Sorbitol is required in higher amounts than glycerol to achieve similar mechanical properties, due to the larger size (i.e. lower efficiency) of sorbitol (Gennadios *et al*., 1996). Cuq *et al.* (1997) used sorbitol, glycerol and sucrose as plasticizer for fish myofibrillar protein films at the same molecular contents. In fish myofibrillar protein films, glycerol had a slightly higher plasticizing effect than sorbitol and sucrose at the highest amount used. These results suggested that the smaller molecular size made it easier to be inserted within the three-dimensional protein network.

5.2.3 Polyethylene glycol (PEG)

Polyethylene glycol (PEG) is a substance, much larger in molecular weight than glycerol and also less hydrophilic (Park *et al.,* 1994). According to Lindsay (1996), a polyol is a substance that contains only hydroxyl groups as functional groups. Because the PEG molecule is much bigger than glycerol, PEG will not diffuse through the film matrix as rapidly and it may be prevented from interacting with some of the hydrophilic sites on a protein chain. It cannot be inserted between protein chains because of stearic hindrance that might exist from adjacent side chains. Therefore, PEG is more likely to cross-link protein chains, promoting hydrophobic intermolecular bonding between them (Park *et al.,* 1994; Siew *et al*., 1999).

5.3 Theories of plasticization

There are three important theories being proposed to explain the plasticizing effects. For the films, they are the lubricity theory, the gel theory and the free volume theory (Sears and Darby, 1982).

5.3.1 Lubricity theory

The resistance of a brittle polymeric substance to deformation because the intermolecular friction that exists between the macromolecules. As a polymer is deformed, the macromolecules move back and forth over each other on internal glide planes. In order to make this back and forth movement of the resin macromolecules easier, a plasticizer is added to lubricate these internal glide planes, acting as oil between two moving parts. The plasticizer therefore gives internal lubricity (Sears and Darby, 1982).

With this theory, it is assumed that no bonding between macromolecules exists, expect for where surface irregularities occur. It is assumed that there is only, if any at all, very weak bonding between the plasticizer molecules and/or the plasticizer molecules and the polymer molecules. This weak bonding could be described as similar to the low interfacial energy that exists between a solid and a liquid lubricant.

5.3.2 Gel theory

The gel theory in food hydrocolloids related to an internal, three-dimensional honeycomb structure. Plasticizer molecules attach along the polymer chains, replacing polymer-polymer attachments at places and hindering the forces holding polymer chains together (Van der Waals, London, Debye, hydrogen bonding, crystal, or primary valence forces). This reduces the rigidity of the gel structure, resulting in increased gel flexibility. Furthermore, plasticizer molecules that are not attached to polymer form aggregated plasticizer domains that facilitate the movement of polymer molecules. This also enhances the gel flexibility (Sothornvit and Krochta, 2001).

5.3.3 Free volume theory

To explain plasticization by means of the free volume theory took a little more time since this theory grew out of the less evident characteristics of materials like crystals, glasses and liquids. This theory depends very strongly on mathematical corroboration for is validity and strength.

Approaches to increasing the free volume of polymer system could include:

(1) Using low M_w polymer, or reducing the M_w of polymer, to increase the number of polymer end groups.

(2) Increasing the length of side chains, thus increasing steric hindrance and lowering chain intermolecular forces to increase polymer chain motion, related to internal plasticization.

(3) Using low M_w plasticizers that are compatible with the polymer molecules to increase the motion of chain ends, side chains and main chain, related to external plasticization

(4) Increasing temperature.

Free volume theory is used to describe many things, such as plasticizing action, T_g, viscosity, cross-linking, diffusion, film drying and film properties (Wicks, 1986).

5.4 Use of plasticizers in biopolymer-based films

Plasticizers are required for biodegradable films especially for polysaccharides and proteins. These films are often brittle and stiff due to extensive interactions between polymer molecules (Krochta, 2002). The addition of plasticizers affects not only the flexibility and other mechanical properties, but also the resistance of films to permeation of vapors and gases (Sothornvit and Krochta, 2000; 2001). Different moisture sorption of various plasticizers led to the differences in film permeabilities and mechanical properties. Thus, the appropriate selection of a plasticizer for a given polymer will allow optimization of the film mechanical properties with a minimum increase in film permeability. Selection of plasticizers needs consideration of the issues of plasticizer compatibility, efficiency, permanence and economics (Sothornvit and Krochta, 2001).

5.4.1 Some plasticizers for polysaccharide-based films

Various polysaccharides used to form films include starch, alginate, cellulose derivatives, chitosan, carrageenan, pectins, dextrins, pullulan and other gums (Sothornvit and Krochta, 2001). The addition of plasticizers to achieve the required mechanical properties. The most effective plasticizers are similar to the polysaccharide structure; hydrophilic plasticizers containing hydroxyl groups are best suited to this use. Therefore, the plasticizers commonly used for polysaccharide-based films are glycerol, sorbitol, xylitol (X), mannitol (M), polyethylene glycol (PEG) (with molecular weight from 400 to 8000), ethylene glycol (EG), and propylene glycol (PG) (Sothornvit and Krochta, 2001). An appropriate plasticizer for starch-based materials should impart flexibility and suppress retrogradation to thermoplastic starch (TPS) during aging (Ma and Yu, 2004). Garcia *et al*. (2005) found that glycerol and sorbitol showed to be compatible with amylose and improved mechanical properties of films, by decreasing intermolecular interaction. The laminated methylcellulose-corn zein films incorporated with fatty acid exhibited lower tensile strength (Park *et al.*, 1994). Bergo *et al*. (2007) reported that the water vapor permeability of tapioca starch films added with glycerol at 30 and 45 g/100 g starch was higher than films added with sorbitol at the same level. Furthermore, the analysis of the mechanical properties of these films indicated that glycerol alone exerted a more effective plasticization. At the same levels, the smaller Mw hydrophilic

plasticizers such as polyethylene glycol and glycerol rendered the films with higher WVP than did the larger Mw plasticizers such as PEG-400 in methylcellulose films (Donhowe and Fennema, 1993). Galdeano *et al*. (2009) reported that hydrophilic plasticizers (glycerol, sorbitol and urea) incorporated increased the permeability and water sorption property of biodegradable oat starch films.

5.4.2 Some plasticizers for protein-based films

Proteins used to form films can be divided into 2 groups: animal or plant proteins. Animal proteins include whey protein, casein, gelatin, collagen, fish myofibrillar protein, egg-white protein and keratin. Plant proteins include wheat gluten, corn zein, soy protein, peanut protein and cottonseed protein (Sothornvit and Krochta, 2001). The plasticizers commonly used for protein-based films are glycerol (Gly), sorbitol (Sor), polyethylene glycol (PEG), ethylene glycol (EG), diethylene glycol (DEG), triethylene glycol (TEG) and sucrose (Jongjareonrak *et al.*, 2006; Vanin *et al*., 2005). The whey protein-based films had decreased mechanical resistance but increased film solubility with increasing level of glycerol added as plasticizer (Galietta *et al*., 1998). The addition of fatty acid and other lipids led to improved tensile strength of fish myofibrillar protein (FMP) films (Tanaka *et al*., 2001), egg albumin (EA) films (Handa *et al.*, 1999), and soybean films (Cao and Chang, 2002). The plasticizing effect of sorbitol, glycerol and sucrose added at the same concentration in myofibrillar protein-based films from Atlantic sardines (*Sardina pilchardus*) did not cause significant differences in properties of film, due to structural similarities between sorbitol, glycerol and sucrose (Cuq *et al.*, 1995). β-Lactoglobulin films were plasticized with different plasticizers (PG, GLY, SOR, PEG-200, PEG-400 and sucrose) to improve the mechanical properties. GLY and PEG-200 were the plasticizers providing most efficiently achieved desirable mechanical properties for films (Sothornvit and Krochta, 2001). Parris and Coffin (1997) reported that glycerol was the most effective plasticizer for zein protein film in terms of tensile properties, but it also gave the highest permeability. Using different plasticizer mixtures such as Gly-PPG 400 and Gly-PEG 400 in zein films helped to decrease films WVP (Parris and Coffin, 1997). Incorporation of fatty acids and their sucrose esters at 75–100% glycerol substitution as plasticizers significantly reduced the water vapor permeability of films of gelatin derived from skin of bigeye snapper

(*Priacanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*) (Jongjareonrak *et al*., 2005).

6. Protein hydrolysate

Protein hydrolysates are small fragments of peptides that generally consist of 2–20 amino acids. The protein hydrolysate can be produced from different protein sources. Its properties are governed by the protease specificity, nature of protein substrate and degree of hydrolysis (Adler-Nissen, 1986). Cleavage of proteins into smaller molecules or peptides during hydrolysis process could improve the functional and nutritional properties of food proteins (Kudo *et al.*, 2009). Protein hydrolysates are used as readily available sources of protein for humans and animals due to their good functional properties (Neklyudov *et al.*, 2000). NolsØe and Undeland (2009) reported that suitable source of protein for humans and animals nutrition due to the balanced amino acid composition.

Among protein hydrolysates, those derived from gelatin from various aquatic animals have been widely prepared (Klompong *et al.*, 2009; Thiansilakul *et al*., 2007; Wu *et al.*, 2003). The manufacturing of gelatin hydrolysates has been currently investigated to improve and develop the functional, nutritional properties of proteins and bioactive peptides (Je *et al*., 2007). Protein hydrolysates including gelatin hydrolysate can be prepared by hydrolysis process under different means such as thermal, acid, alkaline and enzymatic hydrolysis (Adler-Nissen, 1986)

6.1 The production of protein hydrolysates

Chemical and biological methods are used for hydrolysis processes. Chemical hydrolysis has been used in industrial practices due to the lower in cost. Biological processes using enzymes are employed for the high functionality and nutritive value (Kristinsson and Rasco, 2000).

6.1.1 The chemical methods

Chemical hydrolysis of proteins is achieved by cleaving peptide bonds with either acid or alkaline (Hale, 1972). It is relatively inexpensive and quite simple to conduct. However, this method tends to be a difficult process to control (Blendford, 1994). There are many limitations to produce food ingredients since the

process needs extreme temperatures and pH, giving products with reduced nutritional qualities, poor functionality and restricted to use as flavor enhancers (Webster *et al.*, 1982; Leffler, 1986).

6.1.1.1 Acid hydrolysis

Acid hydrolysis of proteins is used more commonly than hydrolysis under alkaline conditions. The acid hydrolysis is also widely utilized to convert under-utilized and secondary raw material from fish into fertilizer due to the low production cost and the resulting extensive hydrolysis. Although the process is harsh and hard to control, it is still the preferred method for hydrolysis of vegetable proteins (Kristinsson and Rasco, 2000). For total hydrolysis of fish protein, acid hydrolysis is carried out using hydrochloric acid or sulfuric acid. A drawback of acid hydrolysis is the destruction of tryptophan, which is an essential amino acid (Kristinsson and Rasco, 2000).

6.1.1.2 Alkaline hydrolysis

Protein hydrolysate can be prepared under alkali condition using sodium hydroxide. The resulting hydrolysates have poor functionality and more importantly can adversely affect the nutritive value of them (Peterson, 1978). During alkaline hydrolysis of fish protein, the rapid cleavage of proteins to water-soluble polypeptides takes place, followed by further degradation at a slower rate. Alkaline hydrolysis results in less degradation of tryptophan. Several deleterious reactions can occur during alkaline hydrolysis. These are initiated by hydrogen abstraction from the alpha carbon of an amino acid and include racemization of L-amino acids, which produces D-amino acids, which are not absorbed by humans (Peterson, 1978; Linder *et al.*, 1995). Also, the formations of lysinoalanine, ornithinoalanine, lanthionine and β-amino alanine can occur via β-elimination reactions (Kinsella, 1976). These may lead to the formation of toxic substances (Lahl and Braun, 1994; Linder *et al.*, 1995). In addition, the resulting hydrolysates have an inhibiting effect on proteolytic enzymes. Hence, future hydrolysis using enzymes might reduce the rate of hydrolysis (Kristinsson and Rasco, 2000).

6.1.2 The enzymatic methods

Biological processes are preferred methods for improving functionality and sensory properties of the native protein without threatening its nutritive value, enzymatic processes with added enzymes are used to hydrolyze food protein (Lahl and Braun, 1994). These processes take place under mild circumstances and do not generate hydrolytic degradation products via racemization reactions occurred with acid and alkaline hydrolysis (Kristinsson and Rasco, 2000). This can be done via proteolytic enzymes already present in the fish viscera and muscle or by adding enzymes from other sources. They are characterized further by their hydrolyzing mechanism into endopeptidases which cleave the peptide bonds within protein molecules or exopeptidases which hydrolyze the terminal peptide bonds (Adler-Nissen, 1986). The enzymatic hydrolysis of fish muscle proteins is characterized by an initial rapid phase, during which a large number of peptide bonds are hydrolyzed. Thereafter, the rate of enzymatic hydrolysis decreases and reaches a stationary phase where no apparent hydrolysis takes place (Shahidi *et al*., 1995). Added enzymes are used to obtain a more selective hydrolysis since proteases are specific for peptide bonds adjacent to certain amino acid residues (Peterson, 1978). Enzymatic hydrolysis potentially affects the molecular size and hydrophobicity, as well as polar and ionisable groups of protein hydrolysates (Mutilangi *et al.*, 1996).

Proteolytic enzymes from microorganisms such as Alcalase, Neutrase, Protease N and Protamex are most suitable to prepare fish protein hydrolysates because of their high productivity (Gildberg *et al.*, 1989; Rebeca *et al.*, 1991; Guerard *et al.*, 2001; Liaset *et al*., 2002). Enzymes from plants and animals such as papain, bromelain, ficin and pepsin are still used for hydrolysis (Aspmo *et al.*, 2005; Liaset *et al.*, 2000). The selection of enzymes is usually based on a combination of efficacy and economics (Lahl, 1994).

6.2 Functional properties of protein hydrolysate

Hydrolysis reaction can improve functional characteristics of proteins (Klompong *et al.*, 2007). This process increases the number of polar groups with increasing the solubility of the protein hydrolysate. The functional properties of proteins are those physicochemical properties that govern their performance and

behavior in food systems during their preparation, processing, storage and consumption and also influence the use of protein hydrolysate as an ingredient in food (Sathivel *et al*., 2005). The main functional properties of fish protein hydrolysates include solubility, water holding, emulsifying, foaming and sensory properties (Kristinsson and Rasco, 2000). Functional properties of protein were affected directly by the characteristic of protein hydrolysates obtained (Klompong *et al*., 2007). Adler-Nissen (1986) and Kristinsson and Rasco (2000) reported that hydrolysis process directly influences the molecular size, hydrophobicity and polar group of the hydrolysate. Moreover, antioxidant activity has been reported for protein hydrolysates prepared from various fish protein sources such as whole capelin, tuna cooking juice, yellowfin sole frame, Alaska pollack frame, round scad muscle or Pacific hake muscle (Amarowicz and Shahidi, 1997; Jao and Ko, 2002; Je *et al*., 2005; Jun *et al*., 2004; Thiansilakul *et al*., 2007). The hydrolysis products are also reported to have antioxidative and antimicrobial activities (Hammami *et al*., 2009).

6.2.1 Solubility

Solubility is one of the most important of protein and protein hydrolysate functional properties. In many protein-based formulations, such as emulsification and foaming, good solubility for the protein is usually required (Wilding *et al*., 1984). The major factors that influence the solubility of proteins include hydrophobic and ionic interactions. Enzymatic hydrolysis is very important in increasing the solubility of soluble peptides by the cleavage of proteins into small peptide units (Yin *et al*., 2008). The enhanced solubility of the hydrolysates is due to the newly exposed ionizable amino and carboxyl groups of the amino acids that increase the hydrophilicity (Mahmoud, 1994). However, at high DH leads to high solubility, the negative effects on the other functional properties (Gbogouri *et al*., 2004). Protein hydrolysates from yellow stripe trevally (*Selaroides leptolepis*) meat, hydrolysed by Alcalase and Flavourzyme with different DHs (5–25%), also showed high solubility (>85%) in the pH range of 2–12 (Klompong *et al*., 2007). Gbogouri *et al*. (2004) found that the solubility of salmon hydrolysate was high at pHs 6 to 7 and was low at pHs 3 to 4. The pH influences the charge on the weakly acidic and basic side-chain groups. Thus, proteins and hydrolysates display low solubility at their isoelectric point. Nalinanon *et al.* (2011) studied that the functional properties of

hydrolysates from the muscle of ornate threadfin bream with different DHs at various pHs. All hydrolysates were soluble over a wide pH range, in which more than 70% solubility was obtained. The hydrolysates were generally soluble in alkaline pH to a greater extent, compared with acidic pH, except at pH 3 for hydrolysate with 10% DH.

6.2.2 Emulsifying properties

Hydrolysates are water-soluble and surface active and can stabilize the oil-in-water emulsions, due to their exposed hydrophilic and hydrophobic functional groups (Wilding *et al.,* 1984). Gelatin peptides contain mainly hydrophobic amino acids and the abundance of these amino acids is associated with higher affinity to oil and better emulsifying ability (Mendis *et al.,* 2005). Proteins adsorb to the surface of the freshly formed oil droplets during homogenization and form a protective membrane that prevents droplets from coalescence (Demetriades *et al.,* 1997). Lin and Chen (2006) proposed that the emulsification process includes two steps: (1) deformation and disruption of droplets which increase the specific surface area of emulsion and (2) stabilisation of this newly-formed interface by emulsifier or surfactant. Emulsifying property was affected by the solubility (Pacheco-Aguilar *et al.,* 2008). Emulsifying capacity and emulsifying stability are two parameters generally used to measure the ability of protein hydrolysates to form and stabilize emulsions (Sathivel *et al*., 2003; Gbogouri *et al*., 2004; Šližyte *et al.,* 2005). The controlling of hydrolysis extent can improve emulsifying properties of protein hydrolysate. Mahmoud (1994) showed that the emulsifying activity of the hydrolysates decreased linearly with increasing DH (25-67%). Quaglia and Orban (1990) and Kristinsson and Rasco (2000) also reported that the emulsifying capacity of fish waste protein decreased with increasing DH. The optimum molecular size or chain length for peptides exhibited good foaming and emulsifying properties. Mutilangi *et al.* (1996) postulated that higher contents of higher MW peptides or more hydrophobic peptides contribute to the stability of the emulsion. In the emulsion, large peptides with greater hydrophobicity can absorb to the oil surface and induce the formation of small oil droplets, which are of higher stability than large oil droplets induced by small peptides. Klompong *et al.* (2007) reported that both emulsifying activity index (EAI) and emulsion stability index (ESI) of protein

hydrolysates from yellow stripe trevally meat decreased with increasing DH. Moreover, Nalinanon *et al.* (2011) reported that at 0.10% protein, hydrolysate with 10% DH showed the highest EAI and ESI, when compared with those having higher DHs. Moreover, Fuente-Betancourt *et al.* (2009) reported that EAI of jumbo squid protein hydrolysate continuously decreased with increasing temperature due to protein aggregation. Therefore, emulsifying properties of hydrolysate were governed by peptide molecular characteristics and peptide chain length (Kristinsson and Rasco, 2000). Environmental conditions such as pH, ionic strength, temperature, etc. also have an effect on the emulsification properties (Gauthier *et al.*, 1993).

6.2.3 Foaming properties

Foaming properties are physicochemical characteristics of proteins to form and stabilise foams (Thiansilakul *et al.,* 2007). Food foams consist of air droplets dispersed in and enveloped by a liquid containing a soluble surfactant lowering the surface and interfacial tension of the liquid (Kinsella, 1976). Foaming ability of protein hydrolysates governed by their surface property. Fish protein hydrolysate from herring with its reduction in molecular weight presented an improved foamability (Liceaga-Gesualdo and Li-Chan, 1999). Limam *et al*. (2008) reported that the production of smaller molecular size peptides, resulting in enhanced foaming properties. The small size of both squid and sole peptides allow them to adsorb quickly to the air–water interface, lowering the surface tension and giving rise to a similar foam expansion. Philips *et al*. (1994) reported that the proteins with low molecular weight are contributory to rapid foam formation, but they may not be ideal in forming the protein-protein interactions that give rise to stable foams.

