Improvement of Properties of Edible Film Based on Gelatin from Cuttlefish (*Sepia pharanois*) Skin

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

Doctor of Philosophy in Food Science and Technology

Prince of Songkla University

2011

Thesis Title	Improvement of Properties of Edible Film Based on Gelatin from Cuttlefish (*Sepia pharanois*) Skin
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Academic Year	2010

ABSTRACT

Effects of heat treatment at different temperatures (40-90 ºC) of film forming solution (FFS) containing 3% gelatin from cuttlefish (*Sepia pharaonis*) ventral skin and 25% glycerol (based on protein) on properties and molecular characteristics of resulting films were investigated. The film prepared from FFS heated at 60 and 70 ºC showed the highest tensile strength (TS) with the highest melting transition temperature (T_{max}) (p<0.05). Nevertheless, film from FFS heated at 90 ºC had the highest elongation at break (EAB) with the highest glass transition temperature ($T_{g)}$ (p<0.05). With increasing heating temperatures, water vapor permeability (WVP) of films decreased (p<0.05), but no differences in L*-value and transparency value were observed (p>0.05).

When skin gelatin with different degree of hydrolysis (DH: 0.40, 0.80 and 1.20%) was used along with glycerol as plasticizer at various levels (10, 15 and 20%, based on protein), films prepared from gelatin with all DH had the lower TS and EAB but higher WVP, compared with the control film (without hydrolysis) (p<0.05). At the same glycerol content, both TS and EAB decreased, while WVP increased (p<0.05) with increasing %DH. Based on FTIR spectra, with the increasing %DH, higher amplitudes for Amide-A and Amide-B peaks were observed, compared with control film due to the increased -NH_2 group caused by hydrolysis. TGA analysis indicated that film prepared from gelatin with 1.20% DH exhibited the higher heat susceptibility and weight loss, compared with control film.

To improve properties of film from cuttlefish skin gelatin without and with partial hydrolysis (1.2% degree of hydrolysis), ethanolic extracts of cinnamon (CME), clove (CLE) and star anise (SAE) at a level of 1% were incorporated. Films with different herb extracts (without and with oxidation) had higher TS but lower

EAB, compared with the control film (without addition of herb extracts) (p<0.05). Lower WVP and L*- value but higher b*- and ΔE*-values were observed when the extracts were incorporated (p<0.05). Oxidized extracts yielded films with higher TS and WVP than those without oxidized herb extracts (p<0.05). Electrophoresis study and FTIR spectra indicated that protein-polyphenol interactions were involved in the film. Thermogravimetric analysis revealed that films incorporated with SAE or SAE with oxidation (OSAE) exhibited lower heat susceptibility and weight loss, compared with control film.

Properties of film from cuttlefish skin gelatin without and with partial hydrolysis (1.2% degree of hydrolysis) as influenced by H_2O_2 and Fenton's reagent at different levels were also investigated. Films added with H_2O_2 (0.01-0.04 M) and Fenton's reagent [H_2O_2 (0.01-0.04 M) + $FeSO_4$ (0.001-0.004 M)] had higher TS but similar or lower EAB, compared with the control film (without addition of H_2O_2 and Fenton's reagent) (p<0.05). Slight differences in WVP were observed for all films. Films added with Fenton's reagent possessed lower L*- value but higher a*-, b*- and ΔE*-values, compared with the control film. Films added with Fenton's reagent had lower solubility in water than did those added with H_2O_2 as well as the control (p<0.05). Electrophoresis study and FTIR spectra suggested that interaction of protein was induced by H_2O_2 and Fenton's reagent. However, fragmentation of gelatin molecules more likely took place when Fenton's reagent at higher level was used.

Blend films based on cuttlefish skin gelatin (CG) and mungbean protein isolate (MPI) at different blend ratios (CG/MPI = 10:0, 8:2, 6:4, 4:6, 2:8 and 0:10, w/w) prepared at pH 11 using 50% glycerol (based on total protein) as plasticizer were characterized. CG films incorporated with MPI at increasing amounts had the decreases in TS (p<0.05). The increases in EAB were observed when CG/MPI ratios of 6:4 or 4:6 were used (p<0.05). Decreased WVP was obtained for films having the increasing proportion of MPI (p<0.05). Electrophoretic study revealed that disulfide bond was present in MPI and CG/MPI blend films. Differential scanning calorimetry result suggested solid-state morphology of CG/MPI (6:4) blend film that consisted of amorphous phase of partially miscible CG/MPI mixture and the coexisting two different order phases of individual CG and MPI domains.

Stability of cuttlefish skin gelatin film (CG) and film incorporated with Fenton's reagent (H_2O_2 0.02 M + Fe_2SO_4 0.002 M) (FG) was evaluated after 21 days of storage at 50% relative humidity and 25 °C. No changes in mechanical property were observed for CG but slight increase in TS was found for FG after storage (p<0.05). Furthermore, WVP increased for both films (p<0.05). DSC and TGA study revealed that molecular reorganization with higher thermal stability were formed in the film matrix during storage. When CG and FG were used to cover chicken meat powder, the samples covered with both films had lower moisture content, peroxide values (PV) and thiobarbituric acid reactive substances (TBARS), compared with control samples (without cover) (p<0.05). However, both films were poorer in preventing moisture migration and retarding the color changes of chicken meat powder than low-density polyethylene (LDPE) films.

Therefore, cuttlefish skin gelatin film can be used as an alternative packaging after appropriate modification, however the further improvement of water barrier property is still needed to widen its application.

ACKNOWLEDGEMENT

First and the foremost, I would like to express my profound sense of obligations, gratefulness, unending appreciations, heartfelt thanks and praise to the "Almighty Allah", Who has enabled me to complete research work, submit the thesis and fulfillment for the degree of Doctor of Philosophy in Food Science and Technology, Prince of Songkla University, Thailand.

I would like to express my boundless gratification, respect and sincere thanks to my advisor, Prof. Dr. Soottawat Benjakul of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University for his affectionate encouragement, sincere co-operation, kindness, active guidance and assistance, scholar supervision and constructive criticism during my study since the first day of being his student. His generous contribution in my academic and personal life, working hard throughout research period, preparation of manuscripts and thesis dissertation, determination and perseverance to train me to be a good researcher with responsibility, vigilance and honesty are deeply appreciated.

It is my profound privilege to express my gratitude and sincere thanks to my co-advisor, Asst. Prof. Dr. Thummanoon Prodpran of the Department of Material Product Technology, Faculty of Agro-Industry, Prince of Songkla University for his affectionate encouragement, sincere co-operation, kindness, assistance, scholastic guidance, helpful guidance in consultation, suggestion and support during my academic and personal life in Thailand.

I am grateful to my examining committee, Assist. Prof. Dr. Manee Vittayanont of the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, Assoc. Prof. Dr. Rungsinee Sothornvit of the Department of Food Engineering, Kasetsart University and Dr. Supachai Pisuchpen of the Department of Material Product Technology, Faculty of Agro-Industry, Prince of Songkla University for their valuable time, kindness, helpful suggestion and advices on my thesis.

I would like to express my heart felt gratitude and indebtedness to Prof. Dr. A.K.M. Nowsad Alam and all other faculty members in the Department of Fisheries Technology, Faculty of Fisheries, Bangladesh Agricultural University,

Mymensingh for their valuable lessons, suggestions, encouragements to complete my study. I am highly thankful to all the teachers in Sheikh Fazilatunnesa Mujib Fisheries Collage, Melandah, Jamalpur, Bangladesh for their instruction, suggestions and encouragements during my study.

I would like to expresses my immense indebtedness, sincere appreciation and utmost gratitude to my beloved parents, sisters, brothers, uncles, aunts, relatives and friends for their unstinted faith and confidence in me, endless sacrifice, patience, affection, love and blessings in all phases of my academic and personal life in home and aboard.

I would like to express my truly thanks, heart felt gratitude and best wishes to Dr. Tanong Aewsiri, Dr. Sutheera Khantaphant, Dr. Sitthipong Nalinanon, Dr. Amjad Balange, Dr. Palanivel Ganesan, Dr. Sajid Maqsood, Mr. Chodsana Sriket, Ms. Yaowapa Thainsilakul, Ms. Rossawan Intarasirisawat, Mr. Phanat Kittiphattanabawon, Mr. Phakawat Tongnuanchan, Ms. Suthasinee Yarnpakdee, Ms. Kusaimah Manheem, Ms. Sochaya Chanavat, Ms. Pimchanok Kaewudom, Ms. Meita Priana Indah Puri, Mr. Nilesh Nirmal, Mr. Mehraj Ahmed and all other Thai and International friends and colleagues of Fish Chemistry and Biochemistry Laboratory (2205). I would like to thanks all of my International and Thai friends in Prince of Songkla University, to whom I know in Thailand, for their suggestion, share, support and encouragements during my study.

I would like to thanks all faculty members of Agro-Industry and its staff, students for their kind co-operation during my study period. The sincere, accurate and active services of all professional scientist and technical personnel in Scientific Equipments Center, Prince of Songkla University, are highly appreciated.

Finally, without the Scholarships from the Graduate School of Prince of Songkla University and support from the National Research University Project of Thailand's Office of the Higher Education Commission, Ministry of Education, Thailand, this study could not be succeeded. The financial supports are highly acknowledged.

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CHAPTER 1

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Cephalopods, including cuttlefish, squid and octopus, are one of seafood products, which have become an important income generator for Thailand with high export value. In Thailand, cuttlefish is processed mostly as a frozen product (Thanonkaew *et al*., 2006). As a consequence, a large amount of skin, as a byproduct from processing, are generated and disposed or mainly used as animal feed. This colllagenous source can be served as an alternative raw material for the collagen and gelatin production for further uses. It has been known that marine animal skins have paid more attention as the safe source with the lower risk of mad cow disease, foot and mouth diseases etc. Additionally, an increase in demand for kosher and halal foods has led to the limitation of by-products of mammalians as source of collagen and gelatin (Cho *et al.,* 2005; Muyonga *et al.,* 2004a). Collagen is the major structural protein in connective tissue of animal skin and bone (Cho *et al.*, 2004; Kołodziejska *et al.*, 2004; Foegeding *et al*., 1996). Thermal denaturation of these collagens generally produces gelatin, which is a marketable value added product (Foegeding *et al*., 1996). Generally, gelatin has wide range applications in cosmetics, biomedical, pharmaceutical, leather, encapsulation and edible film industry (Jongjareonrak *et al*., 2005; Cho *et al.*, 2004; Segtnan *et al*., 2003; Ogawa *et al*., 2004; Slade and Levine, 1987). In food industries, gelatin have been widely used as a beverage clarifier, a thickener in dessert, a texturizer in confectionary and a stabilizer in ice cream, cream cheese and cottage cheese as well as in food foams and fruit salad, etc (OMRI, 2002). Gelatin has been attracted the attention for the developments of edible films due to its abundance and biodegradibility (Jongjareonrak *et al*., 2006b).

The physical and structural properties of gelatin are mainly influenced by the molecular weight distribution and amino acid composition that play a vital role in the rheological and barrier properties of the resulting films (Gómez-Guillén *et al*.,

2009). 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 intramolecular and intermolecular disulfide bonds (Gennadios and Weller, 1991). Interconnection of protein molecules during the drying process leads to the formation of film matrix. Therefore, the extension or unfolding of protein molecule could favor the interaction among molecules, in which the junction zones could be formed to a higher extent. Unfolding of proteins by heat treatment is thus a promising approach to improve the film-forming ability. Protein-based films have good oxygen barrier properties but have higher water absorptivity, owing to hydrophilicity of amino acids in protein molecules and to the significant amounts of hydrophilic plasticizers, such as glycerol and sorbitol, incorporated into films to impart adequate flexibility (Gennadios *et al*., 1993; McHugh and Krochta, 1994a). Since protein films do not have ideal mechanical properties and water vapor barrier, their applications as edible film and biomaterials have been limited. The important functional characteristic of edible films and coatings is to hinder the migration of moisture, oxygen, carbon dioxide, to inhibit microbial or solute transmission and to prevent collapse of products (Artharn *et al*., 2009). Controlled enzymatic hydrolysis of proteins may produce a series of small polypeptides which can modify the mechanical property by lowering the strong interaction between the molecules. As a result, the lower hydrophilic plasticizer might be used, which can be associated with the lower hydrophillicity of resulting film.

Chemical and physical treatments can be applied to modify the polymer network through cross-linking of the polymer chains to improve protein film functionality. The chemical agents used for cross-linking agents include aldehyde, gossypol, calcium salt and different types of phenolic acid (Cao *et al*., 2007a). Aldehyde which can bind quickly to protein is usually used to protein cross-linking but it has toxicity (Bigi *et al*., 2001). Polyphenols are known to react under oxidizing conditions with side chain amino group of peptides, leading to formation of protein cross-linking (Strauss and Gibson, 2004). Free radicals formed from ferulic acid can react with tyrosine and with itself to form a diferulic acid, which act as a bridge between protein molecules (Oudgenoeg *et al*., 2001). Free radical-mediated protein

modification could be an alternative approach to modify the properties of protein films. Fenton reaction is another approach to generate the active radical, hydroxyl radicals ($OH^{\bullet}$), from H_2O_2 in the presence of Fe^{2+} (Kocha *et al*., 1997). Hydroxyl radicals are reactive species which can alter protein composition and configuration (Liu and Xiong, 2000).

Furthermore, polymer blending is a well-used technique to modify the properties of polymer, due to its easy, uncomplicated and low cost procedure (Wang *et al*., 2009). With the proper miscibility or compatibility, polymer blend render new materials with better properties, compared to similar materials made from the respective pure polymers (Tang *et al*., 2003; Li *et al*., 2006; Cao *et al*., 2007b; Pérez-Mateos *et al*., 2009; Limpan *et al*., 2010). Blending of gelatin with other compatible biopolymers would be an alternative means to improve the properties of resulting blend films. Therefore, the improvement of protein-based film, especially from gelatin via different safe and environmental friendly approaches to lower its limitations could broaden its application.

1.2 Review of literature

1.2.1 Collagen and gelatin

1.2.1.1 Collagen

Collagen is abundant in tendons, skin, bone, the vascular system of animals, and the connective tissue sheath surrounding muscle, contributing to toughness of muscle. About 10% of mammalian muscle protein is collagen but the amount in fish is generally much less (Foegeding *et al.*, 1996; Karim and Bhat, 2009). The collagen monomer is a long cylindrical protein about 2,800 Å long and 14-15 Å in diameter (Foegeding *et al.*, 1996). The triple helix of collagen assembles from specific polypeptide chain (α chains), which has Gly-X-Y repeat with the frequent occurrence of proline and hydroxyproline in the X and Y position, respectively. Hydroxyproline and hydroxylysine are found only in position Y, while proline can be found in either the X- or Y- position (Fratzl, 2008). Each α-chain coil is a left-handed helix with three residues per turn, and the three chains are twisted right-handed to form tropocollagen. The triple helix is held together by hydrogen bonding (Figure 1). Each α-chain contains ~1,000 amino acid residues and varies in amino acid compositions (Wong, 1989) and has a molecular mass of about 100,000 Da, yielding a total molecular mass of about 300,000 Da for collagen (Foegeding *et al.*, 1996).

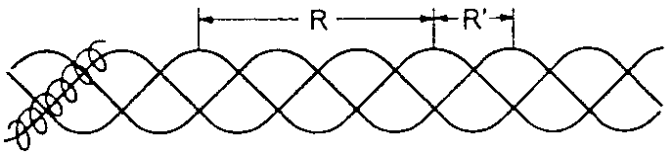


Figure 1. Schematic representation of the conformation of tropocollagen

Source: Burghagen (1999)

The presence of proline stabilizes the helix structure by preventing rotation of the N-C bond. Hydroxyproline also stabilizes the collagen molecule, and collagen that has small concentrations of both imino acids, denatures at lower temperatures than do those with high concentrations (Foegeding *et al.*, 1996). In general, fish collagens have lower imino acid contents than mammalian collagens, and this may be the reason for the denaturation at low temperature (Karim and Bhat, 2009).

Collagen has been extracted from fish skin and type I collagen is the dominant collagen (Table 1) (Nalinanon *et al*., 2011; Ahmad *et al*., 2010; Muyonga *et al.,* 2004a). Collagen was also extracted and characterized from the skin of mollusk such as squid (Uriarte-Montoya *et al*., 2010), scallop (Xuan *et al*., 2007), etc.

Table 1. Sources of Type I collagen from different fish species

Fish species	Extracted collagen	Yield (wet weight basis	References
Unicorn leatherjacket (*Aluterus monoceros*)	ASC	4.19%	Ahmad *et al*. (2010)
Ornate threadfin bream (*Nemipterus hexodon*)	-	-	Nalinanon *et al*. (2011)
Striped catfish (*Pangasianodon hypophthalmus*)	ASC PSC	ASC: 5.1% PSC: 7.7%	Singh *et al*. (2011)
Brownbanded bamboo shark (*Chiloscyllium punctatum*),	ASC PSC	ASC: 9.38% PSC: 8.86%	Kittiphattanabawon *et al*. (2010a)
Largefin longbarbel catfish (*Mystus macropterus*)	ASC PSC	ASC: 16.8% PSC: 28.0%	Zhang *et al*. (2009)
Silver carp (*Hypophthalmichthys Molitrix*)	-	-	Rodziewicz-Motowidło *et al*. (2008)
Bigeye snapper (*Priacanthus tayenus*)	ASC PSC	ASC: 5.31% BSP:18.74% (dry basis)	Nalinanon *et al*. (2007)
Walleye pollock (*Theragra chalcogramma*)	ASC	-	Yan *et al*. (2008)
Young and adult Nile perch (*Lates niloticus*)	ASC	58.7-63.1 %	Muyonga *et al*. (2004a)
Channel catfish (*Ictalurus punctaus*)	ASC PSC	ASC: 25.8% PSC: 38.4%	Liu *et al*. (2007)

ASC: acid soluble collagen; PSC: pepsin soluble collagen

1.2.1.2 Gelatin

Gelatin is a denatured protein derived from collagen by thermo-hydrolysis and has a rheological property of thermo-reversible transformation between sol and gel (Cho *et al*., 2005). The process involves the disruption of non-covalent bonds and it is partially reversible in agreement with the gelling properties of gelatin (Karim and Bhat, 2009). Collagen fibrils shrink to less than one-third of their original length at a critical temperature, known as the shrinkage temperature. This temperature varies, depending on species from which the collagen is derived (Burghagen, 1999). This shrinkage involves a disassembly of fibers and a collapse of the triple-helical arrangement of polypeptide subunits in the collagen molecule. Essentially the same type of molecular change occurs when collagen is heated in solution, but at a much lower temperature (Foegeding *et al.*, 1996). The midpoint of the collagen-to-gelatin transition is defined as the melting temperature (Figure 2). During the collagen-to-gelatin transition, many non-covalent bonds are broken along with some covalent inter- and intra-molecular bonds (Schiff's base and aldo condensation bonds). This results in conversion of the helical collagen structure to a more amorphous form, known as gelatin. These changes lead to the denaturation of the collagen molecule but not to the point of a completely unstructured product (Foegeding *et al.*, 1996).

After gelatin is produced and the temperature is lowered to below the critical value, there is a partial renaturation of the collagen molecule, involving what is called the "Collagen fold". Apparently, those parts of collagen that are rich in proline and hydroxyproline residues regain some of their structure, following which they can apparently interact (Foegeding *et al.*, 1996). When many molecules are involved, a three-dimensional structure is produced and responsible for the gel observed at low temperatures. The strength of the gel formed is proportional to the square of the concentration of gelatin and directly proportional to molecular weight (Cho *et al.*, 2004). Circular dichroism analysis reveals that gelling involves a refolding of denatured collagen chains into the typical triple helix conformation and conversely unfolding upon reheating. The folding process seems to be directly related in the stabilization of the gels without disregarding its role in triggering the gelation process (Gómez-Guillén *et al.*, 2002).

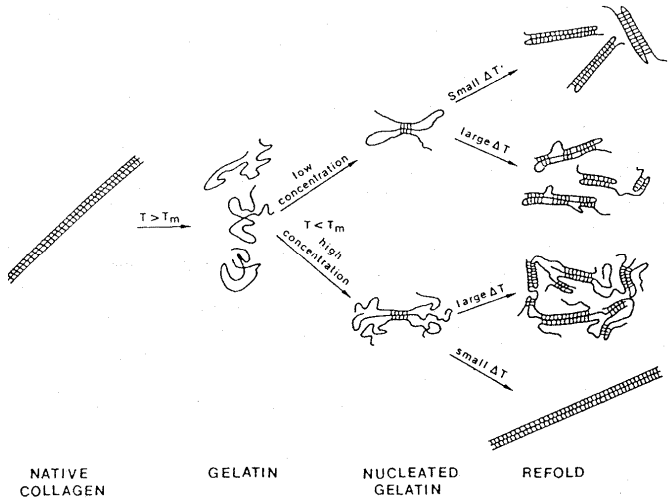


Figure 2. Collagen conversion into gelatin
Source: Wong (1989)

1.2.1.2.1 Composition of gelatin

Gelatin is a heterogeneous mixture of water-soluble proteins of high molecular weight (Kantaria *et al.*, 1999). On a dry weight basis, gelatin consists of 98 to 99% protein. The molecular weight of these large proteins typically ranges between 20,000 and 250,000 Da. However, some aggregates weigh in the millions (Poppe, 1997). Coils of amino acids are joined together by peptide bonds. The predominant amino acid sequence is Gly-Pro-Hyp (Fratzl, 2008). Gelatin contains relatively high levels of these following amino acids: glycine (Gly) 26-34%; proline (Pro) 10-18%; and hydroxyproline (Hyp) 7-15% (Poppe, 1997). Other significant amino acids include alanine (Ala) 8-11%; arginine (Arg) 8-9%; aspartic acid (Asp) 6-7%; and glutamic acid (Glu) 10-12% (Poppe, 1997). Gelatin is not a nutritionally complete protein. It contains no tryptophan and is deficient in isoleucine, threonine, and methionine (Potter and Hotchkiss, 1998). Sulfur-containing amino acids, cysteine and cystine are also deficient in gelatin. Water varies between 6 and 9% (Alais and Linden, 1991; US FDA, 1997). Table 2 shows the amino acid composition of gelatin from different sources. Generally, gelatin from fish skin shows a wider variety in amino acid compositions than those of mammalian gelatin. Fish gelatin has lower imino acid contents (proline and hydroxyproline) than mammalian gelatin (Grossman and Bergman, 1992). The proline and hydroxyproline contents are approximately 30%

for mammalian gelatins, 22–25% for warm-water fish gelatins (tilapia and Nile perch), and 17% for cold-water fish gelatin (cod) (Muyonga *et al.*, 2004).

Avena-Bustillos *et al.* (2006) reported that cold-water fish gelatins have significantly lower hydroxyproline, proline, valine, and leucine residues than mammalian gelatins, but significantly possess more glycine, serine, threonine, aspartic acid, methionine, and histidine residues. However, both cold-water fish and mammalian gelatins have the same proportion of alanine, glutamic acid, cysteine, isoleucine, tyrosine, phenylalanine, homocysteine, hydroxylysine, lysine, and arginine residues (Avena-Bustillos *et al.*, 2006). Squid gelatin contained higher content in Hyp than cod, sole megrim and hake, while the total imino acid (Pro+Hyp) content was similar (Gómez-Guillén *et al*., 2002). A special feature of squid gelatin was its high Lys hydroxylation degree that frequently found in highly insoluble collagens with a high cross-linking degree (Montero *et al.*, 1990).

Table 2. Amino acid composition of gelatins from different sources (residues/1000 total amino acid residues)

Amino acid	Cod[a]	Hake[b]	Megrim[a]	Tilapia[b]	Giant squid[c]	Pork[d]
Ala	96	119	123	123	82	112
Arg	56	54	54	47	61	49
Asx	52	49	48	48	61	46
Cys	0	0	0	0	10	0
Glx	78	74	72	69	83	72
Gly	344	331	350	347	332	330
His	8	10	8	6	7	4
Hyl	6	5	5	8	17	6
Hyp	50	59	60	79	74	91
Ile	11	9	8	8	11	10
Leu	22	23	21	23	27	24
Lys	29	28	27	25	12	27
Met	17	15	13	9	10	4
Phe	16	15	14	13	10	14
Pro	106	114	115	119	89	132
Ser	64	49	41	35	43	35
Thr	25	22	20	24	26	18
Trp	0	0	0	0	0	0
Tyr	3	4	3	2	8	3
Val	18	19	18	15	37	26
Imino acid	156	173	175	198	163	223

[a] Gómez-Guillén *et al.* (2002); [b] Sarabia *et al.* (2000); [c] Giménez *et al.* (2009b); [d] Eastoe and Leach (1977).

1.2.1.2.2 Gelatin structure

Primary structure

The primary structure of gelatin closely resembles the parent collagen. Small differences are due to raw material sources together with pretreatment and extraction procedures. These can be summarized as follows (Johnston-Banks, 1990):

1. Partial removal of amide groups of asparagines and glutamine, resulting in an increase in the contents of aspartic acid and glutamic acid. This increases the number of carboxyl groups in the gelatin molecule and thus lowers the isoelectric point. The degree of conversion is related to the severity of the pretreatment process.

2. Conversion of arginine to ornithine in more prolonged treatments experienced during long liming processes. This takes place by removal of a urea group from the arginine side-chain.

3. There is a tendency for trace amino acids, such as cysteine, tyrosine, isoleucine, serine, etc., to be found in lower proportions than in their parent collagens. This is due to the inevitable removal of some telopeptide during cross-link cleavage, which is then lost in the pretreatment solutions.

Secondary structure

Gelatin is not completely polydispersed, but has a definite molecular weight distribution pattern corresponding to the α-chain and its oligomers (Table 3). One to eight oligomers may be detected in solution, but it is possible that higher numbers exist. Doublets, known as β-chains, are formed from both α1- and α2-chains, giving rise to β11- and β12-molecules (Johnston-Banks, 1990). Oligomers of three α-chains will mainly exist as intact triple helix, but a certain proportion will exist as extended α-polymers bonded randomly by end-to-end or side-to-side bonds (Johnston-Banks, 1990). The structure of oligomers of greater than four α-chain units obviously becomes increasingly more complex. Molecular-weight spectra normally relate with physical properties of gelatin (de Wolf, 2003; Karim and Bhat, 2009).

Differences can be detected between commercial gelatin from the different raw materials. In general, the sum of the α- and β-fractions, together with their larger peptides, is proportional to the bloom strength, and the percentage of

higher molecular weight material is related with the viscosity (Karim and Bhat, 2009). The setting time is increased for the peptide fractions below α-chain, but a certain proportion of the very high molecular weight "Q" fraction can reduce the setting time markedly (Johnston-Banks, 1990). The melting point also increases with higher molecular weight content (Cho *et al.*, 2004; Karim and Bhat, 2009).

Table 3. Molecular weight distribution showing the major structural components of gelatin

Molecular fraction	Description
Q	Very high molecular weights, of 15-20 x 10^6 daltons and thought to be branched in character owing to their inability to penetrate the gel successfully.
1-4	Oligomers of α-chains, levels of five to eight.
X	Oligomers of four α-chains.
γ	285,000 daltons, i.e. 3 x α-chain.
β	190,000 daltons, i.e. 2 x α-chain.
α	95,000 daltons.
A-peptide	86,000 daltons.
α-, β- and γ- peptides	Seen as tailing their parent peaks.

Source: Johnston-Banks (1990)

1.2.1.3 Fish gelatin

Gelatin from marine sources (warm- and cold-water fish skins, bones, and fins) is a possible alternative to bovine gelatin (Kim and Mendis, 2006). One major advantage of fish gelatins is that they are not associated with the risk of outbreaks of Bovine Spongiform Encephalopathy. Fish gelatin is acceptable for Islam, and can be used with minimal restrictions in Judaism and Hinduism (Cho *et al.*, 2005). Furthermore, fish skin, which is a major byproduct of the fish-processing industry, causing waste and pollution, could provide a valuable source of gelatin (Badii and Howell, 2006). Fish skin contains a large amount of collagen. Nagai and Suzuki (2000) reported that the collagen contents in the skin of Japanese sea-bass, chub mackerel, and bullhead shark were 51.4, 49.8, and 50.1% (dry basis), respectively. Production of fish gelatin is actually not new as it has been produced since 1960 by acid extraction (Norland, 1990). Gelatin has been extracted

from skins and bones of various cold-water (e.g., cod, hake, Alaska pollock, and salmon) and warm-water (e.g., tuna, catfish, tilapia, Nile perch, shark and megrim) fish as shown in Table 4.

Table 4. Different sources of fish gelatin

Fish species	References
Unicorn leatherjacket (*Aluterus monoceros*)	Ahmad and Benjakul (2011)
Bamboo shark (*Chiloscyllium punctatum*), blacktip shark (*Carcharhinus limbatus*)	Kittiphattanabawon *et al.* (2010b)
Baltic cod (*Gadus morhua*), salmon (*Salmo salar*), herrings (*Clupea harengus*)	Kołodziejska *et al.* (2008)
Catfish (*Ictalurus punctatus*)	Liu *et al.* (2008)
Grass carp (*Ctenopharyngodon idella*)	Kasankala *et al.* (2007)
Atlantic salmon (*Salmo salar*)	Arnesen and Gildberg (2007)
Skate (*Raja kenojei*), Yellowfin tuna (*Thunnus albacares*)	Cho *et al.* (2006)
Bigeye snapper (*Priacanthus macracanthus*), brownstripe red snapper (*Lutjanus vitta*)	Jongjareonrak *et al.* (2006a)
Sin croaker (*Johnius dussumieri*), shortfin scad (*Decapterus macrosoma*)	Cheow *et al.* (2007)
Alaska pollock (*Theragra chalcogramma*)	Zhou and Regenstein (2005)
Nile perch (*Lates niloticus*)	Muyonga *et al.* (2004b)
Flounder (*Platichthys flesus*)	Fernández-Díaz *et al.* (2003)
Black tilapia (*Oreochromis mossambicus*), red tilapia (*Oreochromis nilotica*)	Jamilah and Harvinder (2002)
Megrim (*Lepidorhombrus boscii*) (Risso), Hake (*Merluccius merluccius*), Dover sole (*Solea vulgaris*)	Gómez-Guillén *et al.* (2002)

1.2.1.3.1 Extraction of fish gelatin

Generally, gelatin manufacturing processes consist of three main stages: pretreatment of the raw material, extraction of the gelatin, and purification and drying (Karim and Bhat, 2009). Depending on the method in which the collagens are pretreated, two different types of gelatin (each with differing characteristics) can be produced. Type A gelatin (isoelectric point at pH 6–9) is produced from acid-treated collagen, and type B gelatin (isoelectric point at approximately pH 5) is produced from alkali-treated collagen (Stainsby, 1987). Acidic treatment is most suitable for the less covalently cross-linked collagens found in pig and fish, while alkaline treatment is suitable for the more complex collagens found in bovine hides. The extraction process can influence the length of the polypeptide chains and the functional properties of the gelatin. This depends on the processing parameters (temperature, time, and pH), the pretreatment, and the properties and preservation method of the starting raw material (Karim and Bhat, 2009).

Gelatin can be extracted from many fish species by non-collagenous protein elimination, demineralization and swelling with acid solution prior to conversion of collagen to gelatin by heating in the presence of water, and finally recovery of gelatin in the final form (Foegeding *et al*., 1996). For raw material constituting high content of lipid, it is more important to degrease before another pretreatment and extraction (Holzer, 1994). Gelatin extraction normally takes place under either acid or neutral conditions at the minimum temperature to give a high yield of gelatin (Jones, 1987). Type of acid used, ionic strength and pH strongly influences swelling process and solubilization of collagen as well as the extraction of gelatin (Giménez *et al*., 2005). Gómez-Guillén and Montero (2001) reported that acetic- and propionic-acid pretreated skin of megrim (*Lepidorhombus boscii*) rendered the gelatins with the highest elastic modulus, viscous modulus, melting temperature, and gel strength. Gómez-Guillén *et al*. (2002) compared the rheological characteristics (viscoelasticity and gel strength) and chemical/structural properties (amino acid composition, molecular weight distribution and triple helix formation) of different fish skin gelatins. Gelatins from flat-fish species (sole and megrim) presented the best gelling ability and the gels were more thermostable than those from cold-adapted fish (cod and hake). This different behavior may be determined by the

amino acid composition, the α_1/α_2 collagen-chain ratio, and the molecular weight distribution. Cod gelatin contained a lower alanine and imino acid content, and a decreased proline hydroxylation degree. Cod and hake gelatins had a low α_1/α_2 ratio (~1), whereas hake gelatin showed a highly significant decrease in β-components and other aggregates (Gómez-Guillén *et al*., 2002). The squid gelatin had the α-chains with slightly different mobility on SDS-PAGE from other fish species (Gómez-Guillén *et al*., 2002). Very low content of β- components and an almost disappearance of higher molecular aggregates was observed in squid gelation.

Type and concentration of base and acid during pretreatment, and the extraction temperature and time strongly influenced the total yield and rheological properties of pollack skin gelatin (Zhou and Regenstein, 2003). Gelatins extracted from the skin of unicorn leatherjacket (*Aluterus monoceros*) pretreated with 0.2 M acetic acid or 0.2 M phosphoric acid had the yields of 5.23-9.18 or 6.12-11.54% (wet weight basis), respectively. The gel strength of gelatin from skin pretreated with phosphoric acid was higher than that of gelatin from skin pretreated with acetic acid (Ahmad and Bejakul, 2011). The combination of 0.1 N $Ca(OH)_2$ or NaOH with 0.05N acetic acid or 0.025N citric acid improved the gel strength of gelatin (Zhou and Regenstein, 2003). Although increasing extraction temperature and time (above 40 °C and 180 min) could slightly increase the total yield of gelatins, the gel strength decreased. The total yield of gelatin from pollock skin was more than 12% with a hydroxyproline content around 7%. Moreover, pollock skin gelatin extraction was also affected by 4 variables, pretreatment temperature, concentration of OH^-, concentration of H^+, and extraction temperature. Based on response surface methodology, a concentration of OH^- at 0.25 M, a concentration of H^+ at 0.09 M, a pretreatment temperature at 2 °C, and an extraction temperature at 50 °C, gave the gelation with the highest yield (18%), gel strength (460 g), and viscosity (6.2 cP) (Zhou and Regenstein, 2003). The gelatin extraction efficiency was improved by an acid-swelling process in the presence of smooth hound crude acid protease extract (SHCAP). The yields of gelatins from cuttlefish skin pretreated with acid and with crude acid protease (15 units/g alkaline-treated skin) for 48 h were 2.21% and 7.84%, respectively (Balti *et al*., 2011).

Type-A gelatins extracted from skins and bones of young and adult Nile perch with the sequential extraction temperature at 50, 60, 70 and 95 °C had the bloom strength in descending order: adult fish skins > young fish skins > adult fish bones > young fish bones. Bloom gel strength was 81-229 and 134-179 g, respectively, for skin and bone gelatins (Muyonga *et al*., 2004b). Gelatin from adult Nile perch skins exhibited higher viscosity and lower setting time than those from bone and the young fish skin gelatins. Skin gelatins were found to exhibit higher film tensile strength but lower film percent elongation than bone gelatins (Muyonga *et al*., 2004b). Gelatins from winter and summer fish skins were extracted at 60, 70 and 80 °C. The gelatins from summer fish presented higher melting points and gel strengths as well as better viscosity properties than the winter equivalents (Duan *et al*., 2011).

Gel strength of yellowfin tuna gelatin (426 Bloom) was higher than bovine and porcine gelatins (216 Bloom and 295 Bloom, respectively) (Cho *et. al.,* 2005). Gelatin extraction from shark *(Isurus oxyrinchus*) cartilage was optimized with response surface methodology by Cho *et al*. (2004) with a maximum yield of 79.9%, in which the optimum conditions were alkali treatment with 1.6 N NaOH for 3.16 days and hot-water extraction at 65 °C for 3.4 h. Gelatins from the skins of brownbanded bamboo shark (BBS; *Chiloscyllium punctatum*) and blacktip shark (BTS; *Carcharhinus limbatus*) were extracted using the distilled water at different temperatures (45, 60 and 75 °C) and times (6 and 12 h) with the yield of 19.06–22.81% and 21.17–24.76% (based on wet weight), respectively. Gelatins from both species extracted at 45 °C for 6 h exhibited the highest bloom strength (206–214 g), which was higher than that of commercial bovine bone gelatin (197 g) (Kittiphattanabawon *et al*., 2010b).

The extraction process can influence the length of the polypeptide chains and the functional properties of the gelatin. This depends on the processing parameters (temperature, time, and pH), the pretreatment, and the properties and preservation method of the starting raw material (Karim and Bhat, 2009). Pretreatment with alkaline solutions of $Ca(OH)_2$ and/or acetic acid (HAC) of sturgeon (*Acipenser baeri*) skin provided gelatin with a favourable color. Pretreatment with alkali removed noncollagenous proteins effectively, whilst acid induced some loss of

collagenous proteins. Gel strength and viscosity of gelatin pretreated with HAC or alkali followed by HAC were as high as gelatin extracted in the presence of protease inhibitors *(*Hao *et al*., 2009). Yang *et al*. (2008) studied that correlation between the physical properties and nanostructure of gelatins made of channel catfish (*Ictalurus punctatus*) skins pretreated with sodium hydroxide, acetic acid, or water, and then extracted with hot water before the measurement. The acid pretreatment group showed the highest gel strength and protein yield, and a reasonable viscosity. The water pretreatment group showed the lowest values for all of the physical properties. Shark cartilage gelatin had lower concentration of hydroxyproline than the two porcine skin gelatins. Kittiphattanabawon *et al*. (2004) reported that the gelatin extraction from bigeye snapper skin and bone was carried out by deproteinization the skin in 0.025 N NaOH for 1 h with 2 repetitions. Only deproteinized bone was then subjected to demineralization with either 1.2 M citric acid for 4 h or 0.6 M HCl for 2 h. Swelling process was carried out by soaking the pretreated bone and skin in 0.05-0.2 M citric or acetic acid for 40 min with 3 repetitions. Gelatin was then extracted using hot water (45 °C) for 12 h. The yields of skin and bone gelatin were 6.29-7.76% and 1.19-2.25% (wet basis), respectively. The highest bloom strength of gelatin gel from skin was obtained when skins were swollen with 0.2 M acetic acid prior to extraction. Gelatin extracted from the skin of farmed giant catfish (*Pangasianodon gigas*) contained a high number of imino acids (proline and hydroxyproline) (211 residues per 1,000 residues). The bloom strength of the gelatin gel from giant catfish skin gelatin (153 g) was greater than that of calf skin gelatin (135 g) ($P<0.05$). Viscosity, foam capacity and foam stability of gelatin from giant catfish skins were in general greater than those of the gelatin from calf skin (Jongjareonrak *et al*., 2010).

Gelatin was extracted from the bigeye snaper (*Priacanthus macracanthus*) skin in water without and with 0.001 mM soybean trypsin inhibitor (SBTI) using a skin/water ratio of 1:7 at different temperatures (35, 40, 45, 50, 55 and 60 °C) for 12 h. In the presence of SBTI, the degradation was markedly inhibited. However, β-chain disappeared and α-chains underwent degradation to some extent at temperature above 50 °C. Generally, a higher yield of gelatin was obtained as the extracting temperature increased ($P<0.05$) (Intarasirisawat *et al*., 2007). Moreover, the degradation of gelatin components was markedly prevented, when SBTI at a

concentration of 0.1μM was incorporated during the gelatin extraction from bigeye snapper (*Priacanthus tayenus*) skin (Nalinanon *et al*., 2008). Hydroxyproline content and bloom strength of gels treated with bigeye snapper pepsin and porcine pepsin were similar, but their bloom strength was greater than the gelatin extraction from the bigeye snapper skin by the conventional process, which had a substantial degradation of gelatin components (Nalinanon *at el.,* 2008). Gelatin was extracted from unicorn leatherjacket skin using distilled water at 50 °C for 12 h in the presence and absence of SBTI. In the presence of 0.04 mM SBTI, the degradation was markedly inhibited, but a lower gelatin extraction yield was obtained (Mehraj *et al*., 2011). Higher gel strength (320.68 ± 3.02 g) was obtained in gelatin extracted with SBTI, compared with that of gelatin extracted without SBTI (288.63 ±1.44 g). High emulsifying activity index but lower emulsifying stability index was observed in the gelatin extracted with SBTI (Mehraj *et al*., 2011).

Bleaching using 2% and 5% H_2O_2 could improve not only the color of gelatin by increasing the L*-value and decreasing a*-value but also enhanced the bloom strength, and the emulsifying and foaming properties of the resulting gelatin from dorsal and ventral skin of cuttlefish (Aewsiri *et al*., 2009). When different raw naterials were used for gelatin extraction, gelatin from dried channel catfish skin exhibited higher gel strength. This can be explained by the large α-chains content of gelatin from the dried skins. The gelling point and melting point of dried channel catfish skin gelatin solution were similar to those of fresh skin gelatin solution, but distinctly different from those of frozen skin gelatin (Liu *et al*., 2008). Flounder skins were frozen at -12 or -20 ° C, and the resulting gelatin was compared with a gelatin extracted from fresh skins. Gelatin from skins frozen at -12 °C had the lower gel strength when compared to that from fresh skins but showed the highest melting point value (Fernández- Díaz *et al*., 2003).

Gel strength and gel melting point are the major physical properties of gelatin gels. These are governed by molecular weight, as well as by complex interactions determined by the amino acid composition and the ratio of α /β-chains present in the gelatin (Cho *et al*., 2004). Gelatin from salmon contained slightly more hydroxyproline and proline (16.6%) than cod gelatin (15.4%). Salmon gelatin expressed slightly higher gelling temperature (12 °C) than cod gelatin (10 °C), and

higher initial gel strength (Arnesen and Gildberg, 2007). Gelatins extracted from the skins containing fine scales of two species of bigeye snapper, *Priacanthus tayenus* (GT) and *Priacanthus macracanthus* (GM) had high content of imino acids (proline and hydroxyproline) (186.29–187.42 mg/g). The bloom strength of GM (254.10 g) was higher than that of GT (227.73 g), but was slightly lower than that of commercial bovine gelatin (293.22 g) (Benjakul *et al*., 2009). Shortfin scad gelatin had higher melting and gelling temperatures than those of sin croaker gelatin. The bloom strengths of gelatins from sin croaker and from shortfin scad were 125 and 177 g, respectively, compared to 240 g for commercial bovine gelatin (Cheow *et al*., 2007). Giant squid (*Dosidicus gigas*) inner and outer tunics were subjected to hydrolysis with pepsin prior to gelatin extraction (G1 gelatin) by a mild-acid procedure. Furthermore, a second gelatin extraction (G2 gelatin) from the collagenous residues that remained from the first extraction. G1 exhibited good gel forming ability but G2 showed poor viscoelastic behaviour and low gel strength. G2 showed a considerably higher content of low molecular weight components (Giménez *et al.*, 2009b). The dynamic storage modulus and bloom value for all types of gelatin increased with increasing average molecular weight. Type-A and type-B gelatins with similar average molecular weight exhibited different dynamic storage modulus (G') and different bloom values. This is most probably due to a different molecular weight distribution as well as the presence of different hydrolytic fragments (Eysturskar *et al.,* 2009). The dynamic storage modulus, gelling and melting temperatures and helix content are related and increase with increasing average molecular weight up to about 250 kg/mol (Eysturskar *et al*., 2009).