Foaming ability of both sole and squid gelatin hydrolysates increased when hydrolysate concentration increased (Giménez *et al.,* 2009). Thiansilakul *et al.* (2007) reported that an increase in the concentration of protein hydrolysate prepared from round scad mince gave rise to a higher foam expansion, possibly due to an increase in the rate of diffusion (Sánchez and Patino, 2005). Lawal (2004) postulated that an increase in foam stability with increasing concentration was a result of formation of stiffer foams. Foam stability is enhanced by flexible protein domains, which enhance viscosity of the aqueous phase, protein concentration and film thickness (Phillips *et al.,* 1994). The high foam stability resulted from the high degree

of some amino acid residues in protein structure, e.g. proline and lysine, which leads to a higher ability to establish protein-protein interactions via hydrogen bonds, resulting in a denser network that favors foam stabilization (Giménez *et al.,* 2009).

6.3 Antioxidative activity of protein hydrolysates

Protein hydrolysate consist of amino acids and peptides, which have been found to show antioxidative activity. The amino acid composition, sequence and chain length are the factors governing the antioxidative activity (Klompong *et al.,* 2007; Phanturat *et al.,* 2010). Different radical scavenging activity is determined by amino acid composition and special amino acid sequences (Kim *et al.,* 2001; Mendis *et al.,* 2005). Fish protein hydrolysates have been found to possess the radical scavenging activity (Sathivel *et al.,* 2003). Gelatin hydrolysates with the antioxidative activity have been produced from gelatin from the skin of Alaska Pollack (Kim *et al.,* 2001), hoki (Mendis *et al.,* 2005), cobia (Yang *et al*., 2008) and sole (Giménez *et al.,* 2009). Those hydrolysates were prepared by proteolytic enzymes. Ngo *et al*. (2010) reported that gelatin hydrolysate from gelatin from Nile tilapia (*Oreochromis niloticus*) had the antioxidative activity. Nalinanon *et al.* (2011) studied the antioxidant properties of protein hydrolysates from the muscle of ornate threadfin bream treated with pepsin from skipjack tuna. The highest ABTS and DPPH radical-scavenging activities were found in hydrolysates with 20% DH. Therefore, peptides in hydrolysates with various DH might differently scavenge two different radicals, ABTS and DPPH radicals. Protein hydrolysates contained proteins or peptides, which were hydrogen donors and could react with radicals to convert them to more stable products, thereby terminating the radical chain reaction (Khantaphant and Benjakul, 2008). Wu *et al*. (2003) studied the antioxidant properties of mackerel hydrolysates prepared by autolysis and with Protease N and found that antioxidant property increased gradually with the increasing hydrolysis time.

Several amino acids are generally accepted to be antioxidative and exhibit higher antioxidative activities when incorporated into peptides (Saito *et al*., 2003). Dávalos *et al.* (2004) indicated that tryptophan, tyrosine and methionine showed the highest antioxidant activity among the amino acids, and followed by cysteine, histidine and phenylalanine. Mendis *et al.* (2005) reported that proline,

alanine, valine and leucine in jumbo squid skin hydrolysate contributed to the higher antioxidative activities and phenylalanine and leucine at N- and C- terminals of peptide could contribute to the high activity. In addition, tripeptides containing tryptophan or tyrosine residues at the C-terminus had strong radical scavenging activities, but very weak peroxynitrite scavenging activity (Saito *et al.*, 2003). Yellowfin sole frame protein hydrolyzed with mackerel intestine crude enzyme and pepsin were also fractionated into five major types with varying antioxidative activities. Fraction-I (10-30 kDa) exhibited the highest activity in the linoleic acid autoxidation system. The sequence of the purified peptide was Arg-Pro-Asp-Phe-Asp-Leu-Glu-Pro-Pro-Tyr and molecular weight was 13 kDa (Jun *et al.*, 2004). Nalinanon *et al.* (2011) found that the fraction containing peptides with a MW of 1.3 kDa of hydrolysate prepared using skipjack tuna pepsin showed the highest ABTS radical-scavenging activity. Table 4 shows characteristics and antioxidative activities of some peptides derived from collagen and gelatin of different sources.

Table 4. Characteristics and antioxidative activities of some peptides derived from collagen and gelatin.

Source	Characteristic	Proteases	Activity	Reference
Fish skin gelatin (Alaska Pollack)	Gly-Glu-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-ProHyp-Gly-Pro-Hyp-Gly Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-Pro-Hyp-Gly-ProHyp-Gly	Serial digestion (Alcalase, Pronase E, collagenase)	Inhibition of lipid peroxidation Increase of cell viability exposed to t-BHP	Kim *et al.* (2001)
Squid skin gelatin *(Dosidicus gigas)*	Phe-Asp-Ser-Gly-Pro-Ala-Gly-Val-Leu Asn-Gly-Pro-Leu-Gln-Ala-Gly-Gln-Pro-Gly-Glu-Arg	Trypsin	Radical scavenging Increase of cell viability exposed to t-BHP	Mendis *et al.* (2005)
Squid tunic gelatin *(Dosidicus gigas)*	Gly-Pro-Leu-Gly-Leu-Leu-Gly-Phe-Leu-Gly-ProLeu-Gly-Leu-Ser	Alcalase	Radical scavenging Ferric reducing power	Aleman *et al.* (2011)
Fish skin gelatin *(Jonius belengerii)*	His-Gly-Pro-Leu-Gly-Pro-Leu	Trypsin	Radical scavenging Inhibition of lipid peroxidation Increase of antioxidative enzyme levels in hepatoma cells	Mendis *et al.*(2005)
Tuna backbone	Val-Lys-Ala-Gly-Phe-Ala-Trp-Thr-Ala-Asn-Gln-GlnLeu-Ser	Pepsin	Radical scavenging Inhibition of lipid peroxidation	Je *et al.* (2007)

6.4 Antimicrobial activity of protein hydrolysates

The antimicrobial activity of the peptide fractions depends on several factors such as amino acid composition, sequence and molecular weight of amino acid as well as bacterial species (Di Bernardini *et al*., 2011). Gómez-Guillén *et al.* (2010) reported antimicrobial activity in peptide fractions from tuna and squid skin gelatins within a range of 1-10 kDa and <1 kDa. The bacteria species manifesting the most susceptible to those peptides fractions were *Lactobacillus acidophilus, Bifidobacteria lacti, Shewanella putrefaciens and Photobacterium phosphoreum*. It is generally agreed that peptides need to interact with cell membranes as part of their action against microbes (Hancock and Patrzykat, 2002). The reduced molecular weight in the peptide fractions, which was related to the elimination of aggregates, better exposure of the amino acid residues and their charges, as well as structure acquisition, were suggested as factors facilitating the interaction with bacterial membranes (Gómez-Guillén *et al.,* 2010). However, Molinero *et al.* (1988) analyzed dipeptides with a surfactant-like behaviour by condensation between Nα-lauroyl arginine and amino acids coming from a collagen hydrolysate. A higher antimicrobial activity against both Gram-positive and Gram-negative bacteria was found for those dipeptides having a more pronounced cationic character.

The hydrophobic properties of amino acids would let peptides enter the bacterial membrane, as the positive charge would initiate the peptide interaction with the negatively charged bacteria surface (Wieprecht *et al.,* 1997). However, Floris *et al.* (2003) found that differences existing in membrane composition have implications in the mode of action and the specificity of the antibacterial compounds. Patrzykat and Douglas (2005) reported that the degree of lipopolysaccharide (LPS) binding is neither directly nor inversely proportional to peptide activity because, following the rupture of the outer membrane, peptide activity would depend on its ability to interact with the bacterial cytoplasmic membranes. Therefore, both the sequence and concentration of the peptide and the composition of the bacterial membranes would influence the mode of interaction.

6.5 Use of protein hydrolysate in biodegradable films

There exists few reports on use of protein hydrolysate in biopolymer-based films. Recently, Salgado *et al.* (2011) studied the properties of soybean (SPI) and sunflower protein isolate (SFPI) based films incorporated with bovine plasma hydrolysates (BPH). Both types of films showed a decrease in strength and elastic modulus, and an increase in elongation at break with increasing BPH level from 0-40 g /100 g protein isolate. The BPH added had a plasticizing effect on SPI and SFPI films properties. Moreover, BPH also conferred important antioxidant properties to SPI and SFPI films. The antioxidant capacity of the films was increased when BPH in the range of 0-40 g/100 g protein isolate was added. However, the SFPI films had higher antioxidant capacity than SPI films.

Giménez *et al.* (2009) observed that the increase of squid gelatin hydrolysates content (0-10%) in squid skin gelatin films led to an increase in antioxidant activity of the films, as measured by ABTS and FRAP assays, although in detriment of mechanical properties and the water vapor permeability but increase in extensibility. Small peptides could be easily inserted in the protein network and form hydrogen bondings with the gelatin chains in detriment of chain–chain interactions, thereby decreasing the density of intermolecular interactions and increasing the free volume between gelatin chains (Arvanitoyannis *et al.,* 1998).

7. Sealing of films

Sealing can be defined as a process of bonding together two or more surfaces of polymer films (Abdorreza *et al.,* 2011). The bonding between the polymer films or plastic sheet can be carried out by several ways, depending on various factors, including the use of mechanical force, heat, ultrasonic and adhesive (Briston, 1983). Heat-sealing process is an important and widely used method for sealing thermoplastic films. To increase the strength of the seal during the heat-sealing process, temperature, pressure and heat-sealing time are the basic factors to be considered (Chukhlanov and Tereshina, 2007). Several sealing techniques such as bar, band, impulse, wire or knife, ultrasonic, friction, gas, contact, hot-melt, pneumatic, dielectric, magnetic, induction, radiant, adhesive (or glue) and solvent sealing methods have been implemented (Brody and Marsh, 1997).

7.1 Important sealing methods

7.1.1 Heat sealing

Heat sealing is defined as a process of joining two or more thermoplastic films or sheets by the heating of areas in contact with each other to a temperature at which fusion occurs; this is usually aided by pressure. The interact process between surface areas of thermoplastic films is generally required optimal time (Abdorreza *et al.,* 2011). The heat-sealing property is normally evaluated mechanically by peel strength testing as determined by two aspects: the heat-sealing process and the microstructure of the heat-sealing part. To enhance the sealing strength during the heat-sealing process, the pressure, temperature, and heat-sealing time are the three basic parameters to be considered. The initial pressure makes an intimate squeeze between two films; then, the adhesion of two films is promoted by the action of heat from the outside with sufficient time (Brody and Marsh, 1997). It is well known that the mechanism of heat sealing is that the intimate contact of the sealing surfaces occurs after the molecular segments diffuse across the interface and form entanglement. Moreover, the cooling and re-crystallization greatly affect the heat seal strength by chains diffusion and entanglement at the interfaces.

Heat sealing quality depends on the processing conditions such as temperature, time and pressure as well as previous film treatments (López *et al.*, 2011). Sealing strength is also influenced by film thickness. The optimum sealing is that providing the layers of material melted into a homogeneous without causing the decrease of material's thickness and property of material remains unchanged. There are 2 main types of heat sealing: thermal heat sealing as a continuous heat sealer and impulse heat sealing as a heating elements are not continuously heated (Euapitaksakul, 1996).

7.1.1.1 Thermal heat sealing

Thermal heat sealing is sealing under continuous heating with constant temperature. Heat source which can be used includes steam, hot oil, hot water, hot air, heating oil and electricity. For heat sealing process, two films are pressed together between heated plates or dies under pressure (Patton, 1976). The pressure makes an intimate press between two thermoplastic films; then, the adhesion of films is

supported by the action of heat, but this process requires time. Mechanism of heat sealing is that the intimate contact of the sealing surfaces occurs after the molecular segment diffuse across the interface (Aithani *et al*., 2006). The increasing of seal bar temperature and sealing time led to the seal strength increases, and it will increase until it reaches appropriate seal bar temperature and sealing time, and then seal strength will be constant. For appropriate heat-sealing, seal bar temperature should be slightly higher than the melting point of sealant. Moreover, the optimal pressure of sealing should be in the way that prevent seal distortion (Euapitaksakul, 1996). Thus, heat sealing quality depends on temperature, time, pressure and film samples.

7.1.1.2 Impulse heat sealing

Impulse heat sealing are sealing technique in which the heating elements are not continuously heated at certain temperature. In this technique, jaws are heated to fusion temperature by a short powerful electric impulse. During the heat-sealing process, temperature of sealing jaws (bar or wire) increases but decreases when seal cools. Therefore, seal bars wire should have a low specific heat capacity which involves rapid heating and cooling (Young, 1986). When the polymeric film is placed between seal jaws, the seal jaws will be closed under the pressure and then the current flows through Nichrome wire. The resistance of the Nichrome wire turns electricity into heat making films fuse together. After that, heat is removed from a system so that Nichrome wire and seal of films are cooled down. Normally, impulse wire sealing was selected over impulse bar sealing since it allows cooling the zip, enhancing its strength, before the jaws are opened. Sealing factors that must be controlled include temperature, pressure and time. Sealing time used depends on the thickness and polymer film type. The increase in seal width generally requires a higher pressure. Also, whenever the temperature increases, it must increase the cooling rate (Young, 1986).

7.1.2 Ultrasonic sealing

The sealing with ultrasonic occurs when high frequency mechanical energy is transferred into two layers of polymer films. This method is commonly used with a thicker material due to difficult heat transfer problem or the high of sealed area. It is commonly used for plastics, and especially for bonding of difference materials (Bongaerts, 1988).

7.1.3 The sealing using electrodes, magnet and electromagnetic induction

These methods make a rapid of heat seal area due to principles of heat. For heating with electrodes, high-frequency electric field is transferred through polymeric materials. This approach is suitable for electrode packaging due to the rapid alignment of molecule. For packaging with magnetic components, magnets are arranged by the magnetic field and heat occurred. Magnetic field anneals magnet along with surface of packaging (Bongaerts, 1988).

7.1.4 The sealing using adhesive or solvent

Several adhesives or glues and solvents can be utilized for sealing of polymeric films. In this technique, appropriate adhesive or solvent is smeared on surface of the packaging. When the adhesive or solvent reacts on the surface, it can make the surfaces of packaging stick together. Contacted surface of packaging is pressed and integrated homogeneously. After that, removal of solvents from samples by evaporation for increasing the strength of the joint seal. However, the excessive solvent used might result in reduced strength (Bongaerts, 1988).

7.2 Heat sealing properties of some biodegradable/edible films

The sealing ability is another important property of such protein-based films that greatly influences the application as packaging material. As compared with available sealing methods, heat-sealing is the most versatile method, with the advantages of safety, convenience, good productivity and high mechanical strength. Among heat sealing, impulse sealing technique is widely used to zip flexible synthetic films especially biopolymer-based films (Brody and Marsh, 1997; Su *et al.*, 2012). However, a limited numbers of studies concerning sealing of edible and biodegradable films have been reported. A successful heat-sealing of edible films would be based on the thermoplastic behavior that would allow them to be heat-sealed, if appropriate temperature, pressure and dwell time conditions are used (Su *et al.*, 2012).

Kim and Ustunol (2001) studied the thermal properties and heat-sealability of solution-cast whey protein and whey protein–lipid composite films, plasticized with either glycerol or sorbitol. They reported that films were heat-sealable at temperatures close to the onset of the thermal transitions.

Hernandez-Izquierdo and Krochta (2009) investigated the conditions necessary for efficient heat-sealing of whey protein–glycerol films obtained by both solution-casting and extrusion methods. Films were heat-sealed using an impulse heat-sealer at an effective jaw pressure of 293.31 kPa, a voltage of 15 V and a cooling time of 4 s. Various impulse times, ranging from 1.5 to 2.5 s for films formed by solution-casting and extrusion, respectively, were used to heat-seal the films. Glycerol-plasticized whey protein films obtained by both solution-casting and extrusion displayed thermoplastic behaviour that allowed them to be heat-sealed at temperatures around those melting transitions. With shorter times (lower sealing temperatures), the seals delaminated. A longer time (higher sealing temperature) was necessary to achieve a heat-seal of extruded films as compared with solution-cast films, due to the greater thickness in extruded films (0.18 ± 0.02 mm) as compared with solution-cast films (0.13 ± 0.01 mm). Morevoer, heat-sealing temperatures ranged between 126.1 ± 9.0 and 204.0 ± 5.4 °C for the shortest and longest impulse times, respectively. In addition, film thickness appeared to have an effect on seal strength; higher strengths were achieved for thinner films. Thicker films required a longer minimum impulse time to achieve a heat-seal. Greater film thickness along with lower protein content appeared to result in decreased seal strengths.

Farris *et al*. (2009) studied the influence of temperature, dwell time and bar pressure on heat seal strength of oriented polypropylene films coated with a gelatin-based thin layer as a heat sealant. They reported that bar pressure and bars temperature turned out to be the most influencing factors on the strain energy of the film tested. The bar pressure rather than the sealing temperature was the factor affecting the most the maximum force and strain energy, considered as a measure of the strength necessary to break the bond across the sealed interface. Moreover, whereas the bar pressure negatively affected the seal strength of coated polypropylene films, the sealing temperature had a positive effect. Dwell time did not have any significant influence as a main factor, while influencing negatively the seal strength as an interaction term (i.e. time x pressure), together with the further interaction of temperature x pressure.

López *et al.* (2011) evaluated the heat sealing capacity of native and acetylated corn starch based films. The heat seals were produced using an impulse-

wire thermo-sealer working at 220 V, leading to a fixed temperature of 154.3 ± 8.5 °C and the times varied between 0.83 ± 0.05 and 2.03 ± 0.05 s. They found that all films showed a good heat sealing capacity. Acetylated starch addition in film formulation reinforced the sealing resistance. Mode of failure of seal depended on samples. Unplasticized heat-sealed films presented adhesive failures while those containing glycerol showed a rupture near the zip. They reported that the best zip quality obtained by heat sealing of the developed starch films was achieved at 154.3 ± 8.5 °C during 1.84 ± 0.01 s of dwell time.

Su *et al*. (2012) investigated the heat-sealing ability of SPI/carboxymethyl cellulose (CMC) blend films. The films were heat-sealed at heat-sealing temperatures in the range of 170–250 °C for 0.1 s with a 150 kPa pressure. Peel strength and tensile strength measurements showed that the Maillard reactions more likely were the main effect of enhancing the heat-sealing ability above the melting temperature. The heat-sealing ability of SPI/CMC blend films was superior to that of pure SPI films. In addition to the CMC content in the blend films, heat-sealing temperature was a main factor controlling the mechanical properties of films. The long-chain molecules of CMC blending with SPI and the occurrence of Maillard reactions caused entanglement and inter-molecularly cross-linked structures, which enhanced the melting temperature and heat of fusion of films. They also found that in the heat-sealing process involving melting and diffusing of polymers through the laminate interface, was strongly dependent on temperature. When the heat-sealing temperature was lower than 170 °C, all films showed peeling "separation" and did not meet heat-sealing requirements. SPI/CMC sample could not provide sufficient entanglement at the lower temperature. When heat-sealing was performed at temperatures higher than 180 °C, the peel strength for all samples sharply increased, attributed to Maillard reactions occurring at a higher melting temperature for these blends. The molecules in laminated films were softened at 180 °C and adhered to each other in a state of conglutination; then, the molecules inserted into and entangled with each other at the higher temperature (220 °C). The dependence of peel strength on heat-sealing temperature also differed substantially for blends with different compositions (Su *et al*., 2012).

Objectives

1. To study the role of gelatin hydrolysate as a plasticizer in fish myofibrillar protein film.
2. To study the effect of degree of hydrolysis (DH) and level of gelatin hydrolysate incorporated on the properties of fish myofibrillar protein film.
3. To investigate the combined effect of gelatin hydrolysate and glycerol on the properties of fish myofibrillar protein film.
4. To comparatively characterize and study some properties of fish myofibrillar protein films added with glycerol and gelatin hydrolysate.
5. To examine the stability of fish myofibrillar protein film added without and with gelatin hydrolysate during storage.
6. To study seal ability of fish myofibrillar protein film added with gelatin hydrolysate.

CHAPTER 2

MATERIALS AND METHODS

1. Materials

1.1 Raw material

Fresh red tilapia (*Oreochromis niloticus*) with an average weight of 400-500 g/fish were purchased from a local market in Hat Yai, Songkhla province, Thailand. Fish were kept in ice with a fish/ice ratio of 1:2 (w/w) and transported to the Department of Material Product Technology, Prince of Songkla University within 30 min. Upon the arrival, fish were immediately washed, filleted and minced to uniformity, using a mincer with a hole diameter of 0.5 cm. Fish gelatin produced from tilapia skin (~240 bloom) was purchased from Lapi Gelatine S.p.A (Empoli, Italy).