To improve the gelatin properties, various chemicals and enzymes have been used. Gelatin from the skins of Baltic cod (*Gadus morhua*) was modified using transglutaminase. A gelatin solutions (5%) formed gel at room temperature in the presence of microbial transglutaminase (MTGase) (0.15- 0.7mg of enzyme protein/ml) depending on the reaction time (Kolodziejska *et al*., 2004). The addition of MTG ase at concentration up to 0.005% and 0.01% (w/v) increased the bloom strength of gelatin gel from bigeye snaper (*Priacanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*), respectively ($p<0.05$). SDS-PAGE of gelatin gel added with MTGase showed the decrease in band intensity of protein components,

especially, β and γ-components (Jongjareonrak *et al*., 2006a). Norziah *et al*. (2009) also found the similar result when the gel of gelatin extracted from wastes of herring (*Tenualosa ilisha*) was added with MTGase. Gelatin gel contained α_1-chains and 53 kDa but in gels added with higher concentration of transglutaminase, these protein bands disappeared. Alaska pollack (*Theragra chalcogramma*) and Alaska pink salmon (*Oncorhynchyncus gorbuscha*) skin gelatin had the improved gelation and melting behavior as well cross-linking behavior upon the addition of genipin and glutaraldehyde. Pollock gelatin was cross-linked faster with glutaraldehyde than with genipin (Chiou *et al*., 2006). Gel strength of gelatin from walleye pollock (*Theragra chalcogramma*) skin increased with increasing gallic acid concentration up to 20 mg/g dry gelatin, and then decreased at further elevated gallic acid concentration. However, gel strength continuously increased with increasing levels of rutin (Yan *et al*, 2011). Table 5 shows extraction process of gelatin from different sources and their bloom strength.

Table 5. Extraction, yield and bloom strength of gelatin from skin of different fish species

Fish species	Pretreatment	Extraction condition	Yield	Bloom strength	References
Megrim (*Lepidorhombus boscii*)	1) Stir with cold (2 °C) 0.2 N NaOH and then with 0.2 N sulphuric acid (1:6 w/v) for 40 min of each (both repeated 3 times). Then treat with 0.7% citric acid for 40 min with continuous stirring (GM1) 2) Clean with 0.8 N NaCl and then swollen with 0.05 N acetic acid (1:10 w/v) at 25-28 °C for 3 h (GM2) (Rinse with tap water after treatment of both processes).	Distilled water overnight at 45 °C.	-	GM1: 220 GM2: 350	Montero and Gómez-Guillén (2000).
Megrim (*Lepidorhombus boscii*), Cod (*Gadus morhua*), Dover sole (*Solea vulgaris*), Hake (*Merluccius merluccius*) and Squid (*Dosidicus gigas*).	Swollen in 0.05 M acetic acid.	Distilled water overnight at 45 °C and for squid at 80 °C.	Megrim: 7.4% Cod: 7.2% Dover sole: 8.3% Hake: 6.5% Squid: 2.6%	Megrim: ~310g Cod: ~ 90g Dover sole: 350 g Hake: ~110 g Squid: ~15g	Gómez-Guillén *et al*. (2002).
Black tilapia (*Oreochromis mossambicus*) and red tilapia (*Oreochromis nilotica*)	Soak in 0.2% (w/v) NaOH solution for 40 min, followed by soaking in 0.2% sulphuric acid and 1.0% citric acid.	Distilled water at 45 °C for 12 h.	Black tilapia: 5.39% Red tilapia: 7.81%	Black tilapia: 180.76g Red tilapia: 128.11g	Jamilah and Harvinder (2002)
Baltic cod (*Gadus morhua*)	-	Water 45 °C (1:6) with gently stirred for 15–120 min.	12.3%	-	Ko1odziejska *et al*. (2004)
Nile perch (*Lates niloticus*)	Acidify with 0.01 M H_2SO_4 (pH of 2.5–3.0) with skin/acid ratio of 1:2 (w/v) and wash until a final pH of 3.5–4.	Three sequential 5 h extractions at 50, 60 and 70 °C, followed by boiling for 5 h	Young Nile perch: 12.3%, Adult Nile perch: 16.0% (wet weight basis)	Young Nile perch: 217g Adult Nile perch: 240g	Muyonga et al. (2004a)

Table 5: (Continued)

Fish species	Pretreatment	Extraction condition	Yield	Bloom strength	References
Bigeye snapper (*Priacanthus macracanthus*) and brownstripe red snapper (*Lutjanus vitta*)	1) Soak in 0.2M NaOH with a skin/solution ratio of 1:10 (w/v) at 4 °C with a gentle stirring. Change solutions every 30 min for 3 times. 2) Soak in 0.05M acetic acid with a skin/solution ratio of 1:10 (w/v) for 3 h at 25-28 °C with a gentle stirring.	Distilled water with a skin/water ratio of 1:10 (w/v) at 45 °C for 12 h with a continuous stirring.	Bigeye snapper: 6.5%, Brownstripe red snapper: 9.4%, (wet weight basis).	Bigeye snapper:105.7g, Brownstripe red snapper: 218.6g	Jongjareonrak *et al*. (2006a)
Sin croaker (*Johnius dussumeiri*),and shortfin scad (*Decapterus macrosoma*)	Soak in 0.2% (w/v) NaOH solution for 40 min, with 0.2% (w/v) H_2SO_4 for 40 min, followed by soaking with 1.0% (w/v) citric acid for 2 h (repeat for 3 times each step)	Distilled water at 40–50 °C for 12 h.	Sin croaker: 14.3% Shortfin scad : 7.25%	Sin croaker: 124.94g, Shortfin scad: 176.92g	Cheow *et al*. (2007)
Baltic cod (*Gadus morhua*) fresh, cold-smoked salmon (*Salmo salar*)	Wash with 0.45 M NaCl solution.	Water (1:6, w/v) for 15–120 min at 45, 70 or 100 °C.	Fresh salmon: 74-98%, Smoked salmon: 84-95%	-	Ko1odziejska *et al*. (2008)
Bigeye snapper (*Priacanthus tayenus*)	1) Mix with 10 volumes of 0.025M NaOH, stir for 2 h at 25–28 °C. Change solution every hour. 2) Soak in 0.2M acetic acid with a solid/solvent ratio of 1:10 (w/v), in the presence of BSP (0-15 units/g alkaline treated skin), then stir at 4 °C for 48 h.	Water at 45 °C for 12 h with continuous stirring.	22.2- 40.3% (at different BSP levels)	Typically extracted process: 56g, Using 15 units/g BSP: 135g	Nalinanon *et al*. (2008)
Channel catfish (*Ictalurus punctatus*)	Soak in eight volumes (v/w) of 50-mM acetic acid at 15 °C for 18 h, then wash with distilled water until pH reached 3.5–4.0.	Distilled water at 45 °C for 7 h.	-	Gel strength 256g	Liu *et al*. (2008)
Cuttlefish (*Sepia pharaonis*)	1) Soak in 0.05 N NaOH with a skin: solution ratio of 1:10 (w/v) for 6 h with gentle stirring at 26–28 °C. 2) Bleach in 2% and 5% H_2O_2, using a sample: solution ratio of 1:10 (w/v) for 24 and 48 h at 4 °C.	Distilled water at 60 °C for 12 h, with a sample: water ratio of 1:2 (w/v) with continuous stirring.	Dorsal skin: 36.83-49.65%, Ventral skin: 58.91-72.88 % (on dry basis, at different treatments)	Dorsal skin: 126g Ventral skin: 137 g (bleached with 5% H_2O_2 for 48 h)	Aewsiri *et al*. (2009)

Table 5: (Continue)

Fish species	Pretreatment	Extraction condition	Yield	Bloom strength	References
Bigeye snapper, *Priacanthus tayenus* (GT) and *Priacanthus macracanthus* (GM)	1). Soak in 0.025 M NaOH (1:10 w/v) with gentle stirring for 2 h. Change solution every hour. Wash alkaline-treated skins with tap water until neutral or faintly basic pHs were obtained. 2). Soak the skin in 0.2 M acetic acid (1:10 w/v) with gentle stirring for 2 h. Change solution every 40 min. Wash acid-treated skins with tap water.	Distilled water with a skin/water ratio of 1:10 (w/v) at 45 °C for 12 h, with continuous stirring.	GT: 7.93% GM: 6.41%	GT: 227.73 g GM: 254.10 g	Benjakul *et al*., (2009)
Bamboo shark (*Chiloscyllium punctatum*), blacktip shark (*Carcharhinus limbatus*)	Soak the skin in 0.1M NaOH (1:10 w/v) with gentle stirring for 6 h, followed by washing with tap water until neutral basic pH was obtained. Demineralize pretreated skins using 1 M HCl (1:10 w/v) with gentle stirring for 1 h, followed by washing with tap water until neutral basic pH. Swollen the skin using 0.2 M acetic acid (1:10 w/v).	Distilled water at 45, 60 aand 75 °C for 6 and 12 h with a continuous stirring.	Bamboo shark: 19.06-22.81% Blacktip shark: 21.17-24.76% (based on wet weight)	Both species: 206-214 g	Kittiphattanabawon *et al.* (2010b)
Unicorn leatherjacket (*Aluterus monoceros*)	Treat with 0.2 M acetic acid (GAA) or 0.2 M phosphoric acid (GPA)	Distilled water at 45 °C for 4 and 8 h.	GAA: 5.23-9.18% GPA : 6.12-11.54% (wet weight basis)	GPA: 121.92-149.77 g GAA: 51.73-56.90	Ahamd and Benjakul (2010)
Cuttlefish (*Sepia officinalis*)	1). Soak the skin in 0.05 M NaOH (1:10) with stirring for 2 h at room temperature. Change solution every 30 min. Wash the alkaline-treated skins with tap water until neutral pH wash water was obtained. 2). Soak alkaline-treated skins in 0.2 M acetic acid (1:10 w/v). Stir the mixtures for 48 h at 4°C. Adjust pH to 7.5 using 10 M NaOH.	Water at 50 °C for 18 h with continuous stirring.	2.21-7.84% (based on wet weight)	181 g	Balti *et al.*, (2011)

Table 5: (Continued)

Fish species	Pretreatment	Extraction condition	Yield	Bloom strength	References
Saithe (*P. virens*)	Soak the skin in 0.1 M NaOH (1:10 w/v) for 24 h with gentle shaking followed by washing with MILLLI-Q water. Bleach skins in 1% (v/v) H_2O_2 with ratios of 1:10 (w/v) for 30 min with gentle shaking. Wash bleached skins with MILLI-Q water.	0.01 and 0.1 M acetic acid for 12,18 and 24 h at 22, 45 and 65 °C.	8.9% (on wet weight basis)	-	Eysturskrə *et al*. (2009)
Red tilapia (*O. nilotica)*, walking catfish (*C. batrachus*) and striped catfish *(P. sutchi fowler*	Soak the skin in the saturated lime solution [$Ca(OH)_2$] (1:2) at the concentration of 27 g L^{-1}, at 20 °C for 14. Wash with abundant tap water (1:10) to remove excessive $Ca(OH)_2$.	Distilled water at 48 °C for over night.	Red tilapia :39.97 % Walking catfish: 32.06% Striped catfish: 26.23%	Red tilapia:384.9g Walking catfish: 238.9g Striped catfish: 147.4g	Jamilah *et al*. (2011)
Carps (*Cyprinus carpio*)	Mix skins with 0.1 M NaOH (1:8 w/v) for 6 h with continuous stirring. Change alkali solution every 3 h. Wash with cold distilled water until neutral pH of washing water was obtained. Soak the skins in 10% butyl alcohol with a solid/solvent ration of 1:10 (w/v) overnight to remove fat, and then wash with cold distilled water repeatedly.	Distilled water at 60, 70, and 80 °C using solid/distilled water ratio of 1:15 for 4 h.	Winter carp skin: 48.2-55.1% Summer carp skin: 47.6-55.8%	Winter carp skin: 30-61 g Summer carp skin: 46-76 g	Duan *et al*. (2011)

1.2.2 Biodegradable film

Biodegradable film and coating have been received the increasing attention owing to their biocompatibility and alternative packaging to synthetic polymers or plastics. Almost food packagings are generally made from plastics, which are non-biodegradable synthetic polymers and have the negative impact on environment (Kester and Fennema, 1986; Krochta and De Mulder-Johnston, 1997). Biodegradable or compostable packaging is preferable to recyclable packaging because recyclable packaging, though better than non-recyclable packaging, still requires external energy to be provided to bring about the recycling process. Nevertheless, biodegradable or compostable packaging is difficult to be recycled (Cuq *et al.*, 1995; Guilbert and Gontard, 1995; Guilbert, 2002). Over the last decade, there has been a widespread interest in films made from renewable and natural polymers which can degrade naturally and more rapidly than petroleum-based plastics. Among all biopolymers, proteins have been paid increasing attention as a potential material for biodegradable films and coating.

Biopolymeric materials used for biodegradable films can be divided into 4 categories: biopolymer hydrocoilloids (proteins and polysaccarides), lipids, resins and composites (Krochta *et al.*, 1994). Physical and chemical characteristics of the biopolymers greatly influence the properties of resulting films and coatings (Sothornvit and Krochta, 2000). In general, plasticizers are required to increase the flexibility of film by lowering the extension between polymers. Films can be incorporated with other additives for different purposes (Table 6).

Table 6. Materials used for edible films and coatings.

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

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

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

1.2.2.1 Proteins as film forming material

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

1.2.2.2 Composite biopolymer as film forming materials

Biopolymer composites can modify film properties and create desirable film structures for specific applications. Similar to multi-layered composite plastic films, biopolymer films can be produced as multiple composite layers, such as protein coatings (or film layers) on polysaccharide films, or lipid layers on protein/polysaccharide films. This multi-layered film structure optimizes the characteristics of the final film. Composite films can also be created by mixing two or more biopolymers, yielding one homogeneous film layer (Debeaufort *et al.*, 1998; Were *et al.*, 1999; Yildirim and Hettiarachchy, 1997). Various biopolymers can be mixed together to form a film with unique properties that combine the most desirable attributes of each component (Wu *et al.*, 2002). Different blend/composite materials for film formation are shown in Table 7.

Table 7. Different blend/composite materials for film formation

Composite/blend materials	Ratio	References
Gelatin/Soy protein isolate (SPI)	SPI:Gelatin (0, 25, 50, 75, 100% [w/w])	Denavi *et al.* (2009)
Gelatin/Sunflower oil	Sunflower oil: 0. 0.3, 0.6 and 10% of gelatin	Pérez-Mateos *et al*. (2009)
†Gelatin/Corn oil	-	Wang *et al*. (2009)
†Gelatin/poly(vinyl alcohol)	-	Carvalho *et al*. (2009)
Gelatin/Nanoclay	Nanocaly 5% (w/w) of gelatin	Bae *et al.* (2009)
Whey protein isolate (WPI) /Mesquite gum (MG)	WPI to MG (100:0, 75:25,50:50, 25:75, 0:100)	Osés *et al*. (2009)
Gelatin/PVA	10, 20, 30 and 40 g of PVA/100 g of macromolecules (gelatin+PVA)	Mendieta-Taboada *et al*. (2008)
Gelatin/ Soy protein isolate (SPI)	SPI to gelatin (10:0, 8:2, 6:4, 4:6, 2:8, 0:10).	Cao *et al*. (2007)
Gelatin/Gellan and κ-carrageenan	Gellan and κ-carrageenan at 1 and 2 g/100 g of gelatin, respectively.	Pranoto *et al*. (2007)
Konjac glucomannan (KGM)/Gelatin (GEL)	KGM/GEL: 1/9, 2/8, 3/7, 4/6, 5/5, 6/4, 7/3, 8/2 and 9/1 (w/w)	Li *et al*. (2006)
Gelatin/casein	Casein–gelatin blend (100:0, 75:25, 50:50, 25:75 and 0:100)	Chambi and Grosso (2006)
Sodium caseinate (NaCas) / whey protein isolate (WPI)	NaCas/WPI 100:0, 75:25,50:50, 25:75, 0:100	Longares *et al*. (2005)
Gellan/Gelatin	Gellan to gelatin (100:0, 80:20, 60:40, 40:60 and 20:80 (V))	Lee *et al*. (2004)
Soy protein isolate (SPI)/ Carboxymethylated konjac glucomannan (CMKGM)	5% wt SPI 2 wt % CMKGM	Tang *et al*. (2003)

†Response surface methodology

1.2.2.3 Plasticizers

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

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

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

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

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

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

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

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

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

1.2.2.4 Film formation processes

There are two categories of film formation processes; dry and wet (Guilbert *et al.*, 1997) (Figure 3). The dry process of edible film production does not

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

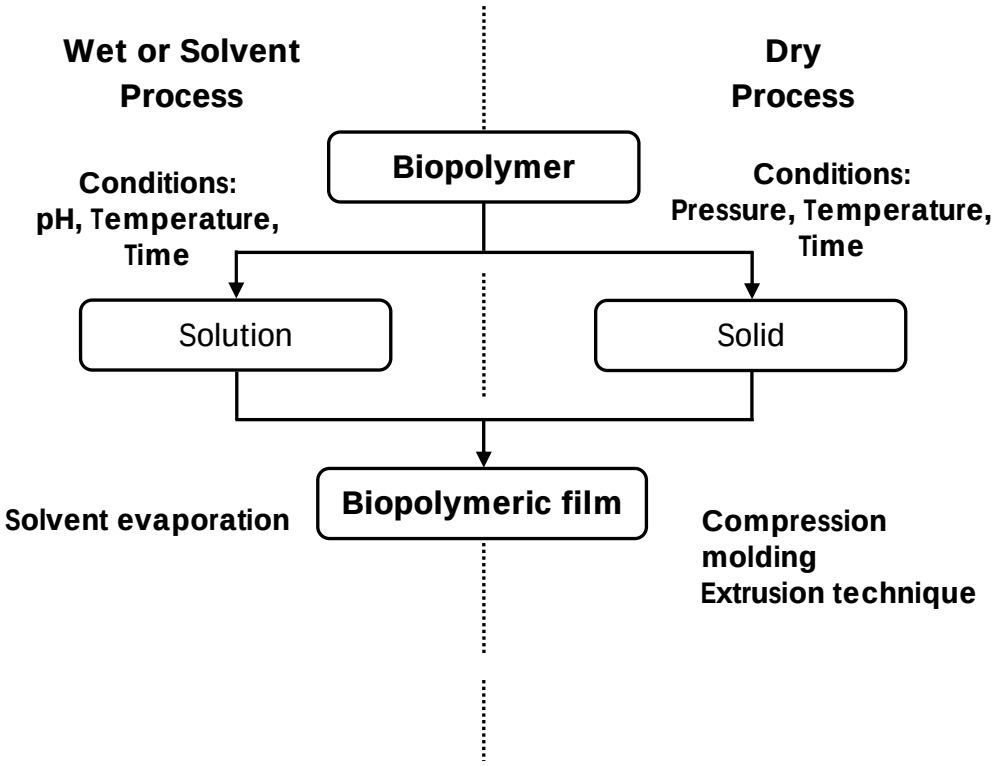


Figure 3. Wet (or solvent) and dry process for film preparation
Source: Adapted from Guerrero *et al.* (2010)

1.2.2.5 Mehanism of protein film formation and its properties

Protein based-film can be formed in three steps (Figure 4) (Marquie and Guilbert, 2002):

1. Intermolecular bonds (non-covalent and covalent bonds) that stabilize polymers in their native forms are broken by using chemical or physical rupturing agents (by solubilization or thermal treatment). Polymer chains become mobile.
2. Mobile polymer chains arrange and orient in the desired shape.
3. New intermolecular bonds and interactions are formed 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 (Cuq *et al*., 1995).

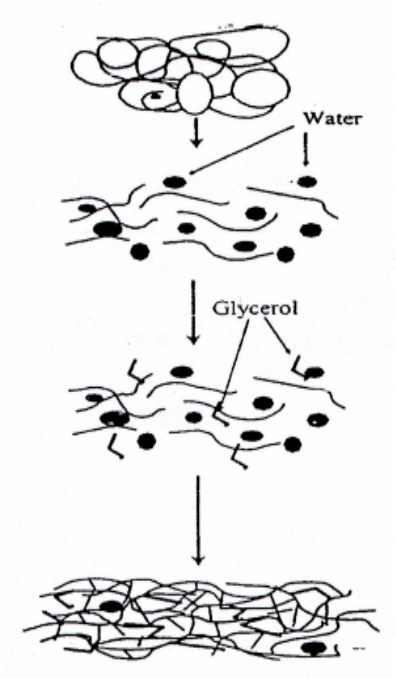


Figure 4. Mechanism of film formation
Source: Adapted from Marquie and Guilbert (2002).

1.2.3 Gelatin films

Gelatin films have been used in various fields such as the delivery system for a wide range of medicine. In food industry, it has been used as a sausage casing component or coating materials (Donhowe and Fenema, 1994). Gelatin has been attracted the attention for the development of edible films due to its abundance, biodegradability and uniquiness of film forming ability (Bigi *et al.*, 2002; Jongjareonrak *et al.,* 2006b). Gelatin-based films with various additives have good potential for applications in a number of integrated optics devices such as holographic recording materials (Iwamoto *et al*., 1999; Arvanitoyannis *et al*., 1998).

Protein content, plasticizers type and concentration have been reported to affect the properties of gelatin based films (Vanin *et al*., 2005). TS of gelatin film from shark (*Prionace glauca*) skin were affected by the protein concentration (1, 2 and 3%) of the film-forming solution (FFS). TS of the film from a 2% protein FFS was the highest. EAB and water vapor permeability (WVP) increased with increasing FFS protein concentration. The addition of glycerol improved flexibility and enhanced the UV barrier property at 280 nm. However, transparency at the visible range and WVP increased with increasing glycerol content (Limpisophon *et al.*, 2009). Sobral *et al*. (2001) studied the effects of sorbitol content on mechanical, water vapor barrier and thermal properties of edible films based on bovine hide gelatin (BHG) and pig skin gelatins (PSG). Increased sorbitol content (from 15 to 65 g sorbitol/100 g gelatin) decreased the puncture force from 16.0 to 8.2 N and from 16.2 to 9.0 N, for the films of BHG and PSG, respectively. The increase of sorbitol content from 15 g to 65 g sorbitol/100 g gelatin content increased the puncture deformation of both BHG and PSG films from 1.2 to 5.3% (Sobral *et al*., 2001).

TS of gelatin film from the skin of brownstripe red snapper (*Lutjanus vitta*) and bigeye snapper (*Priacanthus macracanthus*) decreased with increasing glycerol concentration from 25 to 75% (Jongjareonrak *et al.,* 2006b). Cuq *et al*. (1997) reported that the proteins network becomes less dense, and consequently more permeable with the plasticizer incorporation. The increase in free volume of the system also raises the solvent mobility, thereby increasing the water diffusion in the matrix of the film (Cuq *et al*., 1997). Moreover, at the same plasticizer concentration, fish skin gelatin from the two different species plasticized with glycerol (Gly) showed the greatest EAB whereas ethylene glycol (EG) plasticized film showed the highest TS ($p<0.05$) (Jongjareonrak *et al.,* 2006c). Cao *et al*. (2009) also studied the effects of different kinds of plasticizer including oligosaccharide-sucrose, some organic acid and polyethylene glycol (PEG) with different molecular weight (300, 400, 600, 800, 1500, 4000, 10000, 20000) where PEG of lower molecular weights exhibited better plasticizing effects for gelatin film and such films had better visual properties. In addition, among the four polyols (glycerol—GLY, propylene glycol—PPG, di- DTG and ethylene glycol—ETG) used as plasticizer with five different concentrations: 10, 15, 20, 25, and 30 g plasticizer/100 g of gelatin, GLY showed the greater plasticizing

effect and the higher concentrations yielded the film with more flexibility (Vanin *et al.*, 2005). Andreuccetti *et al.* (2011) studied the plasticizing effect of natural surfactants lecithin or yucca extract from *Yucca schidigera* on gelatin-based films. Films containing yucca extract showed higher tensile strength values (~90–40 MPa) and moisture contents (~15%) and less elongation (~5%) and water vapor permeability values (~0.22–0.09 g mm m^{-2} h^{-1} kPa^{-1}), compared to films containing lecithin.

The addition of plasticizer alters some functional and physical properties. The plasticizer causes no apparent tendency to re-crystallization in the film structure but alter the physical properties such as flexibility, interaction between macromolecular chains and susceptibility to humidity (Bergo and Sobral, 2007). The increase in the plasticizer concentration increases the moisture content of the film because of its high hygroscopic character, which also contributes to the reduction of the forces between the adjacent macromolecules. The water vapor permeability of the PSG and BHG films increased linearly from 1.8 to $3.2x10^{-8}$ $g.mmh^{-1}cm^{-2}Pa^{-1}$, and from 1.7 to $3.8x10^{-8}$ $g.mmh^{-1}cm^{-2}Pa^{-1}$, respectively, with an increased sorbitol content from 15 to 65 g sorbitol/100 g gelatin (Sobral *et al.*, 2001). The increase in total plasticizer content (water, glycerol, sorbitol and sucrose) in the polymer matrix was found to result in a proportional increase in water vapor transmission rate (Arvanitoyannis *et al.,* 1998). Andreuccetti *et al.* (2009) reported that gelatin-based films using hydrophobic plasticizers derived from citric acid and soy lecithin as an emulsifier had the increase in tensile strength values (TS) from 36 to 103 MPa. However, the increase in the concentration of plasticizers (acetyltributyl citrate and tributyl citrate) reduced TS by 57% and no relation was observed between plasticizer quantities and the elongation in the quantities tested. TS of film generally decreased with the addition of fatty acid (FA), while gradually increased with increasing amount of fatty acid sucrose esters (FASE) (Jongjareonrak *et al.,* 2006c). Cao *et al*. (2009) studied different plasticizers: oligosaccharides – sucrose, and some organic acids such as oleic acid, citric acid, tartaric acid, malic acid (MA) on the properties of film from bovine bone (type B) gelatin. Only MA could increase the ductility of gelatin film, and the visual appearance of MA modified gelatin film was better. Addition of hydrophobic substances including amaranth oil, rapeseed oil, lanolin, beeswax and

ozococerite at a concentration of 10% decreased WVP of gelatin films by 42, 15, 37, 53 and 36%, respectively. Increasing concentration of these substances up to 60% as emulsifiers caused further improvement of the water barrier properties. The highest decrease of WVP was found in case of amaranth oil and beeswax in which the decreases by 73 and 87% were found respectively, in comparison to the control gelatin films. WVP of chemically modified films in the presence of 60% of beeswax with addition of lecithin was decreased by about 65% (Sztuka and Kolodziejska, 2009). WVP was minimum for films containing 20 g glycerol/100 g gelatin, while flexibility increased from 2.2% to 180.9% and T_g shifted from 137.5 to 21.3 °C, for films plasticized with 80 g glycerol/100 g gelatin (Rivero *et al*., 2011).

The edible films plasticized with 30 g sorbitol /100 g gelatin from Atlantic halibut (*Hippoglossus hippoglosus*) skin, with an intermediate evaporation step at 60 °C in the drying procedure, were transparent, weakly colored, water-soluble and highly extensible. The intermediate evaporation step at 60 °C induced thermal protein degradation, causing the film to be significantly less resistant and more extensible (Carvalho *et al.,* 2008). The DSC traces obtained in the first scan of BHG and PSG films with 15- 35 g sorbitol/100 g gelatin showed a well visible glass transition followed by a sol-gel transition. However, with the increase of sorbitol concentration more than 35 g sorbitol/100 g gelatin, the glass transition become broader, typical characteristic of the system presenting a phase separation (Sobral *et al*., 2001).

Film from bigeye snapper skin gelatin contained the lower content of high molecular weight cross-links with the concomitant increased degradation peptides, compared with the gelatin powder and film from brownstrip red snapper skin gelatin. The addition of 10 and 20 mM EDTA, and 0.01 and 0.1 mM soybean trypsin inhibitor into the film forming solution (FFS) of bigeye snapper skin gelatin totally inhibited and mostly retarded the degradation of gelatin components, respectively (Jongjareonrak *et al.,* 2006b).

1.2.4 Improvement of the property of protein-based film

Chemical, physical and enzyme treatment can be applied to modify the polymer network through the cross-linking of the polymer chains to improve the properties of protein film (Mahmoud and Savello, 1993; Yildriim and Hittarachchy, 1998; Marquie, 2001). Cross-linking agents are natural and synthetic molecules containing at least two reactive groups that are able to form a covalent inter- and /or intra molecular links between protein chains. These agents, when used to prepare protein based-films, strengthen the materials through the formation of new covalent bonds, while reducing film elasticity and solubility in water (Ginnadois and Weller, 1992). To improve the properties of protein film, different chemicals and enzyme with protein cross-linking property including gossypol, formaldehyde, glutaralaldehyde (de Carvalho & Grosso, 2004), glyoxal (Nuthong *et al*., 2009a), transglutaminase (Mariniello *et al*., 2003), physical (Ouattara *et al*., 2002; Jo *et al*., 2005), phenolic compounds (Nuthong *et al*., 2009b; Cao *et al*., 2007), plant or herb extracts (Gómez-Estaca *et al*., 2009a, 2009b) and seaweed extract (Rattaya *et al*., 2009) have been used.

1.2.4.1 Use of aldehydes

Chemical modification has been reported to affect the properties of protein film. With formaldehyde modification, a slight reduction in water vapor permeability (WVP) in gluten protein based films (Micard *et al*., 2000) and film based on soy protein (Ghorpade *et al*., 1995) was found. The action of formaldehyde was more pronounced in lowering the WVP than that of glyoxal in gelatin based films (De Carvalho and Grosso, 2004).

Formaldehyde**,** the simplest cross-linking agents, has the broadest reaction. In addition to amino group of lysine, it reacts with the side chains of cysteine, tyrosine, hidtidine, tryptophan and arginine (Tae, 1983). Audic and Chaufer (2005) showed that chemical cross-linking between formaldehyde and free amino acid groups of sodium caseinate caused the increase in water resistance of triethanolamine (TEA) plasticized films. Although formaldehyde contains a single functional group, it

can react bifunctionally and therefore effectively crosslinks the proteins (Audic and Chaufer, 2005).

The variation in mechanical properties of the formaldehyde modified films depended on the ability of proteins to form chemical and physical bonds simultaneously, and the final effect of these bonds on the orientation of the protein (Fakirov *et al.,* 1996). Wheat gluten based films treated with formaldehyde showed an increase in tensile strength (TS) but a decrease in elongation at break (EAB) as compared to the untreated control films (Micard *et al*., 2000). The addition of formaldehyde did not alter the mechanical property of pea protein isolate films, whereas treatment by immersion of the protein films in an ethanol formaldehyde mixture markedly increased both TS and hydrophobicity character (Geguen *et al*., 1998).

Formaldehyde can be directly added to soy protein isolate film-forming solution or it can be applied by the immersing dried soy protein isolate films into formaldehyde solutions (Rhim *et al*., 2000). Treatment with formaldehyde resulted in the increase in TS and puncture strength by two-fold, but reduced WVP (by about 6%) and water solubility (by about 42%) of soy protein isolate films. The oxygen permeability of the formaldehyde-treated soy protein isolate films increased slightly (Ghorpade *et al*., 1995). Galietta *et al*. (1998) reported that formaldehyde enhanced the mechanical properties and decreased the solubility in water of whey protein isolate films. Formaldehyde and glutaraldehyde addition caused a significant increase in TS of peanut protein films, compared to the control films (Liu *et al*., 2004). The WVP and oxygen permeability of the films decreased after aldehyde treatment. The ability of formaldehyde and glutaraldehyde to promote covalent intermolecular cross-linking of peanut protein film was therefore effective to increase the mechanical and barrier properties of the films.

Glutaraldehyde is more specific than formaldehyde. It can react with lysine, cysteine, histidine and tyrosine. Protein cross-linking by glyoxal involves the reaction with lysine and arginine side chain groups (Marquie and Guilgert, 2002). Bigi *et al*. (2001) studied the influence of glutaraldehyde (GTA) concentration on the stability of GTA –cross-linked gelatin films. Air-dried gelatin films were subjected to

treatment with GTA solution at concentration ranging from 0.05 to 2.5%. At the smallest GTA concentration, the crosslinking degree, determined by trinitrobenzensulfomic acid (TNBS) assay, was about 60% and increased up to values near 100% with GTA concentration ≥ 1wt%. A cross-linking degree of about 85% obtained by using 0.25% GTA, was enough to prevent gelatin release in buffer solution and to provide a significant reduction of the swelling in solution. Furthermore, cross-linking greatly increased the thermal stability of the films (Bigi *et al*., 2001).

In addition, fish gelatin films from pollock and salmon skin were cross-linked with glutaraldehyde at the concentration of 0.25%, 0.50%, and 0.75% (w/w). The addition of cross-linkers had little effect on tensile properties and melting temperatures of fish gelatin films (Chiou *et al.,* 2008). Pollock gelatin films had the lower water vapor and oxygen permeability values than salmon gelatin films. Cross-linking resulted in lower water vapor permeability for salmon gelatin films and higher oxygen permeability for pollock gelatin films (Chiou *et al*., 2008). The films were prepared from gelatin solutions at different concentrations (5, 10 and 15 wt.%) with different amounts of oxidized alginate (0, 1 and 3 wt.% with respect to the weight of gelatin). The extent of crosslinking increased as dialdehyde alginate (ADA) concentration increased (Boanini *et al*., 2010). The mechanical properties of film from bovine hide gelatin (type B) were significantly affected by treatment with formaldehyde, where an increase of approximately 60% with respect to tensile strength was observed for gelatin based film. Greater thermal stability, with an increase in the melting point, was observed for the chemically (glyoxal and formaldehyde) treated films from bovine hide gelatine indicating a greater degree of crosslinking, as confirmed by the number of free α-amine groups remaining after the modification reactions (de Carvalho and Grosso, 2004). The incorporation of glyoxal resulted in the increase in protein cross-links stabilized by both disulfide and non-disulfide covalent bonds (Nuthong *et al*., 2009a).

1.2.4.2 Use of transglutaminase

Transglutaminase catalyzes the formation of intermolecular and intramolecular covalent bonds between and within protein chains, resulting in a broad

size distribution of protein molecules (Amoumahmoud and Savello, 1990). Transglutaminase-catalyzed reactions which cross-link soybean 11S globulin and whey protein isolate, gave bioplymers with improved functionality (Yildirim *et al*., 1996). Cross-linking of proteins provided biopolymers with improved heat stability. Yildirim and Hettaracchchy (1998) compared the properties of transglutaminase cross-linked whey protein isolate films, soybean 11S and a mixture of these two protein (1:1, w/w). TS values of films added with 0.2 units TGase/g were two-fold higher than the control films at pH 3, 4, 6 or 8. Fish gelatin-chitosan film modified with TGase at a concentration of 0.2 mg/l of the film forming solution decreased the solubility of the films at 25 °C from 65% to 28% at pH 6 and from 96% to 37% at pH 3. The gelatin based films modified with TGase, glyxoal and formaldehyde had 20% reduction in solubility for all modified films (de Carvalho *et al.*, 2004). Furthermore, the solubility at pH 3 and 6 of TGase-modified fish skin gelatin film was decreased from 100% to 30%. The water barrier properties of film were not improved by cross-linking of gelatin either with TGase or with EDC. Water vapor permeability of TGase-modified films slightly increased with the increase in glycerol concentration. Plasticization of films cross-linked with EDC affected water vapor permeability into a higher degree. Elongation of enzymatically modified films containing 22.5% of glycerol was about 42% (Piotrowska *et al*., 2008). A substantial increase in the high molecular weight protein components was observed in the film forming solution of gelatin and casein cross-linked with TGase (Chambi and Grosso, 2006). A nanoclay composite film was produced using warm water fish gelatin as a base material with microbial transglutaminase treatment. SDS–PAGE results indicated that the molecular weight of fish gelatin solutions increased after treatment with microbial transglutaminase. Tensile strength decreased from 61.60 (0 min) to 56.42 MPa (30 min), while E% increased from 13.94 (0 min) to 15.78% (30 min) when 2% (w/w) MTGase was added. The oxygen permeability and water vapour permeability were not changed as a function of time after treatment with 2% (w/w) MTGase concentration (Bae *et al.*, 2009). The mechanical resistance of the chitosan-ovalbumin films increased from 24 to 35 MPa and barrier efficiency toward water vapour was slightly improved due to transglutaminase-mediated cross-linking (Pierro *et al*., 2007).

1.2.4.3 Use of Phenols

Polyphenols can interact non-covalently or covalently with proteins. Under non-oxidizing conditions, non-covalent interactions including hydrogen bond and hydrophobic forces stabilize tannin-protein complexes (Chen and Hagerman, 2004). The non-covalent interaction may occur between polyphenols and proteins by hydrogen bonding and by hydrophobic bonding. In some cases, ionic bonding may be possible. Covalent attachment can occur depending on the polarity of the polyphenol (Siebert *et al*., 1996; Hagerman *et al*., 1998; Suryaprakash *et al*., 2000; Rawel *et al*., 2003). The interaction may occur via multisite interaction (several phenolic compounds bound to one protein molecule) or multidentate interaction (one phenolic compound bound to several protein molecules). The type of interaction depends on the type and the molar ratio of both phenolic compound and protein (Prigent *et al*., 2003). Tannin can interact non-specifically with proteins such as bovine serum albumin (BSA) or specifically with protein such as gelatin (Frazier *et al*., 2003). Tannins already bound to protein have an effect on new binding interaction (Frazier *et al*., 2003).

Polyphenols interaction with tryptophan is most likely due to the partial positive charge present at the –NH group of the indole moiety (Suryaprakash *et al*., 2000). Lysine as well as arginine, has hydrophobic section in the side chain which may take part in the interaction. In addition, lysine has positive charge near neutral pH, leading to interaction with the carboxylate group or the aromatic ring of the polyphenols (Surayaprakash *et al*., 2000). Charlton *et al*. (2002) concluded that polyphenols bind through hydrophobic interactions reversibly and relatively weakly at each individual binding site. Procyanidin interaction with proteins increased significantly with the increasing degree of polymerization. Procyanidins tends to have poor affinity for small, tightly folded globular proteins (myoglobin, lysozyme, BSA), whereas they bind preferentially to proteins that have either random-coil or collagen-like helical conformations (Hagerman and Bulter, 1981; de Freitas and Mateus, 2001). The interaction with globular proteins probably involves only surface-exposed aromatic rings, whereas face-to-face stacking of aromatic rings with proline residues are involved with proline-rich proteins (Williamsons, 1994).

The covalent binding of the phenolic compounds to the proteins occurs through oxidized phenolic substances (quinones). Quinones are formed when 1, 2-dihydroxy or 1,2,3-trihydroxy phenolic groups are oxidized (Loomis, 1974). Quinones can bind covalently to neucleophilic functional groups of protein, such as lysine, methionine, histidine, cystine, tyrosine, and tryptophan residues, limiting the digestibility of the protein molecule (Hurrell *et al*., 1982; Carbonaro *et al*., 1996; Rawel *et al*., 2001). The formation of the quinone-protein complexes, as well as formation of the other polyphenol-protein complexes, can lead to polymerization (Loomis *et al*., 1974). Quinones are electrophilic and can form cross-links with either sulfhydryl or amino groups of proteins (O'Connell and Fox, 2001). The protein-quinone interaction can change the isoelectric point of protein to lower pH values due to the introduction of carboxylic groups following the covalent attachment of the phenolic acids, and by the parallel blocking of the lysine residues in protein (Rawel *et al*., 2001).

Principally, five potential types of interaction of phenolics and protein can be proposed: hydrogen bonding, π-bond and hydrophobic, ionic and covalent linkages (Hagermen, 1992; Bianco *et al*., 1997). In order to function as an anti-oxidant, the phenolic compound must be capable of forming a resonance stabilized phenoxyl radicals, which in turns can react with other available radicals (e.g. peroxyl radicals), consequently deactivating them (Robards *et al*.1999; Pedrielli *et al*., 2001). The reactivity of the phenolic compounds has been shown to increase with the higher number of hydroxyl groups, and the reactivity depends also on the position of the hydroxyl groups (Rawel *et al*., 2001).

In addition, the quinones can react very efficiently with lysine residues in proteins (Figure 5) (Hurrell *et al*., 1982; Rawel *et al*., 2001). Phenol-protein complexes formed under oxidizing conditions are more stable than those formed in the absence of oxidants (Chen and Hagerman, 2004). In addition, quinones can oxidize the functional groups of the proteins (Lommis, 1974) or the semiquinoone radical can react with proteins (Hagerman *et al*., 2003).

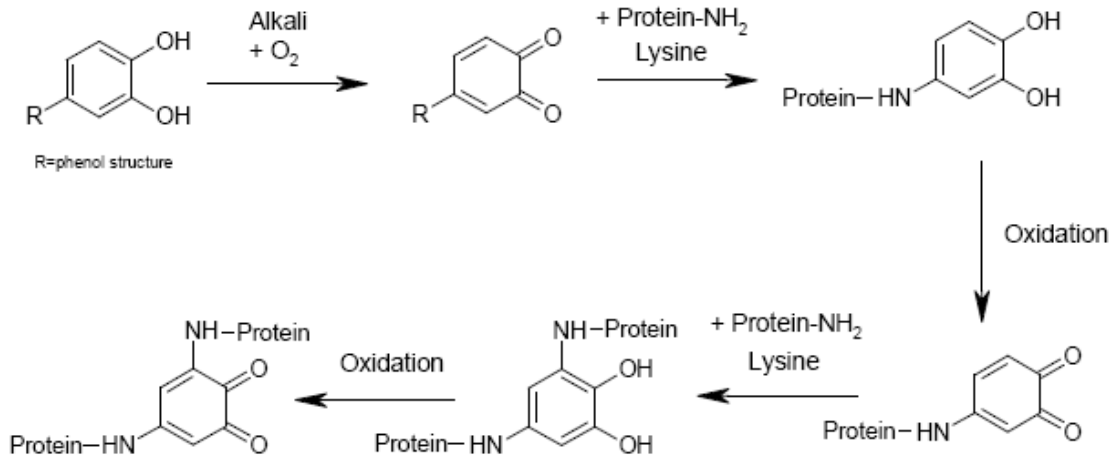


Figure 5. Interaction reaction between lysine side chain of the protein and quinone of polyphenols, leading to polymerization or complex formation.

Sourece: Rawel *et al*. (2001).

The mechanical properties of protein-based films can improve by various phenols. Ferulic acid and tannin acid extracted from plant were able to cross-link gelatin films (Cao *et al*., 2007a). Ferulic acid is a ubiquitous phenolic acid of low toxicity in the plant kingdom. It has such functions as antioxidant, antimicrobial, anti-cancer, lower cholesterol, cross-linking activities, etc. Ferulic acid and its oxide, quinoid ferulic acid, can react with some amino acids in proteins such as tyrosine, lysine, and cystine, leading to the cross-linking of protein molecules (Ou *et al.*, 2004). An optimal concentration of ferulic acid increased the tensile strength, elongation percentage at break and antioxidant activity of soy protein isolate films. The properties of the film were further improved when ferulic acid was oxidized by hydrogen peroxide (Ou *et al*., 2005). The role of ferulic acid in the preparation of SPI based films is that it reacted with amino acids and increased cross-linking of the protein.

In addition to polyphenolic plant extracts, there has also been growing interest in using other natural extracts as protein cross-linker and antioxidant in food systems. Fish skin gelatin films incorporated with seaweed extract at pHs 9 and 10 exhibited the higher elongation at break (EAB) than the control film ($p < 0.05$).

However, no differences in tensile strength (TS) and transparency between films without and with seaweed extract were observed ($p>0.05$). Water vapor permeability (WVP), and film solubility decreased as seaweed extract was incorporated, regardless of pH ($p<0.05$). This was associated with the formation of non-disulfide covalent bond in the film matrix, most likely induced by the interaction between oxidized phenols in seaweed extract and gelatin molecules (Rattaya *et al*., 2009). The polyphenol–protein interaction was found to be more extensive, when tuna-skin gelatin film was employed with oregano and rosemary extracts than bovine-hide gelatin film. Oregano extracts showed the higher total phenolic content and antioxidant activity of film (Gómez-Estaca *et al*., 2009a). The bovine-hide gelatin reacted only slightly with the polyphenols in both the oregano and the rosemary extracts. As a consequence, the attributes (mechanical properties, water solubility, and water vapour permeability) of the films were practically unchanged, compared with the film made without any added plant extract. The tuna-skin gelatin underwent the interactions with the polyphenols in both extracts, thereby altering the attributes of the corresponding films, namely, a higher glass transition temperature, decreased deformability, and increased water solubility (Gómez-Estaca *et al.*, 2009a).