1.2 Chemicals

Glycerol, sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium sulphite, L-Leucine, 2,4,6-trinitrobenzenesulphonic acid (TNBS), di-sodium hydrogen orthophosphate, sodium dihydrogen orthophosphate, 2,2-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picryl hydrazyl (DPPH)**,** Sodium dodecyl sulfates (SDS) and 6-hydroxy-2,5,7,8 tetramethylchroman-2carboxylic acid (Trolox) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Malondialdehyde bis (dimethyl acetal) and β-mercaptoethanol (βME) were purchased from Sigma (St. Louis, MO, USA). Acrylamide, *N,N,N'N'*-tetramethylethylenediamine (TEMED) and bis-acrylamide were obtained from Fluka (Buchs, Switzerland).

2. Equipment

The equipments used in this study are shown in Table 5.

Table 5. List of equipment used in this work.

Equipment	Model	Company/Country
Homogenizer	WIGGEN HAUSER D-500	TE Scientific Sdn. Bhd of Lot 8, Selangor, Malaysia
pH meter	pH/Ion 510	Eutech Instruments Pte Ltd., Singapore
Magnetic stirrer	Ro 15 power	IKA labortechnik, Stanfen, Germany
Shaker	Heidolth Inkubator 10000	Schwabach, Germany
Electrophoresis apparatus	Mini-Protean II	Bio-Rad Laboratory Int., California, USA
Microcentrifuge	MIKRO20	ZENTRIFUGEN, Hettich, Germany
Universal testing machine	LR 30 K	LLOYD Instruments Ltd., Hampshire, UK
Environmental chamber	KBF 115	WTB Binder, Tuttlingen, Germany
Fourier transform infrared spectrometer	Bruker Model Equinox 55	Bruker Co., Ettlingen, Germany
Scanning electron microscope	JSM-5800 LV	JEOL, Tokyo, Japan
Thermo-gravimetric analyzer	TGA 7	Perkin Elmer, Norwalk, CT, USA
CIE colorimeter	Color Flex	HunterLab Reston, Virginia, USA
Impulse sealing machine	ME-300HEM	Taiwan

3. Methods

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 homogenized with 3 volumes of cold distilled water (2-4 ^{o}C) at a speed of 13,000 rpm for 2 min, using an IKA Labortechnik homogenizer followed by filtering through a layer of nylon cloth. The mince was mixed with 5 volumes of cold 50 mM NaCl (2-4 ^{o}C) 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 and protein pattern analysis of fish myofibrillar protein (FMP)

FMP obtained was subjected to compositional analysis for protein, moisture, fat and ash contents according to AOAC (2000). Protein pattern of FMP 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 and incubated at 85 ^{o}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). Proteins (15 μg) determined by the Biuret method (Robinson and Hodgen, 1940) were loaded 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. Molecular weights of proteins were estimated from protein markers.

3.2 Preparation and analysis of gelatin hydrolysate

3.2.1 Determination of preparing condition of gelatin hydrolysate

Gelatin hydrolysate was prepared by acid hydrolysis according to Tsugita and Scheffler (1982) with some modifications, under different conditions. Gelatin was dissolved in hydrochloric acid solution of different concentrations (0.5, 1 and 1.5 M) to obtain the protein concentration of 10% (w/v). The mixtures were incubated at 50 ^{o}C for 15 min. The solutions were then placed in a temperature controlled oil bath at 100 ^{o}C for different times (1, 3, 6, 9 and 12 h). After that, the reaction was terminated by cooling with cold water. The pH of the resulting hydrolysates were adjusted to 7.0 using 2 M NaOH. The DH of gelatin hydrolysates was analyzed at different hydrolysis times as described by Benjakul and Morrissey (1997) (see section 3.2.2.1). The plots between DH and hydrolysis time of each HCl concentration used were constructed. From the relation, the HCl concentrations and times required to hydrolyze gelatin to obtain the desired DHs (30, 60 and 90%) were selected.

3.2.2 Preparation and analysis of some properties of gelatin hydrolysates with different degree of hydrolysis (DH)

Gelatin hydrolysates with different degree of hydrolysis (DH) approximately 30, 60 and 90% were prepared according to the method and the selected condition (concentration of HCl and hydrolysis time) obtained from section 3.2.1. The DH of the resulting hydrolysates was determined as following:

3.2.2.1 Degree of hydrolysis (DH)

The DH of obtained gelatin hydrolysates was analyzed according to the method of Benjakul and Morrissey (1997). Diluted gelatin hydrolysate sample (100 µl) were added with 1.6 ml of 0.2125 M phosphate buffer, pH 8.2 and 0.8 ml of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature controlled water bath at 50 ^{o}C for 30 min in the dark. The reaction was terminated by adding 1.6 ml of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was measured at 420 nm using a uv-vis spectrophotometer and α-amino group was expressed in terms of L-leucine. The DH was calculated as follows (Benjakul and Morrissey, 1997):

$$DH = [(L_s - L_0)/(L_{max} - L_0)] \times 100$$

where L_s is the amount of α-amino groups of gelatin hydrolysate sample. L_0 is the amount of α-amino groups in the original gelatin solution. L_{max} is the total α-amino groups in the original gelatin solution obtained after complete acid hydrolysis using 6 N HCl at 110 ^{o}C for 24 h.

3.3 Study on effect of concentration and DH of gelatin hydrolysate on properties of fish myofibrillar protein (FMP) film

3.3.1 Preparation of film-forming solutions (FFS) and films

The film-forming solutions (FFS) of FMP were prepared according to the method of Prodpran and Benjakul (2005). The 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. The pH of the mixture was adjusted to 3 using 1 N HCl to solubilize the protein. The solution was filtered through a layer of nylon cloth to remove undissolved debris. Gelatin hydrolysates with different DH (≈30, 60 and 90%) at pH 3 were added to the FMP solutions at various levels (30, 40, 50 and 60% based on protein). The solutions were stirred gently for 15 min at room temperature. The control FFS's were also prepared by using glycerol as plasticizer at different level (30, 40, 50 and 60% based on protein).

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 ^{o}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 ^{o}C and 50% RH until used for analyses.

3.3.2 Determination of film properties

3.3.2.1 Film thickness

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

3.3.2.2 Mechanical properties

Prior to the measurement of mechanical properties, films were conditioned for 48 h in a ventilated oven at 25 ^{o}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 by dividing the tensile stress by the tensile strain in the elastic (initial, linear) portion of the stress-strain curve.

3.3.2.3 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 dried silica gel (0% RH) with silicone vacuum grease and rubber gasket to hold the film in place. The cup was placed at 25 ^oC and 50% RH in ventilated oven. The cup was weighed at 2 h intervals over a 16 h period. WVP of the film was calculated as follows:

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

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.

3.3.2.4 Color

Color of film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., Reston, Virginia, USA), working with D_{65} (day light) and a measure cell with opening of 30 mm. The color parameters were expressed as L^* (lightness), a^* (redness/greenness), b^* (yellowness/blueness) values and total difference of color (ΔE^*) was calculated as follows (Gennadios *et al*., 1996):

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

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

3.3.2.5 Light transmittance and transparency value

The light transmittance of films was measured at the ultraviolet and visible range (200–800 nm) using UV–vis spectrophotometer (Shimadzu UV-1800, Tokyo, Japan) as described by Shiku *et al.* (2004). The transparency value of film was calculated using the following equation (Han and Floros, 1997):

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

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

Gelatin hydrolysate with the DH providing the FMP film with the best overall properties (high extensibility and strength and low WVP) was chosen for next study.

3.4 Study on effect of gelatin hydrolysate and glycerol blend on properties of fish myofibrillar protein film

The film-forming solutions (FFS) (protein concentration of 2% (w/v)) were prepared in the same maner as mentioned in section 3.3.1. The blend of glycerol and gelatin hydrolysate (DH≈60%) of varying ratios of glycerol/gelatin hydrolysate (100/0, 75/25, 50/50, 25/75 and 0/100) were added to the FFS at different concentrations (30 and 50% based on protein). The obtained films were subjected to property determination similar to sections 3.3.2.1-3.3.2.5.

The glycerol/gelatin hydrolysate blend at the ratio and concentration yielding FMP film sample which had the best overall properties (high extensibility, and strength and low WVP) was chosen for further study.

3.5 Study on some properties and characteristics of FMP films added with gelatin hydrolysate

Films selected from section 3.3 and 3.4 and the control film (with glycerol but without gelatin hydrolysate) were used for some properties analysis and characterization as following.

3.5.1 Mecanical properties

3.5.1.1 Tensile strength (Iwata *et al.,* 2000) as described in section 3.3.2.2

3.5.1.2 Elongation at break (Iwata *et al.,* 2000) as described in section 3.3.2.2

3.5.1.3 Young's Modulus (Iwata *et al.,* 2000) as described in section 3.3.2.2

3.5.2 Moisture content according to AOAC (1999**)**

3.5.3 Water vapor permeability (WVP) as described in section 3.3.2.3

3.5.4 Color, light transmittance and transparency value as described in section 3.3.2.4 and 3.3.2.5

3.5.5 Protein pattern

Protein patterns of films were determined using SDS-PAGE using 4% stacking gel and 10% running gel according to the method of Laemmli (1970). Muscles (3 g) were solubilised in 27 ml of 5% SDS. The mixture was homogenised for 1 min at a speed of 13,000 rpm, using an IKA homogenizer and incubating at 85 ^{o}C for 1 h to dissolve total proteins. To solubilize the films prior to SDS-PAGE analysis, films were mixed with 20 mM Tris HCl (pH 8.8) containing 2% SDS and 8 M urea in the presence and the absence of 2% β-ME. The mixture was homogenized at 13,000 rpm for 1 min. The homogenate was stirred continuously for 24 h at room temperature (28-30 ^{o}C). Then, the sample was 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.

3.5.6 Film solubility and protein solubility

Film solubility was determined according to the method of Gennadios *et al.,* 1998. The conditioned film samples (2 cm x 5 cm) 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 30 ^{o}C for 24 h. Undissolved debris was removed by centrifugation at 3000xg for 20 min. The pellet was dried at 105 ^{o}C for 24 h using a hot air oven (Binder FED115, Tuttlingen, Germany). Film solubility was calculated by subtracting the weight of insolubilized dry matter from the initial weight of dry matter and expressed as a percentage of the total weight.

To determine the protein solubility, the supernatant obtained from sample preparation for film solubility test were used. 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 determined using the Biuret method (Robison and Hodgen, 1940). Protein solubility was expressed as the percentage of total protein in the film, which was solubilised with 0.5 M NaOH at 30 °C for 24 h.

3.5.7 Protein solubility in various solvents

Protein solubility of films in various solvents was determined as described by Chawla *et al.* (1996) with some modifications, to determine the major associative forces involved in the film matrix. 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 at a speed of 13,000 rpm for 1 min using a homogenizer (IKA Labortechnik, Malaysia). The homogenate with S3 was heated in boiled water (100 ^{o}C) for 2 min and 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). The mixture was then kept at 4 ^{o}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 determined using the Biuret method (Robinson and Hodgen, 1940). To obtain the total amount of protein in the films, films were solubilized in 0.5 M NaOH. The solubility was reported as percentage of the total protein in film.

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

Prior to analysis, films were conditioned in a desiccators containing phosphorus pentoxide (P_2O_5) for two weeks at room temperature to obtain the most dehydrated films (Sobral *et al.,* 2001). The films were scanned with a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45^{o} ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at 25 ^{o}C as described by Nuthong *et al*. (2009). Films were placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra in the range of 650–4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 ^{o}C.

3.5.9 Thermo-gravimetric analysis (TGA)

The conditioned films (as described in 3.5.8) were scanned using a thermo-gravimetric analyzer (TGA7, PerkinElmer, Norwalk, CT, USA) from 30 to 1,000 ^{o}C at a rate of 10 ^{o}C/min (Nuthong *et al*., 2009). Nitrogen gas was used as the purge gas at a flows rate of 20 ml/min.

3.5.10 Scanning electron microscopy (SEM)

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

SPI-Module, PA, USA) in order to make the sample conductive, and photographs were taken at selected magnification.

3.6 Study on stability of fish myofibrillar protein film added without and with gelatin hydrolysate during storage

Films selected from section 3.3 and 3.4 were stored at room temperature (28-30 ^{o}C). Film samples were taken at week 0, 1, 2, 3, 4, 5, 6, 7 and 8 of storage for the following analyses:

3.6.1 Mechanical properties (as described in section 3.3.2.2)

3.6.2 Water vapor permeability (as described in section 3.3.2.3)

3.6.3 Moisture content (AOAC, 1999**)**

3.6.4 Color and film transparency (as described in section 3.3.2.4 and 3.3.2.5, respectively)

3.6.5 Film solubility and protein solubility (as described in section 3.5.6)

3.6.6 Protein solubility in various solvents (as described in section 3.5.7)

3.7 Study on sealing ability of fish myofibrillar protein film added with gelatin hydrolysate

The sealing ability of FMP films added with 50% of glycerol/gelatin hydrolysate blend (25/75) was evaluated. Generally, the film samples were sealed by the aid of different bio-based adhesives in combination with applied heat. The bio-adhesives used included gelatin, soy protein isolate and tapioca flour. The detail of study is as following:

3.7.1 Preparation of adhesives from different bio-based polymers

3.7.1.1 Preparation of adhesive from gelatin

Fish gelatin powder was solubilized in distilled water to obtain the concentration of 10% (w/v). The solution was heated in a water bath at 60 °C for 30 min (Jonjareonrak *et al*., 2006). The gelatin solution was then cooled down to room temperature before use.

3.7.1.2 Preparation of adhesive from soy protein isolate

Soy protein isolate was mixed with 0.1M NaOH solution at the concentration of 10% (w/v). The mixture was heated in a water bath at 80 °C for 30 min followed by cooling to room temperature (Nordqvist *et al*., 2010).

3.7.1.3 Preparation of adhesive from tapioca flour

Tapioca flour at 5% (w/v) was placed in distilled water. The suspension was gelatinized in a water bath at 90 °C for 10 min (Srirod, 1993) and cooled down to room temperature.

3.7.2 Sealing and seal strength determination

Film samples were cut into strips of 5 x 2 cm^2 and subjected to conditioning in an environmental chamber (WTB Binder, Tuttlingen, Germany) at 25 °C and 50% RH for 48 h before sealing. For sealing, adhesive of required amount 0.05±0.02 g of dry basis weight was spread evenly over the area of 2 x 0.5 cm^2 on one film strip. The other one film strip was placed on top of one another and allowed to stand at room temperature for 5 min prior to heat sealing. The glued area of the film samples was heat-sealed using impulse sealer with magnet Model ME-300HIM (S.N.MARK Ltd., Park, Nonthaburi, Thailand) at 150 ºC for 1.5, 2.5, and 3.5 s of heating time and 4 s of cooling time.

All sealed film samples were conditioned in an environmental chamber (WTB Binder, Tuttlingen, Germany) at 25 °C and 50% RH for 48 h prior to determining seal strength. The seal strength was evaluated via the peel tests according to the ASTM F88-00 standard with slight modification as described by Su *et al.* (2012) and Abdorreza *et al.* (2011). The peel strength test was conducted using Universal Testing Machine (Lloyd Instruments, Hampshire, UK). Each leg of the sealed film was clamped to the machine in which the distance between the clamps was 50 mm. A 100 N static load cell and cross-head speed of 90 mm/min was used. Seal strength was calculated as follows:

$$\text{Seal strength} = \frac{\text{Peak force}}{\text{Film width}}$$

The maximum force required to cause seal failure was reported as seal strength in newtons/meter (N/m).

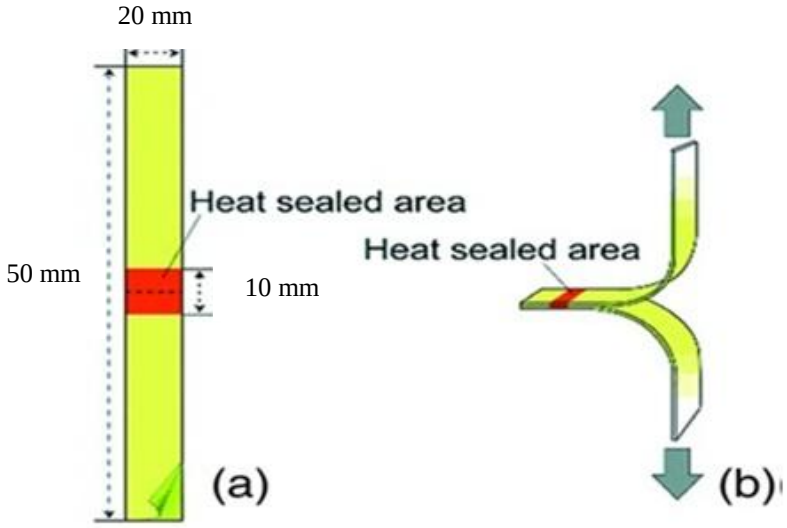


Figure 6. Illustration for the test specimen for seal strength test (peel test).

3.8 Statistical analysis

Experiments were run in triplicate. 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 16.0 for window, SPSS Inc., Chicago, IL, USA.) was used for data analysis.

CHAPTER 3

RESULTS AND DISCUSSION

1. Chemical compositions and protein pattern of FMP

Proximate composition of washed mince or fish myofibrillar protein (FMP) from red tilapia (*Oreochromis niloticus*) muscle is shown in Table 6. Protein content was found as a major component at 13.26 % of wet basis weight or 86.47% of dried basis weight. Commonly, the flesh of fish contains 11-24% (wet weight) of crude protein (Sikorski *et al.,* 1990). The chemical components found in FMP of red tilapia in this study were in agreement with those reported by Tongnuanchan *et al.* 2011. Crude protein content in fish flesh varies, depending on the species, the nutritional condition, the state of nutrition, the productive cycle, as well as the parts of fish (Sikorski *et al.,* 1990; Sikorski, 1994). During washing, sarcoplasmic proteins and other components, including fat or inorganic substances, could be removed (Hultin and Kelleher, 2000). As a result, the myofibrillar proteins concentration increased. Washing process can reduce lipid substrate and prooxidative heme protein (Chaijan *et al.,* 2010). Lanier and Lee (1992) suggested that washing process can remove fat and undesirable materials, such as blood, pigments and odorous substances. As a consequence, the myofibrillar proteins became more concentrated.

Protein pattern of mince (M) and washed mince are depicted in Figure 7. From the result, it indicated that myosin heavy chain (MHC), actin and tropomyosin were found as prominent proteins in unwashed and washed mince. In general, MHC is the dominant protein in fish muscle (Shahidi, 1994). After washing, band intensity of sarcoplasmic protein was decreased, while the band intensity of myofibrillar proteins (MHC and actin) was increased. 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).

Table 6. Proximate composition of fish myofibrillar protein (FMP) from red tilapia muscle.

Composition	**Content (%)**	
	Wet wt.	Dry wt.
Moisture	84.41±0.39	-
Protein	13.48±0.22	86.47±0.35
Fat	1.63±0.34	10.46±0.75
Ash	0.34±0.05	2.18±0.16

Mean ±SD from triplicate determinations.

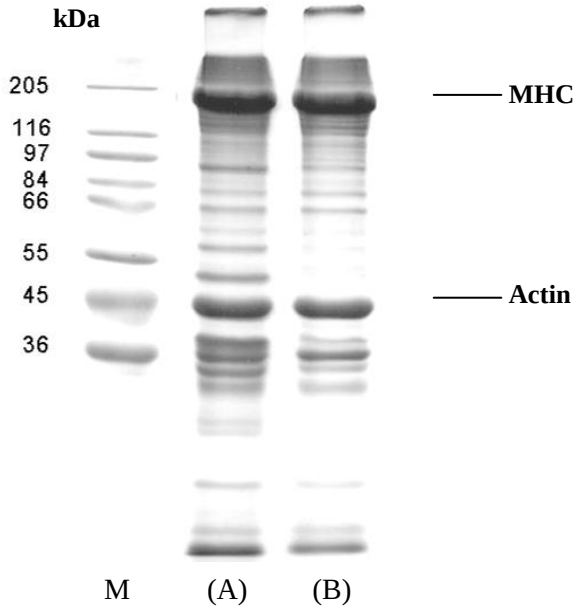


Figure 7. Protein patterns of unwashed mince (A) and washed mince (B) of red tilapia muscle. M: protein markers.