The molecular weight distribution of the tuna-skin gelatin exhibited appreciably higher quantities of β-components (covalently linked α-chain dimers), whereas bovine-hide gelatin showed a certain degradation of α_1-chains being indicative of a greater proteolysis. The incorporation of the borage extract into the films gave rise to a pronounced increase of their antioxidant properties irrespective of the gelatin origin from sole skin gelatin or commercial fish skin gelatin. Minor modifications of their physico-chemical properties decrease of the breaking force and increase of film opacity were observed with the addition of borage extract (Gómez-Estaca *et a*l., 2009b). WVP of the film was significantly different when the aqueous extract of two ecotypes of murta (*Ugni molinae Tuecz*) leaves (Soloyo Grand “SG” and Soloyo Chico “SC”) were used to investigate the antioxidative activity. The addition of murta extracts led to transparent films with increased protection of UV light as well as antioxidant activity (Gómez-Guillén *et al.*, 2007).

Properties of porcine plasma protein-based film incorporated with tannic acid, caffeic acid and ferulic acid at different concentrations (1–3% (w/w) of protein content) were studied. Tensile strength (TS) of resulting film increased by 123.3, 194.3 and 19.5% and elongation at break (EAB) increased by 71.1, 86.3 and 10.2%, respectively, compared with the control film, when tannic acid, caffeic acid and ferulic acid at a level of 3% was added (Nuthong *et al*., 2009b). The use of all phenolic compounds slightly increased water vapor permeability (WVP) of resulting films ($p < 0.05$). The increases in a*- and b*-values of films were observed as the higher concentrations of tannic acid and caffeic acid were used (Nuthong *et al*., 2009b). Considerably improved barrier properties were found for starch-chitosan blend films upon the incorporation of oxidized ferulic acid (Mathew and Abraham, 2008).

The formation of rigid molecular structures by reactions of ortho-quinones with proteins is well known. The diphenol moiety of a phenolic acid or other polyphenol (1) is readily oxidized to an orthoquinone, either enzymatically as in plant tissues, or by molecular oxygen. The quinone forms a dimmer (2) in a side reaction, or reacts with amino or sulfhydryl side chains of polypeptides to form covalent C-N or C-S bonds with the phenolic ring, with regeneration of hydroquinone. The latter can be reoxidized and bind a second polypeptide, resulting in across-link (3). Alternatively, two quinones, each carrying one chain, can dimerize, also producing a cross-link (4). Occurrence of such a cross-linking is supported by the identification of phenolic acid dimmers (Strauss and Gibson, 2004) (Figure 6).

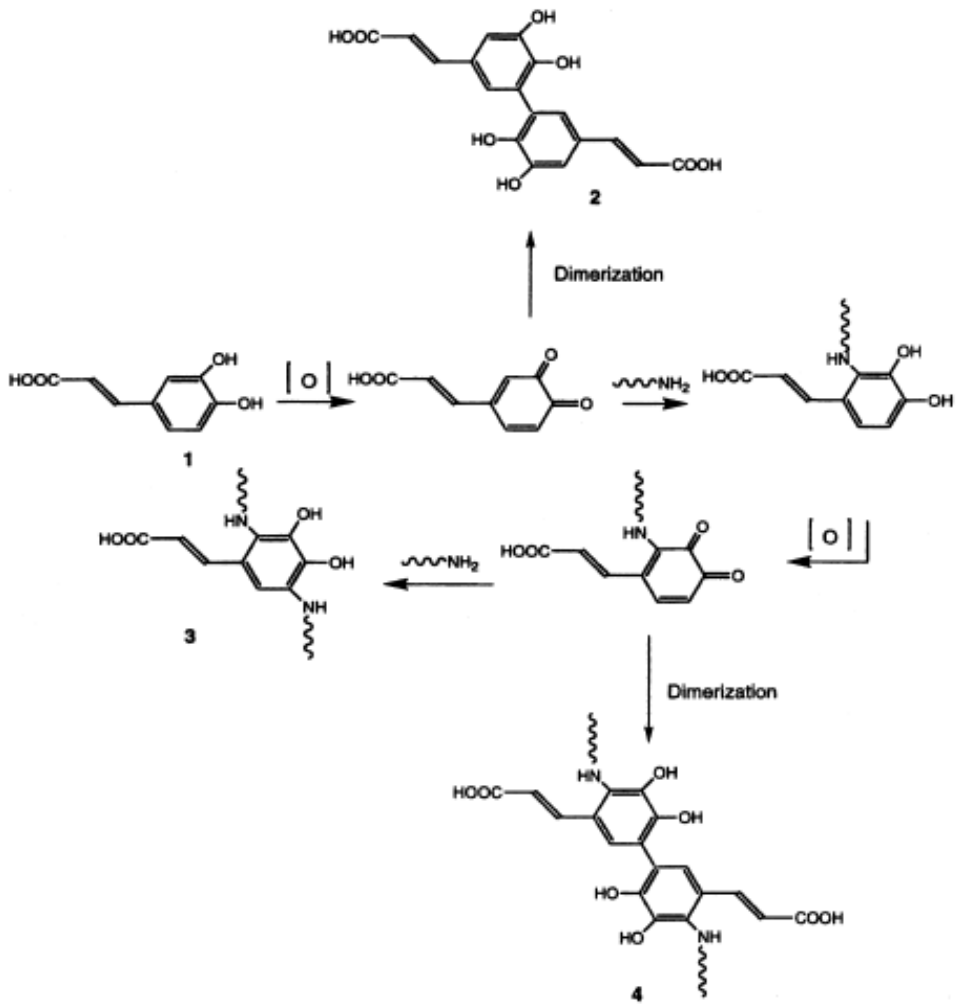


Figure 6. Reactions of a phenolic acid with amino side chains of polypeptides. Source: Strauss and Gibson (2004).

1.2.4.4 Protein based composite or blend film

Properties of gelatin film can be modified by addition of some polysaccharides. Modified starch shows a crucial role in improving the properties of protein-based film. Gelatin does not have ideal water vapor barrier properties. Thus, some chemical treatments can be applied to modify the polymer network through cross-linking of the polymer chains (Arvanitoyannis *et al.*, 1997; Cao *et al.*, 2007b). The structure and properties of SPI edible films were modified and improved by blending with carboxymethyl cellulose (CMC). Increasing CMC content improved the mechanical properties and reduced the water sensitivity of blend films (Su *et al.*, 2009) Parris and coffin (1997) reported that a reduction in WVP when polymeric

dialdehyde starch was added (at 20%w/w of zein) to zein films without the addition of plasticizers. However, addition of polymeric dialdehyde starch did not affect the WVP when glycerol or polypropylene glycols were added to the films. Gennadios *et al*. (1998) reported that TS and EAB increased significantly with increasing amounts of dialdehyde starch to egg white films (optimum dialdehyde starch level ~5% w/w of egg white), suggesting the formation of covalent cross-links between egg white proteins and dialdehyde starch. Soy protein isolate films containing dialdehyde starch at 5 or 10% w/w of soy proteins isolate had the increase in TS by about 20%, compared to the control soy protein isolate films (Rhim, 1998). Parris *et al*. (1995) reported that the films from protein polysaccharide blends (whey proteins and alginate or pectin) had lower WVP than those formed from protein alone. Shih (1994) reported that the poteitial use of propyleneglycol alginate (PGA) in improving water stability of multicomponent films by forming covalent complexes between PGA and soy protein. Rhim *et al*. (1999) reported that different levels (5, 10, 15, 17.5 or 20% w/w of solid) of PGA were incorporated in soy protein isolate (SPI) films to form biodegradable composite films with modified physical properties. Color of the SPI films was affected ($P<0.05$) by the incorporation of PGA. Tensile strength increased ($P<0.05$) with addition of PGA up to 17.5%, while the percent elongation at break decreased with incorporation of PGA of higher levels. WVP and water solubility also decreased by adding PGA up to 10%, but further addition of PGA increased values for these properties. Lee *et al*. (2004) reported that tensile strength (TS) of the gellan and gelatin composite films linearly decreased and tensile elongation (TE) increased with increasing gelatin ratio. Gelatin films cross-linked with genipin provoked a significant reduction of the swelling in physiological solution, and enhanced the thermal stability (Bigi *et al*., 2002).

Furthermore, a variable in pH of film forming solution may be also important factor influencing functional properties of biopolymer films (Anker *et al*., 2002; Banerjee and Chen, 1995). WVP decreased as the pH of the film forming solution was adjusted away from pH 7.0 or corn oil (CO) addition level increased above 27.25% of gelatin based biopolymer films (Wang *et al*., 2009). To improve the hydrophobic properties of the cod gelatin film, sunflower oil was added at levels of 0%, 0.3%, 0.6% and 15% and the resulting film had the decreased WVP (Pérez-

Mateos *et al*., 2009). Oleic acid, pure or mixed with beeswax, had a plasticizing effect in the sodium caseinate films, thereby increasing their elasticity, flexibility and stretchability, and reduced water vapor permeability (Fabra *et al.*, 2008). Moreover, the glycerol presence had negative effect on water vapor permeability value (increase of WVP) where wheat bran had a positive influence (decrease WVP) on wheat gluten film (Mastromatteo *et al*., 2008).

The films of vicilin-rich protein isolates exhibited much less mechanical strength (TS) and elongation at break (EB) but similar film surface hydrophobicity, as compared with those of the SPI film. The heating remarkably improved the TS of these films, and the extent of the improvement much higher than that of the SPI film (Tang *et al*., 2009). Composite films obtained from soybean-protein isolate (SPI) and cod gelatin containing different ratios of SPI: gelatin (0, 25, 50, 75, 100% [w/w]) and plasticized by a mixture of glycerol and sorbitol were prepared by casting. Regardless of the protein concentration, the thickness and water-vapor permeability of the composite films diminished significantly as compared to pure-gelatin films. The formulation containing 25% SPI: 75% cod-skin gelatin had the maximum force at the breaking point, which was 1.8-fold and 2.8-fold greater than those of 100% gelatin and 100% SPI films, respectively (Denavi *et al*., 2009). Cao *et al*. (2007b) reported that with increasing gelatin ratio in the SPI/gelatin composite films, tensile strength (TS), elongation to break (EB), elastic modulus (EM) and swelling property were increased. In addition, the films became more transparent, and easier to handle. When the ratio of SPI:gelatin was 4:6–2:8, TS, EB, and other properties of composite film approached those of gelatin film and were better than those of SPI film. The blend film of konjac glucomannan and gelatin was prepared successfully by using the solvent-casting technique with different blending ratios of the two polymers. The blend film had the best miscibility, a good tensile strength, heatseal and the least water–vapor transmission ratio at the same ratio. All the blend films showed a high water solubility (Li *et al*., 2006). The incorporation of increasing amounts of mesquite gum (MG) in the formulation of whey protein isolate (WPI) and mesquite gum (MG) mixture film resulted in the improved flexibility with significantly lower tensile strength and higher elongation at break. The use of MG

could improve mechanical properties of WPI films and could be used as an alternative of using larger amounts of low molecular weight plasticizers (Osés *et al*., 2009). Physical properties of biodegradable films based on blends of gelatin and poly(vinyl alcohol) (PVA) were governed by PVA hydrolysis degree and concentration (Carvalho *et al*., 2009). However, FFS based on blends of gelatin and poly(vinyl alcohol) (PVA) exhibited a Newtonian behavior at 30°C and the viscosity of FFS was not affected neither by PVA concentration nor by the plasticizer. PVA affected the viscoelastic properties of FFS by dilution of gelatin (Moraes *et al*., 2009). Tensile strength (TS) and elongation at break (EAB) of films was increased with increasing polyvinyl alcohol (PVA) content, when fish myofibrillar protein (FMP) from bigeye snapper (*Priacanthus tayenus*) to PVA were used at different ratio (FMP:PVA; 10:0, 8:2, 6:4, 5:5, 4:6, 2:8, 0:10) and pH levels (3 and 11) (Limppan *et al*., 2010). Moraes et al. (2009) also reported the effect of the PVA concentration and plasticizer presence on the flow behavior, and viscoelastic and thermal properties of FFS based on blends of gelatin and PVA.

Rivero *et al*. (2009) studied the composite, bi-layer and laminated biodegradable films based on gelatin and chitosan. Water vapor permeability (WVP) was independent of film thickness up to 120 μm for gelatin films and 60 μm for chitosan ones. Both bi-layer and laminated systems were the effective alternatives to reduce WVP of composite films (at least 42.5%). Kolodziejska *et al*., (2006) reported that the solubility of fish gelatin films and gelatin chitosan films (4:1, w/v) could be limited by cross-linking of the components with transglutaminase (TGase) or with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). The novel chitosan/gelatin membranes were prepared using the suspension of chitosan hydrogel mixed with gelatin. The morphology of these chitosan/gelatin membranes was found to be very smooth, homogeneous, and good compatibility and interaction between the chitosan and gelatin. The prepared chitosan/gelatin membranes showed good swelling, mechanical and thermal properties (Nagahama *et al*., 2009). The fish gelatin-chitosan films were more water resistant (~18% water solubility for tuna vs 30% for bovine) and more deformable (~68% breaking deformation for tuna vs 11% for bovine) than the bovine gelatine-chitosan films. The breaking strength of gelatin-chitosan films was higher than that of plain gelatin films irrespective to the gelatin origin. The

chitosan containing films exhibited antimicrobial activity against *Staphylococcus aureus*, a relevant food poisoning (Gómez-Estaca *et al.*, 2011). The tensile strength of chitosan-gelatin based composite and bi-layer system did not differ significantly, but elongation at break of composite films was 40% higher than that of bi-layer film. Both *E. coli* and *L. monocytogenes* showed sensitivity to all films forming solutions of Chitosan and Chitosan-Gelatin (Pereda *et al*., 2011). Addition of grapefruit seed extract (GSE) to the rapeseed protein-gelatin (RG) film inhibited the growth of pathogenic bacteria such as *Escherichia coli O157:H7* and *Listeria monocytogenes.* However, the film functionality of chitosan–whey protein blend might be compromised due to the incompatibility between the polysaccharide and protein components within the film matrix (Ferreira *et al*., 2009). Properties of gelatin based film from different fish species has been summerised in Table 8.

Table 8. Properties of gelatin based film from different fish species

Fish Species	Protein Conc.	Plasticizer conc.	Mechanical property		WVP ($x10^{-10}$g m^{-1} s^{-1} Pa^{-1})	References
			TS (MPa)	EAB (%)		
Bigeye snapper (*Priacanthus tayenus*) [1]	2% (w/v)	Glycerol- 50% (w/w) of protein	10.04 – 11.43	12.11 – 25.98	0.89 – 1.28	Rattaya et al. (2009)
Blue shark (*Prionace glauca*)	1 - 3% (w/v)	Glycerol- 50% (w/w) of protein	12.58 – 27.29	61.13 – 74.17	0.4 – 1.12	Limpisophon et al. (2009)
	2% (w/v)	Glycerol- 0, 25 & 50% (w/w)	23.30 – 45.90	1.57 – 80.40	0.71 – 1.05	
Bigeye snapper (*Priacanthus marcracanthus*)	1-4 % (w/v)	Glycerol - 25% (w/w)	28.28 and 44.28	2.67 and 7.0	1.22 and 1.37	Jongjareonrak et al. (2006b)
Brownstripe red snapper (*Lutjanus vitta*)	1-4 % (w/v)	Glycerol- 25% (w/w)	41.09 and 58.09	7.02 and 8.20	1.33 and 1.35	
	3% (w/v)	Glycerol- 0, 25, 50 & 75% (w/w)	18.80 – 67.78	5.24 – 95.40 [b]	1.32 – 2.28	
Giant squid (*D. gigas*) [2*]	Gelatin (4% w/v)	Glycerol- 0.15 and Sorbitol -0.15 (g/g gelatin)	1.57 - 10.51 (N) Puncture force	8.35 – 17.60 Puncture deformation	2.19-3.3	Giménez et al. (2009a)
Giant squid (*Dosidicus gigas*) [3a*]	Gelatin (4% w/v)	Glycerol- 0.15 and Sorbitol -0.15 (g/g gelatin)	4.94 [a]	46 [a]	1.89 [a]	Giménez et al. (2009b)
Giant squid (*Dosidicus gigas*) [3b*]	Gelatin (4% w/v)	Glycerol 0.15 and Sorbitol 0.15 (g/g gelatin)	2.63 [b]	34.7 [b]	1.78 [b]	Giménez et al. (2009b)

Table 8. (Continued)

Fish Species	Protein Conc.	Plasticizer conc.	Mechanical property		WVP ($x10^{-10}$g m^{-1} s^{-1} Pa^{-1})	References
			TS	EAB		
Tilapia fish [4]*	Gelatin 5 g/100 ml	Gellan and *k*-carrageenan (1 and 2 g/100) of gelatin	101.23 – 109.76	5.08 - 6.81	1.75 - 2.4	Pranoto et al. (2007)
Alaska pollock (*Theragra chalcogramma*) [5]	Gelatin 5% (w/w)	-	45.9 – 50.1	3.23. – 3.44	0.73 – 0.86	Chiou et al. (2008)
Alaska pink salmon (*Oncorhynchus gorbuscha*)[6]	Gelatin 5% (w/w)	-	49.7 – 60.0	3.36 – 3.8	0.85 – 1.08	
Tuna-fish (*Thunnus tynnus*) [7]	Gelatin 2 g/100ml	Glycerol 0.25 (g/g protein)	2.75 – 5.91	3.56 – 13.77	1.83 - 2.87	Gómez-Guillén et al. (2007)
Sole (*Solea spp*.)[8]*	Gelatin 4 g/100ml	Sorbitol 0.15 and Glycerol 0.15 (g/g gelatin)	11.4 – 28.5 N Breaking force	18.1- 16.8 Breaking deformation	1.66 – 1.77	Gómez-Estaca et al. (2009b)

[1]FFS with 6% seaweed extract at pH 6 and 9; [2] FFS replaced with hydrolyzed gelatin (0 -10%); [3a] Film from gelatin extracted with distilled water at 60 °C/18 h); [3b] Film from the second gelatin extraction of collagenous residues at 60 °C/18 h; [4] Gelatin solution added with gellan and *k*-carrageenan (1 and 2 g/100) of gelatin; [5]and [6] FFS prepared by adding 0.25%, 0.50%, and 0.75% (w/w) glutaraldehyde; [7]FFS added with murta extracts; [8] FFS added with borage extract at a ratio 1:1 (dissolved gelatin:borage extract); [2*], [3*]and [8*] WVP unit (10^{-8} g mm / cm^2 h Pa); [4*] WVP unit (g mm/m^2 h kPa).

1.2.4.5 Free radical-mediated protein modification

Free radical-mediated protein modification could be an alternative approach to modify the properties of protein. Generation of free radicals during processing or storage can alter the molecular weight of biopolymers (Farahnaky, et al., 2003). Hydrogen peroxide (H_2O_2) is an oxidizing agent used in some food industries. Fenton reaction is another approach to generate the active radical, hydroxyl radicals ($OH^{\bullet}$), from H_2O_2 in the presence of Fe^{2+} (Kocha *et al.* 1997). In the Fenton's reaction, iron (II) catalyzes the decomposition of hydrogen peroxide (H_2O_2) to hydroxyl radicals ($OH^{\bullet}$) (Equation 1). Carbonyl formation from oxidation process could alter protein conformation, increase protein hydrophobicity and enhance nonspecific protein–protein interactions (Mirzaei and Regnier, 2008). Metal-catalyzed oxidation systems induce the fragmentation of proteins (Kocha *et al*., 1997).

$$H_2O_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^{-} + Fe^{3+} \qquad (1)$$

Addition of H_2O_2 to a system with excess iron provides conditions that minimize quenching of $OH^{\bullet}$ (Teel *et al*., 2001). Quenching reactions may include following reactions:

$$OH^{\bullet} + H_2O_2 \rightarrow HO_2^{\bullet} + H_2O \qquad (1)$$

$$OH^{\bullet} + Fe^{2+} \rightarrow OH^{-} + Fe^{3+} \qquad (2)$$

$$OH^{\bullet} + HO_2^{\bullet} \rightarrow O_2 + H_2O \qquad (3)$$

$HO^{\bullet}$ radical can abstract H atoms from amino acid residues to form carbon-centered radical derivatives, which can react with one another, to form C–C protein cross-linked products (Stadtman, 2001). Addition of O_2 to form an alkyl-peroxyl radical, which upon reaction with the protonated form of superoxide anion (HO_2•) gives rise to an alkyl peroxide. Interactions of peroxide with $HO_2^{\bullet}$ leads to the formation of protein alkoxyl radical, which can undergo peptide bond cleavage (Stadtman, 2001) (Figure 7).

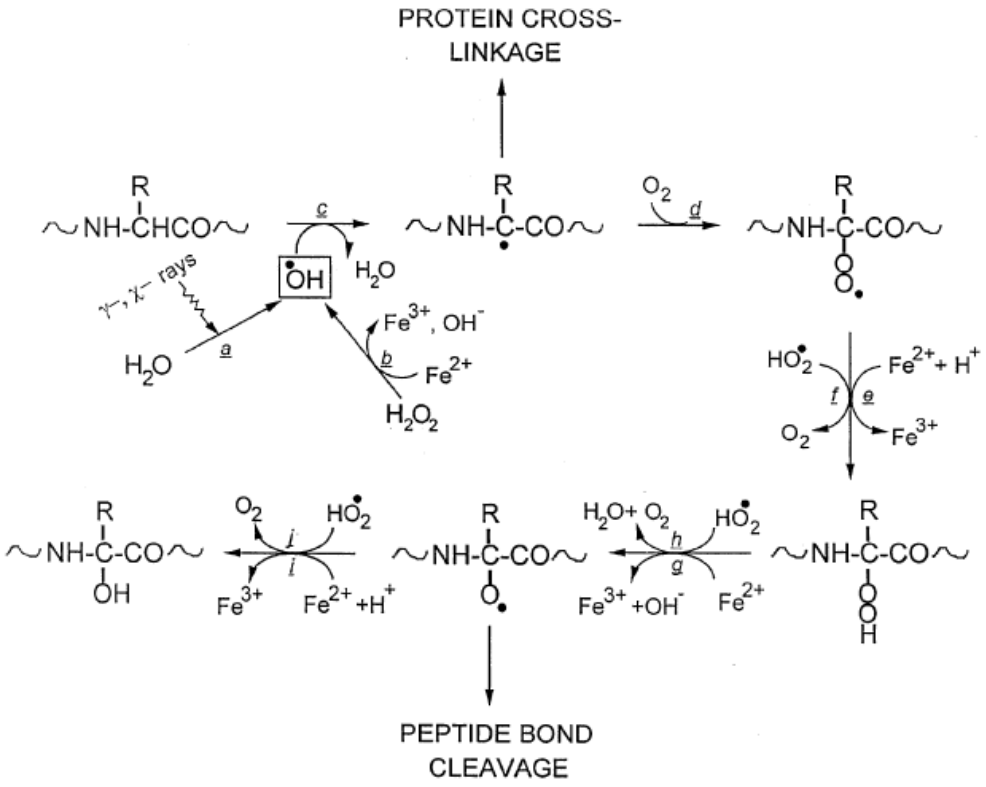


Figure 7. Free radical-mediated protein modifications
Source: Stadtman (2001).

.Stadtman and Berlett (1997) reported that fragmentation of protein is a consequence of direct attack by $HO^{\bullet}$ radical on the polypeptide backbone or on the side chains of glutamyl or prolyl residues. Proline residues of collagen are oxidized to 2-pyrrolidone derivatives with concomitant peptide bond cleavage, according to following reaction**:**

-Gly-Hypro-Pro-Gly-Hypro-Pro + $OH^{\bullet}$ →

-Gly-Hypro-2-Prolidone + Gly-Hypro-Pro-

Amino acid residues of proteins are potential targets by $HO^{\bullet}$ generated by high concentrations of hydrogen peroxide and/or Fe^{2+}. Phenylalanine residues are converted to mono- and dihydroxy derivatives. Tyrosine residues are converted to the 3,4-dihydroxyphenylanine (dopa) derivative, which can undergo redox cycling and thereby production of more ROS. Tyrosine residues are also converted to

nitrotyrosine, chlorotyrosine, and to tyrosyl radicals that can interact with one another to form dityrosine inter- or intra protein cross-linked derivatives (Stadtman, 2001).

The use of wash water containing optimum concentration of H_2O_2 could improve gel-forming ability of surimi, via protein oxidation (Phatcharat *et al*., 2006). Farahnaky et al. (2003) found the decreases in gelatin viscosity when H_2O_2 level exceeded 0.005 M. Aewsiri *et al*. (2009) found that H_2O_2 used as a bleaching agent, induced the oxidation of gelatin, resulting in the formation of gelatin cross-links.

1.2.5 Application of protein based on films

Protein based films have been used to protect and to improve the shelf-life of food products (Krochta and De Mulder-Jounston, 1997). Protein based-films have been applied for coating nuts or used for bakery products (Gennadios and Weller, 1990). Round scad (*Decapterus maruadsi*) protein-based film incorporated with palm oil and chitosan has been used as a packaging material to prevent lipid oxidation of dried fish powder (Artharn *et al*., 2009). Whey protein films are used for coating fresh vegetables as well as the addition in chocolate (McHugh and Krochta, 1994).

Avena-Bustillos *et al*. (1993) studied the use of caseinate-acetylelated-monoglyceride films for peeled carrots and found that water vapor resistance of sample increased, compared with the controls. Xu *et al*. (2001) reported that the shelf-life of kiwifruit coated with edible film comprising soybean protein isolate, stearic acid and pullulan was extended to about 3 times, compared to the control. Casein-lipid (acetylated monoglyceride) emulsion films retarded water loss and browning in cut apple (McHugh and Krochta, 1994).

Soy protein films were used for coating foods to reduce oil uptake during deep-fat frying (Rayner *et al*., 2000). Coated and fried discs of doughnut mix had a notably reduced fat content (by about 55%) compared to non-coated samples. Also, a preference evaluation by an untrained sensory panel indicated no significant difference between coated and non-coated French fries (Rayner *et al*., 2000).

Mallikarjunan *et al*. (1997) reported that coating mashed potato balls with zein prior to frying reduced oil uptake by 59%, compared to non-coated control samples.

Both refrigerated and frozen/thawed round beef steaks wrapped in Coffin collagen film prior to standard retail packaging (permeable film overwrap) or vacuum packaging exhibited significantly less fluid exudate than unwrapped controls (Farouk *et al*., 1990). Tanada-Palmu and Grosso (2005) observed that the bilayer coating of wheat gluten and lipids (beeswax, stearic and palmitic acids) had a significant effect on refrigerated strawberry quality and shelf-life. Retention of firmness and reduced weight loss were obtained. Stuchell and Krochta (1995) showed that frozen king salmon coated with an edible whey protein-lipid solution had the decrease in moisture loss by 42-65% during three weeks of storage at -23 °C. Myofribrillar protein based-film can be used for protecting fish or meat pieces from oxidation or dehydration during storage. For processed meat or fish products e.g sausages and kamaboko, protein film can be the alternative packaging to replace currently used cellulose coatings or plastic films (Cuq, 2002). Collagen film has been used to reduce shrink loss, increase juiciness, and absorb exudate for a variety of cooked meat products including hams and sausages (Gennadois *et al*., 1994). Collagen coatings have also been used to reduce the transport of gas and moisture in meats (Krochta and De Mulder-Johnston, 1997).

Protein based containing antimicrobial activity have been used to extend the shelf-life of food. Min *et al*. (2010) reported that gelatin film incorporated with Nisaplin and Guardian showed antilisterial effects after 16 weeks of storage in the solid media. Nisaplin films showed a 3-log reduction after 1 h and a 6-log reduction in *Listeria* population after 6 to 8 h, compared with the control. The pork loins packed with *Gelidium corneum*–gelatin film containing grapefruit seed extract (GFSE) (0.08%) or or green tea extract (GTE) (2.80%) had a decrease in the populations of *E. coli* O157:H7 and *L. monocytogenes* of 0.69-1.11 and 1.05-1.14 log CFU/g, respectively, compared to the control after 4 days of storage (Hong *et al*., 2009). Jang *et al.* (2011) also reported that 'Maehyang' strawberries wrapped with the rapeseed protein-gelatin (RG) film containing 1.0% antimicrobial grapefruit seed extract (GSE) had the decreased populations of total aerobic bacteria and of yeast and moulds in the strawberries by 1.03 and 1.34 log CFU g^{-1}, respectively, after 14 days

of storage, compared to that of the control. Giménez *et al*. (2011) investigated the lipid oxidation of horse mackerel (*Trachurus trachurus*) patties covered with fish gelatin-based films containing a borage seed extract during 240 days of frozen storage and subsequent thawing and 4 day-chilling. The result suggested that film had protective effects on lipid oxidation of horse mackerel patties throughout frozen storage and particularly after thawing and chilled storage. Furthermore, when compared to vacuum packaging, film showed similar effect until advanced stages of oxidation were reached and exerted enhanced protection once samples were thawed and exposed to air oxygen during chilled storage.

Objectives

1. To investigate the effect of heat treatment of film forming solution on the properties of film from cuttlefish skin gelatin.

2. To prepare and characterize the film from cuttlefish skin gelatin with different degrees of hydrolysis and various levels of plasticizer.

3. To investigate the properties of gelatin-based films incorporated with different herb extracts.

4. To investigate the effect of H_2O_2 and Fenton's reagent on the properties of film from gelatin of cuttlefish skin.

5. To prepare and characterize the blend film based on cuttlefish skin gelatin and mungbean protein isolate.

6. To study the use of cuttlefish gelatin based film for shelf-life extension of chicken meat powder during storage.

CHAPTER 2

EFFECT OF HEAT TREATMENT OF FILM FORMING SOLUTION ON THE PROPERTIES OF FILM FROM CUTTLEFISH (*SEPIA PHARAONIS*) SKIN GELATIN

2.1 Abstract

Effects of heat treatment at different temperatures (40-90 ºC) of film forming solution (FFS) containing 3% gelatin from cuttlefish (*Sepia pharaonis*) ventral skin and 25% glycerol (based on protein) on properties and molecular characteristics of resulting films were investigated. The film prepared from FFS heated at 60 and 70 ºC showed the highest tensile strength (TS) with the highest melting transition temperature (T_{max}) ($p<0.05$). Nevertheless, film from FFS heated at 90 ºC had the highest elongation at break (EAB) with the highest glass transition temperature ($T_{g)}$ ($p<0.05$). With increasing heating temperatures, water vapor permeability (WVP) of films decreased ($p<0.05$), but no differences in L*-value and transparency value were observed ($p>0.05$). Based on FTIR spectra, the lower formation of hydrogen bonding was found in film prepared from FFS with heat treatment. Electrophoretic study revealed that degradation of gelatin was more pronounced in FFS and resulting film when heat treatment was conducted at temperature above 70 ºC. Thus, heat treatment of FFS directly affected the properties of resulting films.

2.2 Introduction

Cuttlefish has become an important fishery product in Thailand as well as other south-east Asian countries, and is mainly exported worldwide. During processing of cuttlefish, skin is generated as a by-product with the low market value. To increase its profitability, cuttlefish skin has recently been used for gelatin extraction (Aewsiri *et al*., 2009).

Gelatin is a proteinaceous compound, commercially obtained from skins and skeletons of cattle and pigs. Gelatin has been used widely in foods, pharmaceutical products and photographic industries (Bigi *et al*., 2004). However, the occurrence of bovine spongiform encephalopathy (BSE), foot and mouth diseases have caused major concerns for human health (Cho *et al*., 2005). Additionally, porcine gelatin can cause objections from some religions. As a consequence, an increasing interest has been paid to other gelatin sources, especially skin and bone from seafood processing by-products (Gómez-Guillén *et al*., 2002; Jongjareonrak *et al*., 2006a). Gelatin has been extensively employed as an ingredient to improve the elasticity, consistency and stability of foods. Additionally, it can be used as a biomaterial for preparing biodegradable films with an excellent gas barrier property (Giménez *et al*., 2009). Fish skin gelatins have been used as a filmogenic agent (Jongjareonrak *et al*., 2006b; Jongjareonrak *et al*., 2006c; Gómez-Guillén *et al*., 2007).

The physical and structural properties of gelatin mainly influenced by the molecular weight distribution and amino acid composition that plays a vital role in the rheological and barrier properties of the resulting films (Gómez-Guillén *et al*., 2009). 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 intramolecular and intermolecular disulfide bonds (Gennadios and Weller, 1991). Interconnection of protein molecules during the drying process leads to the formation of film matrix. Therefore, the extension or unfolding of protein molecule could favor the interaction among molecules, in which the junction zones could be formed to a higher extent. Unfolding of proteins by heat treatment is thus a promising approach to improve the film-forming ability. Heat treatments (at 90 °C over 5 min) of pea protein isolate solutions increased mechanical properties of the

resulting film (Choi and Han, 2002). Perez–Gago *et al*. (2001) found that whey protein isolate (WPI) film showed the stiffer, stronger and more stretchable when FFS was heated with increasing times (5-20 min) and temperatures (70-100 ºC). However, no information regarding the use of cuttlefish skin gelatin for film preparation and the effects of heat pretreatment of FFS on the properties of cuttlefish skin gelatin film has been reported. The objectives of the present study were to extract and characterize the gelatin from cuttlefish skin, to characterize the films from extracted gelatins, as well as to study the effect of heat treatment of FFS at different temperatures on properties of the resulting films.

2.3 Materials and methods

2.3.1 Chemicals

Bovine serum albumin and high molecular weight protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2), glycerol, *p*-dimethylaminobenzaldehyde and tris(hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulphates (SDS), Coomassie Blue R-250 and *N, N, N′, N′*- tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). α-Chymotrypsin was procured from Wako Pure Chemical Industries, Ltd (Tokyo, Japan).

2.3.2 Collection and preparation of cuttlefish skin

Ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from a dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1 x 1 cm^2), placed in polyethylene bags and stored at -20 ºC until use. Storage time was not longer than 2 months. Prior to gelatin extraction, the frozen skin was thawed using a running water (25-26 ºC) until it was completely thawed.

2.3.3 Extraction of gelatin from cuttlefish skin

Gelatin was extracted from cuttlefish skin according to the method of Gómez-Guillén *et al*. (2002) and Aewsiri *et al*. (2009) with slight modifications. Skin was soaked in 0.05 M NaOH with a skin/solution ratio of 1:10 (w/v) with a gentle stirring at room temperature (26–28 ºC). The solution was changed every hour to remove non-collagenous proteins for totally 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching in 5% H_2O_2, using a sample/solution ratio of 1:10 (w/v) for 48 h at 4 ºC. The samples treated with H_2O_2 were washed three times with 10 volumes of water. Gelatin was extracted from bleached skin by distilled water at 60 ºC for 12 h, using a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously using a paddle stirrer (RW20.n, IKA LABORTECHNIK, Staufen, Germany). The extracts were centrifuged at 8,000 x g for 30 min at room temperature using a refrigerated centrifuge (Backman Coulter, Avanti® J-E Centrifuge, ©2007 Backman Coulter, Inc. California, USA.) to remove insoluble material. The supernatant was collected and freeze-dried (Model Duratop™ lP/Dura Dry™ lP, FTS® System, Inc., Stone Ridge, NY, USA). The dry matter was referred to as 'gelatin powder'. The yield of gelatin obtained was calculated and expressed as the percentage of dry matter of gelatin obtained relative to cuttlefish skin. Gelatin was subjected to proximate analysis and used for film preparation.

2.3.4 Proximate analysis of gelatin

The moisture, protein, ash and fat contents of gelatin powder were determined according to the AOAC methods (AOAC, 1999) with the analytical No. of 950.46, 920.153, 928.08 and 960.39, respectively.

2.3.5 Determination of amino acid composition

Gelatin was hydrolyzed under reduced pressure in 4.0 M methanesulfonic acid containing 0.2% (v/v) 3-2(2-aminoethyl) indole at 110 ºC for 24

h and the hydrolysate was neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.4 ml was subjected to an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan).

2.3.6 Effect of heat treatment of FFS at different temperatures on the properties of gelatin films

2.3.6.1 Preparation of gelatin films

Gelatin powder was mixed with distilled water to obtain the protein concentration of 3% (w/v). Glycerol at a concentration of 25% of protein was used as a plasticizer. FFS obtained was incubated at different temperatures (40, 50, 60, 70, 80 and 90 ºC) for 30 min in a temperature-controlled water bath (Memmert, GmbH+Co. KG, D-91126, Schwabach, Germany) with an occasional stirring. Heated solutions were then cooled with running tap water. FFS without and with heating (4 ± 0.01 g) was cast onto a rimmed silicone resin plate (5 x 5 cm^2), air-blown for 12 h at room temperature and dried with an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttalingen, Germany) at the temperature of 25 ± 0.5 ºC and 50 ± 5% relative humidity (RH) for 24 h. Dried films were manually peeled off and subjected to following analyses.

2.3.6.2 Analyses

Prior to testing, films were conditioned for 48 h at 50 ± 5% relative humidity (RH) and 25 ± 0.5 ºC. For ATR-FTIR and DSC studies, films were conditioned in a dessicator containing dried silica gel for 3 weeks at room temperature (28-30 ºC) to obtain the most dehydrated films.

2.3.6.2.1 Film thickness

The thickness of film was measured using a micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mituyoto Corp, Kawasaki-shi, Japan). Ten random locations around each film sample were used for thickness determination.

2.3.6.2.2 Mechanical properties

Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata *et al.* (2000) using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK). Ten samples (2 x 5 cm^2) with the initial grip length of 3 cm were used for testing. The samples were clamped and deformed under tensile loading using a 100 N load cell with the cross-head speed of 30 mm min^{-1} until the samples were broken. The maximum load and the final extension at break were used for calculation of TS and EAB, respectively.

2.3.6.2.3 Water vapor permeability (WVP)

WVP was measured using a modified ASTM *(American Society for Testing and Materials, 1989)* method as described by Shiku *et al*. (2004). The film was sealed on an aluminum permeation cup containing silica gel (0% RH) with silicone vacuum grease. The cup was placed at 30 ºC in a desiccator containing the distilled water. It was then weighed at 1 h intervals over an 8 h period. Six films were used for WVP testing. WVP of the film was calculated as follows (McHugh *et al*. 1993):

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

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

2.3.6.2.4 Color and transparency of the film

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

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

where T_{600} is the fractional transmittance at 600 nm and x is the film thickness (mm). The greater transparency value represents the lower transparency, which is associated with the lower fractional transmittance of film samples tested.

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

FTIR spectra of films prepared from FFS without and with heat treatment at different temperatures were recorded using a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45º ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at room temperature as described by Nuthong *et al.* (2009a). 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 ºC. The spectra obtained were used to determine the differences in functional group frequencies affected by different heating temperatures.

2.3.6.2.6 Differential scanning calorimetry

Thermal properties of films were determined using differential scanning calorimeter (DSC) (Perkin Elmer, Model DSC-7, Norwalk, CT, USA) as per the method of Jongjareonrak *et al.* (2006b). Temperature calibration was performed using the Indium thermogram. The film samples (5–10 mg) were accurately weighed into aluminum pans, sealed, and scanned over the range of -30 to 150 ºC with a heating rate of 10 ºC/min. The dry ice was used as a cooling medium and the system was equilibrated at -30 ºC for 5 min prior to the scan. The empty aluminum pan was used as a reference. The maximum transition temperature was estimated from the endothermic peak of DSC thermogram and transition enthalpy was determined from the area under the endothermic peak. Second scan was also performed in the same manner followed by quench cooling of the sample after completing the first scanning.

2.3.6.2.7 Electrophoretic analysis

Protein patterns of gelatin film were analyzed under reducing condition using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Prior to analysis, the film samples were prepared according to the method of Jongjareonrak *et al.* (2006b) with some modification. Film samples (200 mg) were dissolved in 10 ml of 1% (w/v) SDS. The mixture was stirred continuously at room temperature for 12 h. Supernatants were obtained after centrifuging at 3000xg for 5 min using Hettich Zentrifuge (MIKRO-20, D-78532, Tuttlingen, Germany). For FFSs, they were mixed with 2% (w/v) SDS at a ratio of 1:1 (v/v). The mixture was stirred and the supernatant was obtained in the same manner. The supernatants of both FFS and film were then mixed with sample buffer (0.5 MTris–HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol with 10% (v/v) β-ME) at the ratio of 1:1 (v/v). Samples were loaded onto the polyacrylamide gel made of 7.5% running gel and 4% stacking 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 electrophoresis, gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Protein markers were used to estimate the molecular weight of proteins.

Quantitative analysis of protein band intensity was performed using a Model GS-700 Imaging Densitometer (Bio-Red Laboratories, Hercules, CA, USA) with Molecular Analyst Software version 1.4 (image analysis systems). The intensity of interested protein bands was expressed, relative to those of FFS without heating or film prepared from FFS without heating.

2.3.7 Statistical analysis

Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

2.4 Results and discussion

2.4.1. Proximate composition of gelatin

Gelatin extracted from cuttlefish skin with the yield of 13.21% contained protein (88.21%) as the major constituent. Gelatin consisted of 10.07% moisture, 1.06% fat and 0.61% ash. Moisture content of gelatin commonly varies between 9% and 14% and ash content is lower than 2% (Leach and Eastoe, 1997). Carvalho and Grosso (2004) reported that bovine hide gelatin type B contained 88.92% protein, 0.78% ash and 10.3% moisture. The result suggested that the extraction process used resulted in the low contents of both lipid and inorganic matters in the resulting gelatin.

2.4.2. Amino acid composition of gelatin

The amino acid composition of gelatin expressed as residues per 1000 total amino acid residues is illustrated in Table 9. Glycine was the most abundant amino acid in gelatin (31.1%). Gelatin or collagen contains glycine around 1/3 of total amino acid. Gelatin of cuttlefish skin had imino acid (Pro + Hyp) at a level of 194 residues per 1000 residues. The imino acid content was higher than that of gelatin from squid and giant squid (175 and 163 residues per 1000 residues, respectively) (Gómez-Guillén *et al*., 2002; Giménez *et al*., 2009). The stability of the triple helical structure in renatured gelatins has been reported to be proportional to the total content of imino acids. Pro + Hyp rich regions are likely to be involved in the formation of nucleation zones (Ledward, 1986). Hyp plays a key role in the stabilization of the triple-stranded collagen helix due to its hydrogen bonding ability through its hydroxyl group. Apart from Pro and Hyp, gelatin also contained a high content of Ala, Asp, Asn, Glu and Gln. Ala is found in non-polar regions where the sequences of Gly-Pro-Y predominate and the third positions normally occupied by Hyp or Ala (Ledward, 1986). Gelatin with a higher content of Pro, Hyp and Ala are considered to have higher viscoelastic properties and its ability to develop triple helix structures, which are important for stabilizing the structure of gelatin gel (Gómez-Guillén *et al.*, 2002)

as well as gelatin-based film (Jongjareonrak *et al*., 2006b). The properties of film are largely influenced by the amino acid composition and their molecular weight distribution (Gómez-Guillén *et al*., 2009).

Table 9. Amino acid composition of cuttlefish skin gelatin

Amino acids	No. of residue/1000 residues
Ala	83
Arg	60
Asp/Asn	72
Cys	1
Glu/Gln	92
Gly	314
His	5
Ile	20
Leu	26
Lys	12
Hyl	12
Met	7
Phe	12
Hyp	91
Pro	103
Ser	40
Thr	24
Tyr	7
Try	0
Val	19
Total	1000

2.4.3 Effect of heat treatment of FFS at different temperatures on the properties of gelatin films

2.4.3.1 Thickness

Thickness of gelatin films prepared from FFS heated at different temperatures ranged from 0.037 to 0.041 mm (Table 10). Heating temperatures of FFS showed no impact on thickness of the resulting films ($p>0.05$). Nevertheless, the film prepared from FFS heated at 70 °C showed a slightly higher thickness than that of the control film ($p<0.05$). The slight increase in thickness of film might be due to pretruding structure formed during film formation of gelatin strands. Gelatin molecules heated at 70 ºC might contain the peptides with the particular chain length, in which the compact film network could not be developed. Perez-Gago *et al*. (1999) found no difference in the thickness of film from native and heat-denatured whey protein isolate.