2. Acid hydrolysis of fish gelatin: effect of HCl concentration and hydrolysis time on DH of gelatin hydrolysate

Fish gelatin was hydrolyzed using hydrochloric acid solution of difference concentrations (0.5, 1 and 1.5 M) at 100 ^{o}C. The hydrolysis was carried out for different hydrolysis times. As shown in Figure 8, the DH of gelatin hydrolysate (GH) was increased to higher than 80% within the hydrolysis time of 3 h and 6 h for 1.5 M HCl and 0.5 and 1 M HCl, respectively. After that, the DH of gelatin hydrolysate was slightly increased. At the same hydrolysis time, the DH of gelatin hydrolysate increased with increasing concentration of hydrochloric acid. Normally, the hydrolysis reaction depends on the susceptible of peptide bonds and physical structure of protein molecule (Diniz and Martin, 1998; Tsugita and Scheffler, 1982). The DH can indicate the cleavage of peptide bond (Adler-Nissen, 1976). The hydrolysis of fish gelatin is typically in initial rapid phase (15-20 min) indicated that the numerous of peptide bonds are hydrolyzed; thereafter, the rate of hydrolysis decreases and approaches a stationary phase when no apparent hydrolysis take place (Shahidi *et al.,* 1995).

Gelatin hydrolysate could also be prepared by enzymatic hydrolysis processes using different proteases such as Alcalase, Neutrase, Protease N and Protamex (Gildberg *et al.,* 1989; Guerard *et al.,* 2001). However, the maximum DH of gelatin hydrolysates obtained by enzyme hydrolysis was reported to be in the range of ≈ 40-70% which was lower than those prepared from acid hydrolysate (Giménez *et al.,* 2010).

The gelatin hydrolysates with designed DH (23, 60 and 95%) were prepared by using the selected hydrolysis conditions (HCl concentration and hydrolysis time) as shown in Figure 8. The DH of hydrolysate is typically correlated to the size of hydrolysate or peptide. The higher DH value, the smaller size of peptide. Those obtained gelatin hydrolysate with different DH were used for incorporation into the FMP film in the next study.

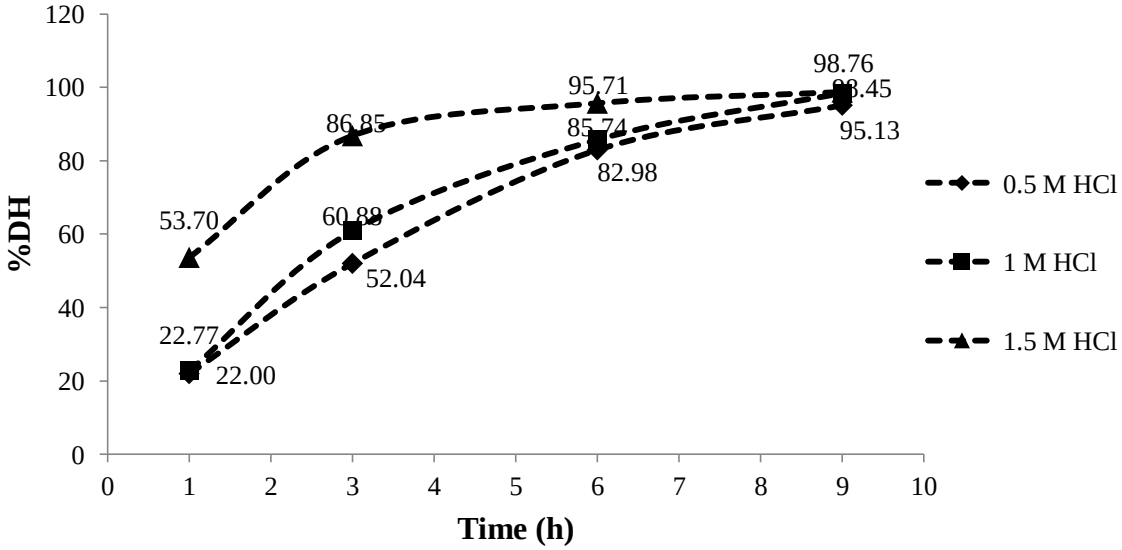


Figure 8. The relationship between hydrolysis time and the degree of hydrolysis (DH) of the gelatin hydrolysate by hydrochloric acid solution of difference concentrations (0.5, 1 and 1.5 M) at 100 ^{o}C. Bars represent the standard deviation (n=3)

3. Effect of concentration and DH of gelatin hydrolysate on properties of myofibrillar protein film

3.1. Thickness and mechanical properties

Thickness of FMP film was in the range of 0.035 – 0.040 mm (data not shown). There was no marked difference in thickness when glycerol or gelatin hydrolysate with all DHs were incorporated, regardless of level added. Mechanical properties of control films using glycerol as a plasticizer and those added with fish gelatin hydrolysates having different DHs at various levels are presented in Figure 9. The control films generally had the decreased E and TS but increased EAB as the levels of glycerol increased ($p<0.05$). In general, plasticizer affects not only the elastic modulus and other mechanical properties but also the resistance of film. Plasticizer plays a role in preventing films from cracking during packing and transportation (Aydinli and Tutas, 2000; Barreto *et al.,* 2003). Plasticizers are able to position themselves between polymer molecules, thereby interfering polymer-polymer

interaction. As a result, they can increase flexibility and processability (Guilbert and Gontard, 1995; Krochta, 2002). Plasticizers have been known to increase the free volume of polymer system and molecular mobility of polymer molecules (Sothornvit and Krochta, 2000).

When FMP films were added with gelatin hydrolysates, E and TS decreased with increasing levels, especially from 30 to 40% ($p<0.05$). Nevertheless, E of film incorporated with 23%DH hydrolysate increased as level of hydrolysate increased from 30 to 40%. The EAB of the films increased when level of hydrolysate having DHs of 23 and 60% increased from 30 to 50% ($p<0.05$). Gelatin hydrolysate added obviously showed plasticizing effect on FMP film as indicated by increased flexibility and extensibility as well as decreased strength of the films, compared to the FMP film without plasticizer addition which was very brittle. The short peptide chains of gelatin hydrolysate may insert between FMP molecules of film matrix, thereby lowering the inter-molecular interactions and increasing the free volume between FMP molecules. Thus, chain mobility of protein and flexibility of film were enhanced. The result was in agreement with Giménez *et al.* (2009) who observed that mechanical resistance of squid skin gelatin films decreased but extensibility increased with increasing levels of gelatin hydrolysates. With higher degree of hydrolysis, short gelatin molecules from cuttlefish skin with the higher mobility of chain exhibited plasticizing effect by preventing protein-protein interaction (Hoque *et al.,* 2011).

At the same gelatin hydrolysate level, films had the decreased E and TS as DH of gelatin hydrolysate increased ($p<0.05$) (Figure1). Smaller peptides might insert between protein chains and impede the protein-protein interaction more effectively. EAB of films varied with DH and level of gelatin hydrolysate used. With 30% gelatin hydrolysate addition, EAB of films increased when DH increased ($p<0.05$). Nevertheless, no difference in EAB was observed between films added with 30% of gelatin hydrolysate having DHs of 60 and 95% ($p>0.05$). When gelatin hydrolysate in the range of 40-60% was added, EAB of films increased with increasing DH from 23 to 60%.

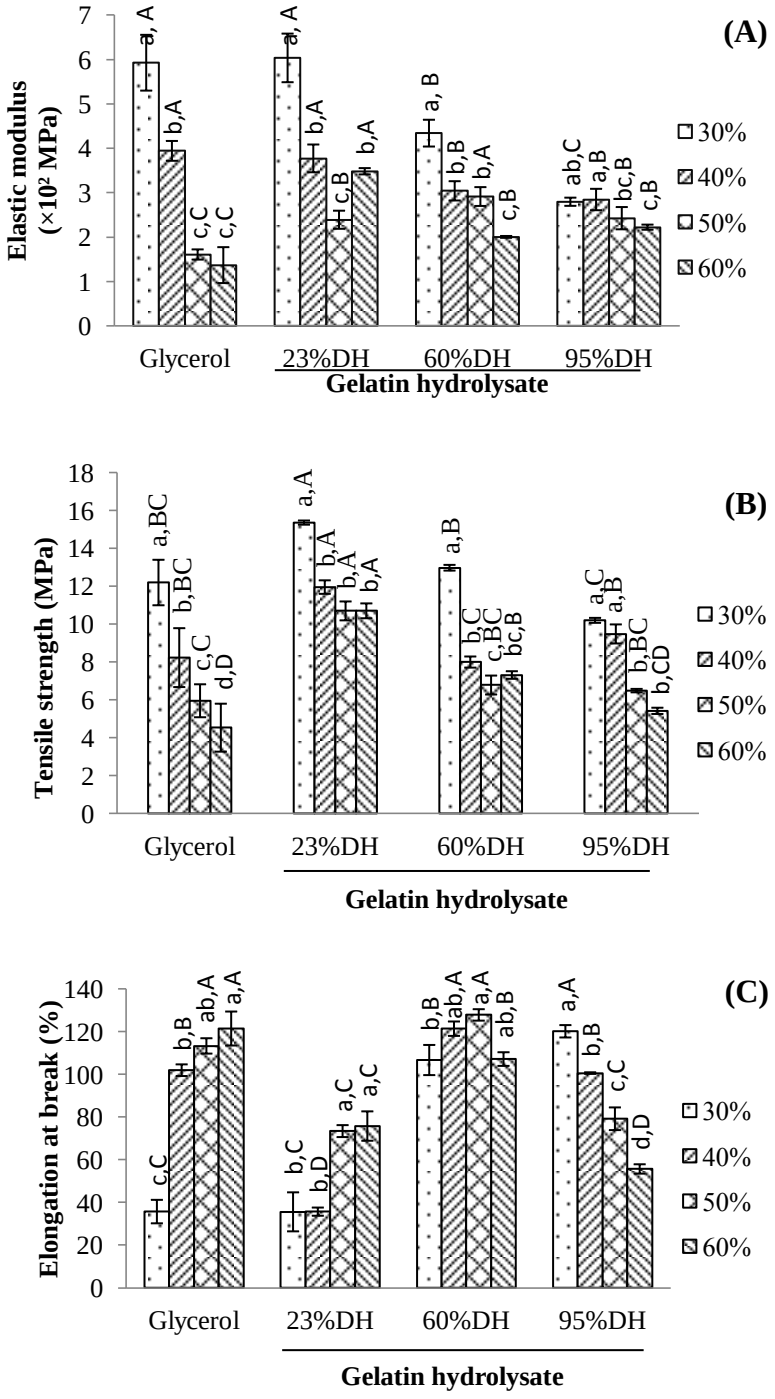


Figure 9. Mechanical properties of FMP films added with glycerol or fish gelatin hydrolysates having different DH at various levels. Bars represent the standard deviation (n = 3). Different lowercase letters on the bars within the same plasticizer indicate significant differences ($p<0.05$). Different uppercase letters on the bars within the same concentration indicate significant differences ($p<0.05$).

However, EAB of films decreased when gelatin hydrolysate of 95%DH in the range of 40-60% was used ($p<0.05$). It was noted that the decrease in EAB with the concomitant decrease in E and TS of films added with increasing level of 95%DH gelatin hydrolysate was mainly because these films were too soft and weak to resist the deformation upon tensile test. When compared to the control FMP films with glycerol as plasticizer at the same level, those added with gelatin hydrolysate at the levels of 50-60% exhibited higher E ($p<0.05$). In particular, films added with 23%DH hydrolysate had higher E and TS but lower EAB than those added with glycerol at the same level ($p<0.05$). The results suggested that glycerol was more effective plasticizer than gelatin hydrolysate especially when compared to the gelatin hydrolysate with low DH. This might be attributable to the varying sizes of peptides of gelatin hydrolysate, which plausibly exhibited varying plasticizing effect. Some chains of peptides molecules may act as plasticizer by inserting between protein chains and some chains would interact with protein chain in film matrix (Giménez *et al.,* 2009). However, films incorporated with 60%DH gelatin hydrolysate at the levels of 30-50% had similar TS to those added with glycerol at the same level ($p>0.05$). Therefore, the addition of gelatin hydrolysate of varying DHs and levels had an impact on the mechanical properties of FMP film. In particular, incorporation of gelatin hydrolysate with the DH of 60% at the level of 40-50% could render the FMP films with good flexibility and strength comparable to those added with glycerol as plasticizer.

3.2 Water vapor permeability (WVP)

Water vapor permeability (WVP) of FMP films added with gelatin hydrolysate and the control films (added with glycerol) at various levels is shown in Figure 10. The control films showed the increase in WVP with increasing glycerol level ($p<0.05$). Glycerol added is highly hydrophilic and hygroscopic plasticizer. Therefore, it can attract water molecules and form a large hydrodynamic of plasticizer-water complex, thus enhancing sorption and permeability of water through the film (Nemet *et al*., 2010). Increase in WVP of protein-based films with increasing plasticizer content was widely reported (Park *et al.,* 1994; Sothornvit *et al.,* 2000). FMP films incorporated with gelatin hydrolysates having DHs of 23 and 60% had slight increase in WVP as the levels of hydrolysate increased. However, no

differences in WVP were observed in FMP films added with 60%DH gelatin hydrolysate at the levels of 40-60% ($p>0.05$). In contrast, films with 95%DH gelatin hydrolysate showed continuous increase in WVP with increasing hydrolysate levels used ($p<0.05$). This result suggested that peptides of gelatin hydrolysate inserted between protein chains caused an increase in the free volume of FMP film. This allowed the water molecules to penetrate through the matrix of film with more ease, leading to the higher WVP of the resulting film. The results were in agreement with Salgado *et al.* (2011) who observed that the addition of bovine plasma hydrolysate (BPH) in soybean and sunflower protein-based films yielded the increase in WVP, especially with increasing BPH contents. Gontard *et al.* (1993) and Cuq *et al.* (1997) reported that increasing plasticizer levels led to the protein network with less dense. Plasticizer modified the molecular three-dimensional organization by decreasing the inter-molecular attractive forces and increasing the free volume and chain mobility and consequently increasing the rate of water diffusion and permeability.

At the same gelatin hydrolysate level, films added with 23%DH and 60%DH hydrolysates generally showed similar WVP ($p>0.05$). However, films added with 95%DH gelatin hydrolysate exhibited higher WVP than did those added with 23%DH and 60%DH hydrolysates at the same level ($p<0.05$). Addition of higher DH hydrolysate which mostly possessed smaller peptides would concomitantly impart greater amounts of hydrophilic groups ($-NH_2$, -COOH, -OH) in the matrix of FMP protein films. As a consequence, the films could absorb more water molecules, leading to increased WVP of the resulting films.

FMP films incorporated with gelatin hydrolysates had much lower WVP than those added with glycerol at the same level ($p<0.05$), regardless of DH of hydrolysate used. Gelatin hydrolysate incorporated might be more compatible and closely interacted with FMP chains, compared to glycerol. This could result in the lower free volume and denser protein network of film with gelatin hydrolysate as plasticizer, compared to that using glycerol. This resulted in lower WVP. Thus, DH and level of gelatin hydrolysate affected the WVP of FMP film.

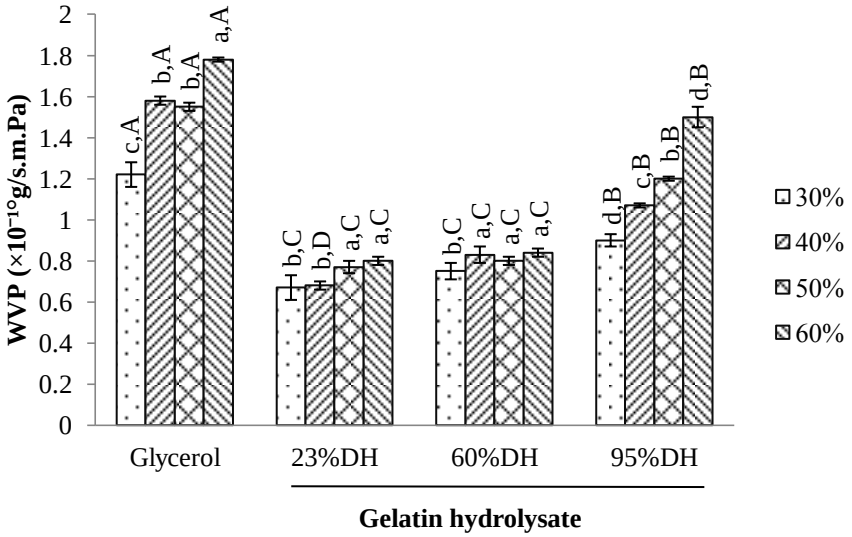


Figure 10. Water vapor permeability (WVP) of FMP films added with glycerol or fish gelatin hydrolysate having different DH at various levels. Bars represent the standard deviation (n = 3). Different lowercase letters on the bars within the same plasticizer indicate significant differences ($p<0.05$). Different uppercase letters on the bars within the same concentration indicate significant differences ($p<0.05$).

3.3 Color and transparency of film

*L**, *a**, *b** and Δ*E** values of FMP films incorporated with fish gelatin hydrolysate and control films are shown in Table 7. In general, FMP films added with gelatin hydrolysate had lower *L** and *a** values but higher *b** and Δ*E** values ($p<0.05$), compared to those added with glycerol at the same level. It is noted here that Δ*E** value relates to overall color of the sample where the higher Δ*E** indicates the more intense color. With the same DH, films containing different levels of gelatin hydrolysate were slightly different in colour parameters. No differences were found in *b** value of FMP film added with 60%DH hydrolysate at different levels ($p>0.05$). However, films incorporated with gelatin hydrolysate having high DH showed higher *b** and Δ*E** values, especially at higher level ($p<0.05$). This indicated that addition of gelatin hydrolysate resulted in the increased yellowness of FMP films. The gelatin hydrolysate, which contains amino group ($-NH_2$) may interact with carbonyl group (C=O) of lipid oxidation products of membrane lipid in FMP via Maillard reaction,

particularly during drying of film (Tongnuanchan *et al.,* 2013; Burghagen, 1999). Normally, the yellow coloration has been reported to be associated with protein-aldehyde interactions via Maillard reaction, and the reaction rate is dependent on the material composition, temperature and pH (Cuq *et al.,* 1997). Salgado *et al.* (2011) also observed that the addition of bovine plasma hydrolysates to soy protein isolate films caused a slight increase in the *b** value, without significantly affecting the *L** and *a** values. Hoque *et al.* (2011) reported that films from cuttlefish skin gelatin with different DH had lower *L** value and higher *b** value, compared with those from gelatin without hydrolysis.

Transparency value of FMP films incorporated with fish gelatin hydrolysate and control films is shown in Table 7. Regardless of DH and level, the transparency value of FMP films added with gelatin hydrolysate was higher than that of the control films added with glycerol ($p<0.05$). Higher transparency value indicates that the film is less transparent. Therefore, addition of gelatin hydrolysate yielded FMP films with decreased transparency. Gelatin hydrolysate might closely interact with FMP molecules, resulting in more compact network structure which could retard the transmission of light as compared to glycerol.

In addition, the transparency values of hydrolysate-added films varied, depending on DH and level of hydrolysate used. For 23%DH and 60%DH hydrolysate addition, films had similar transparency value ($p>0.05$), irrespective of DH and level used. However, transparency value of films added with 95%DH hydrolysate trended to decrease as hydrolysate level increased, indicating an increase in film transparency. Additionally, films added with 95%DH hydrolysate exhibited more transparent (lower transparency value) than those added with 23%DH and 60%DH hydrolysates ($p<0.05$). At high DH, shorter peptides of hydrolysate with higher numbers of chain ends might act as an effective plasticizer by preventing protein-protein interaction, leading to less dense film matrix with more transparency (Hoque *et al.,* 2010; Giménez *et al.,* 2009). Optical properties including colour and transparency of films are important attributes for various applications (Ahmad *et al.,* 2012). Generally, FMP film was more transparent than HDPE film (Hamaguchi *et al.,* 2007; Shiku *et al.,* 2003). It was noted that gelatin hydrolysate-added FMP film in this study was transparent enough for using as packaging film.

Table 7. Color and transparency values of FMP films added with glycerol or fish gelatin hydrolysates having different DH at various levels.

Plasticizer	level (%)	Color				Transparency value
		*L**	*a**	*b**	Δ*E**	
GLY	30	89.32 ± 0.26 aA	-1.69 ± 0.03 bcA	4.88 ± 0.25 cD	6.13±0.25 aC	4.13 ± 0.84 abB
	40	89.17 ± 0.13 aA	-1.65 ± 0.02 cA	4.95 ± 0.16 cC	6.22±0.23 aC	5.30 ± 0.66 aB
	50	89.05 ± 0.48 aA	-1.83 ± 0.01 aA	6.48 ± 0.19 aD	6.33±0.19 aD	3.39 ± 0.23 bC
	60	89.18 ± 0.17 aA	-1.70 ± 0.03 bA	5.67 ± 0.16 bD	6.22±0.22 aD	3.26 ± 0.66 bB
GH 23%DH	30	86.94 ± 0.27 aB	-2.23 ± 0.01 aB	9.08 ± 0.35 cC	7.96±0.15 aB	6.50 ± 0.39 aA
	40	87.05 ± 0.07 aB	-2.26 ± 0.03 aB	9.78 ± 0.23 bB	7.87±0.17 aB	6.96 ± 0.61 aA
	50	88.06 ± 2.53 aA	-1.93 ± 0.66 aA	11.41 ± 0.13 aB	7.04±0.13 bB	5.87 ± 0.54 aA
	60	86.93 ± 0.17 aBC	-2.31 ± 0.01 aC	10.97 ± 0.17 aC	7.98±0.22 aB	6.42 ± 0.61 aA
GH 60%DH	30	87.04 ± 0.12 bcB	-2.22 ± 0.03 aB	9.89 ± 0.27 aB	7.88±0.26 aB	7.43 ± 0.60 aA
	40	86.67 ± 0.08 cC	-2.26 ± 0.03 aB	9.82 ± 0.28 aB	8.18±0.18 aAB	7.14 ± 0.53 aA
	50	88.70 ± 0.18 aA	-2.41 ± 0.04 bA	9.39 ± 0.26 aC	6.68±0.22 cCD	6.54 ± 0.62 aA
	60	87.65 ± 0.95 bB	-2.27 ± 0.02 aC	9.93 ± 0.55 aB	7.41±0.26 bC	6.51 ± 0.64 aA
GH 95%DH	30	86.32 ± 0.05 aC	-2.24 ± 0.09 bB	11.10 ± 0.11 dA	8.46±0.25 aA	7.56 ± 0.90 aA
	40	86.41 ± 0.05 aD	-2.34 ± 0.02 cC	11.83 ± 0.13 cA	8.40±0.17 aA	5.20 ± 0.42 bB
	50	86.42 ± 0.15 aB	-2.20 ± 0.04 bA	12.75 ± 0.07 bA	8.37±0.15 aA	4.64 ± 0.39 bcB
	60	86.50 ± 0.10 aC	-1.90 ± 0.03 aB	13.55 ± 0.32 aA	8.26±0.22 aAB	3.85 ± 0.44 cB

Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column within the same plasticizer indicate significant differences ($p<0.05$). Different uppercase letters in the same column within the same plasticizer content indicate significant differences ($p<0.05$).

Among gelatin hydrolysates used, film added with that having 60% DH exhibited sufficient flexibility comparable to the control film added with glycerol, with the concomitantly lower WVP. Therefore, gelatin hydrolysate of 60% DH was chosen for next study.

4. Effect of glycerol and gelatin hydrolysate blend on properties of fish myofibrillar protein film

FMP films were plasticizered with 50% of glycerol, fish gelatin hydrolysate (GH) having 60% DH or blends of glycerol and gelatin hydrolysate of

various GLY/GH ratios (100/0, 75/25, 50/50, 25/75 and 0/100). All film samples had the average thickness of 0.035±0.005 mm. The films exhibited varying properties as follows:

4.1 Mechanical properties

Mechanical properties including E, TS and EAB of those films are presented in Figure 11. All films tested showed increased film flexibility and resilience as compared to FMP film without any plasticizer which was brittle and could not withstand tensile deformation (data not shown). This indicated the plasticizing effect of all plasticizers tested. They could decrease interactions between FMP molecules in the matrix of film, resulting in increased chain mobility and free volume of the film (Gontard *et al*., 1993) and thus enhancing flexibility of the film. Polar groups of plasticizer molecules (-OH in glycerol as well as -NH_2, -COOH and -OH in gelatin hydrolysate) could form hydrogen bonds between protein and plasticizer to replace the protein-protein interactions in the biopolymer films. The film containing glycerol as plasticizer exhibited the lowest E and TS but highest EAB, compared with those added with GH or GLY/GH blend ($p < 0.05$), regardless of blend ratio. This result suggested that glycerol was more effective plasticizer in FMP film as compared to GH and GH/GLY blend. Typically, glycerol molecule could easily form hydrogen bond with protein chains of film matrix. Moreover, glycerol molecule might be more bulky than gelatin hydrolysate. As a result, it could reduce the intermolecular interaction and increase the mobility of protein chains more effectively, leading to the decrease in TS and E but increase in EAB of films (Gontard *et al*., 1993). Glycerol is commonly used as plasticizing agent in protein films (Chinnabhark *et al.*, 2007; Jangchud and Chinan, 1999; Sothornvit and Krochta, 2000). Jongjareonrak *et al.* (2006) reported that gelatin film from bigeye snapper skin and brownstripe red snapper skin showed the decreased TS and increased EAB with increasing glycerol content (25-75%, based on protein). Cuq *et al.* (1997) reported that puncture force of myofibrillar protein film from Atlantic sardine decreased as the level of glycerol used as plasticizer increased (0 - 40% based on protein).

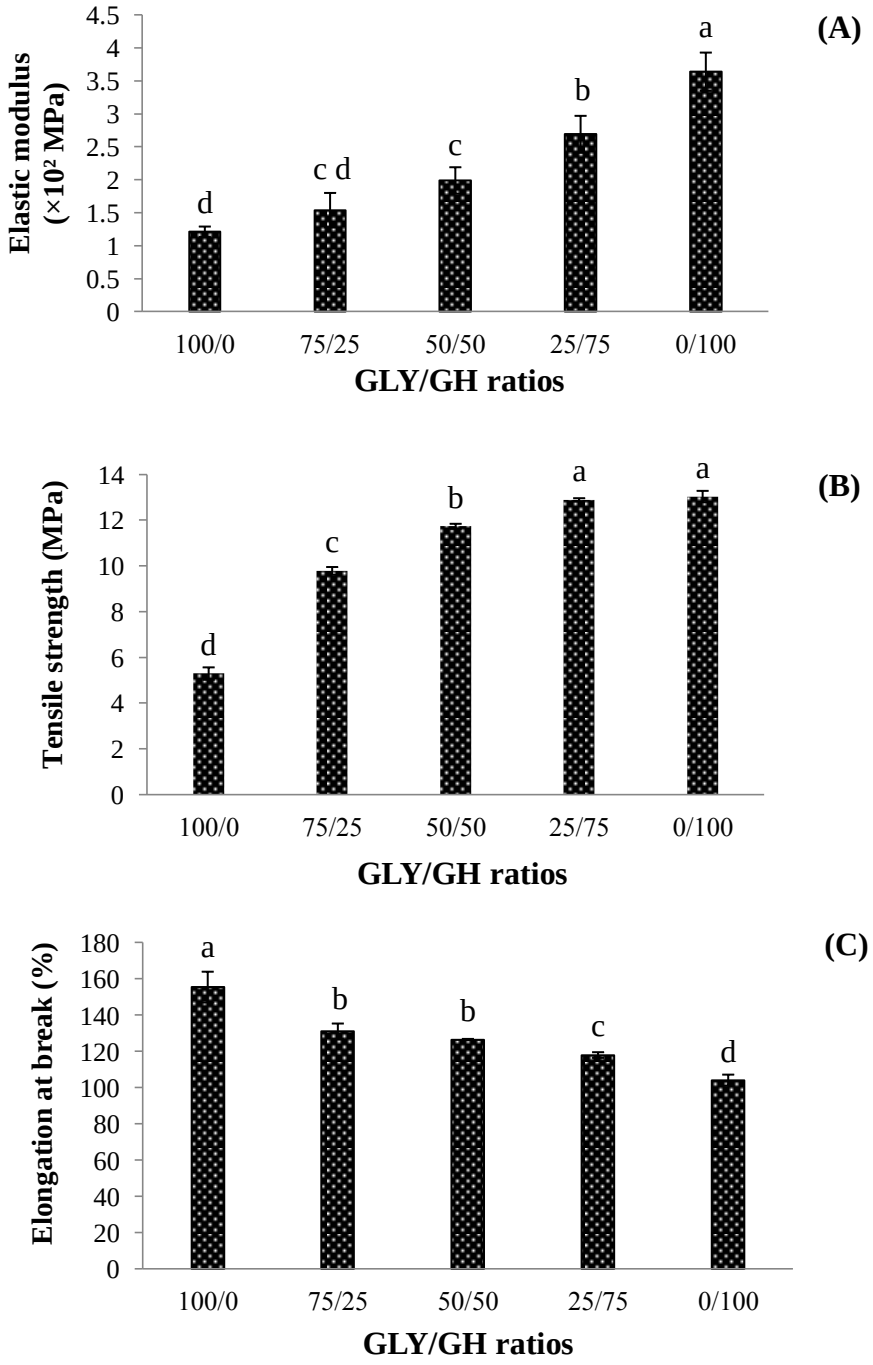


Figure 11. Mechanical properties of FMP films incorporated with glycerol (GLY), fish gelatin hydrolysate (GH) or GLY/GH blend (GLY/GH = 100/0, 75/25, 50/50, 25/75 and 0/100) at 50% of protein. Bars represent the standard deviation (n = 3). Different letters on the bars indicate significant differences ($p<0.05$).

For FMP films added with GLY/GH blend, E and TS increased while EAB of the films decreased as gelatin hydrolysate level in the blend increased (i.e. GLY/GH ratio decreased) ($p<0.05$). However, FMP films plasticized with GLY/GH blend at 25/75 and 50/50 showed similar E and TS ($p>0.05$). Film added with only gelatin hydrolysate had the highest E and TS but lowest EAB ($p<0.05$). The results reconfirmed that gelatin hydrolyasate acting as plasticizer was relatively less effective than glycerol, as previously discussed in section 3. The varying sizes of peptides of gelatin hydrolysate might play a role in varying plasticizing effect. Some chains of peptides molecules may act as plasticizer by inserting between protein chains and some chains would interact with protein chains in film matrix (Giménez *et al.*, 2009). Therefore, blend of glycerol and gelatin hydrolysate had an impact on mechanical properties of FMP films, which was governed by blend composition.

4.2 Water vapor permeability

Water vapor permeability (WVP) of FMP films plasticized with 50% GLY/GH blends having of different blend ratios is shown in Figure 12. The FMP films added with only glycerol (GLY/GH=100/0) showed the highest WVP, compared with those added with GH/GLY blend ($p<0.05$). However, FMP films added with GLY/GH blend exhibited a decrease in WVP with increasing gelatin hydrolysate level in the blend (or decreased GLY/GH ratio) ($p<0.05$). This might be attributable to the less hydrophilic and hygroscopic of gelatin hydrolysate which possibly contains some hydrophobic amino acids, compared to the glycerol. Moreover, gelatin hydrolysate containning carboxylic and amino groups, might closely interact via hydrogen bond with the FMP chain, resulting in decreased free volume and denser protein network of the film matrix, compared to that using glycerol (Hoque *et al.*, 2011). This could retard the diffusion and permeability of water through the film. Different plasticizer types or mixtures added could result in different film hydrophilicity or hydrophobicity, leading to varying water absorptivity and permeability (Sothornvit and Krochta, 2005). Tanaka *et al*. (2001) reported that the increase in WVP of the protein films containing a combination of glycerol (very hydrophilic) and polyethylene glycol (less hydrophilic) was lower than films containing only glycerol. Parris and Coffin (1997) also found that using different

plasticizer mixtures such as GLY-PEG 400 and GLY-PEG 400 in zein protein films could help to decrease film WVP. The results suggested that the film containing highly hygroscopic plasticizer like glycerol could absorb more water from surrounding atmosphere, which had an effect on increased WVP of the film (Nemet *et al*., 2010). Therefore, WVP of FMP films plasticized with GLY/GH blend was lower than that of films plasticized with glycerol, but varied with GLY/GH ratios.

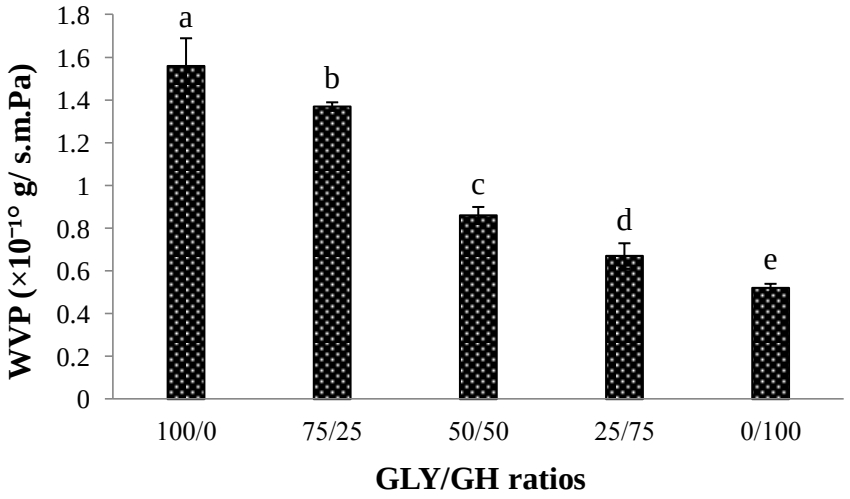


Figure 12. Water vapor permeability (WVP) of FMP films incorporated with glycerol (GLY), fish gelatin hydrolysate (GH) or GLY/GH blend (GLY/GH = 100/0, 75/25, 50/50, 25/75 and 0/100) at 50% of protein. Bars represent the standard deviation (n = 3). Different letters on the bars indicate significant differences ($p<0.05$).

4.3 Color of film

*L**, *a** and *b** values of FMP films added with 50% GLY/GH blend of different GLY/GH blend ratios (100/0, 75/25, 50/50, 25/75 and 0/100) are presented in Table 8. *L** value (lightness) of all films was slightly different ranging from 90.35-90.71. FMP film plasticized with only glycerol showed higher *a**-value (greenness) than those added with GLY/GH blend ($p<0.05$). Similar *a**-value was observed in films containing gelatin hydrolysate as plasticizer in the blend ($p>0.05$). Moreover, FMP films added with GLY/GH blend had higher *b** value (yellowness) than did those added with only glycerol ($p<0.05$), regardless of GH level in the blend.

Yellowness of films with GLY/GH blend seemed to increase with an increase in GH level in the blend (i.e. decreased GLY/GH ratio) ($p<0.05$). This was simply caused by the yellowish color of GH added. The gelatin hydrolysate added in the blend, which contains amino group ($-NH_2$) may interact with carbonyl group (C=O) of lipid oxidation product of membrane lipid in FMP (Tongnuanchan *et al.*, 2013) via Maillard reaction, particularly during drying of film (Burghagen, 1999). Salgado *et al.* (2011) reported that the addition of bovine plasma hydrolysates (BPH) to soy protein isolate films caused an increase in the *b** value. Hoque *et al*. (2011) observed that films from gelatin with DH of 0.40 - 1.20% had higher *b** value when compared to film from gelatin without hydrolysis. Peptides of gelatin which contains some amount of carbohydrates (glucose and galactose) attached to hydroxylysine residues by O-glycosidic bonds, could also react with NH group of FMP molecules, resulting in increased yellowness of the film (Hoque *et al.*, 2011). Therefore, GLY/GH blend ratio had an influence on the color of resulting FMP films, particularly yellowness (*b**-value) of films.

Table 8. *L**, *a** and *b** values of FMP films incorporated with glycerol (GLY), fish gelatin hydrolysate (GH) or GLY/GH blend (GLY/GH = 100/0, 75/25, 50/50, 25/75 and 0/100) at 50% of protein.

GLY/GH	Color parameters		
	*L**	*a**	*b**
100/0	90.58±0.05bc	-2.05±0.01a	5.71±0.05d
75/25	90.49±0.01c	-2.21±0.04b	6.45±0.05c
50/50	90.60±0.03b	-2.18±0.05b	6.48±0.05c
25/75	90.71±0.02a	-2.19±0.06b	6.71±0.10b
0/100	90.35±0.01d	-2.23±0.02b	8.89±0.11a

Values are given as mean ± SD (n = 3).

Different letters in the same column indicate significant differences ($p < 0.05$).

4.4 Transparency of film

Transparency value of FMP films added with glycerol and GLY/GH blend of different blend ratios is shown in Figure 13. FMP film incorporated with gelatin hydrolysate alone exhibited the highest transparency value ($p<0.05$), while that with only glycerol showed the lowest transparency value ($p<0.05$). This suggested that the former was less transparent than the later. Pascoalick *et al.* (2003) observed that the muscle protein films from Nile tilapia became more transparent with glycerol incorporation. Glycerol used is the transparent compound, which could increase the light transmission of the film. The presence of GH at higher level in the GLY/GH blend (i.e. GLY/GH = 25/75, 0/100) resulted in significant decrease in transparency of the FMP film (i.e. increased transparency value) ($p<0.05$). The carboxylic group and amino group of gelatin hydrolysate could form hydrogen bond and hydrophobic interaction with FMP chains in film matrix, resulting in more compact network structure which could retard the transmission of light. However, films added with GLY/GH blend at 0/100, 75/25 and 50/50 exhibited similar transparency as indicated by similar transparency value ($p>0.05$).

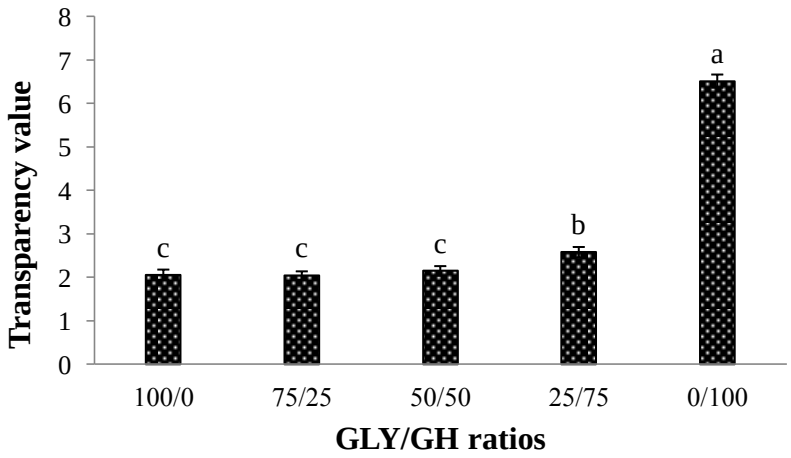


Figure 13. Transparency value of FMP films incorporated with glycerol (GLY), fish gelatin hydrolysate (GH) or GLY/GH blend (GLY/GH = 100/0, 75/25, 50/50, 25/75 and 0/100) at 50% of protein. Bars represent the standard deviation (n = 3). Different letters on the bars indicate significant differences ($p<0.05$).

5. Some properties and characterstics of selected FMP films added with and without gelatin hydrolysate

The selected FMP film samples, including films added with glycerol or gelatin hydrolysate at 30 and 50% and films added with GLY/GH (25/75) blend at 50%, were subjected to further analysis for some characteristics. The results are as following:

5.1 Moisture content, film solubility and protein solubility

Moisture content, film solubility and protein solubility in water of selected films are shown in Table 9. Among all films, FMP films added with 50% glycerol had the highest moisture content and film solubility, followed by those added with glycerol at 30%, GLY/GH blend, 60% DH hydrolysate at 50% and 30%, respectively ($p<0.05$). No differences in moisture content, film solubility and protein solubility were noticed between films added with gelatin hydrolysate and GLY/GH blend ($p>0.05$). It has been known that glycerol is hydrophilic and hygroscopic plasticizer, thus enhancing the solubility of FMP film (Cuq *et al*., 1997). Cuq (2002) and Nemet *et al*. (2010) reported that water-soluble dry content of protein-based films increased with increasing the glycerol content. A linear relationship between water soluble dry matter content and hydrophilic plasticizer content in the film was observed. FMP films added with only gelatin hydrolysate showed lower films solubility, compared to those added with glycerol at the same level ($p<0.05$). This might be due to the lower hydrophilicity of hydrolysate as compared to the glycerol. Water solubility is an indicator of film hydrophilicity. This might also because some short gelatin molecules of hydrolysate underwent interaction with FMP protein chains in the film matrix, leading to the higher compact network structure of film and thus less susceptibility to water. Giménez *et al*. (2009) observed that water solubility of squid gelatin films was not significantly modified by the incorporation of squid gelatin hydrolysates. However, the solubility of the films was generally still high. This was more likely due to the weak bonds between short peptide chains of gelatin and FMP, resulting in high water solubility.