2.4.3.2 Mechanical properties

The mechanical properties of gelatin films prepared from FFS heated at different temperatures are presented in Table 10. TS of films increased with increasing heating temperatures of FFS from 40 to 70 ºC ($p<0.05$). Film prepared from FFS heated at 60 or 70 ºC showed the highest TS (9.66 and 8.90 MPa, respectively). These results suggested that gelatin molecules possibly became more stretched with the sufficient heat. This most likely favored the inter connection of gelatin molecules via hydrogen bonding due to more junction zones during film formation. As a result, film network with increased TS was obtained. The integrity and molecular weight of protein chains might contribute to the network structure of films obtained (Shiku *et al*., 2004). Perez-Gago *et al*. (1999) found that native whey protein films were less stiff, weaker and less extendible than heat-denatured films. However, the decrease in TS was observed as the heating temperatures were higher than 70 ºC ($p<0.05$). The lower TS of film prepared from FFS heated at 90 ºC was obtained, compared with that of the control film ($p<0.05$). As the heating temperature of FFS was higher than 70 ºC, the degradation of gelatin could take place. The shorter chains of gelatin molecules could not form the strong film network, which had the

resistance to the applied mechanical force. Nevertheless, the impact of heating on the properties of film might be varied with the protein sources, particularly bondings involved. Gelatin film was mainly stabilized by the weak bond including hydrogen bond and hydrophobic interaction. There was the negligible disulfide bond formed in the gel of cuttlefish gelatin (Aewsiri *et al*., 2009).

The decrease in EAB was noticeable as the heating temperatures of FFS increased up to 60 ºC (p<0.05). Thereafter, the marked increased in EAB was found when the heating temperatures increased from 70 to 90 ºC (p<0.05). Film prepared from FFS heated at 90 ºC had the increase in EAB by 98.20%, compared with that of the control film. TS, Young's modulus and percentage elongation of whey protein isolate film increased as heating time and temperature of FFS increased (Perez-Gago *et al*., 2001). Films prepared from heated whey protein isolate solutions had the higher percentage elongation, TS and Young's modulus than those from unheated solution (Quinn *et al*., 2003). Additionally, heat-treated pea protein isolate films possessed higher TS and EAB values, compared to those of the non-heated protein films (Choi and Han, 2002). With increasing heating temperature up to 60 ºC, EAB decreased with the coincidental increase in TS. Unfolded or stretched gelatin molecules underwent more interaction as evidenced by the increased TS and the losses in flexibility as indicated by lowered EAB. The increase in EAB of film when FFS was heated at temperature higher than 70 ºC indicated that heat treatment at higher temperatures resulted in the formation of film network with lower rigidity. At high temperature, gelatin might undergo degradation, leading to the formation of short chains. This might lead to the lower interconnection between gelatin molecules. 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. Therefore, heating temperature of FFS had the profound impact on the mechanical properties of film.

Table 10. Mechanical properties, water vapor permeability and thickness of cuttlefish skin gelatin film prepared from FFS heated at different temperatures

Pretreatment Temperature (ºC)	TS† (MPa)	EAB† (%)	WVP† (x 10^{-10} g $s^{-1}.m^{-1}.Pa^{-1}$)	Thickness† (mm)
Control (w/o heating)	6.13 ± 0.20 ^{c}	26.18 ± 3.76 ^{d}	1.07 ± 0.06 ^{c}	0.038 ± 0.001 ^{b}
40	6.37 ± 0.51^{c}	21.83 ± 3.00 ^{e}	1.28 ± 0. 07 ^{ab}	0.039 ± 0.002 ^{ab}
50	7.48 ± 0.50 ^{b}	16.55 ± 2.38 ^{f}	1.30 ± 0.14 ^{a}	0.039 ± 0.002 ^{ab}
60	9.66 ± 0.87 ^{a}	15.56 ± 3.69 ^{f}	1.19 ± 0.06 ^{b}	0.040 ± 0.003 ^{ab}
70	8.90 ± 1.02 ^{a}	32.67 ± 4.54 ^{c}	1.19 ± 0.08 ^{b}	0.041 ± 0.001 ^{a}
80	6.08 ± 0.72 ^{c}	44.10 ± 3.40 ^{b}	0.98 ± 0.03 ^{cd}	0.039 ± 0.001 ^{b}
90	4.99 ± 0.07 ^{d}	51.89 ± 1.91 ^{a}	0.92 ± 0.05 ^{d}	0.037 ± 0.002 ^{b}

† Mean ± SD (n=3). The same superscript in the same column indicates the non-significant difference (p>0.05).

2.4.3.3 Water vapor permeability (WVP)

WVP of gelatin films prepared from FFS heated at different temperatures is presented in Table 10. FFS heated at different temperatures yielded the films with varying WVP (p<0.05). The films prepared from FFS heated at temperature range of 40-70 °C showed the higher WVP, compared with the control film (p<0.05). WVP of film prepared from FFS heated at 80 °C had no differences in WVP, compared with the control film (p>0.05), while the film prepared from FFS heated at 90 °C showed the lower WVP, compared with the control film (p<0.05). Within the heating temperatures used (40–90 °C), the higher temperature of heating generally led to the continuous decrease in WVP of resulting film. Gelatin with hydrophilic characteristics can bind water molecules through hydrogen bridges, resulting in water vapor adsorption. Decreases in WVP of the film prepared from FFS heated at higher temperatures might be due to the exposure of hydrophobic domains of gelatin chains. Cuttlefish gelatin contained both hydrophilic (62% of total amino acid residues) and hydrophobic (38% of total amino acid residues) amino acid residues. Hydrophobic and hydrophilic amino acid ratio, especially on surface of film,

more likely contributed to WVP of film from heat treated FFS differently (Table 9). Furthermore, the regular arrangement of short chain gelatin obtained after heating at higher temperatures led to a compact film matrix during film formation. However, native and heat-denatured whey protein isolate films had similar water vapor permeability (Perez-Gago *et al*., 1999). This might be due to the difference in amino acid composition, molecular weight as well as film network formed between proteinaceous sources.

2.4.3.4 Color and transparency

Color and transparency values of gelatin films prepared from FFS heated at different temperatures are shown in Table 11. Higher L*- value (lightness) was obtained for the control film ($p<0.05$). With all heating temperatures used, the resulting films had the lower L*- values but higher b*-values, indicating the lower lightness with increased yellowness. The result suggested that heat treatment might induce the formation of yellowish pigment in the solution, especially via Maillard reaction. Manzocco *et al*. (2000) reported that color changes due to Maillard reaction are always associated with the heat-induced process. The increased b*-value of fish muscle protein-based film was caused by Maillard reaction (Chinabhark *et al*., 2007). Paschoalick *et al*. (2003) reported that the increased heating temperature resulted in a slight increase in yellowness of film from Nile tilapia muscle protein, possibly due to the occurrence of reaction among the glycerin molecules and the reactive group of lysine. Therefore, heat treatment of FFS affected the color of resulting films from cuttlefish skin gelatin. No differences in transparency value were observed between films from FFS without and with heat treatment at different temperatures ($p>0.05$) (Table 11). Choi and Han (2002) also reported that transparency of film from pea protein isolate was not affected by heat treatment.

Table 11. Color and transparency value of cuttlefish skin gelatin film prepared from FFS heated at different temperatures

Pretreatment Temperature (ºC)	Color†			Transparency value†
	L*	a*	b*	
Control (w/o heating)	91.14 ± 0.37 [a]	-1.64 ± 0.16[a]	2.29 ± 0.11[d]	3.33 ± 0.04 [a]
40	90.44 ± 0.48 [b]	- 1.68 ± 0.04 [ab]	2.71 ± 0.06 [bc]	3.32 ± 0.03 [a]
50	90.34 ± 0.11 [b]	-1.77 ± 0.11[abc]	2.60 ± 0.27 [c]	3.32 ± 0.05 [a]
60	90.53 ± 0.26 [b]	-1.87 ±0.16 [bc]	2.83 ± 0.11 [b]	3.32 ± 0.03 [a]
70	90.49 ± 0.47 [b]	-1.80 ± 0.15[abc]	2.77 ± 0.06 [bc]	3.35 ± 0.03 [a]
80	90.49 ± 0.16 [b]	-1.83 ± 0.07 [abc]	2.87 ± 0.17 [ab]	3.34 ± 0.02 [a]
90	90.39 ± 0.31 [b]	-1.93 ± 0.12 [c]	3.04 ± 0.09 [a]	3.35 ± 0.03 [a]

† Mean ± SD (n=3). The same superscript in the same column indicates the non-significant difference (p>0.05).

2.4.3.5 Infrared spectroscopy

FTIR spectra of gelatin films prepared from FFS heated at different temperatures are shown in Figure 8. Similar spectra of films prepared from FFS heated at various temperatures were noticeable. The bands situated at 3287, 1630, 1539 and 1235 cm^{-1}, corresponding to amide-A and free water, amide-I, amide-II and amide-III, respectively (Aewsiri *et al*., 2009; Jongjareonrak *et al*., 2008). Amide-A represents NH-stretching coupled with hydrogen bonding; amide-I represents C=O stretching/hydrogen bonding coupled with COO; amide-II arises from bending vibration of N-H groups and stretching vibrations of C-N groups; amide-III is related to the vibrations in plane of C-N and N-H groups of bound amide or vibrations of CH_2 groups of glycine (Muyonga *et al*., 2004a; Aewsiri *et al*., 2009). Carvalho *et al*. (2008) reported the similar result for pure pig skin gelatin film where amide-I, amide-II and amide-III showed peaks at the wavenumbers of 1633, 1538 and 1234 cm^{-1} respectively. The peak situated around 1033 cm^{-1} might be related to the possible interactions arises between plasticizer (OH group of glycerol) and film structure (Bergo and Sobral, 2007).

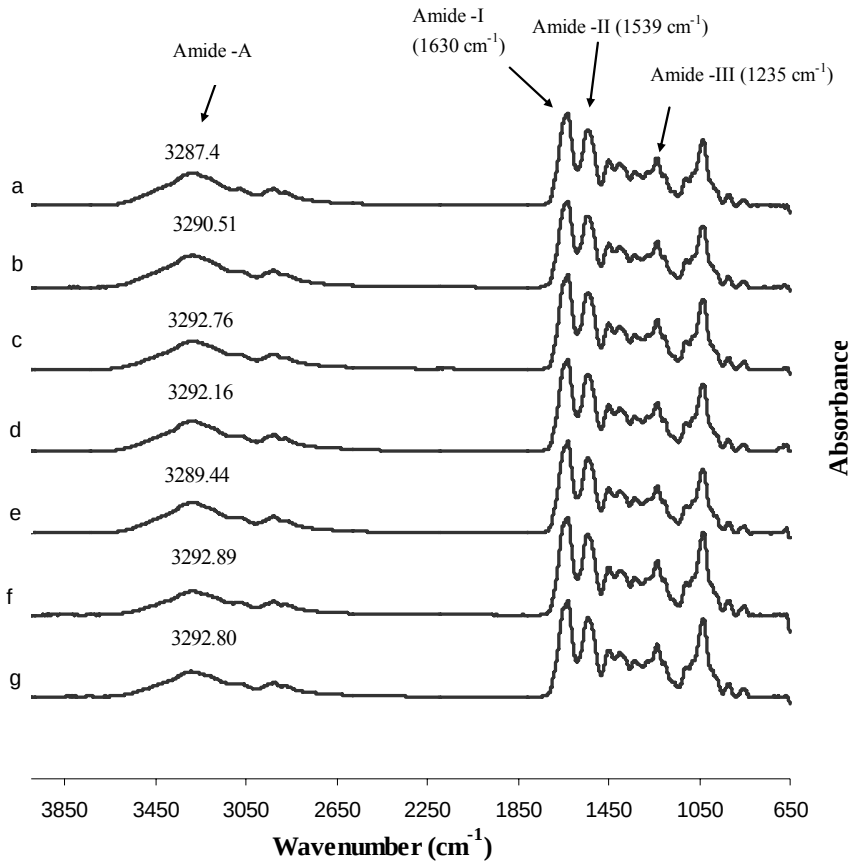


Figure 8. FTIR spectra of films prepared from FFS without heating (a) and from FFS heated at different temperatures, (b) 40 °C, (C) 50 °C, (d) 60 °C, (e) 70 °C, (f) 80 °C, (g) 90 °C.

From the spectra, all films obtained from different heated FFS had no changes in vibrational wavenumber for amide-I, amide-II and amide-III peaks, except for amide-A peak. Typically, decrease in the vibrational wavenumber and broadening of the OH and NH vibration bands could be indicative of a hydrogen bonding interaction between polymer molecules in the film (Xie *et al.,* 2006). However, film prepared from heated FFS had a slight shift to the higher wavenumber of amide-A (3287 - 3292 cm^{-1}), possibly due to the lower formation of hydrogen bonding. This result suggested that other bonds, especially hydrophobic interaction became dominant, when FFS was heated prior to casting. Hydrophobic domain might be more exposed during heating and hydrophobic interaction could be enhanced. Heat-

denatured film from pea protein isolate had the same numbers of peaks at corresponding wavenumbers, in comparison with the native film; however, all the peak heights of the heat-denatured film were lower than those of the corresponding peak of the native film (Choi *et al*., 2002). Water content affected FTIR spectra in the gelatin film without glycerol addition (Yakimets *et al*., 2005). The film in this present study contained glycerol as the plasticizer. As a consequence, some water might be bound with the film matrix, though it was equilibrated over the dry silica gel for 3 weeks. This water might have the influence on FTIR spectra to some degree.

2.4.3.6. Differential scanning calorimetry (DSC)

DSC thermograms of the first heating scan of gelatin films prepared from FFS without heating and FFS heated at different temperatures are illustrated in Figure 9. Glass transition temperature (T_g), melting transition temperature (T_{max}) and enthalpy (ΔH) of gelatin films prepared from FFS without heating or heated at various temperatures are shown in Table 12.

Among all films tested, the control film had the lowest T_{max} and ΔH ($p< 0.05$). Melting transition temperature and enthalpy of films increased as the heating temperature of FFS increased from 40 to 70 °C ($p< 0.05$). However, slight decreases in T_{max} and ΔH were found when films were prepared from FFS heated at 80 and 90 °C. T_{max} of the film indicated the temperature causing the disruption of the protein interaction formed during film formation (Jongjareonrak *et al*., 2006b). The higher T_{max} and ΔH found in films from FFS heated at 60 and 70 °C might be due to the greater inter-chain interaction of heat treated gelatin strands, mostly likely via hydrophobic interaction and hydrogen bond. The higher enthalpy was also required to disrupt the film network. Thermal stability of films was possibly affected by the presence of intermolecular interaction of proteins, such as hydrogen bonds, ionic-interactions, hydrophobic–hydrophobic interactions, which stabilized the film network (Barreto *et al*., 2003). Additionally, partial renaturation of random gelatin strands to the triple-helix structure during film formation was most likely associated with the increased thermal stability of gelatin film (Arvanitoyannis *et al*., 1997). Film from FFS heated at 80 and 90 °C showed the lower T_{max} and ΔH. This might be associated with the degradation of gelatin molecules, which could not form the strong

film network as indicated by the lower TS (Table 10). Weaker film network required the lower enthalpy for destroying the inter-chain interactions. T_{max} of 89.0 ºC of pure gelatin film was previously reported (Mendieta-Taboada *et al*., 2008). Pig skin gelatin film with glycerol as a plasticizer at higher level (10-30 %) showed the lower T_g and T_{max} (Vanin *et al*., 2005). In general, the higher transition enthalpy was coincidentally observed in the films with the higher T_{max}.

Table 12. Glass transition temperature, melting transition temperature and enthalpy of cuttlefish skin gelatin film prepared from FFS heated at different temperatures

Pretreatment Temperature (ºC)	T_g † (°C)	Melting transition	
		T_{max} † (°C)	ΔH † (J/g)
Control (w/o heating)	-6.09 ± 0.31 [a]	83.37 ± 0.71 [d]	10.64 ± 1.05 [b]
40 °C	-4.35 ± 0.22 [b]	85.77 ± 0.62 [c]	11.59 ± 0.99 [cd]
50 °C	-3.34 ± 0.13 [c]	86.92 ± 0.54 [c]	12.94 ± 1.04 [bcd]
60 °C	0.52 ± 0.37 [d]	92.95 ± 0.83 [a]	17.01 ± 1.19 [a]
70 °C	0.87 ± 0.20 [d]	94.53 ± 0.94 [a]	17.54 ± 1.17 [a]
80 °C	3.21 ± 0.58 [e]	90.87 ± 0.71 [b]	14.20 ± 0.48 [b]
90 °C	3.02 ± 0.54 [e]	91.05 ± 0.26 [b]	13.45 ± 0.75 [bc]

† Mean ± SD (n=3).
The same superscript in the same column indicates the non-significant different (p>0.05).

T_g of all films plasticized with 25% glycerol was in the range of -6.09 to 3.21 ºC (Table 12). Slade and Levine (1991) reported that film behaves as a brittle glass at temperature below T_g and film exists in a soft rubbery state at temperature above T_g due to segmented motion of the molecules. The decreased T_g of protein-based films was observed with increasing plasticizer content (Gontard *et al*., 1993; Sobral *et al*., 2001). The plasticizer could localize between the chains of proteins, bind water, and disrupt intermolecular polymer interactions of film matrix (Gontard *et al*., 1993). T_g of gelatin film in this study was lower than room temperature. Therefore,

the films were ductile at room temperature. For the films prepared from FFS with heat treatment, T_g of resulting films increased with increasing heating temperatures (p<0.05) (Table 12). However, it was noted that the marked increase in T_g was observed when FFS was heated at 80 and 90ºC. This was coincidental with the degradation of gelatin in FFS. The degraded gelatin with the shorter chain more likely aligned orderly and closely packed in the film matrix, mainly via weak bonds. This could result in the limiting segmental motion of molecules in the film matrix. However, Carvalho *et al*. (2008) reported that the similar T_g (53 ºC) of films from halibut skin gelatin with and without concentration before drying, indicating that degradation had no impact on the T_g of resulting film. Differences in T_g between both films could be due to the differences in type of plasticizer used and the source of gelatin. In general, the increased crystallinity, molecular weight, ionic degree, and cross linking increase T_g, whereas increasing solvent or plasticizer concentration reduce T_g.

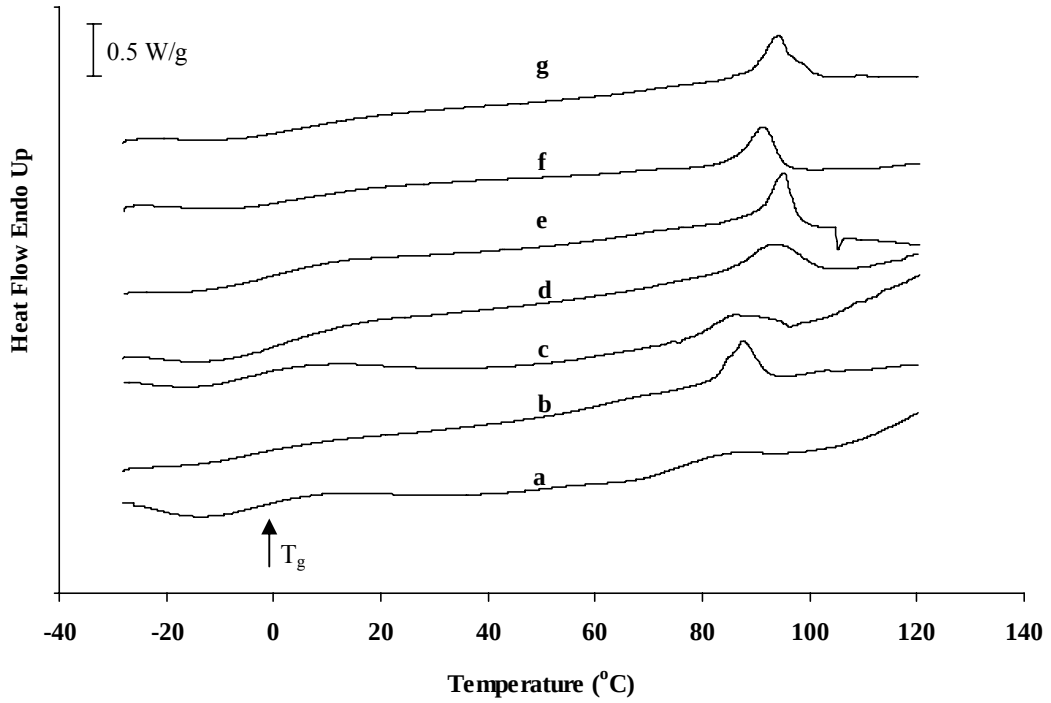


Figure 9. DSC thermograms (1st heating scan) of films prepared from FFS without heating (a) and from FFS heated at different temperatures, (b) 40 °C, (C) 50 °C, (d) 60 °C, (e) 70 °C, (f) 80 °C, (g) 90 °C.

For the second scan, it was found that no transition was observed. It was postulated that absorbed water acting as plasticizer might be removed during the first heating scan. As a consequence, the interaction between gelatin molecules could be enhanced and the more rigid film network could be obtained. Thus, the transition temperature of the film could become too high and could not be detected in the temperature range tested. Therefore, thermal properties of cuttlefish skin gelatin film were affected by heating temperature of FFS.

2.4.3.7 Electrophoresis

The electrophoretic patterns of FFS heated at different temperatures and their corresponding films are shown in Figure 10A and 10B, respectively. Similar protein patterns were observed between the control FFS (without heating) and those heated at temperature range of 40-70 ºC. Proteins with molecular weight of 97 and 118 kDa were found as the dominant proteins in FFS. Anwsiri *et al*. (2009) reported that protein with molecular weight of 97 kDa was the major protein in gelatin extracted from dorsal skin of cuttlefish. In general, the physical properties of gelatin are mainly governed by the source and the extracting conditions (Bigi *et al*., 2004). FFS heated at 80 and 90 ºC showed slight degradation of both proteins. Band intensity of the proteins with molecular weight of 97 and 118 kDa in FFS heated at 90 ºC decreased by 9.9 and 13.2%, when compared with that found in FFS without heating. Degraded gelatin strands might result in the lowered TS of films prepared from FFS heated at both 80 and 90 ºC (Table 10).

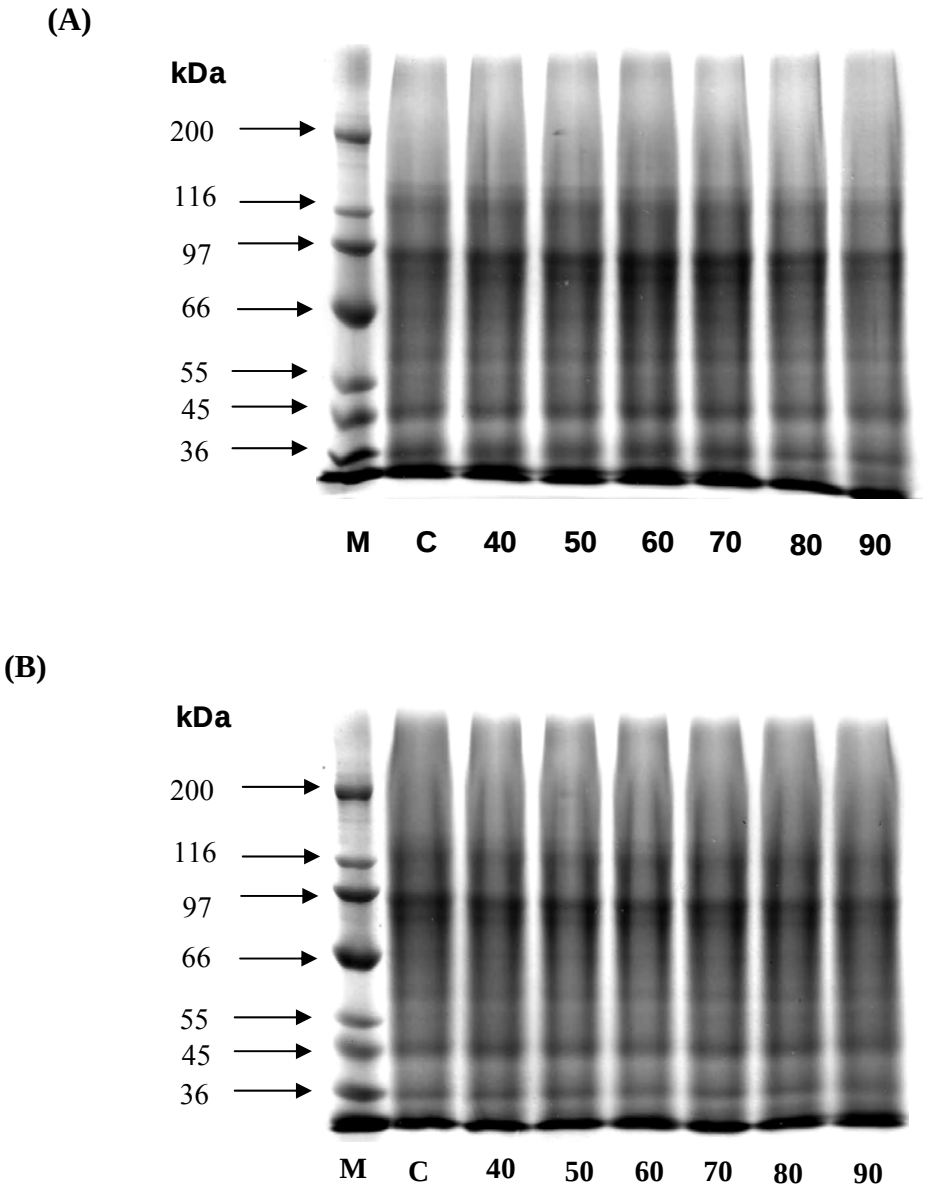


Figure 10. Protein patterns of FFS (A) and corresponding films (B) from cuttlefish skin gelatin: (M) protein marker; (c) control (without heating). Numbers denoted heating temperatures (°C) of FFS.

The similar protein patterns were observed between FFS and their corresponding films. For films prepared from FFS heated at 90 ºC, the band intensity of proteins with molecular weight of 97 and 118 kDa decreased by 6.2 and 7.8%, when compared with that found in the film prepared from FFS without heating. In the

presence of SDS as well as β-ME used for electrophoresis, hydrogen bond, hydrophobic interaction as well as disulfide bond in film network were destroyed. Nevertheless, no disulfide was present in gelatin film. Gelatin contains no cysteine, which can undergo oxidation to disulfide bond. Therefore, gelatin film network without disulfide bond was mainly stabilized by hydrogen bonds and hydrophobic interactions. This was evidenced by the complete solubilization in water (data not shown).

2.5 Conclusion

Gelatin from cuttlefish skin exhibited the good film-forming ability. Properties of gelatin films were affected by the heat treatment of their FFS. Heat treatment at appropriate temperature (70 ºC) brought about the stretching or unfolding of gelatin strands, in which higher inter-chain interaction could be formed via hydrogen bond or hydrophobic interaction and the improved mechanical property was obtained. With the excessive heating, gelatin degradation occurred and the corresponding film showed the increased EAB but lower TS. Thus, heat treatment of FFS directly had the impact on the properties of film from cuttlefish skin gelatin.

CHAPTER 3

EFFECTS OF PARTIAL HYDROLYSIS AND PLASTICIZER CONTENT ON THE PROPERTIES OF FILM FROM CUTTLEFISH (*SEPIA PHARAONIS*) SKIN GELATIN

3.1 Abstract

Properties of film from cuttlefish **(***Sepia pharaonis***)** ventral skin gelatin with different degree of hydrolysis (DH: 0.40, 0.80 and 1.20%) added with glycerol as plasticizer at various levels (10, 15 and 20%, based on protein) were investigated. Films prepared from gelatin with all DH had the lower tensile strength (TS) and elongation at break (EAB) but higher water vapor permeability (WVP), compared with the control film (without hydrolysis) ($p<0.05$). At the same glycerol content, both TS and EAB decreased, while WVP increased ($p<0.05$) with increasing %DH. At the same DH, TS generally decreased as glycerol content increased ($p<0.05$), however glycerol content had no effect on EAB when gelatins with 0.80 and 1.20% DH were used ($p>0.05$). DH and glycerol content had no marked impact on color and total differences in color as compared with white standard (ΔE^*) of resulting films. Electrophoretic study revealed that degradation of gelatin and their corresponding films was more pronounced with increased %DH, resulting in the lower mechanical properties of films. Based on FTIR spectra, with the increasing %DH as well as glycerol content, higher amplitudes for Amide-A and Amide-B peaks were observed, compared with film from gelatin without hydrolysis (control film) due to the increased $-NH_2$ group caused by hydrolysis and the lower interaction of $-NH_2$ group in the presence of higher glycerol. Thermogravimetric analysis indicated that film prepared from gelatin with 1.20% DH exhibited the higher heat susceptibility and weight loss in the temperature range of 50-600 °C, compared with control film. Thus, both chain length of gelatin and glycerol content directly affected the properties of cuttlefish skin gelatin films.

3.2 Introduction

Gelatin is a thermal denatured protein obtained from collagen by acidic or alkaline process. (Gennadios *et al*., 1994; Arvanitoyannis, 2002). Generally, gelatin has the wide range applications in food industries, cosmetics, biomedical, pharmaceutical, leather and encapsulation (Cho *et al*., 2004; Segtnan *et al*., 2003; Slade and Levine, 1987). However, the occurrence of bovine spongiform encephalopathy (BSE), foot and mouth diseases have caused major concerns for human health (Cho *et al*., 2005). Moreover, porcine gelatin can cause objections from some religions. As a consequence, an increasing interest has been paid to other gelatin sources, especially skin and bone from seafood processing by-products (Gómez-Guillén *et al*., 2002; Jongiareonrak *et al*., 2006a; Hoque *et al*, 2010). Cuttlefish are one of the important fishery product in Thailand as well as other south-east Asian countries, and is mainly exported worldwide. During processing of cuttlefish, skin is generated as a by-product with the low market value. To increase its profitability, cuttlefish skin has recently been used for gelatin extraction (Aewsiri *et al*., 2009; Hoque *et al*., 2010). Additionally, gelatin have been used as a material for preparing biodegradable films with an excellent gas barrier property (Gómez-Guillén *et al*., 2009; Jongjareonrak *et al*., 2006b). Biodegradable films made from renewable biopolymers sources can become an important environmental friendly packaging, thereby reducing plastic wastes (Tharanathan, 2003; Prodpran & Benjakul, 2005; Hoque *et al*., 2010).

Gelatin is a heterogeneous protein containing 20 different amino acids in their polypeptide chains (Arvanitoyannis, 2002). The physical and structural properties of gelatin are mainly influenced by the molecular weight distribution and amino acid composition that plays an important role in the rheological and barrier properties of the resulting films (Gómez-Guillén *et al*., 2009). The properties of films also depend on the type and concentration of plasticizer used (Gómez-Guillén *et al*., 2009). Carvalho *et al*. (2008) stated that the mechanical behaviors of gelatin films varied with molecular weight distributions of gelatin. Higher tensile strength and lower elongation values were reported for the film from high molecular weight gelatin, compared with the films of lower molecular weight gelatins (Gómez-Guillén *et al*.,

2009). Gelatin with the lower molecular mass caused by hydrolysis had the reduced thermal and mechanical properties (Langmaier *et al*., 1999). Plasticizer type and concentration is another critical factor affecting the mechanical properties as well as water vapour barrier property of gelatin films (Jongjareonrak *et al*., 2006c; Sobral & Habitante, 2001). Due to hydrophilicity of glycerol, commonly used as plasticizer in the protein based film, lowering the use of glycerol could be a promising means to improve water barrier property of the film. Along with the cleavages of peptide chains, the rigidity of films could be decreased and the lower amount of plasticizer could be required. However, there is no information about the impact of chain length of gelatin from cuttlefish skin, a by-product from cuttlefish processing, and the influence of glycerol content on the properties of resulting film exists. Thus, the objectives of the present study were to study the effects of gelatin from cuttlefish skin gelatin with different degree of hydrolysis (DH) and glycerol content on the properties of films.

3.3 Materials and methods

3.3.1 Chemicals

L-Leucine, 2,4,6-trinitrobenzenesulphonic acid (TNBS), bovine serum albumin and wide range molecular weight protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2), glycerol, *p*-dimethylaminobenzaldehyde and tris(hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfates (SDS), Coomassie Blue R-250 and *N, N, N′, N′*- tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Alcalase 2.4 L was provided by Novozymes (Bagsvaerd, Denmark). All chemicals were of analytical grade.

3.3.2 Collection and preparation of cuttlefish skin

Ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from a dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla

University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1 x 1 cm^2), placed in polyethylene bags and stored at -20 ºC until use. Storage time was not longer than 2 months. Prior to gelatin extraction, the frozen skin was thawed using running water (25-26 ºC) until the core temperature reached 0 - 2 ºC.

3.3.3 Extraction of gelatin from cuttlefish skin

Gelatin was extracted from cuttlefish skin according to the method of Hoque *et al*. (2010). Skin was soaked in 0.05 M NaOH with a skin/solution ratio of 1:10 (w/v) with a gentle stirring at room temperature (26–28 ºC). The solution was changed every hour to remove non-collagenous proteins for totally 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching in 5% H_2O_2, using a sample/solution ratio of 1:10 (w/v) for 48 h at 4 ºC. The skin treated with H_2O_2 was washed three times with 10 volumes of water. Gelatin was extracted from bleached skin by using distilled water at 60 ºC for 12 h, using a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously using a paddle stirrer (RW20.n, IKA LABORTECHNIK, Staufen, Germany). The extract was centrifuged at 8,000 x g for 30 min at room temperature using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble materials. The supernatant was collected and freeze-dried (Model Duratop™ lP/Dura Dry™ lP, FTS® System, Inc., Stone Ridge, NY, USA). The dry matter was referred to as ‘gelatin powder’ and subjected to hydrolysis. Gelatin and its hydrolysates were used for film preparation.

3.3.4 Preparation of gelatin hydrolysate

3.3.4.1 Preparation of gelatin hydrolysate with different degree of hydrolysis (DH)

Gelatin was dissolved in 10 mM phosphate buffer (pH 8) to obtain the protein concentration of 3% (w/v). The pH of the mixture was readjusted to 8.0 using

2 M NaOH. The mixtures were incubated at 50 °C for 10 min. The hydrolysis reaction was started by the addition of Alcalase at the amount calculated from the plot between log enzyme concentration and DH to obtain DH of 0.40, 0.80 and 1.20% as described by Benjakul and Morrissey (1997). After hydrolysis using different levels of Alcalase for 5 min, the resulting hydrolysates were subjected to heat treatment (90 °C) in a temperature controlled water bath (Memmert, GmbH+Co. KG, D-91126, Schwabach, Germany) for 15 min to fully inactivate the enzyme. Protein pattern of obtained hydrolysates was determined using SDS-PAGE made of 4% stacking gel and 12% separating gel. DH of hydrolysate was also determined as per the method of Benjakul and Morrissey (1997).

3.3.4.2 Determination of DH

DH of gelatin hydrolysate was determined according to the method of Benjakul and Morrissey (1997). Hydrolysate samples with appropriate dilution (125 μL) were added with 2.0 mL of 0.2 M phosphate buffer (pH 8.2) and 1.0 mL of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature controlled water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and α-amino group was expressed in terms of L-leucine. The DH was defined as follows (Benjakul & 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 acid hydrolysis (6 N HCl at 100 °C for 24 h).

3.3.5 Effect of partial hydrolysis and glycerol content on the properties of gelatin films

3.3.5.1 Preparation of film from gelatin hydrolysates with different %DHs having various glycerol contents

Gelatin hydrolysate with different DHs was used for the preparation of film forming solution (FFS). Glycerol at different contents (10, 15 and 20 %; based on protein) was used as a plasticizer. FFS (4 ± 0.01 g) was cast onto a rimmed silicone resin plate (5 x 5 cm^2), air-blown for 12 h at room temperature and dried with an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttalingen, Germany) at the temperature of 25 ± 0.5 ºC and 50 ± 5% relative humidity (RH) for 48 h. Dried films were manually peeled off and subjected to analyses.

3.3.5.2 Analyses

Prior to mechanical properties testing, films were conditioned for 48 h at 50 ± 5% relative humidity (RH) at 25 ± 0.5 ºC. For ATR-FTIR and TGA studies, films were conditioned in a dessicator containing dried silica gel for 3 weeks at room temperature (28-30 ºC) to obtain the most dehydrated films.

3.3.5.2.1 Film thickness

The thickness of film was measured using a micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mituyoto Corp, Kawasaki-shi, Japan). Ten random locations around each film sample were used for thickness determination.

3.3.5.2.2 Mechanical properties

Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata et al. (2000) using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK). Ten samples (2 x 5 cm^2) with the initial grip length of 3 cm were used for testing. The samples were clamped and deformed under tensile loading using a 100 N load cell with the cross-head speed of 30 mm min^{-1} until the samples were broken. The maximum load and the final extension at break were used for calculation of TS and EAB, respectively.

3.3.5.2.3 Water vapor permeability (WVP)

WVP was measured using a modified ASTM (American Society for Testing and Materials, 1989) method as described by Shiku *et al*. (2004). The film was sealed on an aluminum permeation cup containing silica gel (0% RH) with silicone vacuum grease. The cup was placed at 30 ºC in a desiccator containing the distilled water. It was then weighed at 1 h intervals over an 8 h period. Six films were used for WVP testing. WVP of the film was calculated as follows (McHugh *et al*., 1993):

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

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

3.3.5.2.4 Color and transparency of the film

Color of film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., Reston, VA, USA). Color of the film was expressed as L^*-, a^*- and b^*-values and total differences in color was expressed as ΔE*. ΔE* values were calculated according to the following equation (Gennadios *et al*., 1996)

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

where ΔL*, Δa* and Δb* are the differentials between the color parameter of the samples and the color parameter of white standard (L^* = 93.61, a^* = -0.93 and b^* = 0.53).

The transparency value of the film was calculated by the following equation (Han and Floros, 1997):

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

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

3.3.5.2.5 Electrophoretic analysis

Protein patterns of gelatin film were analyzed under reducing condition using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Prior to analysis, the film samples were prepared according to the method of Jongjareonrak *et al*. (2006b) with some modifications. Film samples (200 mg) were dissolved in 10 ml of 1% (w/v) SDS. The mixture was stirred continuously at room temperature for 12 h. Supernatants were obtained after centrifuging at 3000xg for 5 min using Hettich Zentrifuge (MIKRO-20, D-78532, Tuttlingen, Germany). For gelatin hydrolysates, they were mixed with 1% (w/v) SDS at a ratio of 1:1 (v/v). Protein content of the prepared solutions of both gelatin hydrolysates and their corresponding film was determined according to the Biuret method (Robinson and Hodgen, 1940). The solutions were then mixed with sample buffer (0.5 MTris–HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol with 10% (v/v) β-ME) at the ratio of 1:1 (v/v). Samples (30 μg protein) were loaded onto the polyacrylamide gel made of 12% running gel and 4% stacking 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 electrophoresis, gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Wide range molecular weight protein markers were used to estimate the molecular weight of proteins.

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

FTIR spectra of films prepared from gelatin without hydrolysis (0% DH) and that with 1.20% DH in the presence of glycerol at different levels were determined using a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45º ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison,

WI, USA) at room temperature as described by Nuthong *et al.* (2009a). Films were placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra in the range of 700–4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 ºC.

3.3.5.2.7 Thermo-gravimetric analysis (TGA)

Conditioned films were scanned using a thermogravimetric analyzer (TGA7, PerkinElmer, Norwalk, CT, USA) from 50 to 600 ºC at a rate of 10ºC/min (Nuthong *et al*., 2009a). Nitrogen was used as the purge gas at a flow rate of 20 ml/min.

3.3.6 Statistical analysis

Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

3.4 Results and discussion

Films from gelatin of cuttlefish skin with different DH containing glycerol at varying levels exhibited the different properties and molecular characteristics.

3.4.1 Thickness

Thickness of films prepared from cuttlefish skin gelatin with different DHs containing various glycerol contents ranged from 0.036 to 0.040 mm (Table 13). At the same DH, glycerol content had no impact on thickness of the resulting films (p>0.05). Vanin *et al*. (2005) also observed the negligible differences in thickness of gelatin-based films with the different levels of glycerol. Nevertheless, the film prepared from gelatin with higher DH (1.20%) showed a slightly lower thickness than

that of the control film (without hydrolysis) (p<0.05). Gelatin with the shorter chain might align themselves to form the ordered network with the less protrusion. This more likely resulted in the lower thickness of obtained film.

3.4.2 Mechanical properties

Mechanical properties of films prepared from gelatin with different DHs in the presence of glycerol at varying contents are presented in Table 13. At the same level of glycerol used, film prepared from the control gelatin (without hydrolysis) showed the highest TS and EAB, as compared with the film obtained from gelatin with partial hydrolysis (p<0.05). TS and EAB decreased as %DH increased when the same level of glycerol was used (p<0.05). These results suggested that gelatin molecules with the shorter chain most likely established the weaker chain-to-chain interaction or less junction zones mainly via hydrogen bond (Figure 11). Furthermore, the increasing number of chain ends of gelatin with partial hydrolysis directly enhanced the mobility of chains. As a result, the weaker film network was formed. Hoque *et al*. (2010) reported that gelatin film mainly stabilized by the weak bond including hydrogen bond and hydrophobic interaction. The result indicated that integrity and chain length of gelatin molecules directly contributed to the formation of film network. A scheme illustrating the impact of chain length of gelatin on the matrix of resulting film is presented in Figure 11.

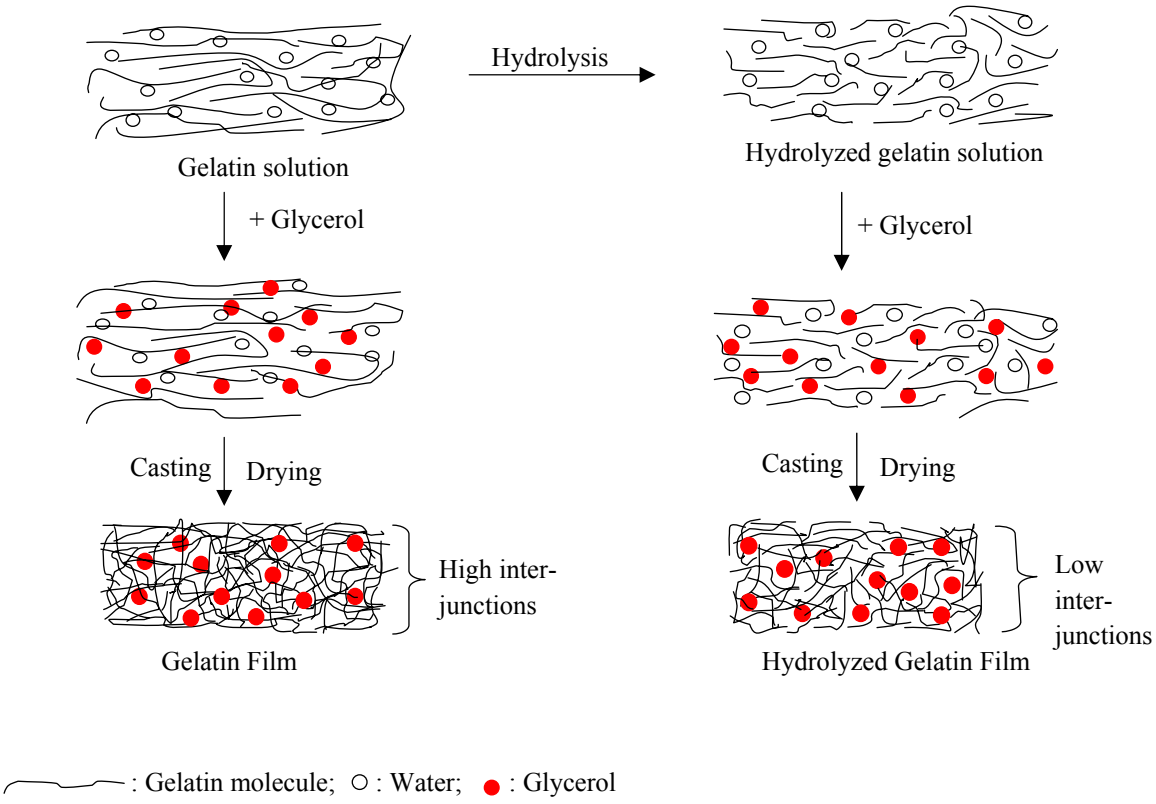


Figure 11. A scheme illustrating the impact of chain length of gelatin on the matrix of resulting film

At the same level of glycerol used, EAB decreased when gelatin with increasing DH was used for film preparation. The lower chain entanglement more likely took place when the shorter chains existed (Figure 11). Therefore, the easier molecular slippage was presumed when the tensile force was applied. As a consequence, both TS and EAB of film with the shorter chains decreased. Since weak bonds were involved in gelatin film (Hoque *et al*., 2010), the interaction of gelatin molecules in the way which provided the longer chain could lower the rigidity of resulting films. Nevertheless, at the same level of glycerol, too short chains could not interact and results could not render the strong and elastic films. Jongjareonrak *et al*. (2006b) 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.

When glycerol at different levels was incorporated, film obtained from the control gelatin and those with different %DH showed the differences in both TS and EAB ($p<0.05$) (Table 13). Regardless of DH, TS decreased with increasing glycerol content ($p<0.05$). The increases in EAB were noticeable when glycerol contents increased ($p<0.05$). The increases in glycerol would lower the interaction between chains. However, EAB values obtained from film with 0.80 and 1.20% DH were not affected by the glycerol content used ($p>0.05$). The results suggested that the chain length was a crucial factor governing the TS and EAB of resulting film. Gontard *et al*. (1993) reported that glycerol molecule could easily form hydrogen bond with protein chain, resulting in a reduction of intermolecular interaction. Also the mobility of macromolecules increased, leading to the decrease in TS and increase in EAB of films. Similar results was reported by Jongjareonrak *et al*. (2006b) who 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). At higher DH (0.80 and 1.20% DH), shorter gelatin molecules with the higher mobility of chain ends might perform plasticizing effect by preventing protein-protein interaction but preferably formed H-bonds with water. Therefore, the mechanical properties of gelatin-based film were largely affected by the chain length of gelatin as well as glycerol content.

Table 13. Mechanical properties, water vapor permeability and thickness of film from gelatin of cuttlefish skin without hydrolysis and with different DH containing glycerol at different levels.

% DH/Glycerol†	TS (MPa)	EAB (%)	WVP ($x10^{-10}$ g $s^{-1}.m^{-1}.Pa^{-1}$)	Thickness (mm)
C/10	45.63 ± 1.10 aA	3.91± 0.64 bA	0.66 ± 0.11 cB	0.037 ± 0.002 bA
C/15	40.39 ± 3.45 bA	4.29 ± 0.59 bA	0.88 ± 0.03 bC	0.039 ± 0.002 abA
C/20	36.57 ± 2.56 cA	5.39 ± 0.16 aA	1.06 ± 0.07 aB	0.040 ± 0.002 aA
0.40/10	23.55 ± 2.38 aB	2.98 ± 0.23 bB	0.89 ± 0.10 bA	0.038 ± 0.002 aA
0.40/15	20.80 ± 2.44 abB	3.23 ± 0.13 bB	1.01 ± 0.06 aAB	0.038 ± 0.001 aAB
0.40/20	18.96 ± 1.84 bB	3.65 ± 0.33 aB	1.08 ± 0.07 aB	0.040 ± 0.002 aA
0.80/10	17.39 ± 2.17 aC	2.50 ± 0.34 aB	0.96 ± 0.08 bA	0.037 ± 0.002 aA
0.80/15	15.84 ± 1.71 aC	2.65 ± 0.26 aC	1.05 ± 0.07 aB	0.038 ± 0.002 aAB
0.80/20	11.64 ± 1.70 bC	2.89 ± 0.45 aC	1.11± 0.04 aAB	0.039 ± 0.002 aAB
1.20/10	15.32 ± 1.77 aC	2.61 ± 0.69 aB	1.01 ± 0.07 bA	0.037 ± 0.001 aA
1.20/15	12.83 ± 1.91 bD	2.66 ± 0.31 aC	1.12 ± 0.06 aA	0.036 ± 0.003 aB
1.20/20	12.99 ± 1.18 bC	2.45 ± 0.29 aD	1.18 ± 0.08 aA	0.037 ± 0.002 aB

† % based on protein content

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

Different capital letters within the same glycerol content in the same column indicate significant differences (p<0.05).

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

3.4.3 Water vapor permeability (WVP)

WVP of films prepared from gelatin without and with hydrolysis at different DH containing different glycerol levels is presented in Table 13. Film with the same glycerol level showed the increases in WVP as %DH increased (p<0.05). The highest WVP was found in film prepared from gelatin with 1.20% DH (p<0.05). These results suggested that the hydrolysis could expose more carboxylic group and amino group, which could form hydrogen bond with the water molecules. This resulted in the increased hydrophilicity of the resulting films. Furthermore, the

shorter gelatin molecules most likely yielded less dense and more permeable film matrix during film formation, leading to the higher WVP of the resulting film. Giménez *et al*. (2009) observed that WVP of film significantly increased ($p<0.05$) when gelatin hydrolysates was incorporated in the films. Film from halibut skin gelatin with lower molecular weight also had the higher WVP, compared with that from the higher molecular weight (Carvalho *et al*., 2008).

When film with the same DH was tested, WVP increased with increasing glycerol content ($p<0.05$). However, no differences in WVP were observed between film with DH of 0.40-1.20% containing 15 and 20% glycerol content ($p>0.05$). These result suggested that hydroxyl group (-OH) of hygroscopic plasticizer, glycerol, which was able to interact with water, increased WVP of the film. Increased WVP was also found when the increasing glycerol content was used in fish skin gelatin film (Jongjareonrak *et al*., 2006b), pig skin gelatin film (Vanin *et al*., 2005) and fish muscle protein film (Paschoalick *et al*., 2003). Thus, degree of hydrolysis and glycerol level had the profound impact on WVP of film from cuttlefish skin gelatin.

3.4.4 Color of film

Table 14 shows the color of films prepared from cuttlefish skin gelatin with different DH and various glycerol levels. Higher L*-value (lightness), lower b*-value (yellowness) and ΔE* were obtained for film prepared from gelatin without hydrolysis, compared with those of films from gelatin with all DH ($p<0.05$). For a*-value, it was noted that similar value was obtained for all films ($p>0.05$). However, films prepared from gelatin with 1.20% DH showed the lower a*-value than film from gelatin without hydrolysis, when glycerol at 15 and 20% was used ($p<0.05$). Hydrolysis process rendered amine group ($-NH_2$), which might undergo interaction with carbonyl group in gelatin via Maillard reaction, particularly during drying of film. Fish and invertebrate collagen and/or gelatin contains some amount of carbohydrates (glucose and galactose), which are attached to hydroxylysine residues of the peptide chain by O-glycosidic bonds. The presence of 2-O-α D-glucosyl-O-β-D-galactosyl-hydroxylysine and O-β-D galactosyl-hydroxylysine was confirmed (Burghagen,

1999). Petibois et al. (2006) also reported that the carbohydrate moieties are attached to the collagenous protein. Hoque *et al*. (2010) found the increased b* -value due to heat treatment of FFS from the cuttlefish skin gelatin film. Therefore, hydrolysis of gelatin showed the impact on the color of resulting films. However, glycerol content did not show the marked effect on color. Gennadios *et al*. (1996) and Vanin *et al*. (2005) reported that neither plasticizer type (glycerol, polyethylene glycol, sorbitol) nor concentration had effect on the color difference of films from egg albumen and gelatin, respectively.

Table 14. Color of film from gelatin of cuttlefish skin without hydrolysis and with different DH containing glycerol at different levels

%DH/Glycerol[†]	L*	a*	b*	ΔE*
C/10	91.13 ± 0.20 aA	-1.29 ± 0.02 aA	2.55 ± 0.19 aB	3.22 ± 0.23 aB
C/15	91.09 ± 0.13 aA	-1.30 ± 0.04 aA	2.65 ± 0.11 aB	3.32 ± 0.13 aB
C/20	91.10 ± 0.19 aA	-1.28 ± 0.03 aA	2.62 ± 0.11 aB	3.29 ± 0.14 aB
0.40/10	90.89 ± 0.21 aB	-1.33 ± 0.05 aA	2.85 ± 0.26 aA	3.59 ± 0.13 aA
0.40/15	90.78 ± 0.22 aBC	-1.34 ± 0.04 aAB	2.95 ± 0.24 aA	3.70 ± 0.33 aA
0.40/20	90.83 ± 0.17 aB	-1.30 ± 0.05 aAB	2.82 ± 0.18 aA	3.63 ± 0.27 aA
0.80/10	90.88 ± 0.23 aB	-1.32 ± 0.05 aA	2.92 ± 0.28 aA	3.66 ± 0.26 aA
0.80/15	90.75 ± 0.08 aB	-1.33 ± 0.04 aAB	2.86 ± 0.19 aA	3.71 ± 0.13 aA
0.80/20	90.69 ± 0.15 aB	-1.31 ± 0.05 aAB	2.80 ± 0.17 aA	3.71 ± 0.20 aA
1.20/10	90.84 ± 0.17 aB	-1.32 ± 0.03 aA	2.81 ± 0.16 aA	3.61 ± 0.17 bA
1.20/15	90.56 ± 0.18 bC	-1.37 ± 0.04 bB	2.92 ± 0.15 aA	3.84 ± 0.10 aA
1.20/20	90.66 ± 0.25 abB	-1.33 ± 0.05 abB	2.87 ± 0.23 aA	3.69 ± 0.22 abA

[†] % based on protein content

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

Different capital letters within the same glycerol content in the same column indicate significant differences (p<0.05).

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

3.4.5 Light transmission and transparency

UV and visible light transmission at wavelength range of 200–800 nm of film from gelatin without hydrolysis and with different DH containing various glycerol contents are presented in Table 15. Light transmission in visible range (350–800 nm) of films was from 54.85 to 87.59%. The transmission of UV light at 280 nm was in the range of 5.83–9.67%. Very low transmission (0.01%) was found at 200 nm. Therefore, gelatin film effectively prevented the UV light, regardless of DH. Jongjareonrak *et al*. (2006b) also reported that the higher UV light barrier capacity of gelatin film from bigeye snapper and brownstripe red snapper skin effectively retarded the lipid oxidation induced by UV light, compared with the synthetic film.

Table 15. Light transmission and transparency (%) values of film from gelatin of cuttlefish skin without hydrolysis and with different DH containing glycerol at different levels

%DH/ Glycerol†	Wavelength (nm)								Transparency Values
	200	280	350	400	500	600	700	800	
C/10	0.01	8.08	62.44	74.83	82.06	84.84	86.50	87.59	3.40 ± 0.03 aA
C/15	0.01	7.03	59.47	72.91	80.47	83.33	85.10	86.27	3.38 ± 0.04 aA
C/20	0.01	8.55	65.69	77.73	83.96	86.04	87.30	88.11	3.37 ± 0.02 aA
0.40/10	0.01	5.83	56.01	69.77	77.90	81.00	82.92	84.22	3.38 ± 0.04 aA
0.40/15	0.01	6.97	58.46	71.41	79.22	82.24	84.13	85.42	3.36 ± 0.07 aA
0.40/20	0.00	6.51	58.67	73.01	80.70	83.20	84.71	85.77	3.34 ± 0.04 aA
0.80/10	0.01	7.01	54.85	67.93	76.69	80.51	82.95	84.64	3.38 ± 0.03 aA
0.80/15	0.01	7.37	59.60	73.81	81.48	84.11	85.71	86.81	3.35 ± 0.03 abA
0.80/20	0.01	8.26	63.49	75.96	82.44	84.59	85.92	86.83	3.33 ± 0.04 bA
1.20/10	0.01	8.79	60.27	72.11	79.46	82.42	84.27	85.53	3.35 ± 0.05 aA
1.20/15	0.01	9.67	61.34	74.63	81.85	84.27	85.74	86.76	3.33 ± 0.05 aA
1.20/20	0.01	8.98	64.52	76.81	83.24	85.26	86.50	87.33	3.33 ± 0.04 aA

† % based on protein content.
Values are given as Mean ± SD (n = 3).
Different capital letters within the same glycerol content in the same column indicate significant differences (p<0.05).
Different letters within the same DH in the same column indicate significant differences (p<0.05).

No differences in transparency value were observed between films from gelatin without hydrolysis and those with various DH, irrespective of glycerol content (p>0.05) (Table 15). Gelatin film has been considered as highly transparent film (Paschoalick *et al*., 2003; Bergo and Sobral, 2006). Highly transparent film was prepared from polyvinyl alcohol and gelatin blended film (Maria *et al*., 2008). Both plasticizer type and concentration had no effect on transparency of film from pig skin gelatin (Vanin *et al*., 2005). Therefore, chain length of gelatin from cuttlefish skin had no impact on light transmission and transparency value of resulting films.

3.4.6 Electrophoretic protein patterns

The electrophoretic patterns of gelatin used for film preparation (without hydrolysis and with different %DH) and their corresponding films containing different glycerol contents are shown in Figure 12A and 12B, respectively. Proteins with the molecular weight (MW) of ~105 and ~97 kDa were observed as two major bands in gelatin. After hydrolysis with Alcalase, those two major bands disappeared with coincidental occurrence of peptides with lower MW. For, 0.40% DH sample, the small band with MW of ~90 kDa and bands with MW lower than ~80 kDa were found. For gelatin with 0.80 and 1.20% DH, band intensity of all proteins became lowered. Gelatin with 1.20% DH contained no proteins with MW greater than ~80 kDa. The results suggested that enzymatic hydrolysis caused the degradation of gelatin molecules, leading to the formation of shorter peptide chains. Those shorter chain molecules yielded the weaker film network with low TS and EAB (Figure 11 and Table 13). Carvalho *et al*. (2008) reported the differences in molecular weight of gelatin from the skin of Atlantic halibut.

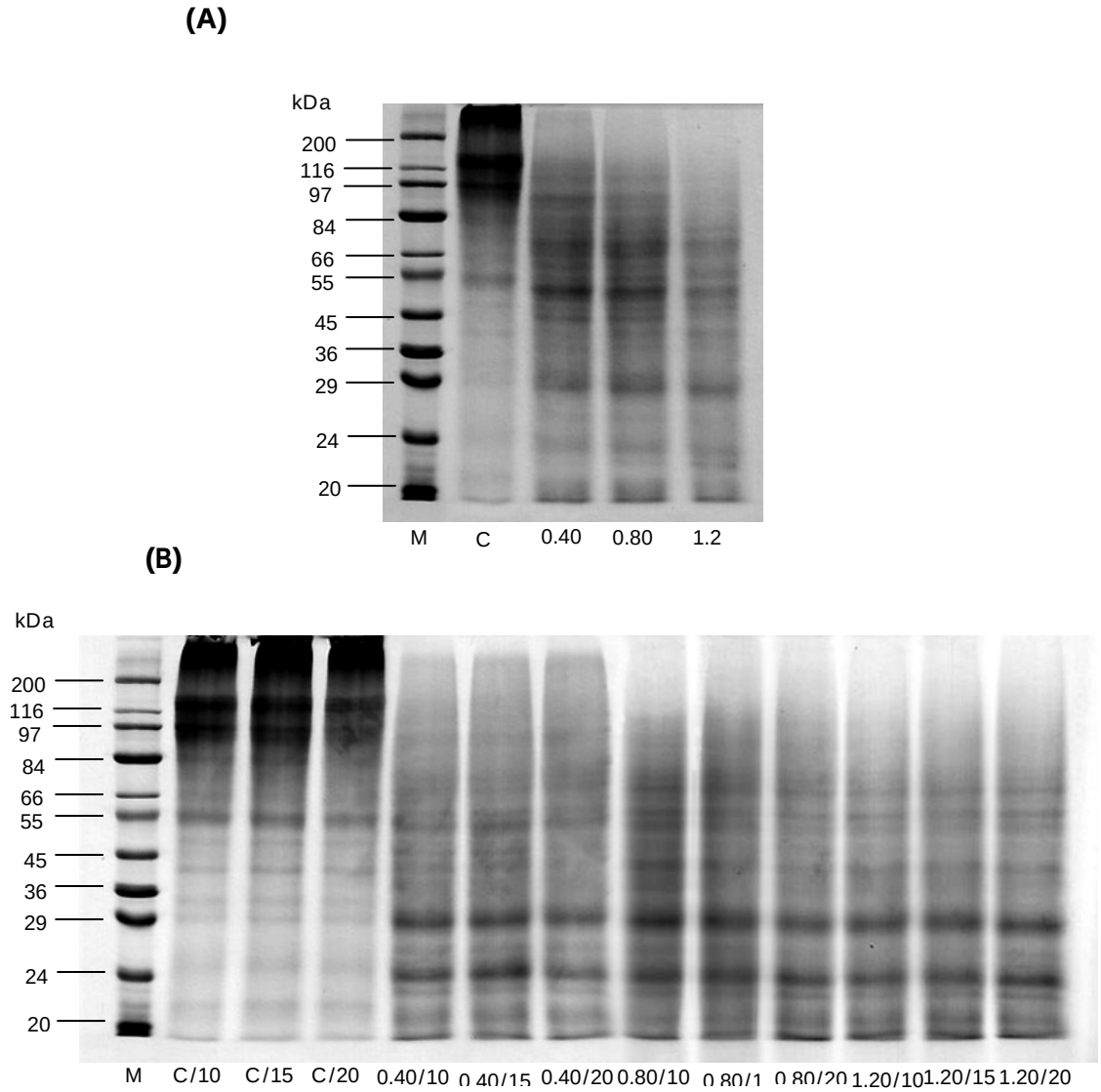


Figure 12. Protein patterns of gelatin without hydrolysis and with different DH (A) and their corresponding films containing glycerol at different levels (B). M: protein marker; C: control (without hydrolysis); 0.40, 0.80 and 1.20 denote %DH; and 10, 15 and 20 represent glycerol level (%).

The similar protein patterns were observed between gelatin samples and their corresponding films (Figure. 12B). Additionally, two protein bands with MW of ~28 and ~24 kDa were observed in all films tested. No differences in protein pattern were observed between films containing different glycerol contents. The result suggested that film of all gelatin samples were most likely stabilized by weak bond, especially hydrogen bond. Limpisophon et al. (2009) found the no differences in the

protein pattern of film with different glycerol and protein content from under-utilized blue shark (*Prionace glauca*) skin gelatin. Hoque *et al*. (2010) also observed the similar protein pattern between FFS and film at different heating temperatures from cuttlefish skin gelatin. In the presence of SDS as well as βME used for electrophoresis, hydrogen bond, hydrophobic interaction as well as non-disulfide covalent bond in film network were destroyed. Nevertheless, no disulfide was present in gelatin film. Gelatin contains negligible amount of cysteine. As a result, no disulfide bond was formed in the resulting film (Hoque *et al*., 2010). This was evidenced by the complete solubilization in water (data not shown). However, different protein components in gelatin with various MW directly influenced the properties of their resulting films, mainly stabilized by weak bonds.

3.4.7 FTIR spectroscopy

FTIR spectra of films prepared from gelatin without hydrolysis and gelatin with 1.20% DH in the presence of glycerol at different levels are shown in Figure 13. The spectra of all films displayed the major bands at 1631 cm^{-1} (amide-I, representing C=O stretching/hydrogen bonding coupled with COO), 1538 cm^{-1} (amide-II, arising from bending vibration of N-H groups and stretching vibrations of C-N groups) and 1235 cm^{-1} (amide-III, representing the vibrations in plane of C-N and N-H groups of bound amide or vibrations of CH_2 groups of glycine) (Muyonga *et al*., 2004a; Aewsiri *et al*., 2009). Yakimets *et al*. (2005) reported the similar result for bovine skin gelatin film where amide-I, amide-II and amide-III peaks were found at the wavenumbers of 1633, 1536 and 1240 cm^{-1}, respectively. The peak situated around 1033 cm^{-1} might be related to the interactions arising between plasticizer (OH group of glycerol) and film structure (Bergo and Sobral, 2007). Generally, similar spectra of all samples were obtained in the range of 1800-700 cm^{-1}, covering Amide-I, II and III.

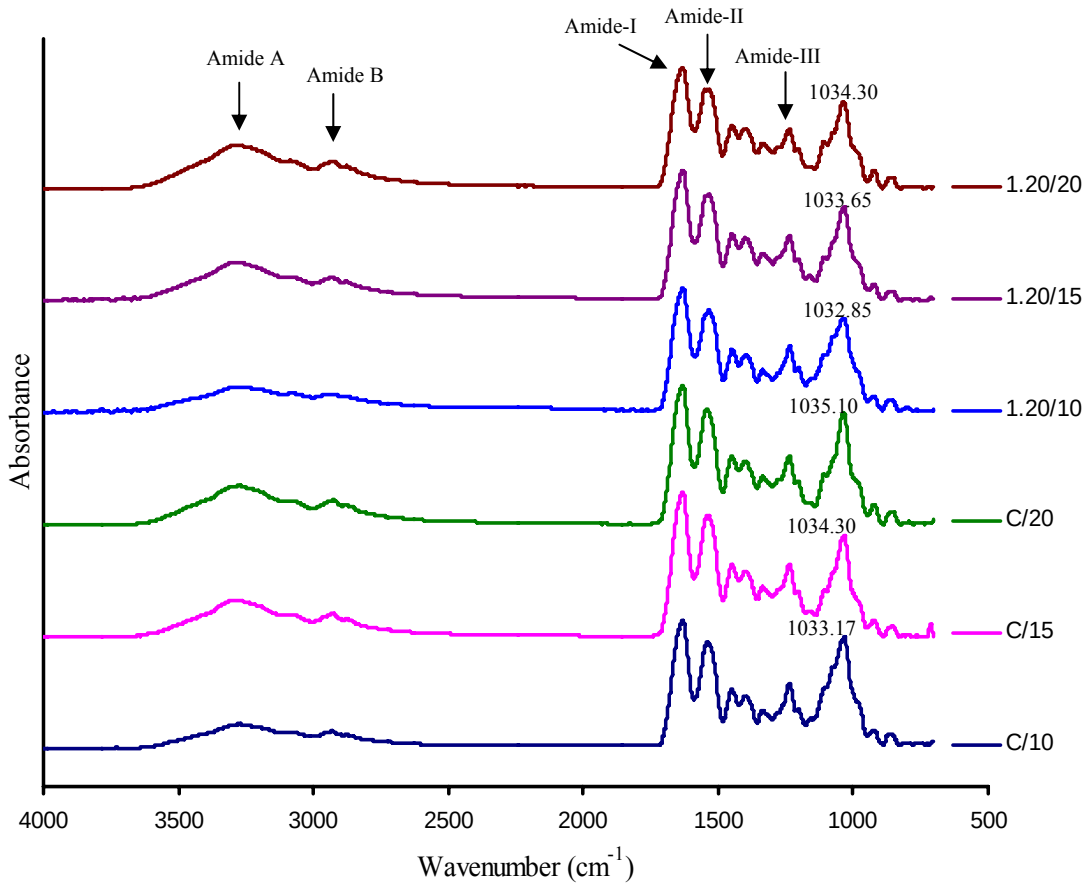


Figure 13. FTIR spectra of films prepared from gelatin of cuttlefish skin without hydrolysis and 1.20% DH containing glycerol at different levels. C: control; 1.20 denote %DH; and 10, 15 and 20 represent glycerol level (%).

Furthermore, amide-A peak was found at 3275 cm^{-1}, representing NH-stretching coupled with hydrogen bonding. Amide-B peak at 2929 cm^{-1}, representing CH stretching and $-NH_2$, was also observed in the spectra. The amide-A and amide-B peaks at wavenumbers of 3458 and 2926 cm^{-1}, respectively were formed in adult Nile perch collagen (Muyonga *et al*., 2004a). At the same glycerol level, spectra of film prepared from gelatin with 1.20% DH showed the higher amplitudes for amide-A peak, compared with that of film prepared from gelatin without hydrolysis. Higher amount of $–NH_2$ or $–NH_3^+$ group obtained from hydrolysis process more likely resulted in the higher amplitude of amide-A and amide-B peaks obtained. For the film prepared from gelatin with the same DH containing different glycerol contents, it was

noted that the higher amplitudes of amide-A and amide-B peaks were obtained as glycerol content increased. The result indicated that in the presence of glycerol at higher content, the interaction between gelatin molecules became less. As a result, the cross-linking of molecules, especially via H-bonds between amino group of either gelatin or gelatin with partial hydrolysis (1.20% DH) and H-acceptors was lowered. This was evidenced by the higher availability of amino group shown by the higher amplitudes of both amide peaks. The film in this present study contained different glycerol level as the plasticizer. The peak situated around 1033 cm^{-1} corresponds to the glycerol. The height of this peak increased with increasing glycerol content. Also, the peak shifted from 1033.17 to higher wavenumber (1034.30 and 1035.10 cm^{-1}) when glycerol increased from 10% to 15 and 20%, respectively. For 1.2% DH gelatin film, peak shifted from 1032.85 to 1033.65 and 1034.30 cm^{-1}, respectively. These changes might be related to the possible interactions between the plasticizer and the film matrix. Bergo and Sobral (2007) observed the similar result for pigskin gelatin film containing 15, 30 and 45% glycerol. Ziani *et al.* (2008) also found the differences in intensities of FTIR spectra of chitosan film added with glycerol or Tween 20. As a consequence, some water might be within the film matrix, though it was equilibrated over the dry silica gel for 3 weeks. Water content affected FTIR spectra in the gelatin film without glycerol addition (Yakimets *et al*., 2005). This water might have the influence on FTIR spectra to some degree. Yakimets *et al*. (2005) also reported that aging did not have a marked effects on hydration behavior to comparison of the increase of water bands for film aged at ambient conditions (22 °C, 40-50% RH) for 1, 5 and 14 days.

3.4.8 Thermo-gravimetric analysis (TGA)

TGA thermograms revealing thermal degradation behavior of films prepared from gelatin without hydrolysis and gelatin with 1.20% DH in the presence of glycerol at different levels are depicted in Figure 14A and 14B. Their corresponding degradation temperatures (Td) and weight loss (Δw) are shown in Table 16. All films from gelatin without hydrolysis and gelatin with 1.20% DH exhibited three main stages of weight loss. For all films, the first stage weight loss

(Δw_1= 2.10-3.70%) was observed approximately at temperature (Td_1) of 50.03-70.00 °C, possibly related to the loss of free water adsorbed in the film. The similar result was reported in collagen hydrolysate film plasticized with glycerol (GLY) and poly(ethylene glycols) at different molecular masses (Langmaier *et al*., 2008) and porcine plasma protein film added with different cross linking agents (Nuthong *et al*., 2009a). The second stage weight loss (Δw_2= 14.94 - 21.70%) appeared at Td_2 (196.30–216.71 °C) of all films, was most likely associated with the loss of glycerol compounds and structurally bound water. For the third stage weight loss, Td_3 of 271.40-290.18 °C were observed in films prepared from gelatin without hydrolysis, whereas Td_3 of 249.47-255.80 °C were found in those from gelatin with 1.20% DH. The lower Td of the later was more likely due to the shorter chains, which could undergo thermal degradation to a high extent, compared with the former.

Table 16. Thermal degradation temperature (T_d, °C) and weight loss (Δw, %) of film from cuttlefish skin gelatin without hydrolysis and 1.20% DH containing glycerol at different levels

%DH/Glycerol†	Δ_1		Δ_2		Δ_3		Residue (%)
	$Td_{1,onset}$	Δw_1	$Td_{2,onset}$	Δw_2	$Td_{3,onset}$	Δw_3	
C/10	70.00	2.10	216.71	14.94	290.18	53.36	29.60
C/15	60.90	2.54	209.68	17.60	286.50	55.25	24.61
C/20	59.51	2.82	202.68	18.81	271.40	55.59	22.78
1.2/10	49.22	3.38	213.89	16.46	255.80	54.71	25.45
1.2/15	50.03	3.57	209.55	19.08	253.32	53.11	24.24
1.2/20	50.18	3.70	196.38	21.70	249.47	51.58	23.02

† % based on protein content

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

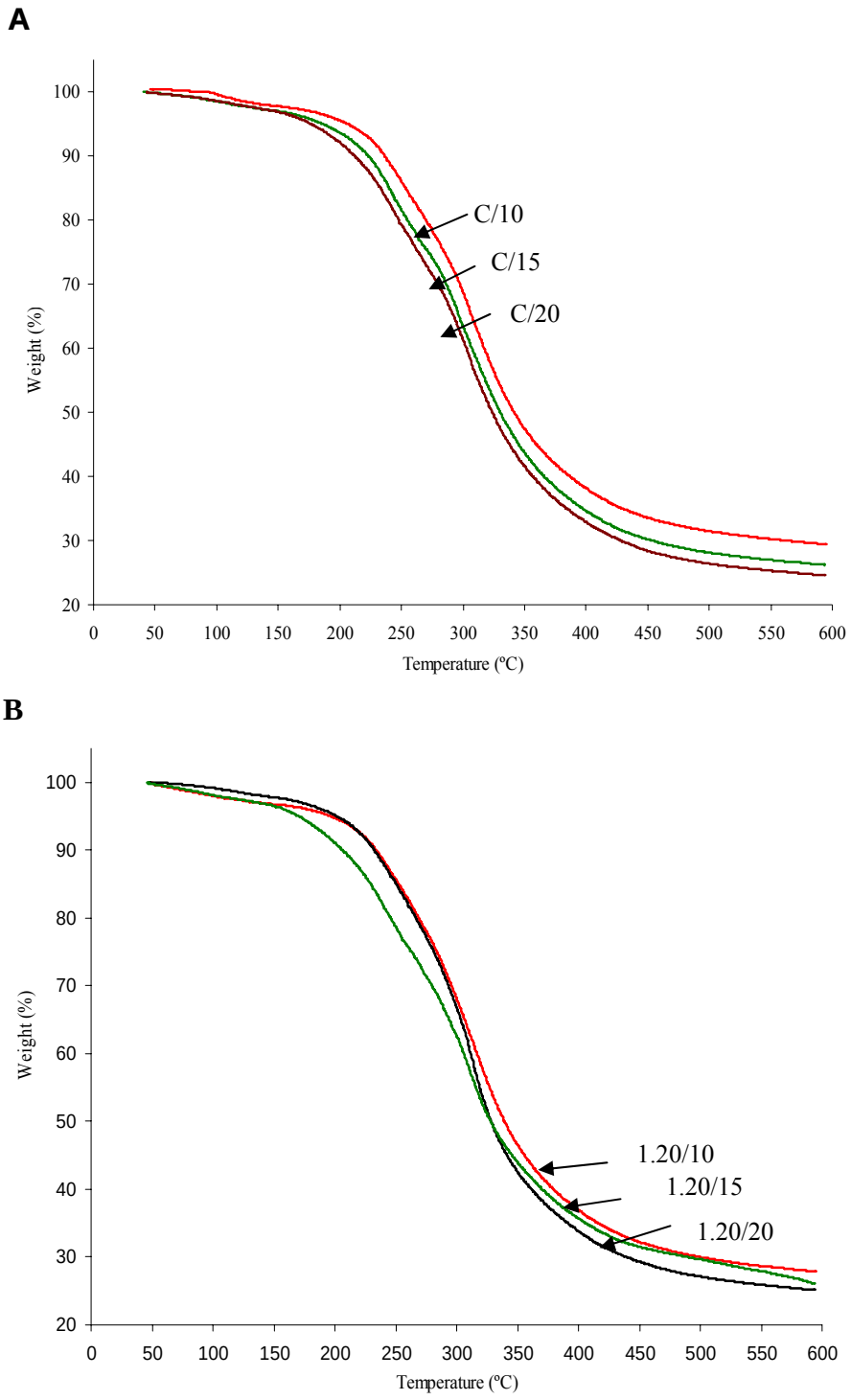


Figure 14. Thermo-gravimetric curve of films prepared from gelatin of cuttlefish skin without hydrolysis (A) and 1.20% DH (B) containing glycerol at different levels. C: control; 1.20 denotes %DH; and 10, 15 and 20 represent glycerol level (%).

The result revealed that at the same glycerol content, film prepared from gelatin with 1.20% DH showed the higher heat susceptibility, compared with film from gelatin without hydrolysis (control film). At any glycerol level, the control films showed the higher thermal degradation temperatures for Td_1, Td_2 and Td_3 and the lower weight loss for Δw_1 and Δw_2, compared with film from gelatin with 1.20% DH (Table 16). The result suggested that weak film network obtained from the gelatin with the shorter gelatin molecules, underwent rapid thermal degradation and higher weight loss, compared with film from without hydrolysates gelatin. At the same DH, the lower Td was obtained in the film containing the higher glycerol content. This confirmed the interfering effect of glycerol on gelatin interaction in the film network. Increased plasticizer contents were responsible for preventing the protein-protein interaction, resulting in the higher heat sensitivity of the films, in which the looser matrix of gelatin molecules was found. In general, higher residue from thermal degradation was obtained in film from gelatin without hydrolysis, compared with those from gelatin with 1.20% DH. The higher residue was also found in film with lower glycerol content. Thus, both chain length of gelatin molecules and glycerol content directly showed the pronounced impact on thermal stability of gelatin.

3.5 Conclusion

Shorter gelatin molecules generated by hydrolysis yielded the film with the lower junction zone or shorter strands via weak bonds during film formation. This led to the lower mechanical properties and thermal stability of their resulting films. Increased amount of $–NH_2$ and –COOH group from hydrolysis process and –OH group of glycerol formed H-bond with water molecules resulted in the increased WVP. Therefore, the chain length of gelatin molecules and plasticizer concentration were crucial factors governing the properties of gelatin-based films from cuttlefish skin.

CHAPTER 4

PROPERTIES OF FILM FROM CUTTLEFISH (*SEPIA PHARAONIS*) SKIN GELATIN INCORPORATED WITH CINNAMON, CLOVE AND STAR ANISE EXTRACTS

4.1 Abstract

Properties of film from cuttlefish **(***Sepia pharaonis***)** ventral skin gelatin without and with partial hydrolysis (1.2% degree of hydrolysis) incorporated with 1% ethanolic extract of cinnamon (CME), clove (CLE) and star anise (SAE) were determined. Films with different herb extracts (without and with oxidation) had higher tensile strength (TS) but lower elongation at break (EAB), compared with the control film (without addition of herb extracts) (p<0.05). Lower water vapor permeability (WVP) and L*- value but higher b*- and ΔE*-values were observed when the extracts were incorporated (p<0.05). Electrophoretic study revealed that cross-linking was pronounced in films containing different herb extracts. Oxidized extracts yielded films with higher TS and WVP than those without oxidized herb extracts (p<0.05). Generally, similar properties were noticeable for films from gelatin with and without partial hydrolysis. Nevertheless, higher mechanical properties were obtained for the latter. FTIR spectra indicated that protein-polyphenol interactions were involved in the film. Thermogravimetric analysis revealed that films incorporated with SAE or SAE with oxidation (OSAE) exhibited lower heat susceptibility and weight loss in the temperature range of 50-600 °C, compared with control film. Films with SAE and OSAE had smoother surface for gelatin without hydrolysis; however, coarser surface were observed in film from gelatin with partial hydrolysis. Thus, the incorporation of different herbs extract directly affected the properties of film from cuttlefish skin gelatin with and without hydrolysis.

4.2 Introduction

Biodegradable films made from renewable biopolymers have become an important environmental friendly packaging, thereby reducing plastic wastes (Tharanathan, 2003; Prodpran and Benjakul, 2005; Hoque *et al*. 2010, 2011a). Most synthetic films are non-biodegradable, leading to environmental pollution and serious ecological problems (Tharanathan, 2003). Gelatin is a natural biopolymer obtained by thermal degradation of collagen (Arvanitoyannis, 2002). Gelatin has been used as a material for preparing biodegradable films with an excellent gas barrier property (Tharanathan, 2003; Jongiareonrak *et al*., 2006b). Protein-based films have good barrier characteristics against gas, organic vapor and oil as compared to synthetic films, which can maintain the quality of food products (Jiang *et al*., 2007). However, the poor mechanical properties and the high water vapor permeability are the main drawbacks of protein films for their application as a packaging material (McHugh and Krochta, 1994).

To modify the properties of protein film, different chemical cross-linking agents and enzyme including gossypol, formaldehyde, glutaralaldehyde (de Carvalho and Grosso, 2004), glyoxal (Nuthong *et al*., 2009a) and transglutaminase (Mariniello *et al*., 2003) have been used. However, the toxicity and high cost of some cross-linking agents have led to their limitation for the application in films (Cao *et al*., 2007a). Polyphenols are known to react with side chain amino groups of peptides, leading to the formation of protein cross-links (Strauss and Gibson, 2004).

Different plant or herb extracts have been incorporated in gelatin films to enhance antioxidant and/or antimicrobial properties such as murta ecotypes leaves extract incorporated in tuna-skin gelatin film (Gómez-Guillén *et al*., 2007), oregano or rosemary aqueous extracts in bovine-hide and tuna-skin gelatins film (Gómez-Estaca 2009a) and borage extract in sole skin or commercial fish skin gelatin (Gómez-Estaca et al., 2009b). Additionally, tuna-skin gelatin film incorporated with oregano and rosemary extracts had altered properties including higher glass transition temperature, decreased deformability and increased water solubility (Gómez-Estaca *et al*., 2009a) and improved light barrier properties (Gómez-Estaca *et al*., 2009c). Rattaya *et al*. (2009) found that fish skin gelatin films incorporated with oxygenated seaweed

(*Turbinaria ornata*) extract exhibited higher EAB, and lower WVP and film solubility than the control film ($p<0.05$). Recently, Hoque et al. (2011a) found that gelatin with shorter chain length produces film with poorer properties. Incorporation of selected herb extracts ingredients may improve the properties of those films. However, there is no information about the effect of incorporation of herb (cinnamon, clove and star anise) extracts on the properties of gelatin-based film. Thus, the objectives of the present study were to study the effects of three different herb (cinnamon, clove and star anise) extracts with and without oxidation on the properties of films from cuttlefish skin gelatin with and without partial hydrolysis.

4.3 Materials and methods

4.3.1 Chemicals

L-Leucine, 2,4,6-trinitrobenzenesulphonic acid (TNBS), bovine serum albumin and wide range molecular weight protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2), glycerol, *p*-dimethylaminobenzaldehyde and tris(hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N, N, N', N'*- tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Folin-Ciocalteu's reagent and Gallic acid were obtained from Fluka (Buchs, Switzerland). Alcalase 2.4 L was provided by Novozymes (Bagsvaerd, Denmark). All chemicals were of analytical grade.

4.3.2 Preparation of herb extract

4.3.2.1 Collection and preparation of herb

Three different herbs namely cinnamon *(Cinnamomum zeylanicum*), clove (*Syzygium aromaticum)* and star anise *(Illicium verum*) were obtained from local market in Hat Yai, Thailand. Herbs were dried using a hot-air oven at 35 °C until the final moisture content of 10-12% was obtained. Dried herbs were then ground and sieved to obtain uniformity using a screen with mesh 35 with an aperture

size of 500 μm, ASTM E11, serial number 5666533 (FRITSCH GMBH, Laborgerätebau, Industriestrasse 8, D-55743 Idar-Oberstein, Germany).

4.3.2.2 Preparation of herb extract

Herb extracts were prepared according to the method of Santoso *et al*. (2004) with some modifications. To prepare herb extracts, the herb powder (25 g) was mixed with 80% ethanol using a powder/solvent ratio of 1:20 (w/v). The mixtures were then stirred continuously at room temperature for 3 h. The mixtures were centrifuged at 5000xg for 10 min at room temperature using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove undissolved debris. The supernatant was collected and filtered through a Whatman filter paper No. 1 (Schleicher & Schuell, Maidstone, England). The solvent in the extract was removed by a rotary evaporator (EYELA, Rotary Vacuum Evaporator, N-1000 series, Tokyo Rikakikai Co., Ltd., Chuo-ku, Tokyo, Japan) at 40 °C to obtain the concentrated herb extract. To remove the residual ethanol, the extract was purged with nitrogen gas. The extract was further dried using a freeze dryer to obtain the dry extract. Dried extract was ground into a powder using a mortar and pestle. Extract powder was referred to as cinnamon extract (CME), clove extract (CLE) and star anise extract (SAE). CME, CLE and SAE powder was placed in amber bottles, closed tightly and stored in a dessicator until use.

4.3.2.3 Determination of total phenolic content of herb extracts

Total phenolic content of herb extracts was determined with Folin-Ciocalteu reagent according to the method of Singleton et al. (1998) using gallic acid as a standard. Extract was dissolved in 25% ethanol to obtain the concentration of 0.5% (w/v). The solution (0.5 mL) was added to 100 μL of Folin-Ciocalteu reagent (two-fold diluted with de-ionized water) and mixed thoroughly. After 3 min, 1.5 mL of 2% Na_2CO_3 was added. The mixture was allowed to stand at room temperature for 30 min. The absorbance was measured at 760 nm using a spectrophotometer (UV-160, Shimadzu, Kyoto, Japan). The concentration of total phenolic compounds in the extract was calculated from a standard curve generated with gallic acid (0.01–0.1 mg/mL) and expressed as mg of gallic acid equivalent (GAE)/g powder.

4.3.2.4 Preparation of oxidized herb extracts

Oxidized herb extracts were prepared as per the method of Strauss and Gibson (2004) with some modifications. Herb extracts (CME, CLE and SAE) were dissolved using 25% ethanol to obtain a concentration of 0.5% (w/v). The solutions were then adjusted to pH 12 by adding 6 M NaOH. The prepared solutions were placed in a temperature controlled water bath (Memmert, GmbH+Co. KG, D-91126, Schwabach, Germany) at 40 °C and subjected to oxygenation by bubbling the solution with oxygen gas with a purity of 99.8% (TTS Gas Agency, Hat Yai, Songkhla, Thailand) for 30 min to convert the phenolic compounds to quinone. After being oxygenated for 30 min, the solution was then neutralized using 2 M HCl and the final volume of the solution was adjusted to 25 ml by using distilled water and was referred to as "oxidized herb extract" named oxidized cinnamon extract (OCME), oxidized clove extract (OCLE) and oxidized star anise extract (OSAE). All oxidized extracts were analyzed for quinone formation using Folin-ciocalteu reagent according to the methods of Balange and Benjakul (2010) with a slight modification. The conversion of phenolic compounds in different herb extracts to quinone was monitored indirectly by determining total phenolic content in different herb extracts before and after oxidation. The differences in the total phenolic content before and after oxidation indicated the formation of quinone and were expressed in percentages.

4.3.3 Collection and preparation of cuttlefish skin

Ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from a dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1 x 1 cm^2), placed in polyethylene bags and stored at -20 ºC until use. Storage time was not longer than 2 months. Prior to gelatin extraction, the frozen skin was thawed using running water (25-26 ºC) until the core temperature reached 0 - 2 ºC.