For protein solubility in water, that of FMP films added with GLY/GH blend and gelatin hydrolysate was higher than that of films added with glycerol at the

same level ($p<0.05$) (Table 9). Films added with gelatin hydrolysate and GLY/GH blend had similar protein solubility ($p>0.05$). Sothornvit and Krochta (2000) reported that plasticizer content (25-35% glycerol) had no effect on soluble protein of hydrolyzed whey protein-based film. For glycerol added films, the greater water solubility was observed, compared with protein solubility. This was likely due to the solubilization or migration of glycerol in the film into water. It was noted that incomplete solubility in water of protein and film of FMP was observed. This was mostly owing to the presence of strong covalent bonds, e.g. disulfide bond. Myofilbrillar protein film was formed through three-dimensional network stabilized by various bondings including hydrogen bond, hydrophobic interaction as well as disulfide and non-disulfide covalent bonds (Chinnabhark *et al*., 2007; Iwata *et al*., 2000).

Table 9. Film and protein solubilities in water of FMP films incorporated with glycerol, 60% DH gelatin hydrolysate or blend of glycerol and gelatin hydrolysate (GLY/GH = 25/75).

Samples	Moisture content* (%)	Film solubility* (%)	Protein solubility* (%)
30%Glycerol	37.64 ± 0.22ab**	82.23 ± 1.19b	75.91 ± 1.08b
50%Glycerol	39.06 ± 0.56a	84.63 ± 0.60a	77.68 ± 1.03ab
30%GH(60%DH)	36.87 ± 0.13b	79.08 ± 0.98c	78.89 ± 1.03a
50%GH(60%DH)	36.31 ± 1.96b	81.48 ± 1.30b	79.10 ± 1.00a
50%GLY/GH(25/75)	36.30 ± 1.34b	82.15 ± 0.82b	78.42 ± 1.27a

* Values are given as mean ± SD (n = 3).

** Different letters in the same column indicate significant differences ($p<0.05$).

5.2 Protein solubility in various solvents

Protein solubility in various solvents of the selected films including those added with glycerol and 60% DH gelatin hydrolysate at 30 and 50% as well as with GLY/GH (25/75) blend is shown in Table 10. The distribution and extents of inter- and intra-molecular interactions between proteins give rise to a three-dimensional network structure of protein-based films (Iwata *et al*, 2000; Cuq, 2002).

The solubility of films in three different denaturing solutions (S1, S2 and S3) was used to determine the major associative forces involved in the film matrix. S1 containing SDS has been known to destroy hydrogen bond (Prodpran *et al*., 2007; Shiku *et al*., 2004). All films tested had non-significant differences in protein solubility in S1 ($p>0.05$) in the range of 74.19-75.86%. High solubility in S1 suggested that the matrix of FMP films with glycerol, gelatin hydrolysate or GLY/GH blend addition was mainly stabilized by hydrogen bonds. With the addition of 8.0 M Urea (S2), hydrophobic interactions can be destroyed (Shiku *et al*., 2004; Prodpran *et al*., 2007). All film samples exhibited the similar protein solubility (80.04-80.96%) in S2 ($p>0.05$). The result suggested that the main forces involved in the formation of all FMP films tested were hydrogen bonds and hydrophobic interactions, as evidenced by the increase in solubility in S2. S3 containing β-mercapthoethanol along with SDS and urea is able to destroy disulfide bond, apart from other weak bonds. The slightly increased protein solubility in S3 compared to that in S2 was observed in all films. This result indicated the presence of disulfide bond in film matrix. A similar result was observed in surimi films from Allaska Pollack (Shiku *et al.,* 2004), porcine-plasma protein film containing different cross-linking agents (Nuthong *et al*., 2009), and films from red tilapia *(Oreochromis niloticus)* protein isolate (Tongnuanchan *et al*., 2013). It was elucidated that hydrogen bond, hydrophobic interaction as well as disulfide bond played an important role in the formation of film from FMP of red tilapia, irrespective of plasticizers used in this study.

Table 10. Protein solubility in various solvents of FMP films incorporated with glycerol at 30 and 50% and with 60%DH hydrolysate at 30 and 50% or blend of glycerol and gelatin hydrolysate.

Samples	Protein solubility (%)*		
	S1***	S2	S3
30%Glycerol	74.53 ± 1.01a**	80.96 ± 1.40a	84.27 ± 0.99ab
50%Glycerol	74.65 ± 1.21a	80.77 ± 0.86a	86.23 ± 1.52a
30%GH(61%DH)	77.10 ± 3.30a	82.65 ± 2.27a	84.14 ± 1.94ab
50%GH(61%DH)	76.00 ± 1.81a	80.04 ± 1.42a	81.42 ± 2.15b
50%GLY/GH(25/75)	75.89 ± 1.84a	80.27 ± 2.83a	83.41 ± 0.29ab

* Values are given as mean ± SD (n = 3).

** Different letters in the same column indicate significant differences ($p<0.05$).

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

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

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

5.3 Protein patterns

Protein patterns of the selected films added with glycerol, 60% DH gelatin hydrolysate and GLY/GH blend at 50% of FMP were determined in comparison with the control FMP film without any plasticizer addition. Protein patterns determined under non-reducing (Figure 14A) and reducing (Figure 14B) conditions indicated that all films showed relatively similar pattern, regardless of plasticizer incorporation. Under non-reducing condition, actin was found as the major constituent and similar band intensity was observed for myosin heavy chain (MHC). The decrease in MHC in films was more likely due to the formation of cross-links during film formation, mainly via strong bonds including disulfide bonds. Band intensity of both MHC and actin was much lower in all films, compared with that found in initial materials (Tongnuanchan *et al*., 2013). The reduction of the protein band might be also due to cross-linking of protein via weak bonds including hydrogen bond and hydrophobic interaction as indicated by high solubilities in S1 and S2 (Table 10). Similar protein pattern suggested that glycerol or gelatin hydrolysate had no effect on protein cross-linking. Those plasticizers with very small size could not form the aggregate directly with main proteins in the matrix. They were presumed to

localize between protein chains and bind with protein matrix, mainly via H-bond, a weak bond, which could be destroyed under electrophoretic condition.

The decreases in band intensity of MHC and actin in FMP films were observed under reducing condition as compared to non-reducing condition. This suggested the role of disulfide bonds in stabilizing proteins or film matrix. Sulfhydryl groups in muscle proteins formed disulphide bonds to yield the film structure upon casting and drying of the film-forming solution (Shiku *et al*., 2003; Prodpran and Benjakul, 2005). However, there was no marked difference in protein patterns between all film samples. Therefore, glycerol, gelatin hydrolysate and their blend did not affect the formation of disulfide bond of film matrix. Owing to small molecules without SH-groups of gelatin hydrolysate or glycerol, they could not form disulfide bridge with muscle proteins.

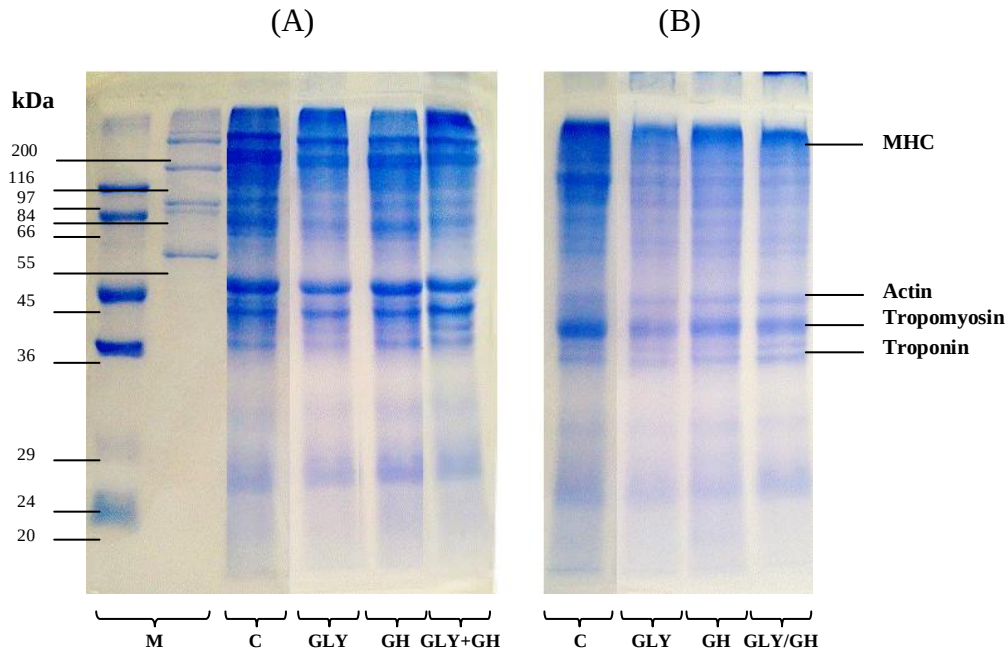


Figure 14. Protein patterns under non-reducing (A) and reducing (B) conditions of FMP films incorporated with 50% of glycerol, 60% DH gelatin hydrolysate (GH) and blend of glycerol and gelatin hydrolysate (GLY/GH = 25/75). M: protein marker; C: control film (without glycerol and fish gelatin hydrolysate); GLY: films added with glycerol at 50%; GH: films added with 60% DH gelatin hydrolysate at 50%, GLY/GH = films added with blend of glycerol and protein hydrolysate.

5.4 Fourier-transform infrared (FTIR) spectroscopy

The FTIR spectra of the selected films are illustrated in Figure 15. FTIR spectra of all films generally showed the similar pattern. The band situated at the wavenumber of 1041– 1042 cm^{-1} in spectra was most likely related to the glycerol added as a plasticizer (Bergo and Sobral, 2007). The spectra of control films with 30% and 50% glycerol addition showed the higher amplitude of peaks at wave number of 1041– 1051 cm^{-1}, corresponding to the glycerol (OH group) added as a plasticizer, compared to the films added with gelatin hydrolysate. For all films, the absorption bands at ~ 1646 cm^{-1} represented Amide-I, illustrating C=O stretching/hydrogen bonding coupled with COO. The vibrations (Amide II) at ~1544 cm^{-1} and 1239 cm^{-1} were associated to the N-H bending and C-N stretching (Amide III) vibrations, respectively. (Schmidt *et al.,* 2005). The similar result was also reported for bigeye snapper *(Priacanthus tayenus)* myofibrillar protein film, where amide-I, II and III bands were found at the wavenumber 1645, 1544 and 1236 cm^{-1}, respectively (Limpan *et al*., 2010). Amide-A and Amide-B bands were observed at the wavenumber of 3289 cm^{-1} and 2927 cm^{-1}, respectively. The Amide-A band represented the NH-stretching coupled with hydrogen bonding and Amide-B band represented the CH stretching and $–NH_3^+$ (Muyonga *et al.,* 2004). For films with GH or GLY/GH blend, it was noticed that peaks of Amide-A and Amide-I appeared at slightly lower wavenumber and the Amide-A peak also became broader, compared to those added with only glycerol. This was more likely attributable to the greater interaction via hydrogen bond between FMP chains and gelatin hydrolysate added in the film matrix. This might be responsible for the strong network and thus higher TS, E and lower (Figure 9) of the films added with gelatin hydrolysate as compared to those added with only glycerol. Moreover, it was noticed also that the Amide-A peak of films incorporated with glycerol or GH at 30% was broader than that of films incorporated with glycerol or GH at 50%. This broader Amide-A peak observed was likely correlated with the higher H-bonding interaction between FMP molecules in films containing the lower amount of plasticizer (glycerol or GH).

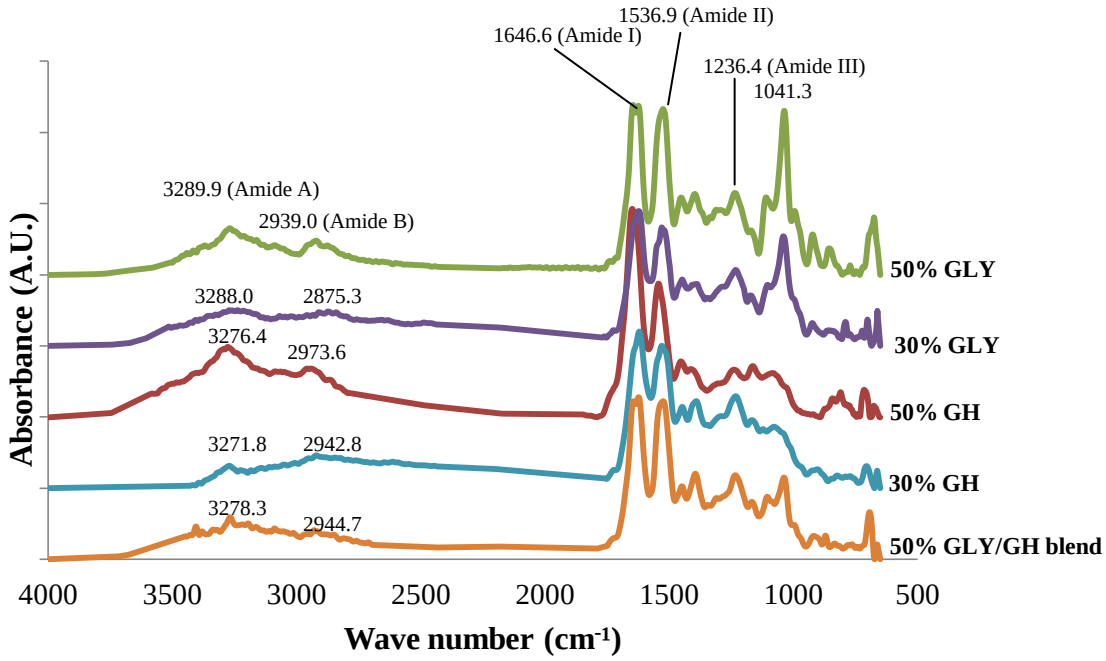


Figure 15. FTIR spectra of FMP films incorporated with glycerol and 61% DH gelatin hydrolysate at 30 and 50% and or blend of glycerol and gelatin hydrolysate (GLY/GH = 25/75) at 50%.

5.5 Thermal properties (TGA measurement)

Thermal degradation behavior of the selected FMP films was studied by using thermogravimetric analyzsis (TGA). The TGA curves of the film tested are illustrated in Figure16. The degradation temperatures (T_d), weight loss (Δw) and residue (%) of the film samples are presented in Table 11. Generally, FMP films exhibited two or three main stages of weight loss depending on samples. For all films, the initial weight loss (Δw_1 = 4.1-6.1% wt) observed at temperature (T_{d1}) in the range of 25.1-43.4 ^{o}C was simply due to the loss of absorbed in the film samples. The similar result was observed in fish skin gelatin film (Hoque *et al*., 2011; Tongnuanchan *et al*., 2012) and porcine-plasma protein film (Nuthong *et al.*, 2009). In films added with glycerol and GLY/GH blend, the second weight loss (Δw_2 = 12.0-18.8%) was observed at temperatures (T_{d2}) ranged from 139.1 ^{o}C to 193.9 ^{o}C and was mostly associated with the loss of glycerol compound (plasticizer) and probably smaller size protein fraction and associated protein chains in the films (Hoque *et al*., 2011). The FMP film added with 50% glycerol showed the highest of weight loss in

second stage simply due to the fact that it contained higher level of glycerol when compared with the other films. This film also showed the most heat susceptibility as indicated by the lower T_{d2} (139.15 ^{O}C) as compared to the others. The third weight loss (Δw_3 = 58.8 – 62.03%) at temperatures (T_{d3}) ranged from 274.15 ^{O}C to 298.8 ^{O}C was also observed in films added with glycerol and GLY/GH blend. This was mainly associated with the degradation of the major protein component or highly associated protein fraction in the film matrix. Major FMP protein and hydrolysate might undergo high interaction by strong covalent bond upon heating at high temperature, resulting in highly thermal stable component.

From the result, as temperature up to 500-800 ^{o}C, the films showed the residual mass (or char) in the range of 12.88-24.84%, depending on film samples. FMP films added with gelatin hydrolysate had higher residue mass compared to those with glycerol.

For films added with only gelatin hydrolysate, the major weight loss (Δw_2 = 71.00-71.19%) observed at degradation temperature of 273.84 and 279.50 ^{o}C for 50% and 30% hydrolysate addition, respectively. This was caused by the degradation of associated major proteins as well as peptides of hydrolysate associated with the major protein in the film matrix. The results showed that FMP films plasticized with gelatin hydrolysate exhibited more thermal stability (higher T_{d2}) than did those plasticized with glycerol. This might be due to the stronger network of the film which associated with the higher interaction between gelatin hydrolysate and FMP chains as compared to the glycerol did. The result was in agreement with the FTIR measurement as well as the lower film solubility (Table 9) and higher strength (Figure 9) shown in films with gelatin hydrolysate as compared to those with glycerol.

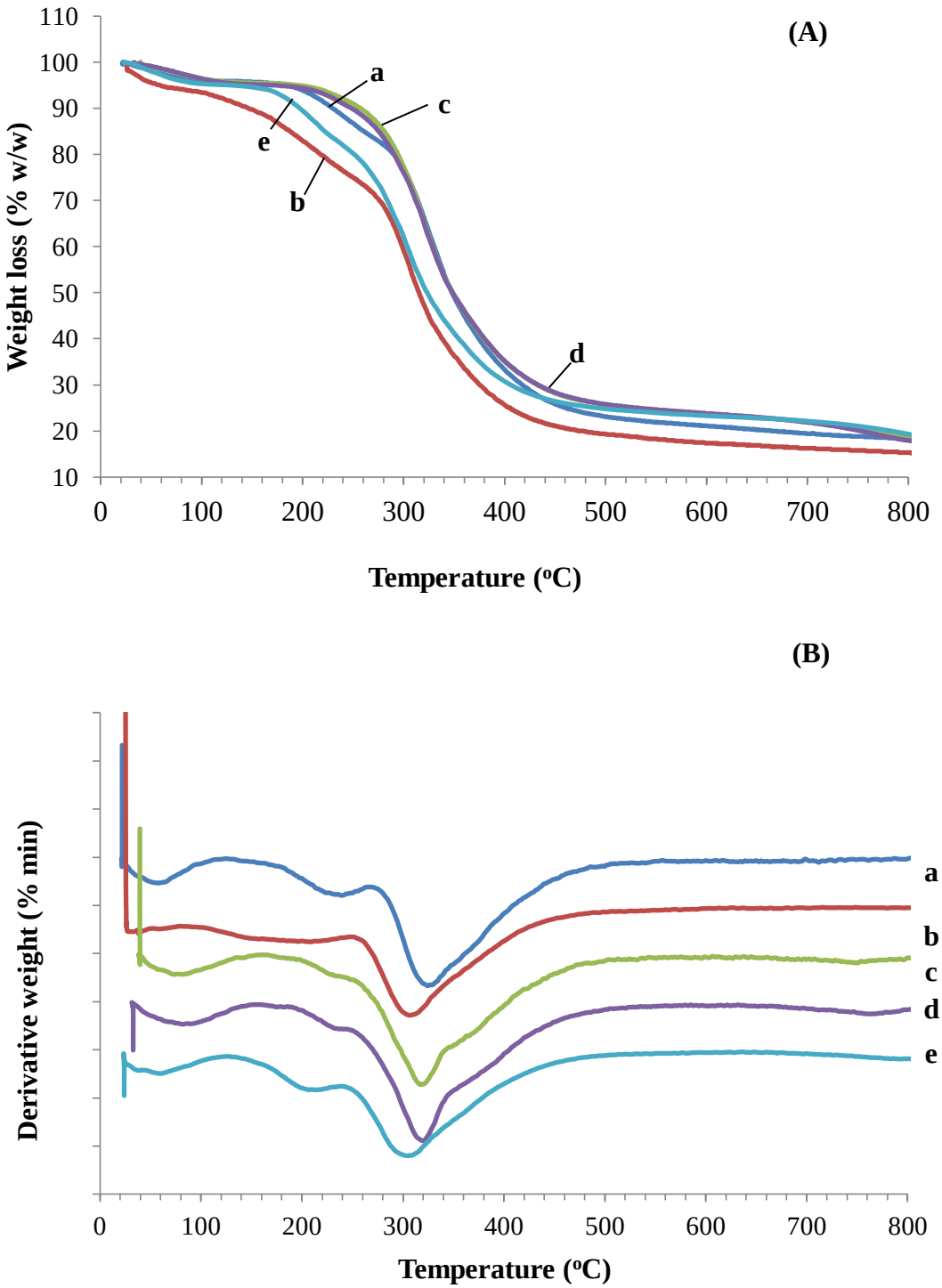


Figure 16. TGA data showing weight loss (A) and derivative weight loss (B) as a function of temperature of FMP films added with glycerol at 30% (a) and 50% (b), with 60% DH gelatin hydrolysate at 30% (c) and 50% (d) and with GLY/GH blend at 50% (e).