4.3.4 Preparation of gelatin and partially hydrolyzed gelatin from cuttlefish skin

Gelatin was extracted from cuttlefish skin according to the method of Hoque et al. (2010). Skin was soaked in 0.05 M NaOH with a skin/solution ratio of 1:10 (w/v) with gentle stirring at room temperature (26–28 ºC). The solution was changed every hour to remove non-collagenous proteins for a totally of 6h. Alkali treated skin was then washed with distilled water until a neutral pH of wash water was obtained. The prepared skin was subjected to bleaching in 5% H_2O_2, using a sample/solution ratio of 1:10 (w/v) for 48 h at 4 ºC. The skin treated with H_2O_2 was washed three times with 10 volumes of distilled water. Gelatin was extracted from bleached skin by using distilled water at 60 ºC for 12 h, using a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously using a paddle stirrer (RW20.n, IKA LABORTECHNIK, Staufen, Germany). The extract was centrifuged at 8,000xg for 30 min at room temperature using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble materials. The supernatant was collected and freeze-dried (Model Duratop™ lP/Dura Dry™ lP, FTS® System, Inc., Stone Ridge, NY, USA). The dry matter was referred to as ‘gelatin powder’.

To prepare gelatin with partial hydrolysis, gelatin was dissolved in distilled water to obtain a final protein concentration of 3% (w/v) and the pH of the mixture was adjusted to 8.0 using 6 M NaOH. The mixtures were incubated at 50 °C for 10 min. The hydrolysis reaction was started by the addition of Alcalase at the amount calculated from the plot between log enzyme concentration and degree of hydrolysis (DH) to obtain DH of 1.2% as described by Benjakul and Morrissey (1997). After hydrolysis by using Alcalase for 5 min, the resulting hydrolysates were subjected to heat treatment (90 °C) in a temperature controlled water bath (Memmert, GmbH+Co. KG, D-91126, Schwabach, Germany) for 15 min to fully inactivate the enzyme. Resulting hydrolysate was neutralized using 6 M HCl and used for film preparation.

4.3.5 Effect of different herb extracts on the properties of gelatin films

4.3.5.1 Preparation of film from gelatin incorporated with different herb extracts

Prior to film preparation, gelatin powder was dissolved in distilled water and heated at 70° C for 30 min (Hoque *et al*., 2010). Glycerol at a level of 20% (based on protein content) was added to solutions of gelatin and gelatin with 1.2% DH both containing 3% protein and mixed thoroughly. The mixtures obtained were referred as 'film-forming solution; FFS'.

Different herb extracts (CME, CLE and SAE) and those subjected to oxidation (OCME, OCLE and OSAE) were incorporated into FFS at a level of 1% (based on protein content). Control films were prepared by omitting herb extracts from FFS. The mixtures were stirred at room temperature for 1 h. FFS (4 ± 0.01 g) was cast onto a rimmed silicone resin plate (5 x 5 cm^2), air-blown for 12 h at room temperature and dried with an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttalingen, Germany) at a temperature of 25 ± 0.5 ºC and 50 ± 5% relative humidity (RH) for 48 h. Dried films were manually peeled off and subjected to analyses.

4.3.6 Analyses

Prior to mechanical properties testing, films were conditioned for 48 h at 50 ± 5% relative humidity (RH) at 25 ± 0.5 ºC. For SEM, ATR-FTIR and TGA studies, films were conditioned in a dessicator containing dried silica gel for 3 weeks at room temperature (28-30 ºC) to obtain the most dehydrated films.

4.3.6.1 Film thickness

The thickness of film was measured using a digital micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mituyoto Corp., Kawasaki-shi, Japan). Ten random locations around each film sample were used for thickness determination.

4.3.6.2 Mechanical properties

Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata et al. (2000) using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK). Ten samples (2 x 5 cm^2) with initial grip length of 3 cm were used for testing. The samples were clamped and deformed under tensile loading using a 100 N load cell with cross-head speed of 30 mm min^{-1} until the samples were broken. The maximum load and the final extension at break were used for calculation of TS and EAB, respectively.

4.3.6.3 Water vapor permeability (WVP)

WVP was measured using a modified ASTM (American Society for Testing and Materials, 1989) method as described by Shiku et al. (2004). The film was sealed on an aluminum permeation cup containing silica gel (0% RH) with silicone vacuum grease. The cup was placed at 30 ºC in a desiccator containing distilled water. It was then weighed at 1 h intervals for up to 8 h. Five films were used for WVP testing. WVP of the film was calculated as follows:

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

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

4.3.6.4 Color, light transmission and transparency of the film

Color of film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., Reston, VA, USA). Color of the film was expressed as L^*-, a^*- and b^*-values. Total difference in color (ΔE*) was calculated according to the following equation (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 corresponding color parameter of the sample and that of white standard (L^*= 93.63, a^*= -0.95 and b^*= 0.46).

Light transmission of films against ultraviolet (UV) and visible light were measured at selected wavelengths between 200 and 800 nm, using a UV–Visible Recording spectrophotometer (model UV-160, Shimadzu Co., Kyoto, Japan) according to the method of Jongiareonrak et al. (2006b).

The transparency value of the film was calculated by the following equation (Han and Floros, 1997):

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

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

4.3.6.5 Film solubility

Film solubility in water was determined according to the method of Gennadios *et al*. (1998) with a slight modification. The conditioned film sample (3x2 cm^2) was weighed and placed in 50 ml centrifuge tubes containing 10 ml of distilled water with 0.1% (w/v) sodium azide. The mixture was shaken continuously at room temperature for 24 h using a shaker (Heidolph UNIMAX 1010, Schwabach, Germany). Undissolved debris film matter was determined after centrifugation at 3000xg for 10 min at 25 ºC and drying them at 105 ºC for 24 h to obtain the dry unsolubilized film matter. The weight of solubilized dry matter was calculated by subtracting the weight of unsolubilized dry matter from the initial weight of dry matter and expressed as the percentage of total weight.

4.3.6.6 Electrophoretic analysis

Protein patterns of all film samples were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Prior to analysis, the film samples were prepared according to the method of Jongjareonrak *et al*. (2006b) with some modifications. Film samples (100 mg) were dissolved in 10 mL of 1% (w/v) SDS. The mixture was stirred continuously at room temperature for 12 h. Supernatants were obtained after

centrifugation at 3000xg for 5 min using Hettich Zentrifuge (MIKRO-20, D-78532, Tuttlingen, Germany). Proteins in supernatants were determined using the Biuret method (Robinson and Hodgen, 1940). The supernatants were then mixed with sample buffer (0.5 M Tris–HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol with 10% (v/v) β-ME) at a ratio of 1:1 (v/v). Samples (30 mg protein) were loaded onto the polyacrylamide gel made of 7.5% (for films from gelatin without hydrolysis) and 12% (for films from gelatin with 1.2% DH) running gel and 4% stacking 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 electrophoresis, gel was stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Wide range molecular weight protein markers were used to estimate the molecular weight of proteins.

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

4.3.7 Characterization of gelatin film incorporated with selected herb extract

Films prepared from gelatin and partially hydrolyzed gelatin incorporated without and with star anise extracts (without and with oxidation) was characterized.

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

FTIR spectra of films without and with addition of SAE and OSAE were collected using a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45º ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at 25 °C as described by Nuthong *et al*. (2009a). Sample was placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The

spectra in the range of 700–4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 ºC.

4.3.7.2 Thermo-gravimetric analysis (TGA)

Conditioned films were scanned using a thermogravimetric analyzer (TGA7, PerkinElmer, Norwalk, CT, USA) from 50 to 600 ºC at a rate of 10 ºC/min (Nuthong *et al*., 2009a). Nitrogen was used as the purge gas at a flow rate of 20 mL/min.

4.3.7.3 Microstructure

Microstructure of upper surface and cross-section of the film samples were visualized using a scanning electron microscope (SEM) (JSM-5800LV, JEOL, Tokyo, Japan) at an accelerating voltage of 15 kV. Prior to visualization, the film samples were mounted on brass stub and sputtered with gold in order to make the sample conductive, and photographs were taken at 8000× magnification. For cross-section, freeze-fractured films were mounted around stubs using double sided adhesive tape, coated with gold and observed at the 3000× magnification.

4.3.8 Statistical analysis

Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

4.4 Results and discussion

4.4.1 Characteristics of herb extracts

Extraction yield and total phenolic content of three different herb extract powders are shown in Table 17. The highest yield was obtained for CLE

(23.2%), followed by CME (19.4%) and SAE (16.0%), respectively. Total phenolic contents of CME, CLE and SAE expressed as gallic acid equivalent, were 138.1, 125.0 and 34.4 mg/g powder, respectively. CME generally contained the highest content of phenolic compounds, though it had the lower yield, when compared with CLE. Su *et al*. (2007) found the phenolic content in cinnamon methanolic extract at a level of 148 mg GAE/g powder. Among all extracts, SAE showed the lowest phenolic content and yield. Thus, different herbs rendered the extracts with different extractable phenolic compounds. Kim and Lee (2004) also reported significant differences in total phenolic content from six different herbs due to genotypic and environmental differences namely, climate, location, temperature, fertility, diseases and pest exposure within species, choice of parts tested, time of taking samples and determination methods.

Different L*-, a*-, b*- values and ΔE* were observed among different extract solutions (0.5%, w/v) (Table 17). The differences in color of different extract solutions might be attributed to the variation in pigments, composition and response to the polarity of the solvent (Tian *et al*., 2009).

Table 17. Total phenolic content and color of three different herbs extracts

Herb	Extracted dry powder†	Total phenolic content		Color of extract solution ††††							
				L*		a*		b*		ΔE*	
		before††	after†††	w/o	w	w/o	w	w/o	w	w/o	w
Cinnamon	19.4	138.1	49.5	3.2	1.4	0.6	0.5	-2.0	0.0	90.5	92.3
Clove	23.2	125.0	52.2	1.5	1.1	1.1	0.8	-1.0	-0.5	92.2	92.8
Star Anise	16.0	34.8	11.9	2.5	1.4	0.9	0.5	-1.9	-0.7	91.3	92.3

† Yield of extracts was calculated as percentage (w/w) of initial herbs weight.
†† Total phenolic content (before oxidation) was expressed as mg gallic acid equivalents/g powder (mg GAE/g of powder).
††† Total phenolic content (after oxidation) was expressed as mg gallic acid equivalents/g powder (mg GAE/g of powder).
†††† 4 ml of 0.5% (w/v) extract solution were used for color determination.
w/o: without oxidation; w: with oxidation.

The formation of quinones, an oxidized form of phenolic compounds, in different herb extracts varied (Table 17). After oxidation process, phenolic compounds were converted to quinone by 64.2, 58.2 and 65.8% for CME, CLE and

SAE, respectively. The greatest extent of quinone formation occurred with SAE. The results suggest possible formation of quinones. Quinones react with amino or sulfhydryl side chains of polypeptides to form C–N or C–S covalent bonds (Strauss and Gibson, 2004). Additionally, changes in color were observed for oxidized herb extracts. Oxidized herb extract solutions had lower L*- values and higher b*- and ΔE* values, as compared with those of herb extracts without oxidation. The changes in color of extracts upon oxidation were more likely related with the formation of quinone. Quinones are brown or dark in color. Discolorations due to conversion of phenol to quinone were reported by Pospíšila *et al.* (2002).

4.4.2 Effects of incorporation of different herb extracts on the properties of film from cuttlefish skin gelatin

Films from both gelatin and partially hydrolyzed gelatin incorporated with three different herb extracts with and without oxidation exhibited different properties and molecular characteristics.

4.4.2.1 Thickness

Thickness of films prepared from cuttlefish skin gelatin incorporated with different herb extracts is shown in Table 18. For films from both gelatin and partially hydrolyzed gelatin, no differences in thickness were found between films incorporated without and with different herb extracts ($p>0.05$). Rattaya *et al*. (2009) found similar thickness of gelatin film incorporated without and with oxygenated seaweed extracts. Films from both gelatin and partially hydrolyzed gelatin had similar thickness, regardless of herb extract incorporated.

4.4.2.2 Mechanical properties

Mechanical properties of films prepared from gelatin and partially hydrolyzed gelatin incorporated with different herb extracts without and with oxidation is presented in Table 18. Films obtained from gelatin incorporated with CME, CLE and SAE showed higher TS, but lower EAB, as compared with the control film (without addition of herb extracts) ($p<0.05$). TS of films were increased by 19.3, 19.1 and 16.2% when CME, CLE and SAE at a level of 1% were incorporated,

respectively. For the films prepared from partially hydrolyzed gelatin, those with SAE had increase in TS by 33.1%, compared with that of the control film. However, there were no changes in TS of films containing other non-oxidized extracts (p>0.05). No changes in EAB of resulting films were noticeable when different herb extracts were incorporated (p>0.05). The results suggested that interactions between proteins and phenolic compounds in herb extracts were determined by the chain length of gelatin. Gelatin without hydrolysis, which possessed the higher chain length, more likely provided more reactive groups for interaction with phenolic compounds via hydrogen bonds and hydrophobic interactions, leading to film strengthening. As a result, inter-connection between gelatin molecules was more pronounced. Polyphenol contained hydrophobic groups which entered into hydrophobic region of protein by hydrophobic interaction (Shi and Di, 2000). Furthermore, hydroxyl group of polyphenol was able to combine with hydrogen acceptor in gelatin molecules by hydrogen bonds. A scheme illustrating the impact of herb extracts without and with oxidation on the cross-linking of gelatin molecules via several bondings in the film matrix is presented in Figure 15. Rattaya *et al*. (2009) also reported increased mechanical properties of gelatin film incorporated with seaweed extracts through protein-polyphenol hydrophobic interactions and hydrogen bonds. Protein-polyphenol interactions thus altered the properties of the gelatin films incorporated with different plant extracts including oregano and rosemary extracts (Gómez-Estaca *et al*., 2009a) and borage extract (Gómez-Estaca *et al*., 2009b). Increases in TS were reported in ferulic acid treated gelatin films at pH 7 and tannic acid treated films at pH 9 (Cao *et al*., 2007a), tannic acid, caffeic acid and ferulic acid treated porcine-plasma protein films at pH 7-10 (Nuthong *et al*., 2009b) and ferulic acid treated soy protein isolate films (Ou *et al*., 2005).

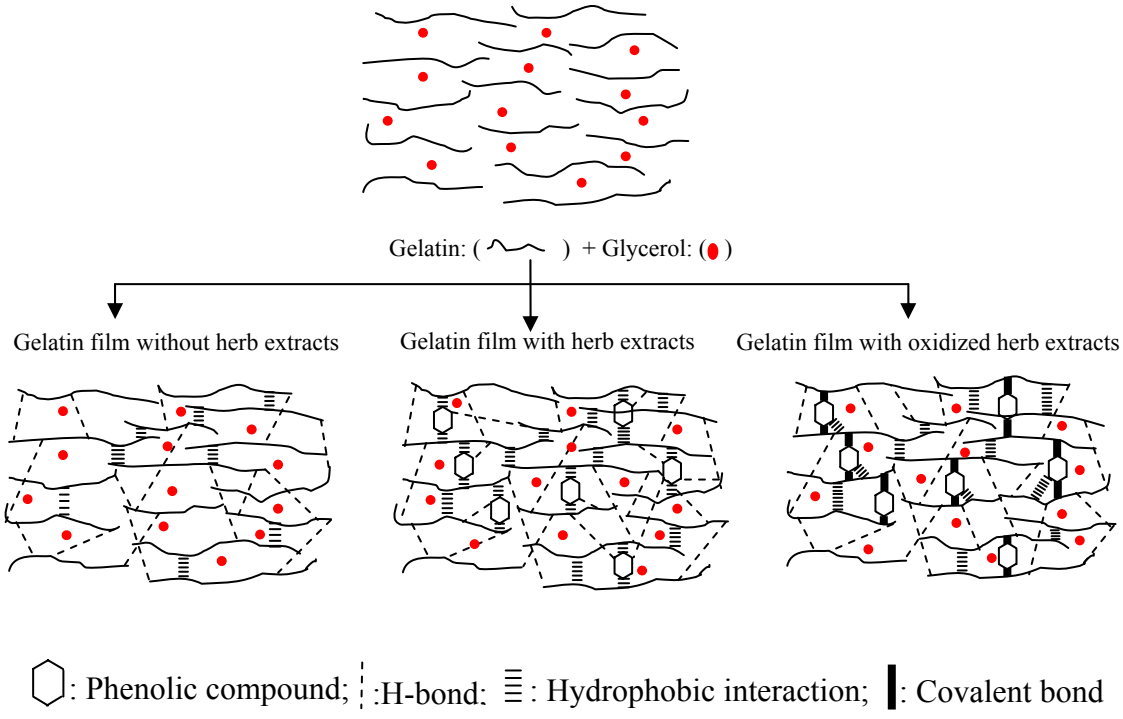


Figure 15. A scheme illustrating the impact of herb extracts without and with oxidation on the cross-linking of gelatin molecules in the film matrix.

Table 18. Mechanical properties, water vapor permeability and thickness of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with herb extracts without and with oxidation.

Source of materials	Herb extracts	TS (MPa)	EAB (%)	WVP ($x10^{-10}$ g $s^{-1}.m^{-1}.Pa^{-1}$)	Thickness (mm)
Gelatin	C	32.78±3.10 d	5.92±0.70a	0.96±0.03 a	0.032±0.003 a
	CME	39.11±1.73 c	4.31±0.41 b	0.80±0.06 c	0.030±0.003 a
	CLE	39.04±1.43 c	4.68±0.39 b	0.77±0.07 c	0.030±0.002 a
	SAE	38.08±1.90 c	4.73±0.28 b	0.79±0.06 c	0.031±0.003 a
	OCME	44.06±1.96 b	4.82±0.22 b	0.88±0.05 b	0.030±0.002 a
	OCLE	43.13±1.82 b	4.69±0.26 b	0.88±0.05 b	0.029±0.003 a
	OSAE	46.96±2.03 a	4.28±0.30 b	0.90±0.02 b	0.029±0.002 a
Partially hydrolyzed gelatin	C	11.10±2.06 c	2.0±0.41 a	1.05±0.05 a	0.031±0.002 a
	CME	13.33±1.27 bc	1.68±0.67 a	0.85±0.06 c	0.030±0.002 a
	CLE	13.01±1.06 bc	1.74±0.41 a	0.82±0.04 c	0.029±0.002 a
	SAE	14.77±2.10 b	1.83±0.15 a	0.83±0.05 c	0.029±0.002 a
	OCME	19.74±1.83 a	1.76±0.55 a	0.95±0.04 b	0.030±0.001 a
	OCLE	18.34±1.54 a	1.88±0.22 a	0.96±0.07 b	0.029±0.003 a
	OSAE	20.31±1.76 a	1.68±0.31 a	0.94±0.05 b	0.030±0.002 a

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

Different letters in the same column within the same source of gelatin indicate the significant differences (p<0.05).

C: control (without herb extracts); CME, CLE, SAE: cinnamon, clove and star anise extracts, respectively.

OCME, OCLE, OSAE: the oxidized cinnamon, clove and star anise extracts, respectively.

When oxidized herb extracts were incorporated, greater increases in TS were obtained, in comparison with those found with the addition of extracts (without oxidation). With the addition of oxidized herb extracts, TS of gelatin film were increased by 34.4, 31.5 and 43.2% for OCME, OCLE and OSAE incorporated film, respectively, as compared with the control film. For the films from partially

hydrolyzed gelatin, the addition of OCME, OCLE and OSAE increased TS of resulting films by 77.8, 65.2 and 82.9%, respectively, as compared with the control. The result suggested that under alkaline pH and in the presence of oxygen, phenolic compounds were converted to quinone. The quinone as a protein-crosslinker could interact with nucleophilic amino group of gelatin (Strauss and Gibson, 2004). Quinones react with amino or sulfhydryl side chains of polypeptides to form covalent C–N or C–S bonds (Strauss and Gibson, 2004). Oxidized phenolic compounds in different herb extracts might contribute to the formation of non-disulfide covalent bond. Thus, the incorporation of oxidized herb extracts effectively improved TS of resulting film. With the addition of OSAE, film prepared from partially hydrolyzed gelatin had higher percentage increase (82.9%) in TS than the film prepared from gelatin (43.2%). The result suggested that the hydrolysis could expose more amino groups at N-termini. Those amino groups could serve as the nucleophilic domain for the attack of oxidized polyphenols, in which subsequent cross-links were formed. Therefore, the mechanical properties of gelatin-based film were largely affected by the addition of herb extracts and chain length of gelatin. Additionally, the oxidized herb extracts exhibited greater strengthening effects on resulting films.

4.4.2.3 Water vapor permeability (WVP)

WVP of films prepared from gelatin containing different herb extracts without and with oxidation is presented in Table 18. Decreases in water vapor permeability (WVP) of film were observed, compared with the control film for both gelatin and partially hydrolyzed gelatin, when different herb extracts were incorporated. Increasing amount of crosslinks via hydrogen and hydrophobic interaction might form film network with decreased free volume of the polymeric matrix, resulting in the lower WVP of the resulting films. Gelatin film incorporated with seaweed extract had significantly lower WVP, compared with the control (without addition of seaweed extract) (Rattaya *et al*., 2009). Gómez-Guillén *et al*. (2007) also found decreased WVP of tuna-fish (*Thunnus tynnus*) gelatin-based edible films incorporated with murta (*Ugni molinae Turcz*) leaves (Soloyo Chico) extracts. A decrease in WVP of soy protein isolate films cross-linked with ferulic acid was also reported (Ou et al., 2005). No differences in WVP were observed among films

incorporated with the three non-oxidized herb extracts (p>0.05). When oxidized herb extract was incorporated, the resulting films showed higher WVP, compared with their non-oxidized counterpart (p<0.05). Nevertheless, no differences in WVP were found among films incorporated with oxidized extract from different herbs (p>0.05). These results might be due to the presence of higher number of polar groups of oxidized phenolic compounds in the extracts dispersed in the films. The barrier characteristics of film were affected by the chemical nature of the macromolecule, structural/morphological characteristics of the polymeric matrix, chemical nature of the additives and degree of cross-linking (McHugh and Krochta, 1994). Thus, incorporation of cinnamon, clove and star anise extract had the profound impact on WVP of film from cuttlefish skin gelatin. The extract and oxidized extracts had an impact on WVP of film from cuttlefish skin gelatin.

4.4.2.4 Color of film

Table 19 shows the color of films prepared from cuttlefish skin gelatin containing different herb extracts without and with oxidation. Lower L*-value (lightness), higher b*-value (yellowness) and ΔE* (color difference) were obtained for films containing all herb extracts, compared with the control (without addition of herb extracts) (p<0.05). When comparing the color of control films from gelatin and partially hydrolyzed gelatin, it was found that the former had the higher L*- value but lower b*- and ΔE*-values (p<0.05). Moderate changes in L*-, a*- and ΔE*-values were observed in the films incorporated with CLE and SAE (both without and with oxidation). The changes in color were more pronounced in the films containing CME (both without and with oxidation), as evidenced by the lowest L*-value, and the highest b*- and ΔE*-values, compared with those of control films and films incorporated with other herb extracts (p<0.05). For all films, the incorporation of oxidized herb extracts resulted in the greater changes in color of resulting films than the extract without oxidation. This was evidenced by the lower L*-value and the increases in b*- and ΔE*-values. These results suggested that the changes in color of resulting films were most likely attributed to the color components in the different herbs extracts (Table 17). Changes in color of film were also observed in gelatin film incorporated with seaweed extracts due to pigments in the seaweed extracts (Rattaya

et al., 2009). Porcine plasma protein-based film incorporated with oxidized caffeic acid showed increase in b*-value (Nuthong *et al*., 2009b). Therefore, incorporation of different herb extracts had an impact on the color of resulting films from gelatin.

Table 19. Color of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with herb extracts without and with oxidation.

Source of materials	Herb extracts	Color			
		L*	a*	b*	ΔE*
Gelatin	C	91.29±0.06 a	-1.31±0.06 d	2.71±0.14 e	3.27±0.12 e
	CME	89.77±0.08 e	-0.95±0.07 b	4.61±0.30 c	5.67±0.22 b
	CLE	90.52±0.27 c	-1.68±0.08 e	4.56±0.14 c	5.38±0.09 c
	SAE	90.83±0.09 b	-1.20±0.04 c	3.77±0.14 d	4.35±0.13 d
	OCME	88.13±0.11 f	-0.21±0.04 a	7.61±0.22 a	9.06±0.23 a
	OCLE	90.23±0.12 d	-1.72±0.06 e	5.00±0.41 b	5.57±0.09 b
	OSAE	90.15± 0.10 d	-1.26±0.13 cd	4.82±0.14 bc	5.59±0.08 b
Partially hydrolyzed gelatin	C	90.65±0.28 ab	-1.32±0.05 c	2.92±0.19 d	3.70±0.19 d
	CME	89.89±0.15 c	-0.90±0.08 a	4.54±0.36 b	5.54±0.30 b
	CLE	90.82±0.65 a	-1.47±0.11 d	3.52±0.38 c	4.20±0.71 cd
	SAE	90.43±0.20 ab	-1.21±0.05 c	3.51±0.29 c	4.44±0.18 c
	OCME	88.15±0.10 d	-0.92±0.08 b	6.92±0.29 a	8.47±0.28 a
	OCLE	90.29±0.34 bc	-1.65±0.09 e	4.26±0.45 b	5.12±0.51 b
	OSAE	90.19±0.09 bc	-1.33±0.11 c	4.53±0.31 b	5.35±0.29 b

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

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

C: control (without herb extracts); CME, CLE, SAE: cinnamon, clove and star anise extracts, respectively.

OCME, OCLE, OSAE: the oxidized cinnamon, clove and star anise extracts, respectively.

4.4.2.5 Light transmission and transparency

Transmission of UV and visible light at wavelength range of 200–800 nm of films from gelatin and partially hydrolyzed gelatin containing herb extracts without and with oxidation are shown in Table 20. The transmission of UV light was found very low at 200 nm and at 280 nm for all films. Therefore, gelatin films effectively prevented the transmission of UV light at these wavelengths, regardless of herb extract incorporation and degree of hydrolysis of gelatin used for film preparation. Jongjareonrak *et al*. (2006b) also reported higher UV light barrier capacity of gelatin film from bigeye snapper and brownstripe red snapper skin, compared with the synthetic film. The films obtained from bigeye snapper (*Pricanthus tayenus*) surimi proteins (Chinabhark *et al*., 2007) also showed excellent UV protection. In general, light transmission in visible range (350–800 nm) for all films were in the range of 52.12 - 89.96%. With the addition of different herb extracts, moderate variations in light transmission of resulting films were observed in the visible range. In general, the incorporation of herb extracts showed no marked effects on transmission of visible light of the resulting films. On the other hand, increase in transparency value of gelatin films incorporated with herb extracts were observed, compared with the control ($p<0.05$). However, no differences in transparency value were observed among all films treated with herb extract with and without oxidation. The increased transparency value indicated the lowered transparency of films. The variation in light transmission and transparency of films incorporated with different herb extracts might be due to the pigments which might affect the color, transparency as well as overall appearance of resulting films differently. Therefore, the addition of different herb extracts either non-oxidized or oxidized form had no profound impact on light transmission and transparency value of resulting films.

Table 20. Light transmittance (%) and transparency values of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with herb extracts without and with oxidation.

Source of materials	Herb extracts	Wavelength (nm)								Transparency values†
		200	280	350	400	500	600	700	800	
Gelatin	C	0.00	8.19	61.40	74.65	82.23	84.66	86.02	86.85	3.42 ± 0.03 b
	CME	0.01	10.31	61.47	72.12	80.39	83.64	85.42	87.42	3.47 ± 0.04 a
	CLE	0.00	9.48	58.02	76.18	86.58	88.47	89.39	89.96	3.49 ± 0.04 a
	SAE	0.01	13.92	65.84	78.41	85.09	86.88	87.81	88.40	3.48 ± 0.02 a
	OCME	0.00	6.53	52.12	67.79	78.51	83.31	85.74	87.75	3.48 ± 0.04 a
	OCLE	0.01	10.63	56.24	73.11	84.10	86.29	87.47	88.80	3.48 ± 0.02 a
	OSAE	0.00	9.03	62.77	76.61	85.60	87.94	89.01	89.65	3.47 ± 0.02 a
Partially hydrolyzed gelatin	C	0.00	8.47	65.47	75.54	83.28	85.76	86.87	87.75	3.49 ± 0.04 b
	CME	0.01	10.43	62.78	76.80	83.61	85.69	86.77	87.82	3.55 ± 0.05 a
	CLE	0.00	7.78	54.22	70.67	82.37	84.61	85.81	87.90	3.57 ± 0.04 a
	SAE	0.00	8.60	65.72	76.43	83.64	85.94	86.89	88.05	3.53 ± 0.04 ab
	OCME	0.00	5.72	52.73	70.33	81.82	85.46	87.92	88.45	3.56 ± 0.02 a
	OCLE	0.01	13.60	67.62	77.97	86.31	88.03	88.89	89.25	3.57 ± 0.06 a
	OSAE	0.01	11.29	66.61	77.43	83.76	85.94	86.88	88.65	3.54 ± 0.05 ab

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

† Different letters in the same column within the same source of gelatin indicate the significant differences ($p<0.05$).

C: control (without herb extracts); CME, CLE, SAE: cinnamon, clove and star anise extracts, respectively.

OCME, OCLE, OSAE: the oxidized cinnamon, clove and star anise extracts, respectively.

4.4.2.6 Film solubility

Solubility of films from gelatin and partially hydrolyzed gelatin containing herb extracts without and with oxidation is presented in Table 21. With the addition of different herb extracts, solubility of non-hydrolyzed gelatin film was lowered compared with that of the control film ($p<0.05$), except for the films containing CME, where no differences were observed ($p>0.05$). Control film showed the highest film solubility (96.02%), while films incorporated with the oxidized herb extracts exhibited lower film solubility. Among all films incorporated with oxidized

herb extracts, that treated with OSAE had the lowest film solubility (p<0.05). The lower solubility of film incorporated with oxidized herb extract might result from the stronger structure of film network through higher extent of protein-polyphenol interaction as evidenced by the higher TS of film. However, all films obtained from partially hydrolyzed gelatin were completely solubilized, irrespective of addition of different herbs extracts, either non-oxidized or oxidized forms. Shorter gelatin molecules could not be able to form strong film network to resist the water during solubilization process for 24 h. Furthermore, the shorter chains were able to be leached out with ease. Film solubility can be viewed as a measure of the water resistance and integrity of a film (Rhim *et al*., 2000). Therefore, addition of different oxidized herb extracts could enhance the interaction between gelatin molecules by non-disulfide covalent bond, leading to the lower solubility (Figure 15). It was noted that gelatin contained negligible content of cysteine. Thus, disulfide bond formation may not have taken place in the gelatin films.

Table 21. Solubility of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with herb extracts without and with oxidation.

Source of materials	Herb extracts	Film solubility[†] (%)
Gelatin	C	96.02±0.61 a
	CME	95.04±0.74 ab
	CLE	94.45±0.42 b
	SAE	94.61±0.98 b
	OCME	92.91±1.19 c
	OCLE	92.86±0.41 c
	OSAE	91.67±0.78 d
Partially hydrolyzed gelatin	C	100
	CME	100
	CLE	100
	SAE	100
	OCME	100
	OCLE	100
	OSAE	100

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

[†] Different letters in the same column within the same source of gelatin indicate the significant differences (p<0.05).

C: control (without herb extracts); CME, CLE, SAE: cinnamon, clove and star anise extracts, respectively.

OCME, OCLE, OSAE: the oxidized cinnamon, clove and star anise extracts, respectively.

4.4.2.7 Electrophoretic protein patterns

Protein patterns of films prepared from gelatin and partially hydrolyzed gelatin incorporated with different herbs extract (both without and with oxidation) are shown in Figure 16A and 16B, respectively. Proteins with molecular weight (MW) of ~117 and ~95 kDa were found as the major proteins in film from gelatin. Aewsiri *et al*. (2009) and Hoque *et al*. (2010) also reported that protein with molecular weight of 118 and 97 kDa was the dominant component in gelatin extracted from ventral skin of cuttlefish. Phenolic compounds in the extracts might contribute to protein-polyphenol cross-linking. Band intensities of those two major protein bands of gelatin films incorporated with all extracts (without oxidation) were similar to those of control film (without addition of herb extracts). For films with herb extracts, weak bonds stabilizing film network may have been destroyed by all denaturants, leading to the dissociation of cross-links. However, the slight decreases in those protein bands were observed when oxidized extracts were incorporated. Band intensity of protein with MW of 97 kDa in film added with OCME, OSAE and OCLE were decreased by 24.62, 26.8 and 25.2%, respectively. This might be due to the formation of large aggregate as evidenced by the appearance of protein band on the stacking gel. These indicated that non-disulfide covalent bonds induced by oxidized extracts were formed to some extent. For films from partially hydrolyzed gelatin, similar results were obtained. However, it was noted that no proteins with MW of 118 and 97 kDa were present in the films, regardless of addition of herb extracts. Band intensity of protein with MW of 29 kDa of film incorporated with OCME, OSAE and OCLE was decreased by 12.05, 24.8 and 17.5%, respectively. This change was concomitant with the formation of high MW protein. Greater contents of cross-links formed were related with the increases in TS (Table 18).

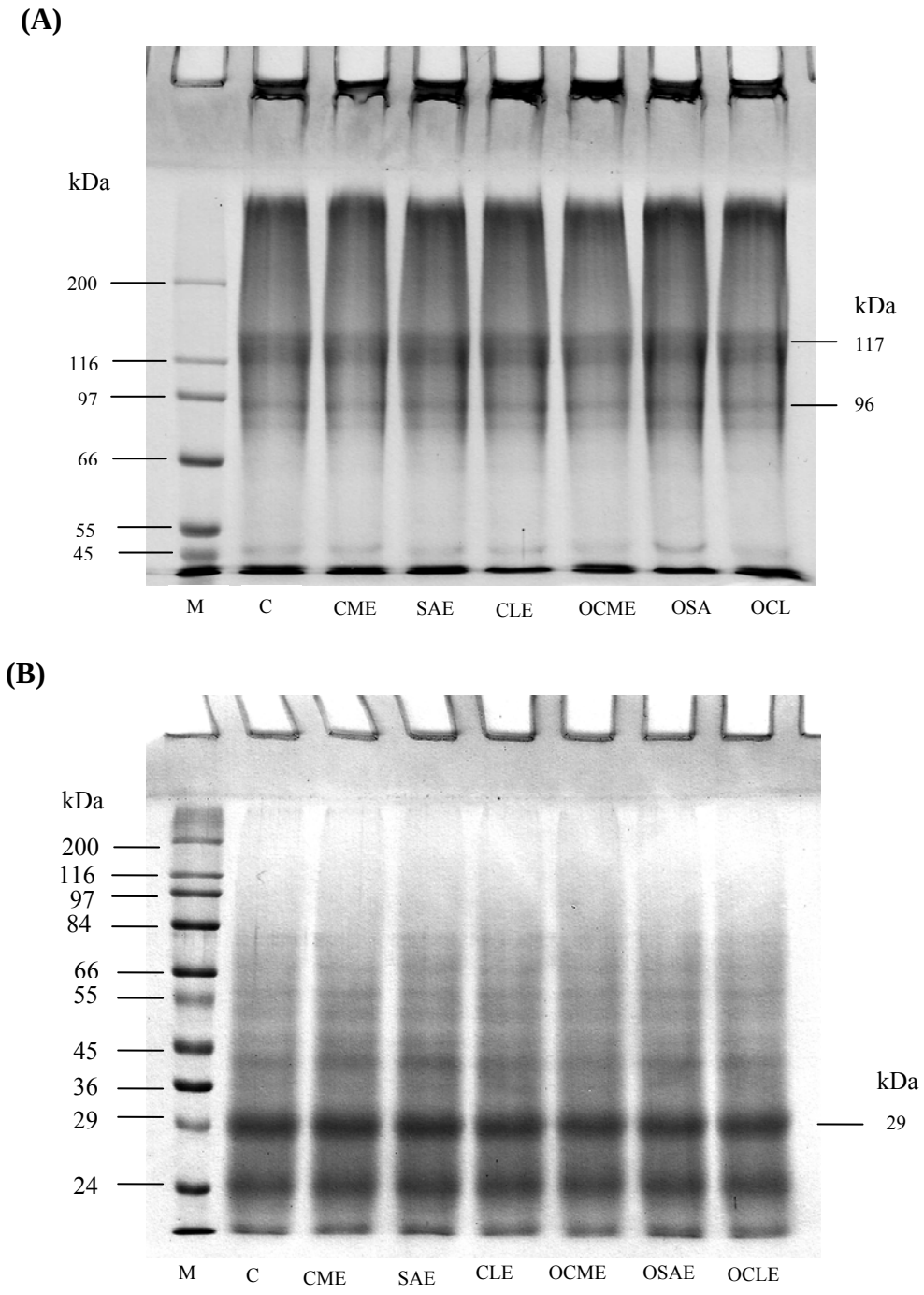


Figure 16. Protein patterns of films from cuttlefish skin gelatin (A) and partially hydrolyzed gelatin (B) containing different herb extracts. M: protein marker; C: control (without addition of herb extracts); CME: cinnamon extract; SAE: star anise extract; CLE: clove extract; OCME: oxidized cinnamon extract; OSAE: oxidized star anise extract; OCLE: oxidized clove extract.

4.4.3 Characteristics of film incorporated with star anise extracts

Films incorporated with star anise extracts, both without and with oxidation, showed higher mechanical properties and lower b*-value as compared with the film incorporated with other herb extracts. Therefore, the films incorporated with star anis extracts were further characterized.

4.4.3.1 FTIR spectroscopy

FTIR spectra of films prepared from gelatin and partially hydrolyzed gelatin incorporated with star anise extracts (without and with oxidation) are shown in Figure 17. The spectra of all films exhibited the major bands at 1631 cm^{-1} (amide-I, representing C=O stretching/hydrogen bonding coupled with COO), 1536 cm^{-1} (amide-II, arising from bending vibration of N-H groups and stretching vibrations of C-N groups) and 1234 cm^{-1} (amide-III, representing the vibrations in plane of C-N and N-H groups of bound amide or vibrations of CH_2 groups of glycine) (Muyonga *et al*., 2004a; Aewsiri *et al*., 2009). Pranoto *et al*. (2007) reported a similar result for fish gelatin film, where amide-I, amide-II and amide-III peaks were found at the wavenumbers of 1656, 1550 and 1240 cm^{-1}, respectively. The peak situated around 1033 cm^{-1} might be related to the interactions arising between plasticizer (OH group of glycerol) and film structure (Bergo and Sobral, 2007). Generally, similar spectra were obtained for films without and with SAE or OSAE in the range of 1800-700 cm^{-1}, covering amide-I, amide-II and amide-III observed at similar wavenumber for both films from gelatin and partially hydrolyzed gelatin.

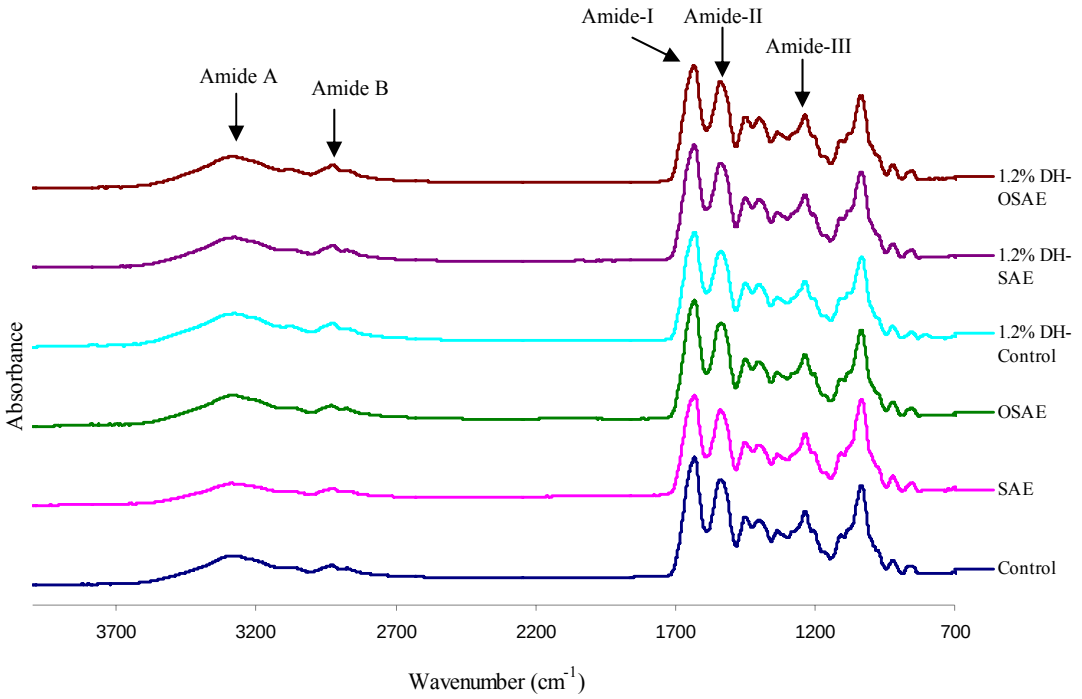


Figure 17. FTIR spectra of films prepared from gelatin and partially hydrolyzed (1.2% DH) gelatin from cuttlefish skin containing star anise extracts without and with oxidation. Control: film without addition of the extract; SAE: film added with star anise extract; OSAE: film added with oxidized star anise extract.

Furthermore, amide-A peak was found at 3275 cm^{-1}, representing NH-stretching coupled with hydrogen bonding. Amide-B peak at 2928 cm^{-1}, representing CH stretching and NH_3^+, was also observed in the spectra (Muyonga *et al.*, 2004a). For film from gelatin, the wavenumber of amide-A peak shifted from 3275 cm^{-1} for the control to 3277 and 3287 cm^{-1} for films incorporated with SAE and OSAE, respectively. For film from partially hydrolyzed gelatin with SAE and OSAE, wavenumber of amide-A peak shifted from 3276 cm^{-1} for the control to 3278 and 3290 cm^{-1}, respectively. The amplitude of amide-A peak of the control gelatin film (Abs = 0.15) decreased markedly in film incorporated with SAE (both without and with oxidation) (Abs = 0.11-0.13). For film prepared from partially hydrolyzed gelatin, the amplitude of amide-A peak decreased from 0.17 to 0.14-0.15 when the

SAE without and with oxidation was incorporated. In general, film with OSAE had slightly higher amplitudes, in comparison with film with SAE. Similar result was observed for amide-B peak. These results suggested that SAE and OSAE induced interaction between phenolic compounds and NH_2 group of gelatin. This led to cross-linking of gelatin. Protein cross-linking led to higher diffraction of film, which affects the spectra obtained using ATR techniques. Nuthong *et al*. (2009a) also observed similar results in porcine plasma protein films incorporated with 2% glyoxal and 3% caffeic acid with or without oxygenation. However, it was also noted that higher amplitudes of amide-A and amide-B peaks were observed in film from partially hydrolyzed than film from gelatin. The result reconfirmed the changes of peptides in partially hydrolyzed gelatin. Higher amount of $–NH_2$ or $–NH_3^+$ group obtained from hydrolysis process more likely resulted in the higher amplitude of amide-A and amide-B peaks obtained. Protein cross-linking was prominent in film network with the addition of SAE and OSAE as indicated by the changes in functional group and conformation of proteins elucidated by the changes in FTIR spectra (Ahmad and Benjakul, 2010).

4.4.3.2 Thermo-gravimetric analysis (TGA)

TGA thermograms revealing thermal degradation behavior of films prepared from gelatin and partially hydrolyzed gelatin in the presence of star anise extract (without and with oxidation) are depicted in Figure 18A and 18B. Their corresponding degradation temperatures (Td) and weight loss (Δw) are shown in Table 22. Three main stages of weight loss were observed for all films from both gelatin and partially hydrolyzed gelatin. For all films, the first stage of weight loss (Δw_1= 2.13 - 4.07%) was observed approximately at temperature (Td_1) of 46.24 - 61.31 °C, possibly associated with the loss of free water adsorbed on the film. A similar result was found in porcine plasma protein film containing different cross-linking agents (Nuthong *et al*., 2009a) and in collagen hydrolysate film plasticized with glycerol and poly(ethylene glycols) (Langmaier *et al*., 2008). The second stage of weight loss (Δw_2= 17.25 - 22.60%) appeared in the region of Td_2 (242.38 - 252.39 °C) for all films. This was most likely associated with the loss of lower molecular weight protein fractions, glycerol compounds and also structurally bound

water. For the third stage of weight loss, Td_3 of 270.18-282.10 °C were observed in films prepared from gelatin, whereas Td_3 of 251.80-258.90 °C were found in those from partially hydrolyzed gelatin. These changes mainly resulted from the degradation of the gelatin with the larger size. The lower Td of the latter was more likely due to the shorter chains, which could undergo thermal degradation to a higher extent, compared with the former.

Table 22. Thermal degradation temperature (T_d, °C) and weight loss (Δw, %) of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with star anise extracts without and with oxidation

Source of materials	Herb extracts	Δ_1		Δ_2		Δ_3		Residue (%)
		$Td_{1,onset}$	Δw_1	$Td_{2,onset}$	Δw_2	$Td_{3,onset}$	Δw_3	
Gelatin	C	53.30	3.53	245.90	21.10	270.18	61.10	25.10
	SAE	58.82	2.92	250.40	18.56	278.45	60.45	29.61
	OSAE	61.31	2.13	252.39	17.25	282.10	59.50	31.78
Partially hydrolyzed gelatin	C	46.24	4.07	242.38	22.60	251.80	58.92	21.45
	SAE	48.21	3.65	244.15	18.78	256.25	55.23	23.20
	OSAE	50.48	3.40	245.98	18.20	258.90	53.57	24.92

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

SAE and OSAE: star anise extracts without and with oxidation, respectively.

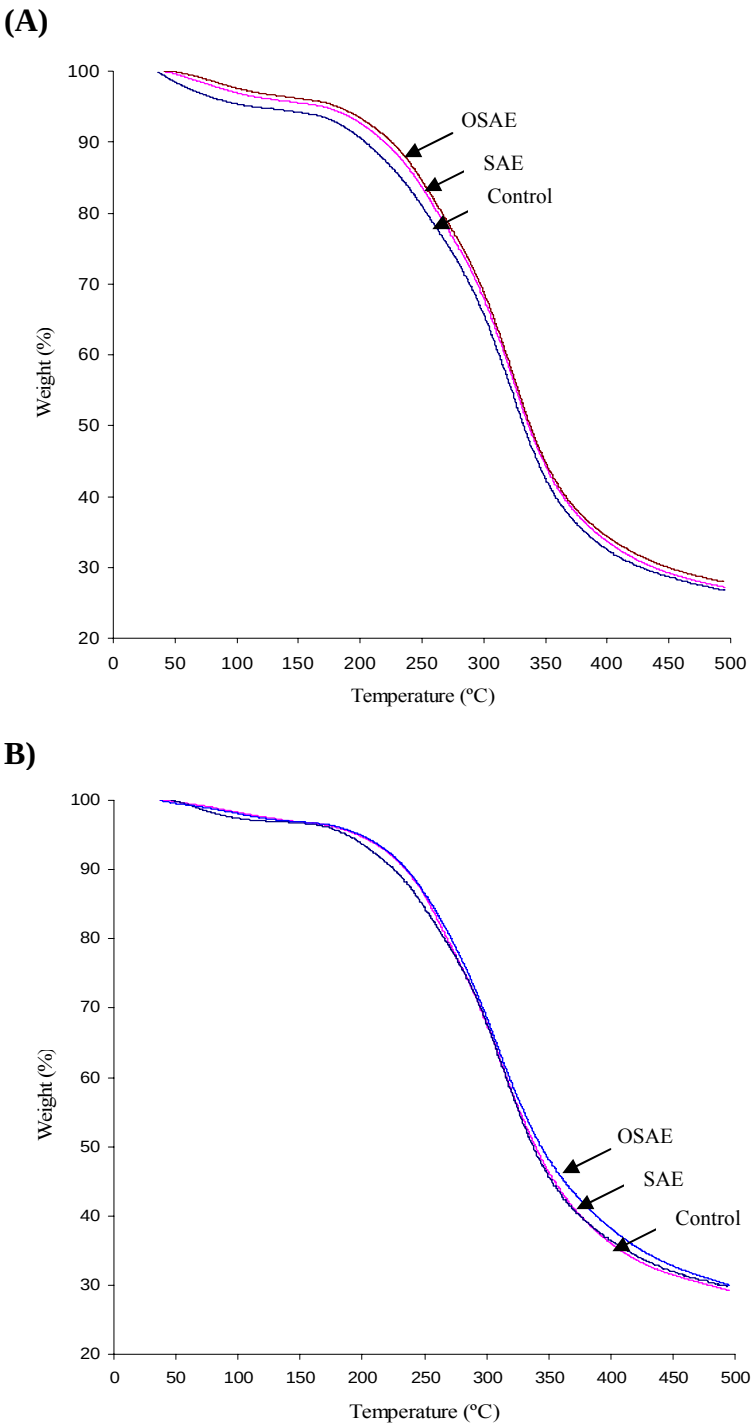


Figure 18. Thermo-gravimetric curves of films prepared from cuttlefish skin gelatin (A) and partially hydrolyzed gelatin (B) containing star anise extract without and with oxidation. Control: film without addition of the extract; SAE: film added with star anise extract; OSAE: film added with oxidized star anise extract.

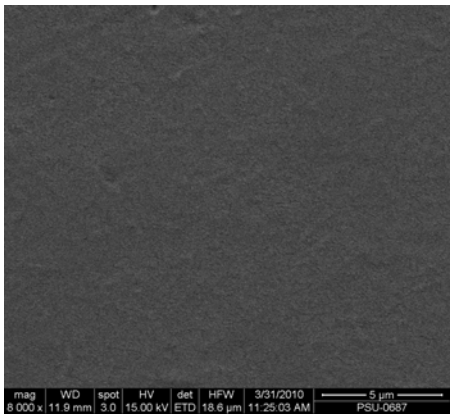
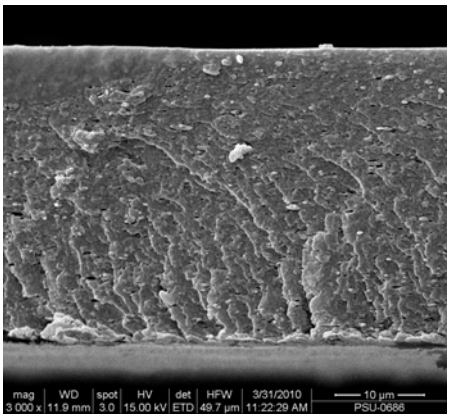
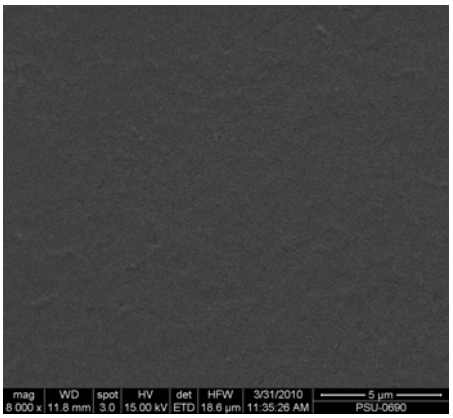
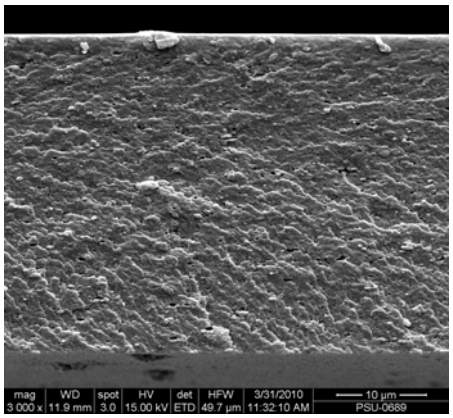
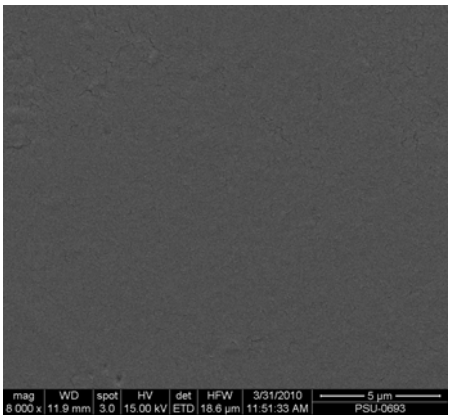
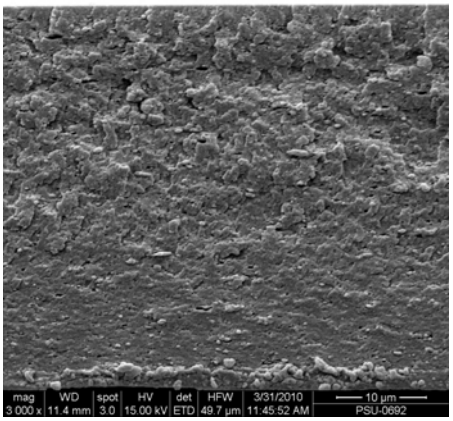
The result suggested that the film with SAE showed higher heat resistance than the control films (without addition of star anise extracts) from both gelatin and partially hydrolyzed gelatin. For both gelatin materials, the control films showed lower thermal degradation temperatures for Td_1, Td_2 and Td_3 and higher weight loss for Δw_1, Δw_2 and Δw_3, as compared to films with SAE and OSAE (Table 6). The result suggested that higher amount of bonding between phenolic compounds of star anise extracts and gelatin molecules yielded stronger film network, leading to the higher heat resistance of the resulting films. For the same materials, films with OSAE had higher Td_1, Td_2 and Td_3 with lower Δw. Thus, OSAE most likely increased the thermal stability of resulting film. This was due to the increased stronger bonds induced by OSAE in the film matrix. Non-disulfide covalent bonds may be predominant in films incorporated with OSAE, compared to those with SAE. However, at the same condition, film from partially hydrolyzed gelatin added with star anise extract showed higher heat susceptibility, compared with film from gelatin. The result suggested that film network obtained from the gelatin with the shorter gelatin molecules underwent rapid thermal degradation and had higher weight loss, compared with film from gelatin. Also, herb extracts showed the pronounced impact on thermal stability of gelatin film via protein-polyphenol cross-linking.

4.4.3.3 Microstructure

SEM micrographs of the surface and cross-section of films from gelatin and partially hydrolyzed gelatin incorporated with SAE and OSAE are illustrated in Figure 19A and 19B, respectively. For the control film, smooth surface was observed for both films from gelatin and partially hydrolyzed gelatin. Smooth surface was also obtained in the film prepared from gelatin incorporated with SAE and OSAE. However, a rough surface was obtained in the film from partially hydrolyzed gelatin when SAE and OSAE were added. The rough surface was more obvious in film with OSAE, compared to that with SAE. For cross section, film obtained from gelatin incorporated with SAE showed a more compact-structure as compared with the control. Increased number of non-covalent protein-polyphenol interaction might contribute to the formation of compact film structure. However, OSAE addition resulted in coarser cross-sectional structure of their resulting films. The roughness

might be governed by conversion of phenol to quinone, which induced the large aggregation in the film matrix via non-disulfide bonds. Nuthong *et al*. (2009b) also found rougher cross-section of film with oxygenated caffeic acid than that with non-oxygenated caffeic acid.

(A)

	Surface	Cross-section
Control		
Added with SAE		
Added with OSAE		

(B)

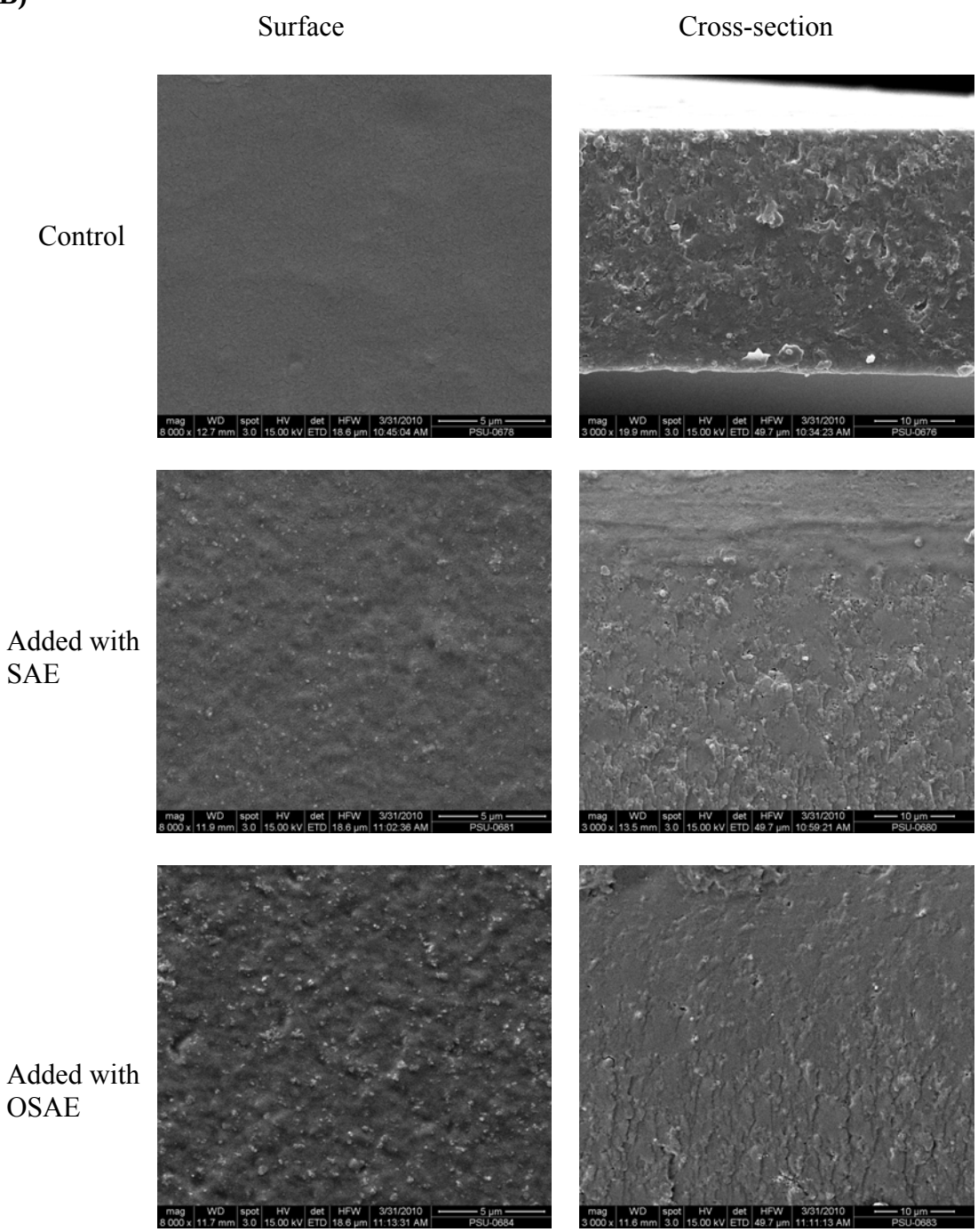


Figure 19. SEM micrographs of surface and cross-section of films from cuttlefish skin gelatin (A) and partially hydrolyzed gelatin (B) containing star anise extract without and with oxidation. Control: film without addition of the extract; SAE: film added with star anise extract; OSAE: film added with oxidized star anise extract.

Coarser surfaces observed in films prepared from partially hydrolyzed gelatin after the incorporation with SAE and OSAE was probably caused by the protruding of polymerized chains. However, the long chain could form the junction zone throughout the film network, leading to the smoother surface irrespective of SAE and OSAE incorporation. For the cross-section, the smaller micro-voids with the denser internal structure were noticed in the film treated with SAE, compared with the control film.

4.5 Conclusion

Incorporation of herb extracts including cinnamon, clove and star anise extracts into gelatin and partially hydrolyzed gelatin increased the TS and decreased WVP of resulting films. However, those extracts could affect the color of resulting films to some degree. Star anise extract was the most effective in improving the mechanical properties and water barrier property of the gelatin films. Extracts with oxidation showed the greater efficiency in increasing the strength of films than non-oxidized counterpart. However, the molecular weight or chain length of gelatin played a role in cross-linking mediated by phenolic compounds in the extracts and also determined the microstructure of resulting film. Therefore, herb extracts can be used as the natural protein cross-linkers, which is able to modify the properties of film from gelatin or other proteins.

CHAPTER 5

EFFECTS OF HYDROGEN PEROXIDE AND FENTON'S REAGENT ON THE PROPERTIES OF FILM FROM CUTTLEFISH (*SEPIA PHARAONIS*) SKIN GELATIN

5.1 Abstract

Properties of film from cuttlefish **(***Sepia pharaonis***)** ventral skin gelatin without and with partial hydrolysis (1.2% degree of hydrolysis) as influenced by H_2O_2 and Fenton's reagent at different levels were investigated. Films added with H_2O_2 (0.01-0.04 M) and Fenton's reagent [H_2O_2 (0.01-0.04 M) + $FeSO_4$ (0.001-0.004 M)] had higher tensile strength (TS) but similar or lower elongation at break (EAB), compared with the control film (without addition of H_2O_2 and Fenton's reagent) (p<0.05). Slight differences in water vapor permeability (WVP) were observed for all films. Films added with Fenton's reagent possessed lower L*- value but higher a*-, b*- and ΔE*-values, while the films added with H_2O_2 had lower b*-value (p<0.05), compared with the control film. Electrophoretic study revealed that cross-linking was pronounced in films added with H_2O_2 or Fenton's reagent. Films added with Fenton's reagent had lower solubility in water than did those added with H_2O_2 as well as the control (p<0.05). FTIR spectra suggested that interaction of protein was induced by H_2O_2 and Fenton's reagent. However, fragmentation more likely took place when Fenton's reagent at higher level was used. Differential scanning calorimetry and thermogravimetric analysis indicated that films incorporated with H_2O_2 and Fenton's reagent exhibited the increased heat stability with lowered weight loss in the temperature range of 50-500 °C, compared with the control film. Generally, similar result was noticeable between films from gelatin with and without partial hydrolysis. The coarser cross-sectional structure was observed in films when H_2O_2 and Fenton's reagent were incorporated. Thus, the H_2O_2 and Fenton's reagent directly affected the properties of film from cuttlefish skin gelatin with and without hydrolysis.

5.2 Introduction

Cuttlefish has become an important fishery product in Thailand as well as other south-east Asian countries. During processing of cuttlefish, skin is generated as a by-product with the low market value. To increase its profitability, cuttlefish skin has recently been used for gelatin extraction (Aewsiri *et al*., 2009; Hoque *et al*., 2010). Gelatin has been used as a material for preparing biodegradable films with an excellent gas barrier property (Jongiareonrak *et al*. 2006b). Protein-based films generally have good barrier characteristics against gas, organic vapor and oil, compared to synthetic films (Jiang *et al*., 2007). However, the poor mechanical properties and the high water vapor permeability are the main drawbacks of protein films for their application as a packaging material (McHugh and Krochta, 1994). Film property has been reported to be governed by the chain length of proteins in film matrix (Gómez-Guillén *et al*., 2009; Hoque *et al*., 2011a). The shorter chains yielded the film with lower mechanical property (Hoque *et al*. 2010b). To improve the properties of protein film, different chemicals and enzyme with protein cross-linking property including gossypol, formaldehyde, glutaralaldehyde (de Carvalho and Grosso, 2004), glyoxal (Nuthong *et al*., 2009a), transglutaminase (Mariniello *et al*., 2003), phenolic compounds (Nuthong *et al*., 2009b; Cao *et al*., 2007a), plant or herb extracts (Gómez-Estaca *et al*., 2009a, 2009b) and seaweed extract (Rattaya *et al*., 2009) have been used.

Free radical-mediated protein modification could be an alternative approach to modify the properties of protein films. Generation of free radicals during processing or storage can alter the molecular weight of biopolymers (Farahnaky *et al*., 2003), either by polymerization or fragmentation (Kocha *et al*., 2008). Several chemicals have been known to induce protein oxidation and fragmentation. Hydrogen peroxide (H_2O_2) is an oxidizing agent used in some food industries as the processing aid such as bleaching agent and can affect the property of food proteins (Farahnaky *et al*., 2003; Phatcharat *et al.,* 2006; Aewsiri *et al*., 2009). Fenton reaction is another approach to generate the active radical, hydroxyl radicals ($OH^{\bullet}$), from H_2O_2 in the presence of Fe^{2+} (Kocha *et al*., 1997). Hydroxyl radicals are reactive species which can alter protein composition and configuration (Liu and Xiong, 2000). Thus,

incorporation of H_2O_2 or Fenton's reagent may improve the properties of those films. However, there is no information about the impact of H_2O_2 and Fenton's reagent on the properties of gelatin-based film. Thus, the objective of this investigation was to study the properties of films from cuttlefish skin gelatin with and without partial hydrolysis as influenced by H_2O_2 and Fenton's reagent.

5.3 Materials and methods

5.3.1 Chemicals

L-Leucine, 2,4,6-trinitrobenzenesulphonic acid (TNBS), bovine serum albumin and wide range molecular weight protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Iron (II) sulfate, glycerol, *p*-dimethylaminobenzaldehyde and tris(hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Analytical hydrogen peroxide (30%) was obtained from BDH, VWR International Ltd (Leicestershire, England). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N,N,N′,N′*-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Alcalase 2.4 L was provided by Novozymes (Bagsvaerd, Denmark). All chemicals were of analytical grade.

5.3.2 Collection and preparation of cuttlefish skin

Ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from a dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1 x 1 cm^2), placed in polyethylene bags and stored at -20 ºC until use. Storage time was not longer than 2 months. Prior to gelatin extraction, the frozen skin was thawed using running water (25-26 ºC) until the core temperature reached 0 - 2 ºC.

5.3.3 Preparation of gelatin and partially hydrolyzed gelatin from cuttlefish skin

Gelatin was extracted from cuttlefish skin according to the method of Hoque *et al*. (2010). Skin was soaked in 0.05 M NaOH with a skin/solution ratio of 1:10 (w/v) with a gentle stirring at room temperature (26–28 ºC). The solution was changed every hour to remove non-collagenous proteins for totally 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching in 5% H_2O_2, using a sample/solution ratio of 1:10 (w/v) for 48 h at 4 ºC. The skin treated with H_2O_2 was washed three times with 10 volumes of distilled water. Gelatin was extracted from bleached skin using distilled water at 60 ºC for 12 h, with a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously using a paddle stirrer (RW20.n, IKA LABORTECHNIK, Staufen, Germany). The extract was centrifuged at 8,000xg for 30 min at room temperature using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble materials. The supernatant was collected and freeze-dried (Model Duratop™ lP/Dura Dry™ lP, FTS® System, Inc., Stone Ridge, NY, USA). The dry matter was referred to as 'gelatin powder'.

To prepare gelatin with partial hydrolysis, gelatin powder was dissolved in distilled water to obtain the final protein concentration of 3% (w/v) and the pH of the mixture was adjusted to 8.0 using 6 M NaOH. The mixtures were incubated at 50 °C for 10 min. The hydrolysis reaction was started by the addition of Alcalase at the amount calculated from the plot between log enzyme concentration and DH to obtain DH of 1.2%. The plot was prepared using the gelatin as substrate and the hydrolytic condition was pH 8.0 at 50ºC for 5 min (Hoque *et al*., 2011a). After hydrolysis by Alcalase for 5 min, the resulting hydrolysate was subjected to heat treatment (90 °C) in a temperature controlled water bath (Memmert, GmbH+Co. KG, D-91126, Schwabach, Germany) for 15 min to fully inactivate the enzyme. Resulting hydrolysate was neutralized using 6 M HCl and used for film preparation.

DH of gelatin hydrolysate was determined according to the method of Benjakul and Morrissey (1997). Hydrolysate samples with appropriate dilution (125

μL) were added with 2.0 mL of 0.2 M phosphate buffer (pH 8.2) and 1.0 mL of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a temperature controlled water bath at 50 °C for 30 min in the dark. The reaction was terminated by adding 2.0 mL of 0.1 M sodium sulfite. The mixtures were cooled at room temperature for 15 min. The absorbance was read at 420 nm and α-amino group was expressed in terms of L-leucine. The DH was defined as follows (Benjakul nad 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 acid hydrolysis (6 N HCl at 100 °C for 24 h).

5.3.4 Effect of H_2O_2 and Fenton's reagent on the properties of gelatin films

5.3.4.1 Preparation of film from gelatin incorporated with H_2O_2 and Fenton's reagent

Gelatin powder was dissolved in distilled water and heated at 70° C for 30 min (Hoque *et al.,* 2010). Solutions of gelatin or gelatin with 1.2% DH containing 3% protein were prepared. H_2O_2 was added to obtain the final concentrations of 0.01 M (H-1), 0.02 M (H-2) and 0.04 M (H-4). Fenton's reagent (a mixture of H_2O_2 and $FeSO_4$) were added to other set of gelatin solution to yield the Fenton's reagent with different concentrations including 1) 0.01 M H_2O_2 + 0.001 M $FeSO_4$ (F-1); 2) 0.02 M H_2O_2 + 0.002 M $FeSO_4$ (F-2) and; 3) 0.04 M H_2O_2 + 0.004 M $FeSO_4$ (F-4). The mixtures were then added with glycerol at a level of 20% (based on protein content) and mixed thoroughly. The mixtures were stirred at room temperature for 1 h. The mixtures obtained were referred to as 'film-forming solution; FFS'. FFS from partially hydrolyzed gelatin with corresponding concentrations of H_2O_2 and Fenton's reagent were also prepared in the same manner. The samples were referred to as 'PH-

1, PH-2 and PH-4' for those added with H_2O_2 and as 'PF-1, PF-2 and PF-4' for those incorporated with Fenton's reagent.

FFS incorporated with different concentrations of H_2O_2 or Fenton's reagent were used for film casting. Control films were prepared by omitting H_2O_2 and Fenton's reagent from FFS. FFS (4 ± 0.01 g) was cast onto a rimmed silicone resin plate (5 x 5 cm^2), air-blown for 12 h at room temperature and dried in an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttalingen, Germany) at the temperature of 25 ± 0.5 ºC and 50 ± 5% relative humidity (RH) for 48 h. Dried films were manually peeled-off and subjected to analyses.

5.3.4.2 Analyses

Prior to mechanical properties testing, films were conditioned for 48 h at 50 ± 5% relative humidity (RH) at 25 ± 0.5 ºC. For SEM, ATR-FTIR, DSC and TGA studies, films were conditioned in a dessicator containing dried silica gel for 2 weeks and 1 week in dessicator containing P_2O_5 at room temperature (28-30 ºC) to obtain the most dehydrated films.

5.3.4.2.1 Film thickness

The thickness of film was measured using a digital micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mitutoyo Corp., Kawasaki-shi, Japan). Ten random locations around each film sample were used for thickness determination.

5.3.4.2.2 Mechanical properties

Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata *et al*. (2000) using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK). Ten samples (2 x 5 cm^2) with the initial grip length of 3 cm were used for testing. The samples were clamped and deformed under tensile loading using a 100 N load cell with the cross-head speed of 30 mm min^{-1} until the samples were broken. The maximum load and the final extension at break were used for calculation of TS and EAB, respectively.

5.3.4.2.3 Water vapor permeability (WVP)

WVP was measured using a modified ASTM (American Society for Testing and Materials, 1989) method as described by Shiku *et al*. (2004). The film was sealed on an aluminum permeation cup containing dried silica gel (0% RH) with silicone vacuum grease. The cup was placed at 30 ºC in a desiccator containing the distilled water. It was then weighed at 1 h intervals for up to 8 h. Five films were used for WVP testing. WVP of the film was calculated as follows:

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

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

5.3.4.2.4 Film solubility

Film solubility in water was determined according to the method of Gennadios *et al.* (1998) with a slight modification. The conditioned film sample (3x2 cm^2) was weighed and placed in 50 ml-centrifuge tube containing 10 ml of distilled water with 0.1% (w/v) sodium azide. The mixture was shaken continuously at room temperature for 24 h using a shaker (Heidolph UNIMAX 1010, Schwabach, Germany). Undissolved debris film matter was determined after centrifugation at 3000xg for 10 min at 25 ºC using a centrifuge (Allegra 25R Centrifuge, Beckman Coulter, Krefeld, Germany) and drying them at 105 ºC for 24 h to obtain the dry unsolubilized film matter. The weight of solubilized dry matter was calculated by subtracting the weight of unsolubilized dry matter from the initial weight of dry matter and expressed as the percentage of total weight.

5.3.4.2.5 Color, light transmission and transparency of the film

Color of film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., Reston, VA, USA). Color of the film was expressed as L^*-, a^*- and b^*-values. Total difference in color (ΔE^*) was calculated according to the following equation (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 corresponding color parameter of the sample and that of white standard (L^*= 93.63, a^*= -0.92 and b^*= 0.42).

Light transmission of films against ultraviolet (UV) and visible light were measured at selected wavelengths between 200 and 600 nm, using a UV–Visible spectrophotometer (model UV-160, Shimadzu, Kyoto, Japan) according to the method of Jongiareonrak *et al*. (2006b).

The transparency value of the film was calculated by the following equation (Han and Floros, 1997):

Transparency value = (-log T_{600})/x

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

5.3.4.2.6 Electrophoretic analysis

Protein patterns of all film samples were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Prior to analysis, the film samples were prepared according to the method of Jongjareonrak *et al*. (2006b) with some modifications. Film samples (200 mg) were dissolved in 10 mL of 5% (w/v) SDS. The mixture was stirred continuously at room temperature for 12 h. Supernatants were obtained after centrifugation at 3000xg for 5 min at room temperature. Protein contents in the supernatants were determined using the Biuret method (Robinson and Hodgen, 1940). The supernatants were then mixed with sample buffer (0.5 M Tris–HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol with 10% (v/v) β-ME) at the ratio of 1:1 (v/v). Samples (30 μg protein) were loaded onto the polyacrylamide gel made of 7.5% (for films from gelatin without hydrolysis) and 12% (for films from gelatin with 1.2% DH) separating gel and 4% stacking 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 electrophoresis, gels were stained with 0.05% (w/v)

Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Wide range molecular weight protein markers were used to estimate the molecular weight of proteins.

5.3.5 Characterization of gelatin film incorporated with H_2O_2 and Fenton's reagent at selected concentration

Films prepared from gelatin and partially hydrolyzed gelatin incorporated with 0.02 M H_2O_2 or Fenton's reagent (0.02 M H_2O_2 + 0.002 M $FeSO_4$) was further characterized, in comparison with the control film as follows:

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

FTIR spectra of films samples were determined using a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45º ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at 25 °C as described by Nuthong *et al*. (2009a). Samples were placed onto the crystal cell and the cell was clamped into the mount of FTIR spectrometer. The spectra in the range of 700–4000 cm^{-1} with automatic signal gain were collected in 32 scans at a resolution of 4 cm^{-1} and were ratioed against a background spectrum recorded from the clean empty cell at 25 ºC.

5.3.5.2 Differential scanning calorimetry

Thermal properties of films samples were determined using differential scanning calorimeter (DSC) (Perkin Elmer, Model DSC-7, Norwalk, CT, USA). Temperature calibration was performed using the Indium thermogram. The film samples (2–5 mg) were accurately weighed into aluminum pans, sealed, and scanned over the temperature range of -50 to 150 ºC with a heating rate of 10 ºC/min. The dry ice was used as a cooling medium and the system was equilibrated at -50 ºC for 5 min prior to the scan. The empty aluminum pan was used as a reference. The maximum transition temperature was estimated from the endothermic peak of DSC thermogram

and transition enthalpy was determined from the area under the endothermic peak. The second scan was also performed in the same manner followed the quench cooling of the sample after completing the first scanning.

5.3.5.3 Thermo-gravimetric analysis (TGA)

Conditioned films were scanned using a thermogravimetric analyzer (TG A-7, Perkin Elmer, Norwalk, CT, USA) from 50 to 500 ºC at a rate of 10 ºC/min (Nuthong *et al*., 2009a). Nitrogen was used as the purge gas at a flow rate of 20 mL/min.

5.3.5.4 Microstructure

Microstructure of upper surface and freeze-fractured cross-section of the film samples were visualized using a scanning electron microscope (SEM) (Quanta400, FEI, Tokyo, Japan) at an accelerating voltage of 15 kV. Prior to visualization, the film samples were mounted on brass stub and sputtered with gold in order to make the sample conductive, and photographs were taken at 8000× magnification for surface. For cross-section, freeze-fractured films were mounted around stubs using double sided adhesive tape, coated with gold and observed at the 5000× magnification.

5.3.6 Statistical analysis

Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

5.4. Results and discussion

5.4.1 Effects of H_2O_2 and Fenton's reagent on the properties of film from cuttlefish skin gelatin

5.4.1.1 Thickness

Films added with H_2O_2 at all levels used had lower thickness, compared with the control film (p<0.05) (Table 23). However, higher thickness was observed when films were added with Fenton's reagent at all concentrations used (p<0.05). Similar results were observed between films from gelatin without and with hydrolysis. H_2O_2 and Fenton's reagent could affect the film matrix differently, in which the pretruded or more compact film matrix was developed when H_2O_2 and Fenton's reagent were added, respectively.

5.4.1.2 Mechanical properties

Films obtained from gelatin added with H_2O_2 showed higher TS and lower EAB, compared with the control film (without addition of H_2O_2 and Fenton's reagent) (p<0.05), except for H-2 which had similar EAB to the control film (p>0.05) (Table 23). TS of films were increased by 13.7, 12.4 and 6.9% for H-1, H-2 and H-4 samples, respectively. For the films prepared from partially hydrolyzed gelatin, all films including PH-1, PH-2 and PH-4 also had higher TS than did the control film (p<0.05). An increase in TS by 32.0, 47.9 and 20.3% was obtained for PH-1, PH-2 and PH-4 samples, respectively. However, no changes in EAB of resulting films were noticeable when different concentrations of H_2O_2 were incorporated in partially hydrolyzed gelatin (p>0.05). In general, films from partially hydrolyzed gelatin had lower TS and EAB, compared with those from gelatin, regardless of the addition of H_2O_2 and Fenton's reagent. The result suggested that H_2O_2 was able to increase the TS of resulting film. Generation of $HO^{\bullet}$ radical from H_2O_2 during mixing or casting might induce the abstraction of hydrogen from amino acid residues to form a carbon centered radical. Those radicals formed more likely underwent interaction each other, in which the protein cross-links could be formed. H_2O_2 is the most powerful oxidizing agent to produce $HO^{\bullet}$ radical, which is able to oxidize most organic compounds such

as proteins (Kocha *et al*., 1997). H_2O_2 caused the formation of highly reactive products: hydroperoxyl anion (HOO^-), hydroperoxyl ($HOO^\bullet$) and hydroxyl ($HO^\bullet$) radicals, which can react with many substances, including protein (Perkins, 1996). It was noted that H_2O_2 at higher levels (0.04 M) resulted in the lower TS, compared with film added with other H_2O_2 concentrations ($p<0.05$). H_2O_2 at high concentration might contribute to the production of excessive amount of $HO^\bullet$ radical, which more likely caused the peptide cleavage of glutamyl side chain and proline residue of protein (Stadtman, 2001). Shorter chains of cleaved gelatin might be associated with the lower TS of film.

When Fenton's reagent was incorporated into films from gelatin and partially hydrolyzed gelatin, higher increases in TS were obtained, in comparison with those found with the addition of H_2O_2 as well as the control ($p<0.05$). However, no differences in EAB were observed when different concentrations of Fenton's reagent were incorporated, in comparison with the control ($p>0.05$). However, the marked decrease in EAB was found in film from partially hydrolyzed gelatin when Fenton's reagent at high concentrations was added (PF-4) ($p<0.05$). With the addition of Fenton's reagent, TS of gelatin film were increased by 21.5, 34.6 and 26.5% for F-1, F-2 and F-4 samples, respectively, compared with the control film. For the films from partially hydrolyzed gelatin, TS of PF-1, PF-2 and PF-4 samples increased by 60.5, 76.2 and 19.9%, respectively, compared with the control. The result suggested that radical-mediated protein cross-linking were more pronounced when film was prepared with the addition of Fenton's reagent, in comparison with H_2O_2 addition. 'Fenton-type' reaction is a metal-catalyzed oxidation system, where the majority of $HO^\bullet$ radicals are produced when certain transition metals react with H_2O_2 (Kocha *et al*. 1997). $HO^\bullet$ radical involves abstraction of the alpha-hydrogen atom from amino acid residues to form a carbon-centered radical derivative. Two different carbon-centered amino acid radicals can react with one another to form –C–C– protein cross linked products (Stadtman, 2001). Dean *et al*. (1997) also reported the $OH^\bullet$ radical mediated protein cross-linking of bovine serum albumin. Metal-catalyzed oxidations induced dityrosine formation, leading to intra- and inter- molecular protein cross-linking (Kato *et al*., 2001). Thus, the Fenton's mediated protein cross linking contributed to the increases in TS of film.

Table 23. Thickness, mechanical properties, water vapor permeability and solubility of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with H_2O_2 or Fenton's reagent

Source of materials	Film sample	Thickness (mm)	TS (MPa)	EAB (%)	WVP ($x10^{-10}$ g $s^{-1}.m^{-1}.Pa^{-1}$)	Film solubility (%)
Gelatin	C	0.036±0.002 b	30.70±1.88 e	5.88±0.89 ab	1.05±0.04 b	95.09±1.42 a
	H-1	0.031±0.002 d	34.90±1.26 c	5.09±0.42 c	1.06±0.09 b	92.87±1.77 ab
	H-2	0.032±0.002 cd	34.49±0.86 c	5.23±0.25 abc	1.07±0.09 ab	91.27±2.13 b
	H-4	0.033±0.001 c	32.81±0.84 d	5.12±0.37 c	1.16±0.07 a	90.96±1.71 b
	F-1	0.040±0.001 a	37.30±1.37 b	6.12±0.53 a	1.04±0.09 b	80.38±2.72 c
	F-2	0.041±0.002 a	41.31±2.56 a	5.91±0.49 ab	0.94±0.06 c	61.61±3.61 e
	F-4	0.042±0.002 a	38.84±1.60 b	5.32±0.25 bc	1.14±0.05 ab	74.09±2.86 d
Partially hydrolyzed gelatin†	PC	0.033±0.002 C	11.71±2.02 E	3.0±1.12 A	1.12±0.06 B	100 A
	PH-1	0.031±0.001 CD	15.46±2.00 CD	3.27±0.44 A	1.08±.06 BC	100 A
	PH-2	0.030±0.002 D	17.33±1.35 BC	2.80±0.45 A	1.01±.05 C	100 A
	PH-4	0.030±0.001 D	14.09±1.58 D	3.04±0.60 A	1.14±0.06 AB	100 A
	PF-1	0.036±0.002 B	18.79±1.25 AB	3.36±0.72 A	1.07±0.07 BC	90.26±1.46 B
	PF-2	0.037±0.002 B	20.63±1.45 A	3.32±0.83 A	1.02±0.04 C	91.17±2.97 B
	PF-4	0.040±0.002A	14.04±1.84 D	1.78±0.25 B	1.21±0.06 A	87.42±2.52 C

Values are given as Mean ± SD (n=3).

Different small letters in the same column within the same source of gelatin indicate the significant differences ($p<0.05$).

Different capital letters in the same column within the same source of gelatin indicate the significant differences ($p<0.05$).

†Partially hydrolyzed gelatin: 1.20% degree of hydrolysis

C, PC: control films from gelatin and partially hydrolyzed gelatin, respectively (without addition of H_2O_2 and Fenton's reagent).

H-1, H-2 and H-4: films from gelatin added with 0.01, 0.02 and 0.04 M H_2O_2, respectively; PH-1, PH-2 and PH-4: films from partially hydrolyzed gelatin added with 0.01, 0.02 and 0.04 M H_2O_2, respectively.

F-1, F- 2 and F- 4: films from gelatin added with Fenton's reagent containing 0.01 M H_2O_2 + 0.001 M $FeSO_4$, 0.02 M H_2O_2 + 0.002 M $FeSO_4$ and 0.04 M H_2O_2 + 0.004 M $FeSO_4$, respectively; PF-1, PF-2 and PF-4: films from partially hydrolyzed gelatin added with Fenton's reagent containing 0.01 M H_2O_2 + 0.001 M $FeSO_4$, 0.02 M H_2O_2 + 0.002 M $FeSO_4$ and 0.04 M H_2O_2 + 0.004 M $FeSO_4$, respectively.

However, film added with high concentration of Fenton's reagent (F-4 or PF-4) showed lower TS, compared with the film added with Fenton's reagent at other concentrations (p<0.05). The excessive formation of $HO^{\bullet}$ radicals at higher concentration of Fenton's reagent was presumed. Higher amount of $HO^{\bullet}$ radical could induce protein fragmentation. Peptide bond cleavage can occur by ROS (reactive oxygen substance)-mediated oxidation of glutamyl side chains (Stadtman, 2001). Lysine, arginine, proline and threonine residues of proteins are particularly sensitive to metal-catalyzed oxidation. Carbonyl derivatives and peptide carbonyl derivatives are formed as fragmentation products of peptide bond cleavage reactions (Dean *et al*., 1997; Stadtman, 2001). Kocha *et al*. (1997) found that the degradation of albumin was initiated by the H_2O_2/Fe^{2+}/EDTA oxidation system, which resulted in marked production of $HO^{\bullet}$ radicals. Therefore, the mechanical properties of gelatin-based film were largely affected by the addition of H_2O_2 and Fenton's reagent as well as chain length of gelatin. Additionally, the concentration of H_2O_2 and Fenton's reagent was shown to be crucial for strengthening the film from either gelatin or partially hydrolyzed gelatin.

5.4.1.3 Water vapor permeability (WVP)

Films prepared from both gelatin and partially hydrolyzed gelatin added with H_2O_2 and Fenton's reagent showed slight changes in WVP, compared with the control film, depending upon the concentrations used (Table 23). H-1 and H-2 samples showed similar WVP to the control film (p>0.05), while H-4 sample had higher WVP (p<0.05). For partially hydrolyzed gelatin films, PH-2 sample showed lower WVP, compared with the control and other films added with H_2O_2 at other levels (p<0.05). Additionally, films added with Fenton's reagent also showed the slight changes in WVP. For gelatin film, F-2 sample had the lowest WVP, compared with other films (p<0.05). For film from partially hydrolyzed gelatin, PF-4 showed the increase in WVP, compared with the control film (p<0.05). The result suggested that H_2O_2 and metal catalyzed Fenton-type reaction affected the matrix of film to different degrees. At appropriate concentration, protein modification induced by oxidizing agent on radicals might take place in the way which the ordered and fine matrix was formed. This led to the stronger and compact film network, which

contributed to restrict in water diffusion and permeability. However, films from both gelatin and partially hydrolyzed gelatin had the increase in WVP when either H_2O_2 or Fenton's reagent at level of 0.04% was used, respectively. The result suggested that an excessive amount of $HO^{\bullet}$ radical, when higher concentrations of H_2O_2 and Fenton's reagent were used, caused the peptide fragments. The fragmented N-terminal portion of protein might form H-bond with water, resulting in the increases in WVP of film. Additionally, excessive cross-linking might lead to the larger aggregate with voids in the matrix, thereby favoring the migration of water molecules. Hoque *et al*. (2011a) also found the increase in WVP of gelatin film with increasing degree of hydrolysis. In general, films obtained from hydrolyzed gelatin tended to have the higher WVP than those from gelatin, regardless of the addition of H_2O_2 or Fenton's reagents. Thus, the impact of H_2O_2 or Fenton's reagent on WVP of film from cuttlefish skin gelatin was governed by the levels of both chemicals used as well as the chain length of gelatin.