Table 11. Thermal degradation temperature (T_d, ^oC) and weight loss (Δw, %) of FMP films incorporated with glycerol, 60% DH gelatin hydrolysate or GLY/GH blend

Film samples	Δ_1(moisture loss)		Δ_2 (degradation)		Δ_3 (degradation)		Residual mass (%)
	T_{d1}	Δw_1	T_{d2}	Δw_2	T_{d3}	Δw_3	
30%GLY	30.00	4.15	193.92	12.03	298.87	62.03	21.79
50%GLY	25.12	6.10	139.15	18.8	283.61	59.09	12.88
30%GH	29.48	4.17	279.50	71.19	-	-	24.64
50%GH	43.44	4.85	273.84	71.00	-	-	24.15
50%GLY/GH (25/75) blend	29.16	4.99	173.45	13.03	274.15	58.83	23.15

5.6 Film morphology (SEM technique)

SEM micrographs of the surface and freeze-fractured cross-section of FMP films incorporated with glycerol at 30 and 50%, with 60% DH hydrolysate at 30 and 50%, and blend of glycerol and gelatin hydrolysate at 50% are illustrated in Figure 17. Generally, the image of FMP films revealed a homogeneous structure and some roughness distributed along the surface, but without the cracks or pin holes. The FMP film added with glycerol showed slightly rougher surface and cross-section than did those added with hydrolysate. Films added with gelatin hydrolysate especially at 50% exhibited smoother and more homogeneous surface and cross-section, compared to the others. This indicated the more compact network structure of this film, possibly due to the more compatible and greater interaction between FMP and gelatin hydrolysate. The SEM result also agreed with the mechanical properties and WVP of this films, in wich the more compact structure of GH-added FMP films resulted in higher E and TS as compared to those of films added with glycerol at the same level (Figure 9).

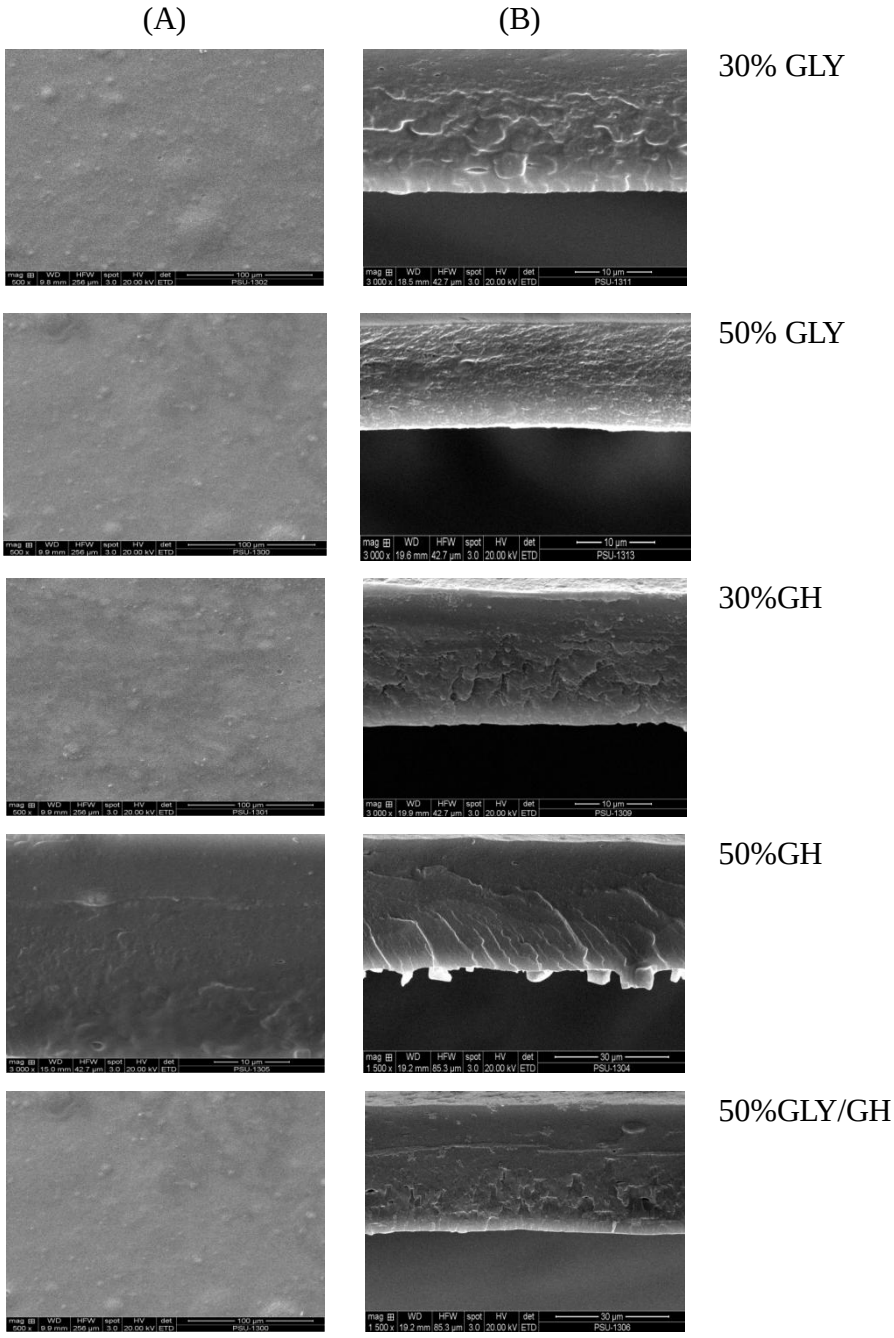


Figure 17. SEM micrographs of the surface (A) and cross-section (B) of FMP films incorporated with glycerol at 30 and 50%, with 60% DH gelatin hydrolysate at 30 and 50% or with GLY/GH blend at 50%.

6. Stability of fish myofibrillar protein film added without and with gelatin hydrolysate during storage

FMP films incorporated with 50% of glycerol, fish gelatin hydrolysate having 60% DH (GH) or glycerol/gelatin hydrolysate blend (GLY/GH) at 25/75 ratio as plasticizer were stored under ambient condition (temp. ≈ 28-30 ^{o}C and 65±5% RH) for 8 weeks. The films exhibited some changes in properties as follows:

6.1 Change in mechanical properties

Mechanical properties including E, TS and EAB of the films during storage are presented in Figure 18. Generally, E, TS and EAB of the films added with 50% of glycerol remained constant during the 8 weeks of storage ($p>0.05$). Cuq *et al.* (1996) also observed that mechanical properties of myofibrillar protein-based films incorporated with saccharose as plasticizer did not change during storage for 8 weeks at 20 ^{o}C and 58% RH. Limpan *et al*. (2012) reported that FMP film from bigeye snapper (*Priacanthus tayenus*) stored at 28-30 ^{o}C and 65±5% RH showed the increased TS and E at the beginning of the storage time (0-2 weeks) and then seemed to constant. Tongnuanchan *et al.* (2011) observed film of myofibrillar protein from red tilapia prepared at alkaline pH during storage of 20 days at room temperature and found that film had no changes in TS and EAB after storage. Park *et al.* (1994) found that the changes in mechanical properties of film from wheat gluten protein and corn-zein mixtures added with glycerol as plasticizer for 20 days of storage at 25 ^{o}C and 50% RH were the results from the slowly migration of plasticizers to surface.

For the FMP films added with gelatin hydrolysate (GH) and added with GLY/GH blend, E and TS seemed to increase but EAB decreased upon the storage for 8 weeks ($p<0.05$). These results suggested that the films were more rigid with increasing storage time. This could be associated to the more compact structure, which could be resulted from the enhanced interaction between peptide molecules from fish gelatin hydrolysate and FMP in the film matrix. It was noticed that films with GLY/GH blend showed slightly lower rate of changes, compared to those with only GH. This suggested that gelatin hydrolysate might interact with FMP and align themselves with a greater extent as compared to glycerol. Gelatin hydrolysate containing amino and carboxylic groups, might closely interact via hydrogen bond

with the FMP chain during storage, resulting in more compact and denser protein network of the film matrix (Hogue *et al.*, 2011), compared to that using glycerol.

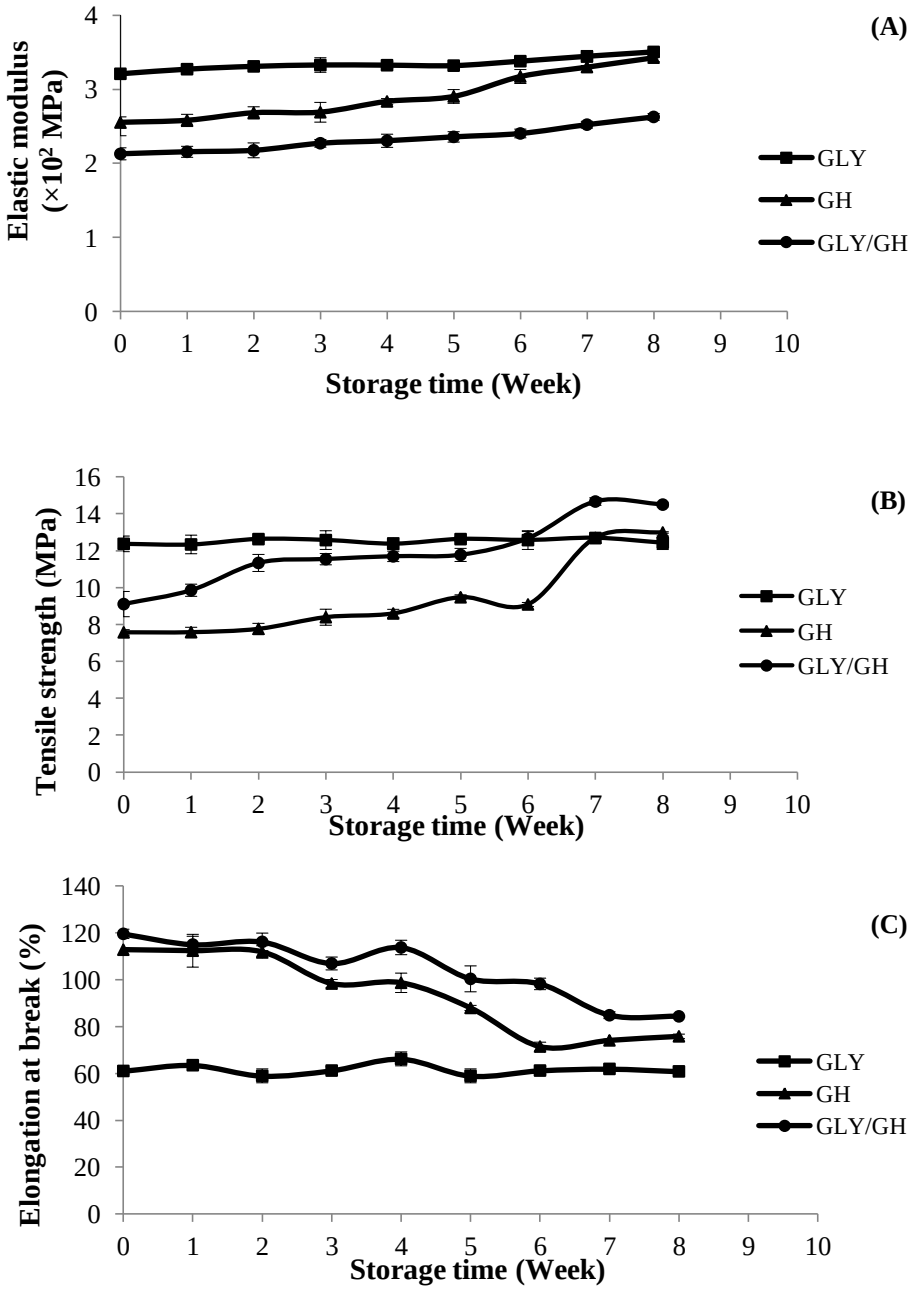


Figure 18. Changes in elastic modulus (A), tensile strength (B) and elongation at break (C) of FMP films incorporated with 50% of glycerol, fish gelatin hydrolysate (GH) having 60% DH or blend of glycerol and gelatin hydrolysate (GLY/GH=25/75) during the storage at room temperature

(28-30 ^{o}C) for 8 weeks. Bar represents the standard deviation (n=3).

6.2 Changes in moisture content and water vapor permeability

Moisture content and water vapor permeability of the selected films during the storage of 8 weeks are shown in Figure 19A and 19B, respectively. Moisture content of all films was continuously reduced during 0-8 weeks of storage. Among films tested, those added with 50% glycerol showed the highest moisture content over the storage time ($p<0.05$). This was more likely due to the hygroscopic nature of glycerol added. However, films added with GH and GLY/GH blend had similar moisture content throughout the storage ($p>0.05$). Limpan *et al.*, (2009) reported that FMP film from bigeye snapper exhibited the decrease in moisture content during 0-4 weeks and tended to be constant thereafter.

WVP of FMP films added with 50% glycerol also tended to reduce during storage ($p<0.05$), while that of films containing GH and GLY/GH blend exhibited a slight decrease with extended storage time. The decrease in moisture content and WVP of all films during storage was in accordance with the increased TS and E of the films (Figure 18) and was mostly attributable to the continuous increase in inter-molecular interaction and molecular alignment during the extended storage. As a consequence, the film matrix became denser, which could retard the absorption and diffusion of water. Anker *et al.* (2001) and Hernandez-Munoz *et al*. (2004) also found the decrease in WVP with increasing storage time of glycerol-plasticized whey protein isolate films and glutenin-rich films. They assumed that this was due in part to the glycerol migration. However, Artharn *et al*. (2009) reported that no difference in WVP was observed throughout the storage time for the round scad protein-based films.

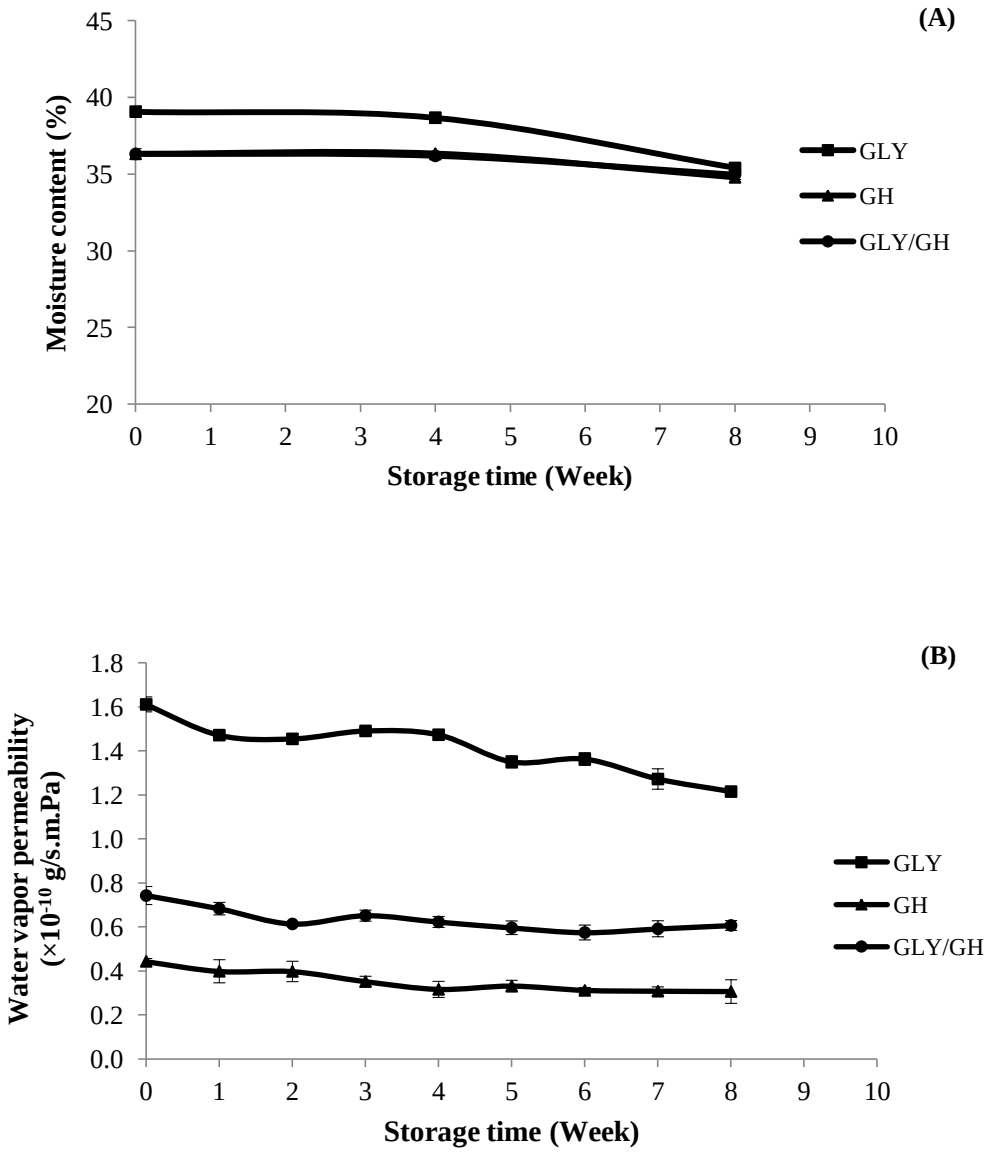


Figure 19. Changes in moisture content (A) and water vapor permeability (B) of FMP films incorporated with 50% of glycerol, fish gelatin hydrolysate (GH) having 60% DH or blend of glycerol and gelatin hydrolysate (GLY/GH=25/75) during the storage at room temperature (28-30 ^{o}C) for 8 weeks. Bar represents the standard deviation (n=3).

6.3 Changes in color and film transparency

The color expressed as *L**, *a** and *b**-values of the selected films during storage at room temperature (28-30 ^{o}C) for 8 weeks is shown in Figure 20. All film samples generally became darker with increasing storage time as evidenced by the decrease in *L** (lightness) and *a**-values (redness) ($p<0.05$). The similar results were found by Arthan *et al.* (2009) who reported that FMP films from round scad incorporated without and with oil or oil/chitosan showed the decreased *L** and *a**-values during storage for 8 weeks at 28-30 ^{o}C. The films were more yellowish (increased *b**-value) as storage time increased. The discoloration of FMP films might be governed by non-enzymatic browning reaction. It has been known that the yellow/brown coloration related 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.,* 1996). The protein might undergo partial degradation during storage, which resulted in increased free amino groups available for Maillard reaction (Arthan *et al.,* 2009). Hoque *et al.* (2011) observed that the hydrolysis process of protein rendered carbonyl group (C=O), which might undergo interaction with amino group in protein chain of film matrix via Maillard reaction during storage. The increase in *b**-value was observed in films stored for a longer time ($p<0.05$). Tongnuanchan *et al.* (2011) also observed that films of FMP from red tilapia became yellowish as demonstrated by the increase in *b**-value during storage of 20 days at room temperature.

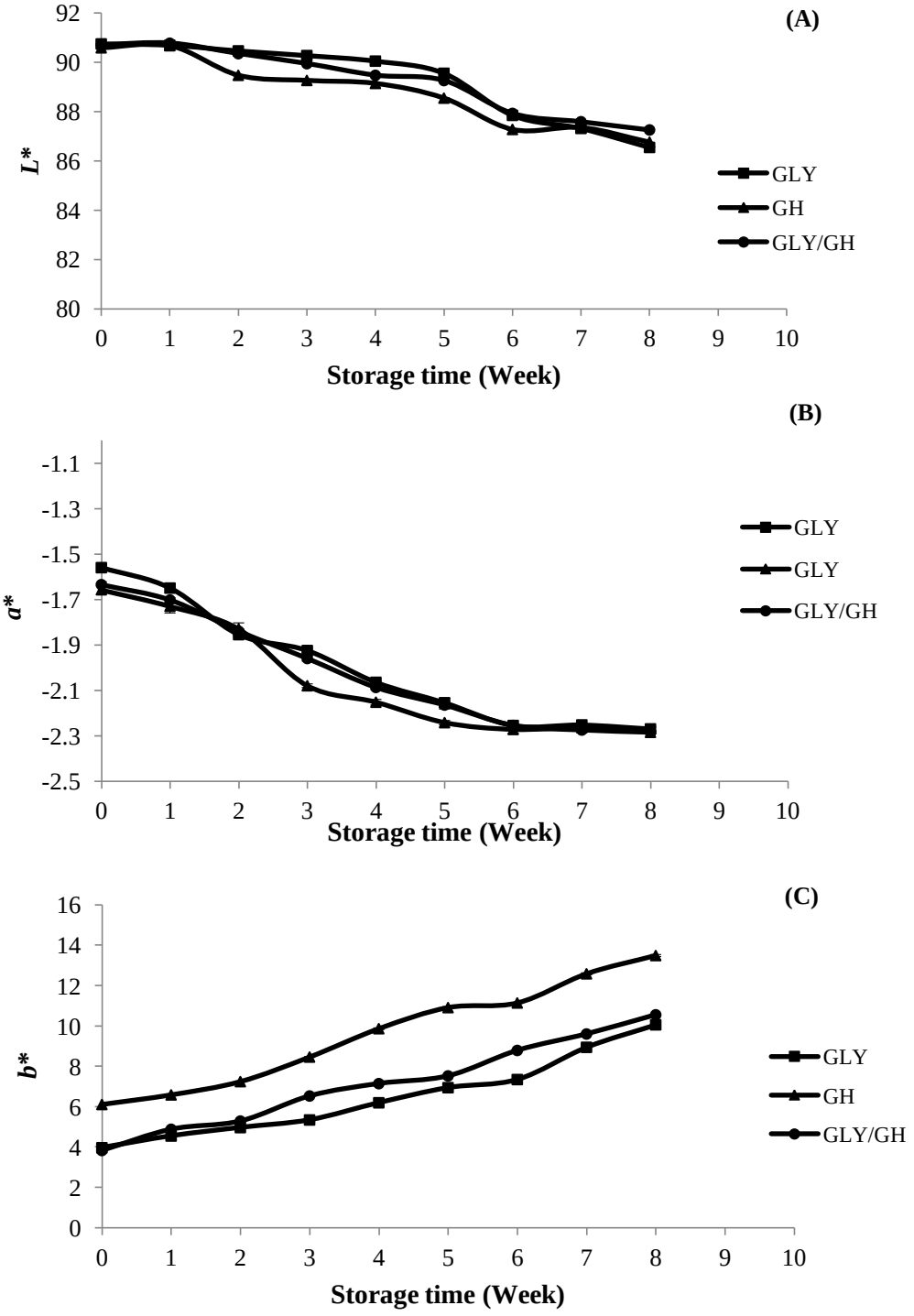


Figure 20. Changes in *L**, *a** and *b** values of FMP films incorporated with 50% of glycerol, fish gelatin hydrolysate (GH) having 60% DH or blend of glycerol and gelatin hydrolysate (GLY/GH=25/75) during the storage at room temperature (28-30 ^{o}C) for 8 weeks. Bar represents the standard deviation (n=3).

Transparency value of the selected films during 8 weeks of storage is shown in Figure 21. With increasing the storage time, all films exhibited a slight increase in transparency value. The increase in transparency value indicated an increase in opacity or decrease in film transparence. This was possibly caused by the orientation or aggregation of FMP molecules in the film matrix throughout the storage. This result is also in accordance with the increase in b*-value of films (Figure 20). Cross-linking of protein via the Maillard reaction might contribute to the increased compactness of film matrix, in which the barrier property to light transmission was gained. The results were in agreement with the finding of Arthan *et al.* (2009) who observed a slight increase in transparency value of round scad protein-based films incorporated without and with oil or oil/chitosan during storage under 54% RH at room temperature (28-30 ^{o}C) for 8 weeks. However, Tongnuanchan *et al.* (2011) reported that no changes in transparency values were found in film samples from red tilapia muscle during storage of 20 days.

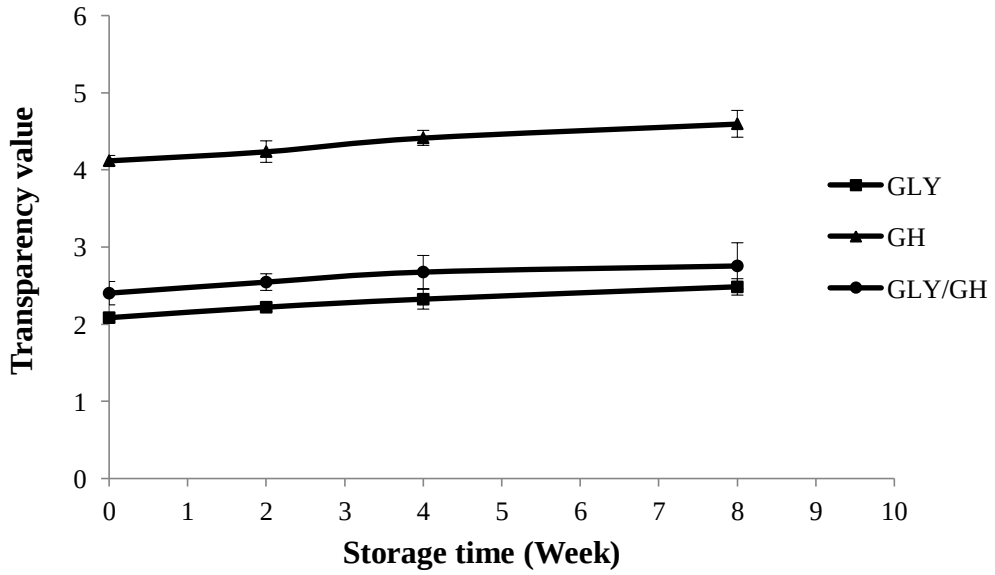


Figure 21. Change in transparency value of FMP films incorporated with 50% of glycerol, fish gelatin hydrolysate (GH) having 60% DH or blend of glycerol and gelatin hydrolysate (GLY/GH=25/75) during the storage at room temperature (28-30 ^{o}C) for 8 weeks. Bar represents the standard deviation (n=3).

6.4 Changes in film solubility and protein solubility

Film solubility and protein solubility in water of the selected film samples during 8 weeks of storage at room temperature (28-30 ^{o}C) are shown in Table 12. Among all films, FMP films added with 50% glycerol had the highest film solubility, followed by those added with GLY/GH blend and 60% DH gelatin hydrolysate, respectively ($p<0.05$), throughout the storage. This was possibly due to the solubilization or migration of glycerol into water with more ease. The solubility of FMP film is in part due to leaching out of plasticizer (glycerol) in water (Denavi *et al*., 2009; Hoque *et al.* 2011). Hydrophilic nature of glycerol will also enhance film solubility in water (Denavi *et al.*, 2009). From the result, FMP films added with gelatin hydrolysate showed the lowest films solubility, compared to those added with glycerol ($p<0.05$). This probably resulted from that some short molecule of gelatin hydrolysate might interact with protein chain of film matrix, resulting in more compact network structure of film matrix. However, protein solubility of FMP films added with gelatin hydrolysate was higher than that of films added with glycerol ($p<0.05$).

During storage, all FMP film samples showed large decreases in film and protein solubilities ($p<0.05$), especially at week 8 of storage which possessed film and protein solubilities as low as 26.90-32.47% and 28.08-30.06%, respectively, depending on samples. This result suggested that protein molecules as well as proteins and peptides of GH in film matrix could undergo higher interaction or cross-linking, in which larger molecular-weight cross-links and therefore more compact network were formed. Besides, aldehydes or carbonyl compounds produced from lipid oxidation can interact with protein amino group via Mallard reaction (Chaijan *et al.*, 2007). These led to stronger and more compact film structure, and thus lowering trendency of water solubility of the protein films upon storage. The results were in agreement with Arthan *et al.* (2009) who reported that film and protein solubility of round scad protein-based films decreased with increasing the storage time. Tongnuanchan *et al.* (2011) also observed the decrease in film and protein solubility of films from red tilapia muscle as the storage time increased.

Table 12. Changes in film solubility and protein solubility of FMP films incorporated with 50% of glycerol, fish gelatin hydrolysate (GH) having 60% DH or blend of glycerol and gelatin hydrolysate (GLY/GH=25/75) during the storage at room temperature (28-30 ^{o}C) for 8 weeks.

Storage time (Week)	Sample	Film solubility (%)	Protein solubility (%)
0	GLY	84.62±0.56aA	79.51±0.72aA
	GH	81.73±0.82bA	81.46±0.95aA
	GLY/GH	82.22±0.40bA	80.06±2.21aA
4	GLY	62.87±1.99aB	59.64±0.59aB
	GH	51.20±1.56bB	60.03±0.70aB
	GLY/GH	52.30±1.85bB	58.81±1.12aB
8	GLY	32.47±0.89aC	29.63±0.41aC
	GH	26.72±1.21bC	30.06±0.58aC
	GLY/GH	26.90±0.21bC	28.08±0.28bC

Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column within the same storage time indicate significant differences ($p<0.05$). Different uppercase letters in the same column within the same plasticizer type indicate significant differences ($p<0.05$).

6.5 Change in protein solubility in various solvents

Protein solubility in various solvents of the selected films at different storage times during the storage at room temperature (28-30 ^{o}C) is shown in Table 13. Among all films tested, slight differences in protein solubility in each solvent (i.e. S1, S2 or S3), suggesting that small difference in bonding stabilizing the film network, regardless of storage time. At day 0, all films had high solubility in S1 in the range of 74.16-76.17%; this indicated that the film matrix was mainly stabilized by hydrogen bond. The slight increase in protein solubilities in S2 and S3 as compared to the solubilities in S1 and S2, respectively, of all films were observed. This revealed the presence of hydrophobic interaction and disulfide bond in the film matrix, in addition to the major hydrogen bond. Protein solubility in various solvents of all films was much decreased during storage of 8 weeks ($p<0.05$), irrespective of film type. The decrease in protein solubility in all solvents tested of the films more likely suggested

the increased interaction and aggregation of polymer molecules in the film matrix, possibly associated with the formation of non-covalent, inter-molecular interactions as well as covalent bonding between the protein-protein molecules. Orliac *et al.* (2002) observed that the proteins in film undergo more aggregation, leading to more cross-linking. It was obvious that all films stored for 4 and 8 weeks had much lower solubility in S3 compared to those at week 0 of storage. Additionally, all films were incompletely soluble in S3. The incomplete solubilization of all films was more pronounced as storage time increased. This result suggested that additional non-disulfide covalent bond was formed in the matrix with a large extent upon the extended storage of FMP films. This could be caused by Maillard reaction taking place during the storage. The decreased solubility was in agreement with the increased yellowness (*b**-value) of FMP films during storage. Therefore, Maillard reaction occurred in FMP films during storage played a crucial role not only on discoloration but also on changes in molecular interactions and bonding in the FMP film added with glycerol and gelatin hydrolysate.

Table 13. Change in protein solubility in various solvents of FMP films incorporated with 50% of glycerol, fish gelatin hydrolysate (GH) having 60% DH or blend of glycerol and gelatin hydrolysate (GLY/GH=25/75) during the storage at room temperature (28-30 ^{o}C) for different times.

Storage time (Week)	Sample	Protein solubility (%)		
		S1	S2	S3
0	GLY	75.66±1.22aA	80.83±0.77aA	84.68±1.43aA
	GH	74.16±1.87aA	78.54±1.43aA	81.35±2.15bA
	GLY/GH	76.17±1.82aA	80.11±2.80aA	83.54±0.28abA
4	GLY	59.58±1.08bB	61.69±1.01aB	67.60±1.15bB
	GH	59.58±1.08bB	61.69±1.01aB	67.60±1.15bB
	GLY/GH	62.16±0.27aB	62.20±0.80aB	70.42±0.82aB
8	GLY	47.76±0.51aC	49.44±0.84aC	53.36±0.51bC
	GH	42.74±1.53bC	46.76±1.45bC	54.30±0.71bC
	GLY/GH	47.40±0.58aC	49.42±0.66aC	56.20±0.54aC

Values are given as mean ± SD (n = 3).

Different lowercase letters in the same column within the same storage time indicate significant differences ($p<0.05$). Different uppercase letters in the same column within the same plasticizer type indicate significant differences ($p<0.05$).

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

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

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

7. Seal ability of FMP film added with gelatin hydrolysate

Seal ability of FMP film added with 60% DH gelatin hydrolysate at 50% was studied by using peel strength test. The preliminary result revealed that FMP films could not be sealed via only heat sealing technique. This was mainly due to the fact that FMP film possessed less thermoplastic behavior and thus limiting its melt flow ability, owing to the presence of highly protein-protein interactions mediated by disulfide bond (Krochta and De Mulder-Johnston, 1997). Therefore, in this study, the sealing of FMP films was carried out by means of applying adhesives based on

natural polymers including tapioca flour (TA), gelatin (GA) and soy protein isolate (SA), followed by heat sealing using an impulse-wire thermo-sealer.

Seal strength at various seal conditions of FMP film added with 60% DH gelatin hydrolysate at the concentration of 50% is shown in Table 14. Sealing times between 1.5 and 3.5 s were used to heat seal the film after applying the adhesives. The FMP films could not be sealed at sealing time less than 1.5 s while sealing at sealing time higher than 3.5 s resulted in degradation of film being heat sealed.

With the same type of adhesive used, films applying with gelatin (GA) and soy protein isolate (SA) adhesives had the increased seal strength with increasing sealing time from 1.5-3.5 s ($p<0.05$). However, no differences in seal strength were observed in FMP films using tapioca flour (TA) and heat sealed at 2.5 and 3.5 s ($p>0.05$). Films using TA as adhesive could not be sealed at sealing time of 1.5 s, resulting in peel separation. The result suggested that seal strength depended on time of sealing. When the sealing time increased, molecules of adhesive might undergo more interaction among themselves and between adhesive and surface of FMP films.

At the same sealing time, FMP films sealed using gelatin as adhesive had significantly higher seal strength than those using soy protein isolate and tapioca flour, respectively ($p<0.05$). Gelatin molecules might be more compatible to FMP molecules as compared to molecules of tapioca flour and soy-protein isolate. As a result, this possibly allowed gelatin to interact with FMP at the interface of film with greater extent. Moreover, the enhancement of seal strength was also attributed to the molecular inter-diffuse and entangle of the adhesive through the FMP film surface, which might take place in some degrees, especially when gelatin was used as adhesive. However, the failure mode of the seal of FMP film exhibited a peeling, a separation between the two layers in contact. This result suggested that molecules in FMP films might not be homogeneously and sufficiently melted. However, to enhance the sealing strength during the heat-sealing process, the pressure, temperature and heat-sealing time are the three basic parameters to be considered (Chukhlanov and Tereshina, 2009). Moreover, type of adhesive and some properties of various films such as structure, composition and melting behavior affect the seal strength of

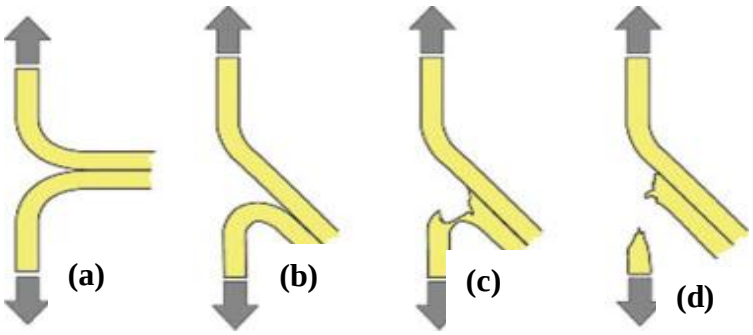
the films. Therefore, sealing ability of film was affected by type of adhesive used as well as the heat-sealing time applied.

Table 14. Sealing ability of FMP films incorporated with 60% DH gelatin hydrolysate at 50%, sealed using various natural adhesives with different heat sealing times.

Sample	Sealing time (s)	Seal strength (N/m)	Peeling processes/ Failure mode**
FMP film/TA	1.5	N/A	separation
	2.5	71.25±6.65e*	peeling
	3.5	73.97±2.35e	peeling
FMP film/GA	1.5	87.59±2.31d	peeling
	2.5	120.95±3.82b	peeling
	3.5	202.35±6.59a	peeling
FMP film/SA	1.5	74.46±1.35e	peeling
	2.5	97.05±2.88c	peeling
	3.5	124.05±2.38b	peeling

*Values are given as mean ± SD (n=3). The different letters indicate significant differences ($p < 0.05$).

** Types of peeling processes/ failure mode are as following



(a) peel separation (b) peeling (c,d) tearing or rupture near the zip

CHAPTER 4

CONCLUSIONS AND SUGGESTION

Gelatin hydrolysate (GH) and blend of glycerol and gelatin hydrolysate (GLY/GH) could be effectively used as plasticizer in FMP film. FMP films added with gelatin hydrolysate or the blend exhibited decreased stiffness and tensile strength with increasing level of gelatin hydrolysate. Incorporation of gelatin hydrolysate also significantly improved water vapor barrier property of the FMP film, compared to using glycerol at the same level. The plasticizing effect and properties of the resulting FMP films depended on DH and levels of gelatin hydrolysate and the composition of the GLY/GH blend used.

The matrix of films from FMP of red tilapia, plasticized with glycerol, gelatin hydrolysate or GLY/GH blend, was stabilized by majorly hydrogen bond as well as hydrophobic interaction and disulfide bond. FMP films incorporated with gelatin hydrolysate possessed higher water and thermal resistances, as indicated by lower film solubility and higher thermal degradation temperature, compared to the films added with glycerol as plasticizer. However, film added with gelatin hydrolysate and the GLY/GH blend exhibited more yellowness and less transparency as compared to that with glycerol.

FMP films incorporated with gelatin hydrolysate or GLY/GH blend possessed lower storage stability at room temperature when compared to those added with glycerol. Upon extended storage time, FMP films with gelatin hydrolysate or GLY/GH blend showed increased stiffness, strength and yellowness, but decreased film solubility and protein solubility in water and various solvents, with a greater extent as compared to the films with glycerol. This was caused by the formation of additional non-covalent bond and non-disulfide covalent bond more likely via the Maillard reaction during the extended storage of film.

FMP films added without or with gelatin hydrolysate could not be sealed by only heat sealing technique. However, the FMP films could be sealed by the aid of using natural adhesive such as gelatin prior to heat sealing at proper sealing time. Sealing ability of FMP film was affected by type of adhesive used and the heat-

sealing time applied. Use of gelatin as adhesive in combination with impulse-wire heat sealing at 3.5 s rendered the seal of FMP films with the highest seal strength.

Suggestions for future work:

- Incorporation of gelatin hydrolysate prepared from enzymatic hydrolysis should be carried out since this hydrolysate may possess antimicrobial and antioxidant activities.
- To obtain in depth knowledge, more molecular characterizations on hydrolysate and the obtained film should be performed.
- Bleaching of gelatin hydrolysate prior use may be implemented, which may decrease the yellowness of the gelatin hydrolysate and the film added with hydrolysate.
- Various approaches for improving the stability of the film added with hydrolysate should be further conducted. For example, use of fish protein isolate may be used instead of washed mince.

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APPENDIX

ANALYTICAL METHODS

1. Determination of moisture content (AOAC, 2000)

Method

1. Dry the empty dish and lid in the oven at 105 ^{o}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 ^{o}C.
4. After drying, transfer the dish with partially covered lid to the desiccator to cool. Reweigh the dish and its dried sample.

Calculation

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

where W1 = weight (g) of sample before drying

W2 = weight (g) of sample after drying

2. Determination of protein content (AOAC, 2000)

Reagents

1. Kjedahl catalyst: Mix 9 part of potassium sulphate (K_2SO_4) with 1 part of copper sulphate (CuSO)
2. Sulfuric acid (H_2SO)
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 is distilled.

6. Remove receiver, wash tip of condenser and titrate excess standard acid distilled with standard NaOH solution.

Calculation

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

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 ^{o}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 ^{o}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

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

4. Determination of fat content (AOAC, 2000)

Method

1. Place the bottle and lid in the incubator at 105 ^{o}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 ^{o}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

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

5. 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 ^{o}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 85 ^{o}C 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 g 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 ^{o}C.
2. Remove the comb without tearing the edge of the polyacrylamide wells.
3. Fill the wells with electrode buffer.
4. Place the upper chamber over the sandwich and lock the upper buffer chamber to the sandwich. Pour electrode buffer into the lower buffer chamber. Place the sandwich attached to the upper buffer chamber into the lower chamber.

5. Fill the upper buffer chamber with electrode buffer so that the sample wells of the stacking gel are filled with buffer.

6. Use a 10-25 µL syringe with a flate-tipped needle; load 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.