5.4.1.4 Film solubility

With the addition of H_2O_2 at 0.02 and 0.04 M (H-2 and H-4), film solubility was lowered, compared with that of the control film ($p<0.05$) (Table 23). Nevertheless, for the partial hydrolyzed gelatin films, H_2O_2 ranging from 0.01 to 0.04 M had no impact on solubility. H_2O_2 might induce the oxidation of protein in which the large protein aggregates could be formed, but the negligible effect was obtained when gelatin with the shorter chain was used. When Fenton's reagent was incorporated, both films from gelatin and partially hydrolyzed gelatin had the decreases in film solubility, compared with the film added with H_2O_2 as well as the control ($p<0.05$). For gelatin films, F-2 sample had the lowest film solubility ($p<0.05$). The result correlated with the highest TS (Table 23), indicating higher amount of protein-protein interaction in F-2 sample. For films from partially hydrolyzed gelatin, the lowest solubility was found in PF-4 sample ($p<0.05$). However, when comparing the solubility between films from gelatin and partially hydrolyzed gelatin, the latter had higher solubility, regardless of type and levels of chemicals added. The results suggested that shorter gelatin molecules might undergo the weaker interaction, leading to poor film matrix. However, aggregations of shorter gelatin molecules upon

interaction with highly reactive $HO^{\bullet}$ radical from Fenton's type reaction, especially at higher concentration, resulted in the decrease in solubility. Furthermore, the shorter chains were able to be leached out with ease. Film solubility can be viewed as a measure of the water resistance and integrity of a film (Rhim *et al*., 2000). Therefore, the addition of H_2O_2 and Fenton's reagent could enhance the interaction between gelatin molecules, especially with long chains, leading to the lower solubility of resulting film.

5.4.1.5 Color of film

Gelatin (without hydrolysis) film added with H_2O_2 showed higher L*-value (lightness) and a*-value (redness/greenness), but lower b*-value (yellowness/blueness) and ΔE* (color difference), compared with the control (without addition of H_2O_2 and Fenton's reagent) (p<0.05) (Table 24). However, no differences in L*-, a*-, b*- values and ΔE* were observed, compared with the control film, when films prepared from partially hydrolyzed gelatin were added with H_2O_2 at all levels used (p>0.05). The changes in color of film added with H_2O_2 might be due to the bleaching effects of H_2O_2. H_2O_2 is widely used as a bleaching agent (Aewsiri *et al*., 2009). Aewsiri *et al*. (2009) also observed the increased L*-value and decreased a*-value of gelatin when 2-5% H_2O_2 solution was used for soaking of cuttlefish skin. On the other hand, gelatin film added with Fenton's reagent exhibited lower L*-values with higher a*-, b*- and ΔE* values, compared with the control (p<0.05). Similar result was also observed in films from partially hydrolyzed gelatin film. It was noticeable that the changes in color of film were more pronounced when the concentration of Fenton's reagent increased (p<0.05). This was evidenced by the lowest L*-value, and the highest a*-, b*- and ΔE*-values, compared with those of control films and films incorporated with Fenton's reagent at other levels (p<0.05). Such changes in color of resulting films were most likely attributed to the color components generated from the reaction between H_2O_2 and ferrous sulfate ($FeSO_4$). The results was in agreement with Barbusiski and Majewski (2003) who reported that only H_2O_2 did not cause the visual discoloration of azo dye acid red 18, but discoloration was noticeable for the mixture of iron and H_2O_2, especially at higher

dose. Therefore, both H_2O_2 and Fenton's reagent had the influence on the color of resulting films from gelatin and partially hydrolyzed gelatin.

5.4.1.6 Light transmission and transparency

The transmission of UV light was very low at 200 nm for all films. At 280 nm, films added with Fenton's reagent, especially at higher levels, showed lower transmission than those incorporated with lower levels of Fenton's reagent (Table 24). Therefore, gelatin film effectively prevented the UV light. The efficiency in preventing UV light became higher when Fenton's reagent was added in comparison with H_2O_2. Hoque *et al*. (2011a) observed the excellent UV protection of film from cuttlefish skin gelatin with and without hydrolysis. Jongjareonrak *et al*. (2006b) also reported higher UV light barrier capacity of gelatin film from bigeye snapper and brownstripe red snapper skin, compared with the synthetic film.

In general, light transmission in visible range (350–600 nm) for all films were in the range of 3.46 - 87.91%. With the addition of H_2O_2 at all level used, only slight changes in light transmission of resulting films were observed in the visible range. Films incorporated with Fenton's reagent showed lower transmission of visible light, compared with the film added with H_2O_2 as well as the control, regardless of degree of hydrolysis of gelatin. Transmission in visible range became lower as the Fenton's reagent concentration increased, particularly in wavelength of 300-500 nm. Fenton's reagent more likely induced the interaction or aggregation of gelatin molecules, in the way which lowered the light transmission.

For transparency values, both gelatin and partially hydrolyzed gelatin films incorporated with H_2O_2 showed the increases in transparency value, compared with the control ($p<0.05$), indicating the decreases in transparency of films. However, Fenton's reagent added films exhibited lower transparency values for both gelatin and partially hydrolyzed films, compared with the control ($p<0.05$), indicating the higher transparency than that from H_2O_2 treated films. Thus, both H_2O_2 and Fenton's reagent not only affected the color (Table 24) but also light transmission and transparency of resulting films.

Table 24. Color, light transmittance and transparency value of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with H_2O_2 or Fenton's reagent

Source of materials	Film sample	Color				Light transmittance (%)						Transparency values
		L*	a*	b*	ΔE*	200	280	350	400	500	600	
Gelatin	C	90.62±0.22 b	-1.32±0.05 e	3.22±0.24 d	4.14±0.27 d	0.00	10.57	63.01	77.44	84.89	86.81	3.46 ± 0.01 c
	H-1	90.92±0.11 a	-1.23±0.02 cd	2.36±0.14 e	3.35±0.13 e	0.01	12.50	69.37	79.22	84.65	86.73	3.58 ± 0.04 a
	H-2	90.77±0.14 ab	-1.27±0.02 de	2.68±0.16 e	3.66±0.12 de	0.01	13.99	68.19	80.17	85.81	87.91	3.51 ± 0.02 b
	H-4	91.01±0.25 a	-1.23±0.04 cd	2.40±0.27 e	3.31±0.22 e	0.01	11.88	64.17	77.50	84.25	86.90	3.50 ± 0.02 b
	F-1	88.61±0.18 c	-1.18±0.04 c	9.19±0.24 c	10.11±0.30 c	0.00	1.40	33.74	60.33	77.82	82.98	3.31 ± 0.01 de
	F-2	86.54±0.35 d	-0.86±0.05 b	15.22±0.85 b	16.41±0.91 b	0.00	0.37	16.52	47.40	72.29	79.78	3.30 ± 0.01 e
	F-4	81.54±0.27 e	0.16±0.07 a	25.50±0.55 a	27.87±0.56 a	0.00	0.02	3.46	28.96	65.02	77.24	3.33 ± 0.04 d
Partially hydrolyzed gelatin†	PC	90.82±0.24 A	-1.23±0.05 CD	2.61±0.18 D	3.58±0.23 D	0.01	10.23	62.30	76.22	83.73	86.17	3.45 ± 0.02 C
	PH-1	90.75±0.41 A	-1.41±0.03 D	2.85±0.11 D	3.81±0.31 D	0.01	15.64	64.46	76.10	82.67	85.74	3.47 ± 0.05 BC
	PH-2	90.65±0.40 A	-1.31±0.04 D	2.60±0.17 D	3.72±0.40 D	0.01	12.35	62.88	76.52	83.64	85.11	3.52 ± 0.05 A
	PH-4	90.64±0.13 A	-1.31±0.05 D	2.45±0.30 D	3.65±0.26 D	0.01	7.76	46.85	68.65	82.14	85.26	3.50 ± 0.03 AB
	PF-1	89.05±0.38 B	-1.09±0.07 C	7.96±1.23 C	8.83±1.25 C	0.00	0.45	14.35	42.22	68.15	79.16	3.38 ± 0.02 D
	PF-2	85.85±0.24 C	-0.79±0.10 B	17.47±0.62 B	18.74±0.64 B	0.01	0.03	13.77	38.55	64.40	77.61	3.33 ± 0.01 E
	PF-4	81.58±0.77 D	0.48±0.39 A	27.02±1.68 A	29.24±1.86 A	0.01	1.64	4.46	35.23	65.10	77.88	3.30 ± 0.06 E

Values are given as Mean ± SD (n=3).

Different small letters in the same column within the same source of gelatin indicate the significant differences ($p<0.05$).

Different capital letters in the same column within the same source of gelatin indicate the significant differences ($p<0.05$).

†Partially hydrolyzed gelatin: 1.20% degree of hydrolysis

See: Table 1's footnote.

5.4.1.7 Electrophoretic protein patterns

Proteins with molecular weight (MW) of ~118 and ~97 kDa were found as the major proteins in film from gelatin (Figure 20A). Aewsiri *et al*. (2009) and Hoque *et al*. (2010) also reported that proteins with MW of 118 and 97 kDa were the dominant components in gelatin extracted from ventral skin of cuttlefish. The formation of numerous high MW protein bands, which appeared as smears or dark bands at the top of the stacking gel, was observed for H-1, H-2, H-4, F-1 and F-2 samples. However, the decrease in proteins with MW of 118 and 97 kDa was noticeable in F-2 and F-4 samples, though there were some polymerized proteins in F-2 sample. The result suggested that H_2O_2 more likely induced protein oxidation, associated with the formation of large MW aggregate. For Fenton's reagent, $HO^{\bullet}$ radicals formed caused both polymerization and fragmentation of gelatin molecules. Those carbonyl groups generated during protein oxidation might undergo Schiff base formation with the amino groups, in which the protein cross-links were most likely formed (Stadtman, 2001). $HO^{\bullet}$ radical can abstract H atoms from amino acid residues to form carbon-centered radical derivatives, which can react with one another, to form C–C protein cross-linked products (Stadtman, 2001). H_2O_2/hemin or H_2O_2/ myoglobin oxidizing system induced the formation of cross-links of myosin by non-disulfide covalent bonds (Bhoite-Solomon *et al*., 1992). On the other hand, fragmentation of gelatin molecules induced by higher amount of $HO^{\bullet}$ radicals generated from H_2O_2 and Fenton's reagent at high concentrations more likely took place. Stadtman and Berlett (1997) reported that fragmentation of protein is a consequence of direct attack by $HO^{\bullet}$ radical on the polypeptide backbone or on the side chains of glutamyl or prolyl residues. $HO_2^{\bullet}$ leads to the formation of protein alkoxyl radical, which can undergo peptide bond cleavage (Stadtman, 2001). Kocha et al. (1997) also found that the degradation of albumin was initiated by the H_2O_2/Fe^{2+}/EDTA oxidation system. Difference in protein pattern between H-4 and F-4 samples might be due to the differences in amount of $HO^{\bullet}$ radicals formed. Bhoite-Solomon *et al*. (1992) reported that H_2O_2 alone could cause myosin to form disulfide-cross-linked aggregates but did not induce fragmentation of myosin. OH radical generated from H_2O_2 and metal catalyzed Fenton's type reaction can cause a

wide variety of reactions on protein molecules, including modification of amino acids, fragmentation, cross linking and aggregation (Liu & Xiong, 2000).

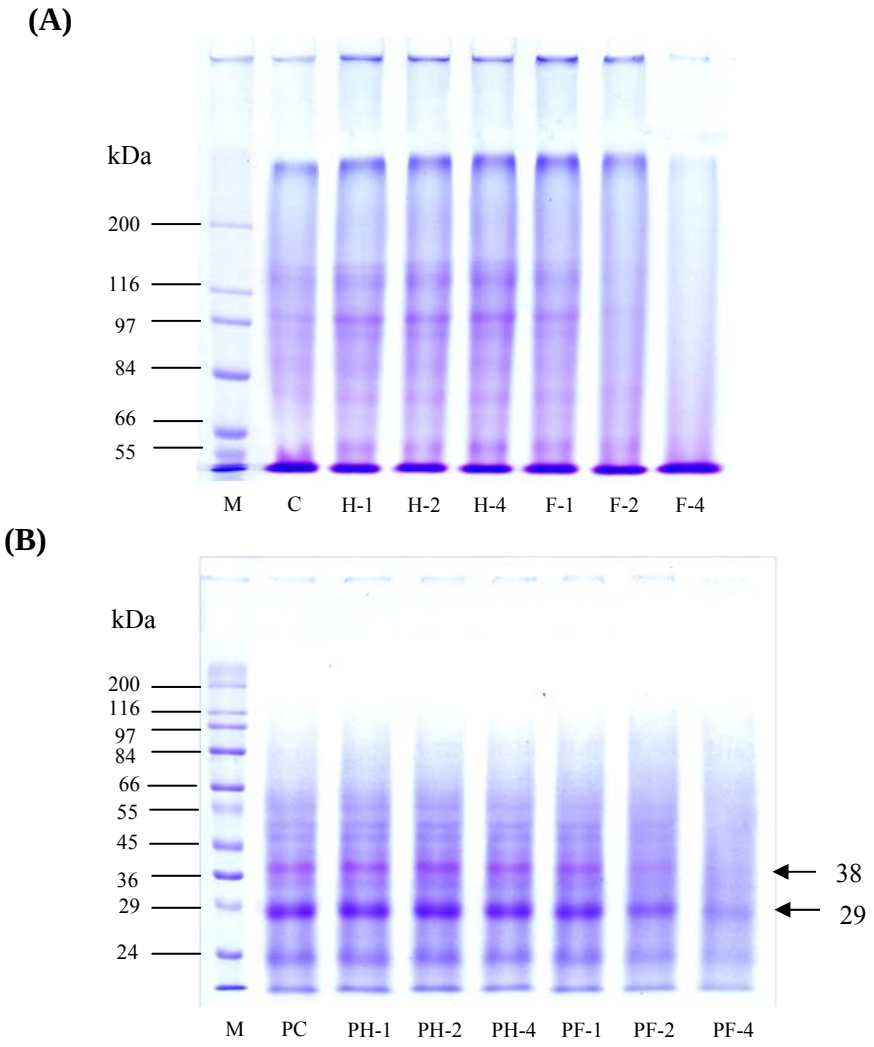


Figure 20: Protein patterns of films from cuttlefish skin gelatin (A) and partially hydrolyzed gelatin (B) containing H_2O_2 and Fenton's reagent at different concentrations. M: protein marker; C, PC: control films from gelatin and partially hydrolyzed gelatin, respectively (without addition of H_2O_2 and Fenton's reagent); H-1, H-2 and H-4: films from gelatin added with 0.01, 0.02 and 0.04 M H_2O_2, respectively; PH-1, PH-2 and PH-4: films from partially hydrolyzed gelatin added with 0.01, 0.02 and 0.04 M H_2O_2, respectively; F-1, F-2 and F-4: films from gelatin added with Fenton's reagent containing 0.01 M H_2O_2 + 0.001 M $FeSO_4$, 0.02 M H_2O_2 + 0.002 M $FeSO_4$ and 0.04 M H_2O_2 + 0.004 M $FeSO_4$, respectively; PF-1, PF-2 and PF-4: film from partially hydrolyzed gelatin added with Fenton's reagent containing 0.01 M H_2O_2 + 0.001 M $FeSO_4$, 0.02 M H_2O_2 + 0.002 M $FeSO_4$ and 0.04 M H_2O_2 + 0.004 M $FeSO_4$, respectively.

For film from partially hydrolyzed gelatin, the similar result was obtained. However, no proteins with MW of 118 and 97 kDa were presented in all films. Proteins with MW of 38 and 29 kDa were found as the major proteins in film from partially hydrolyzed gelatin (Figure 20B). When films were incorporated with H_2O_2, no marked differences in those protein bands were observed, as compared with the control film (PC). On the other hand, films treated with Fenton's reagent had lower band intensity of proteins with MW of 38 and 29 kDa as higher levels of Fenton's reagent were incorporated. Liu and Xiong (2000) reported that oxidation-induced polymerization of myosin or its fragments could be due to the actions of both H_2O_2 and $HO^{\bullet}$ radical, whereas degradation of myosin was probably caused primarily by OH radical. H_2O_2 within the concentration ranged used resulted in the polymerization, while Fenton's reagent exhibited the both protein cross-linking and fragmentation, depending upon the concentrations used.

5.4.2 Characteristics of film incorporated with H_2O_2 and Fenton's reagent

Films incorporated with 0.02 M H_2O_2 or Fenton's reagent (0.02 M H_2O_2 + 0.002 M $FeSO_4$) from both gelatin and partially hydrolyzed gelatin (H-2, F-2, PH-2 and PF-2) having the increased mechanical properties with a little change in color were subjected to characterization in comparison with their corresponding control films.

5.4.2.1 FTIR spectroscopy

FTIR spectra of all films exhibited the major bands at 1631 cm^{-1} (amide-I, representing C=O stretching/hydrogen bonding coupled with COO), 1537 cm^{-1} (amide-II, arising from bending vibration of N-H groups and stretching vibrations of C-N groups) and 1234 cm^{-1} (amide-III, representing the vibrations in plane of C-N and N-H groups of bound amide or vibrations of CH_2 groups of glycine) (Muyonga *et al*., 2004a; Aewsiri *et al*., 2009) (Figure 21). Film obtained from cuttlefish skin gelatin without and with partial hydrolysis showed the similar spectra for amide-I, amide-II and amide-III at their corresponding wavenumber (Hoque *et al*., 2011a). Pranoto *et al*. (2007) also reported that amide-I, amide-II and amide-III peaks

were found at the wavenumbers of 1656, 1550 and 1240 cm^{-1}, respectively. The peak situated around 1033 cm^{-1} might be related to the interactions arising between plasticizer (OH group of glycerol) and film structure (Bergo and Sobral, 2007). Generally, similar spectra were obtained between all gelatin films in the range of 1800-700 cm^{-1}, covering amide-I, amide-II and amide-III. Moreover, similar wavenumbers were observed between treatments for both films from gelatin and partially hydrolyzed gelatin.

Furthermore, amide-A peak was found at 3275 cm^{-1}, representing NH-stretching coupled with hydrogen bonding. Amide-B peak at 2929 cm^{-1}, representing CH stretching and NH_3^+, was also observed in the spectra (Hoque *et al*., 2011a, 2011b; Muyonga *et al*., 2004a). For film from gelatin, wavenumber of amide-A peak shifted from 3284 for the control to 3275 and 3279 for films incorporated with H-2 and F-2, respectively. The amplitude of amide-A peak at these corresponding wavenumbers was decreased markedly from 0.15 for the control to 0.09 and 0.13 for H-2 and F-2, respectively. For partially hydrolyzed gelatin films (PH-2 and PF-2), wavenumber of amide-A peak shifted from 3286 for the control to 3277 and 3282, respectively. The absorbance at these corresponding wavenumbers was decreased from 0.16 for the control to 0.11 and 0.14 for PH-2 and PF-2, respectively. The lower amplitudes were found in sample added with H_2O_2 than did that containing Fenton's reagent for both films from gelatin and partially hydrolyzed gelatin. These results suggested that H_2O_2 and Fenton's type chain reaction might induce the aggregation via NH-domain of the peptides. Coincidentally, some fragmentation, which might generate free amino groups, could lead to the increase in NH, especially when Fenton's reagent was added. Decrease in the vibrational wavenumber could be indicative of a hydrogen bonding interaction between polymer molecules in the film (Xie *et al*., 2006). Liu and Xiong (2000) reported that hydroxyl radical can modify primary structure of proteins.

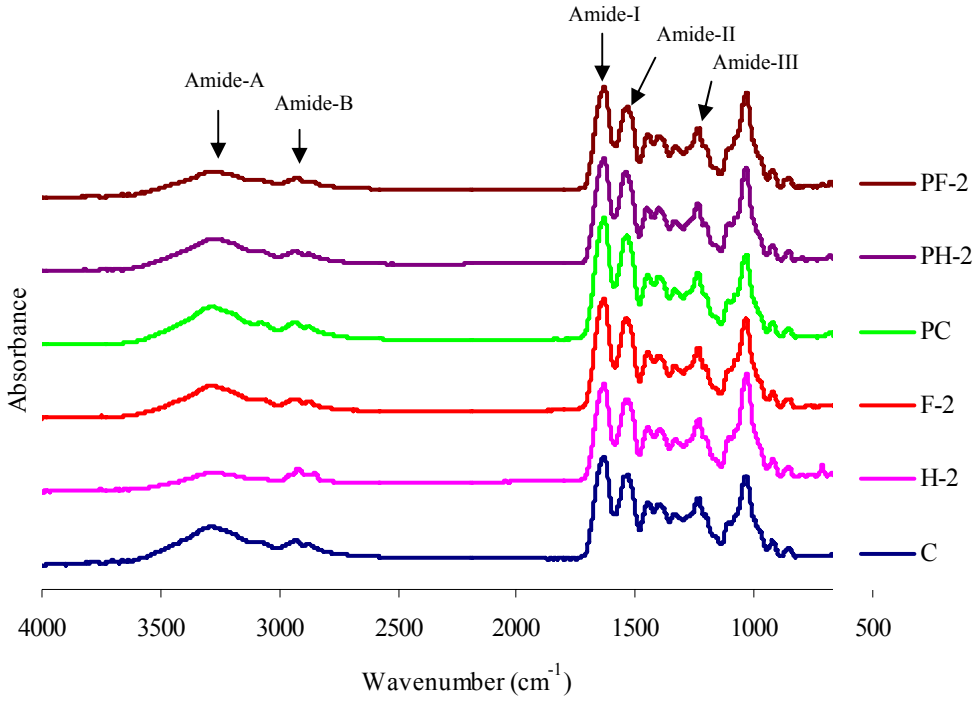


Figure 21. FTIR spectra of films prepared from gelatin and partially hydrolyzed gelatin from cuttlefish skin containing 0.02 M H_2O_2 and Fenton's reagent (0.02 M H_2O_2 + 0.002 M $FeSO_4$). C, PC: control films from gelatin and partially hydrolyzed gelatin (without addition of H_2O_2 and Fenton's reagent); H-2, PH-2: films from gelatin and partially hydrolyzed gelatin added with 0.02 M H_2O_2; F-2, PF-2: films from gelatin and partially hydrolyzed gelatin added with Fenton's reagent containing 0.02 M H_2O_2 + 0.002 M $FeSO_4$; DH: 1.2% degree of hydrolysis.

However, it was also noted that the higher amplitudes of amide-A and amide-B peaks were observed in film from partially hydrolyzed than film from gelatin. The result reconfirmed the presence of higher amount of $–NH_2$ or $–NH_3^+$ group obtained from hydrolysis process. Similar results were observed in film from cuttlefish skin gelatin without and with hydrolysis as affected by both H_2O_2 and Fenton's reagent. Both H_2O_2 and Fenton's reagent could induce the changes in functional group and conformation of proteins as elucidated by the changes in FTIR spectra, especially for amide-A and amide-B regions.

5.4.2.2 Differential scanning calorimetry (DSC)

For films of gelatin, both without and with hydrolysis, the control film had the lowest T_{max} and ΔH than those added with H_2O_2 or Fenton's reagent (Table 25). Film added with Fenton's reagent had higher T_{max} and ΔH than that added with H_2O_2. T_{max} of the film indicated the temperature causing the structural change of film matrix, mainly related with destruction of protein interaction formed during film formation (Jongjareonrak *et al*., 2006b). Higher T_{max} and ΔH found in films added with H_2O_2 and Fenton's reagent might be due to greater interaction of protein molecules induced by radical-mediated protein modification process, which restricted the molecular mobility of gelatin in the film matrix. T_{max} of 89.0 ºC was previously reported for pure gelatin film (Mendieta-Taboada *et al*., 2007). The greater interaction among the gelatin strands resulted in higher T_{max} and ΔH of resulting film (Hoque *et al*., 2010). Thermal stability of films was possibly affected by the presence of intermolecular interaction of proteins, such as hydrogen bonds, ionic-interactions, hydrophobic–hydrophobic interactions and covalent bonds, which stabilized the film network (Barreto *et al*., 2003). In general, films from partially hydrolyzed gelatin showed lower T_{max} and ΔH than those prepared from gelatin, regardless of H_2O_2 and Fenton's reagent incorporated. This might be associated with the shorter gelatin molecules, which could not form the strong film network as indicated by lower TS (Table 23). Hoque *et al.* (2010) also observed lower T_{max} and ΔH of film from thermally degraded gelatin molecules. Weaker film network required lower enthalpy for destroying the inter-chain interactions. Lower thermal stability of film from partially hydrolyzed gelatin was in agreement with poorer mechanical property of film (Table 23). In general, higher transition enthalpy was coincidentally attained in the films with higher T_{max}.

For the second scan, no transition was observed. It was postulated that the absorbed water acting as plasticizer might be removed during the first heating scan. As a consequence, the interaction between gelatin molecules could be enhanced and the more rigid film network was obtained. Thus, the transition temperature of the film could become too high and could not be detected in the temperature range tested. Therefore, thermal properties of cuttlefish skin gelatin film were affected by H_2O_2 and Fenton's reagent to some extent.

Table 25. Melting transition temperature (T_{max}), transition enthalpy (ΔH), thermal degradation temperature (T_d) and weight loss (Δw) of films from gelatin and partially hydrolyzed gelatin from cuttlefish skin incorporated without and with H_2O_2 or Fenton's reagent.

Source of materials	Film sample	Melting transition		Δ_1		Δ_2		Δ_3		Residue (%)
		T_{max} (°C)	ΔH (J/g)	$Td_{1,onset}$ (°C)	Δw_1 (%)	$Td_{2r,onset}$ (°C)	Δw_2 (%)	$Td_{3,onset}$ (°C)	Δw_3 (%)	
Gelatin	C	89.20	12.72	54.25	3.89	213.77	18.20	310.12	52.06	25.85
	H-2	95.70	15.11	57.12	3.02	217.40	16.72	313.40	50.24	30.05
	F-2	102.53	17.72	62.46	2.65	225.55	15.05	318.99	48.12	34.20
†Partially hydrolyze gelatin	PC	85.03	10.74	51.10	4.28	202.76	19.35	302.80	52.41	23.96
	PH-2	92.20	12.20	54.18	3.85	210.05	17.86	308.33	51.15	27.15
	PF-2	100.20	16.89	57.53	2.98	220.26	15.92	315.77	49.28	31.82

†Partially hydrolyzed gelatin: 1.20% degree of hydrolysis

C, PC: control films from gelatin and partially hydrolyzed gelatin, respectively (without addition of H_2O_2 and Fenton's reagent,

H-2: film from gelatin added with 0.02 M H_2O_2; PH-2: film from partially hydrolyzed gelatin added with 0.02 M H_2O_2

F- 2: film from gelatin added Fenton's reagent containing 0.02 M H_2O_2 + 0.002 M $FeSO_4$; PF-2: film from partially hydrolyzed gelatin added with Fenton's reagent containing 0.02 M H_2O_2 + 0.002 M $FeSO_4$.

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

5.4.2.3 Thermo-gravimetric analysis (TGA)

Based on TGA, Three main stages of weight loss were observed for all films from both gelatin and partially hydrolyzed gelatin. For all films, the first stage weight loss (Δw_1= 2.65 - 4.28%) was observed approximately at degradation temperature (Td_1) of 51.10 - 62.46 °Cm, (Table 25), possibly associated with the loss of free water adsorbed in the film. The similar result was found in cuttlefish skin gelatin film with and without hydrolysis (Hoque *et al*., 2011a), porcine plasma protein film added with different cross-linking agents (Nuthong *et al*., 2009a) and in collagen hydrolysate film plasticizing with glycerol and poly(ethylene glycols) (Langmaier *et*

al., 2008). The second stage weight loss (Δw_2= 15.05 - 19.35%) appeared in the region of Td_2 (202.76 - 225.55 °C) for all films. This was most likely due to the loss of lower molecular weight protein fractions, glycerol compounds and also structurally bound water. For the third stage of weight loss (Δw_3= 48.12 – 52.14%), Td_3 of 302.80-318.99 °C were observed for both films from gelatin and partially hydrolyzed gelatin. The results revealed that film added with H_2O_2 and Fenton's reagent showed higher heat resistance than the control films (without addition of H_2O_2 and Fenton's reagent) for both films from gelatin and partially hydrolyzed gelatin. For both gelatin materials, the control films showed lower thermal degradation temperatures for Td_1, Td_2 and Td_3 and the higher weight loss for Δw_1, Δw_2 and Δw_3, compared with the films added with H_2O_2 or Fenton's reagent (Table 25). The result suggested that the incorporation of H_2O_2 and Fenton's reagent yielded stronger film network, leading to higher heat resistance of the resulting films. Moreover, lower Δw_1 of films added with H_2O_2 and Fenton's reagent, compared to that of control film, suggested lower water adsorptivity of the film as also indicated by lower WVP (Table 23). For the same materials, the resulting film added with Fenton's reagent had higher Td_1, Td_2 and Td_3 with lower Δw than that containing H_2O_2. Lower Td and higher weight loss were generally observed for partially hydrolyzed gelatin film, compared with those of gelatin film. Higher heat susceptibility of film from partially hydrolyzed gelatin was more likely owing to the shorter chains, which could undergo thermal degradation to a higher extent, compared with the gelatin. Hoque *et al*. (2011a) also observed the weak film network from gelatin with shorter molecules, which underwent thermal degradation and higher weight loss at higher degree, compared with film from gelatin without hydrolysis. From the results, the gelatin films added with Fenton's reagent had higher residue mass (representing char content) than did those added with H_2O_2 and the control films, respectively. Higher char content most likely resulted from the presence of greater degree of covalent cross-linking of protein in the film network, which was induced by free-radicals generated from H_2O_2 and Fenton's reagent. Thus, H_2O_2 and Fenton's reagent showed the pronounced impact on thermal stability of films from both gelatin with and without partial hydrolysis.

(A)

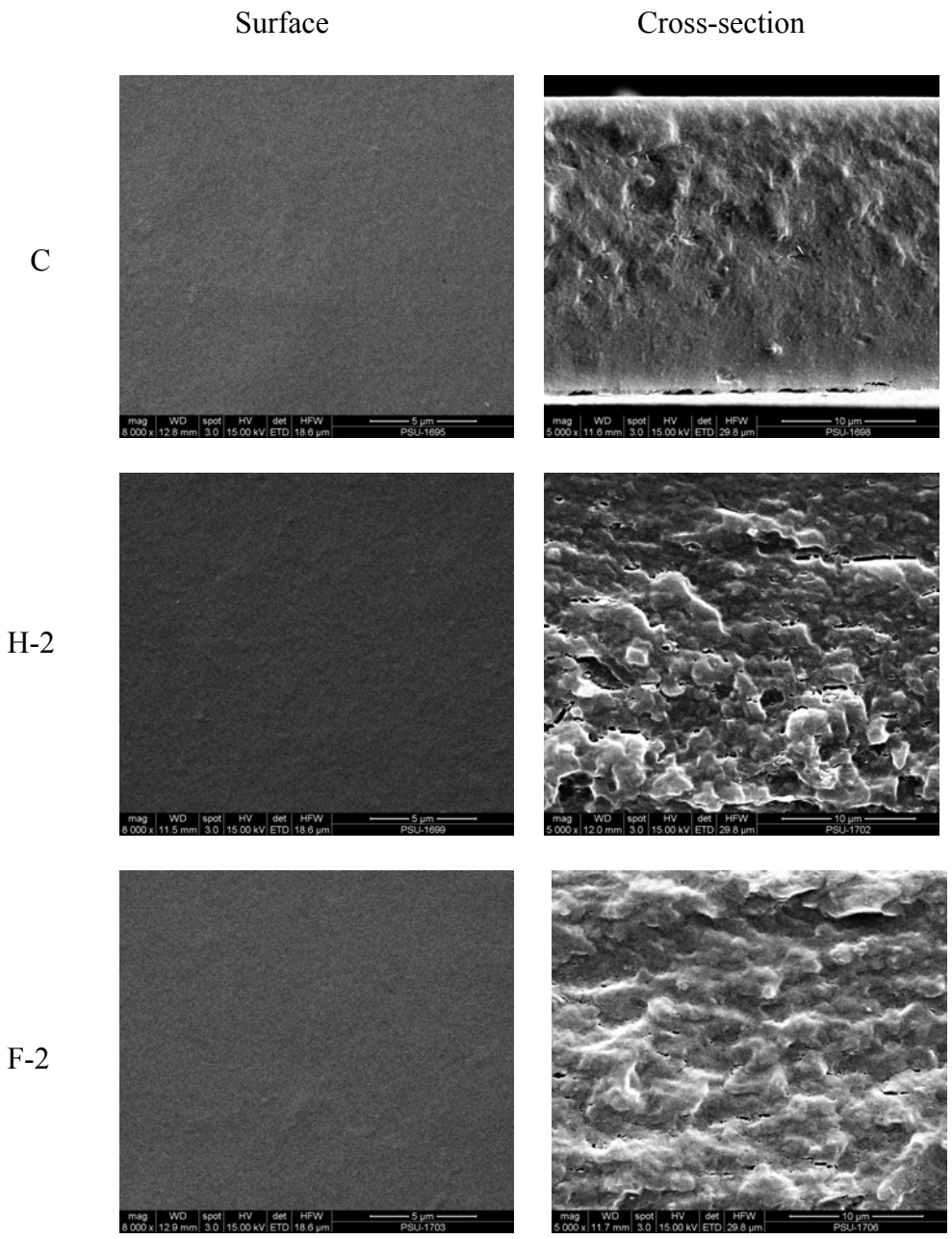


Figure 22: (Continued)

(B)

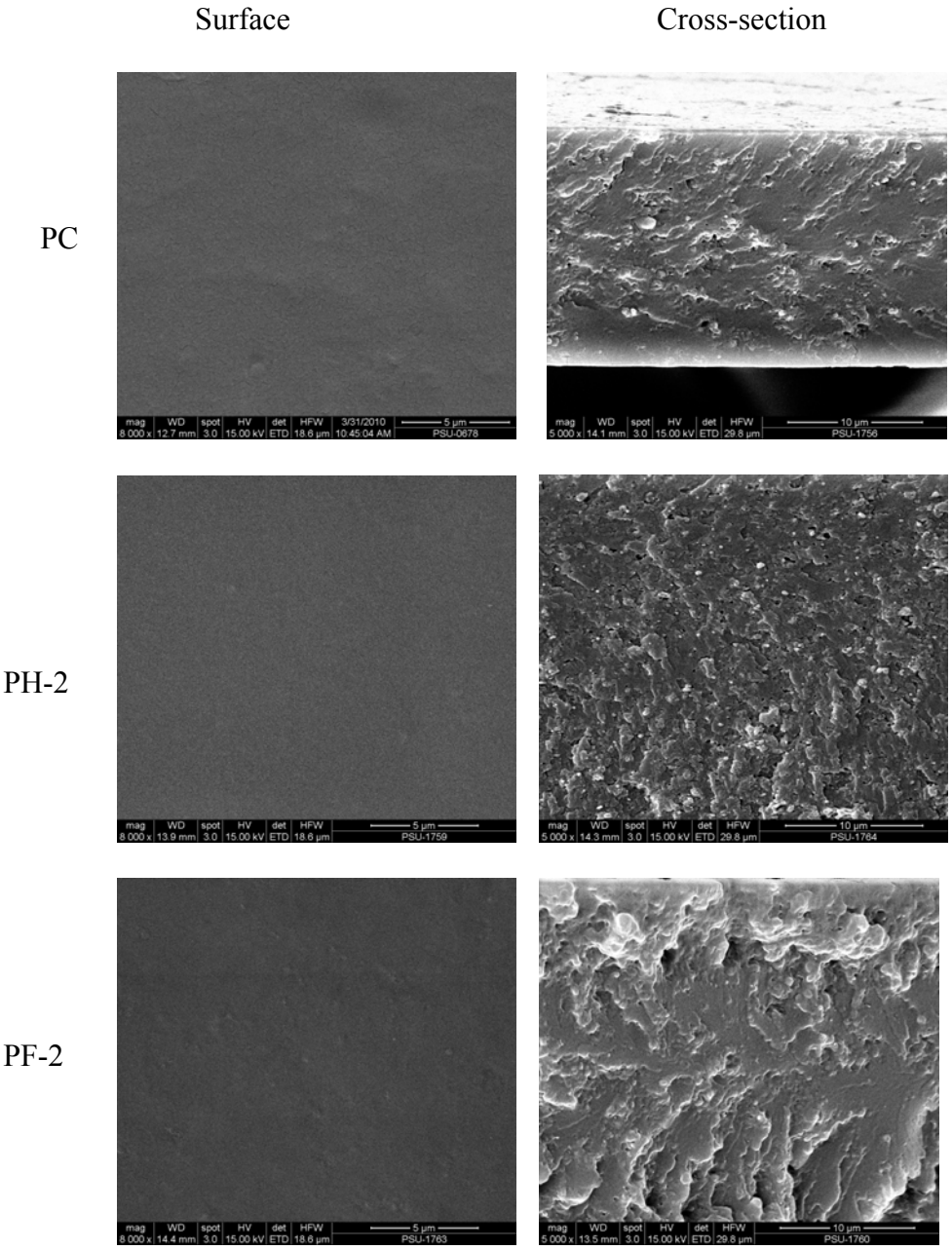


Figure 22. SEM micrographs of surface and freeze-fractured cross-section of films from cuttlefish skin gelatin (A) and partially hydrolyzed gelatin (B) containing 0.02 M H_2O_2 and Fenton's reagent (0.02 M H_2O_2 + 0.002 M $FeSO_4$). C, PC: control films from gelatin and partially hydrolyzed gelatin (without addition of H_2O_2 and Fenton's reagent); H-2, PH-2: films from gelatin and partially hydrolyzed gelatin added with 0.02 M H_2O_2; F- 2, PF-2: films from gelatin and partially hydrolyzed gelatin added with Fenton's reagent containing 0.02 M H_2O_2 + 0.002 M $FeSO_4$; DH: 1.2% degree of hydrolysis.

5.4.2.4 Microstructure

For the control films, smooth surface was observed for both films from gelatin and partially hydrolyzed gelatin (Figure 22). Smooth surface was also obtained in the film prepared from gelatin and partially hydrolyzed gelatin incorporated with H_2O_2 and Fenton's reagent. For cross-section, coarser cross-sectional structure was observed in H-2 and F-2 samples, compared with the control gelatin film. Similar result was found for film prepared from partially hydrolyzed gelatin (PH-2 and PF-2). However, coarser cross-sectional structure was attained for the film from gelatin than that of partially hydrolyzed gelatin, when H_2O_2 and Fenton's reagent were incorporated. Smaller micro-crack and micro-voids with the denser internal structure was observed in gelatin film treated with Fenton's reagent than that with H_2O_2. The results suggested that Fenton's mediated $HO^{\bullet}$ radicals might induce the cross-linking of gelatin molecules, in which the ordered matrix could be formed to a greater extent than H_2O_2. For partially hydrolyzed gelatin film, sandy or rough cross-sectional structure was found in film added with H_2O_2 (PH-2). However, more compact cross-sectional structure was observed in film added with Fenton's reagent (PF-2) than that of control film. Shorter gelatin molecules with higher number of NH_2 group might undergo higher interaction induced by $HO^{\bullet}$ radicals from Fenton's type chain reaction. Thus, the radical-mediated cross-linking of protein molecules determined the microstructure of resulting films.

5.5 Conclusion

Incorporation of H_2O_2 and Fenton's reagent into gelatin and partially hydrolyzed gelatin increased TS and caused the little changes in WVP of resulting films. However, higher concentration of H_2O_2 and Fenton's reagent could affect the color of resulting films to some extent. H_2O_2 (0.02 M) and Fenton's reagent (0.02 M H_2O_2 + 0.002 M $FeSO_4$) were effective in enhancing the molecular interactions, thereby improving the strength of their resulting films. Fenton's reagent showed greater efficiency in increasing the strength, decreasing film solubility and modification of film property than did H_2O_2. Furthermore, chain length of gelatin also determined the properties of resulting film incorporated with both chemicals.

Therefore, H_2O_2 and Fenton's reagent at an appropriate concentration could be used as protein cross-linkers, which is able to modify the properties of film from gelatin and partially hydrolyzed gelatin or other proteins.

CHAPTER 6

PROPERTIES OF BLEND FILM BASED ON CUTTLEFISH (*SEPIA PHARAONIS*) SKIN GELATIN AND MUNGBEAN PROTEIN ISOLATE

6.1 Abstract

Blend films based on cuttlefish (*Sepia pharaonis*) ventral skin gelatin (CG) and mungbean protein isolate (MPI) at different blend ratios (CG/MPI = 10:0, 8:2, 6:4, 4:6, 2:8 and 0:10, w/w) prepared at pH 11 using 50% glycerol (based on total protein) as plasticizer were characterized. CG films incorporated with MPI at increasing amounts had the decreases in tensile strength (TS) (p<0.05). The increases in elongation at break (EAB) were observed when CG/MPI ratios of 6:4 or 4:6 were used (p<0.05). Decreased water vapor permeability (WVP) was obtained for films having the increasing proportion of MPI (p<0.05). CG/MPI blend films with higher MPI proportion had lower film solubility and L*-values (lightness) but higher b*-values (yellowness) and ΔE*-values (total color difference) (p<0.05). Electrophoretic study revealed that disulfide bond was present in MPI and CG/MPI blend films. However, hydrogen bonds between CG and MPI in the film matrix were dominant, as elucidated from FTIR spectroscopic analysis. Moreover, thermal stability of CG/MPI blend film was improved as compared to that of films from respective single proteins. Differential scanning calorimetry result suggested solid-state morphology of CG/MPI (6:4) blend film that consisted of amorphous phase of partially miscible CG/MPI mixture and the coexisting two different order phases of individual CG and MPI domains. Thus, the incorporation of MPI into gelatin film could improve the properties of resulting blend film, which were governed by CG/MPI ratio.

6.2 Introduction

Biodegradable films made from renewable biopolymers have become important environmental friendly materials for packaging, thereby reducing plastic wastes (Tharanathan, 2003; Prodpran and Benjakul, 2005; Hoque *et al*., 2010, 2011a, 2011b). Most synthetic films are non-biodegradable and are associated with environmental pollution and serious ecological problems (Tharanathan, 2003). As a consequence, preparation of biodegradable or edible films from biopolymers to produce environmentally friendly packaging alternative to synthetic plastic packaging films has become important. Among polymers, proteins from different sources have been used to prepare films due to their abundance and the uniqueness in film-forming ability. Specific structure of proteins (based on 20 different monomers) provides a wider range of potential functionalities via different intermolecular bondings (Ou *et al*., 2005; Jongiareonrak *et al*., 2006b; Prodpran *et al*., 2007). The properties of protein films are determined by their microstructure, which significantly varies depending on the protein structure and interaction (Denavi *et al*., 2009). Bondings and degree of interactions involved in the stabilization of a protein film matrix are determined by the amino-acid composition and molecular weight of the proteins (Denavi *et al*., 2009).

Gelatin is a natural biopolymer obtained by thermal degradation of collagen (Arvanitoyannis, 2002). Skin of marine animals such as fish, cuttlefish, etc. can be used as raw materials for gelatin production (Aewsiri *et al*., 2009; Hoque *et al*., 2010; Ahmad and Benjakul, 2010). Gelatin has been used as a material for preparing biodegradable films with high transparency and excellent barrier characteristics against gas, organic vapor and oil, compared to synthetic films (Jongiareonrak *et al*., 2006b; Jiang *et al*., 2007). However, gelatin film possesses poor water barrier and water resistant due to the fact that it contains high amount of hydrophilic amino acids with negligible or no sulfur containing amino acids (Hoque *et al*., 2010; Jongiareonrak *et al*., 2006b; Jiang *et al*., 2007; Denavi *et al*., 2009). This is the main drawback of gelatin films for their application as a packaging material (McHugh and Krochta, 1994; Gómez-Guillén *et al*., 2009).

To improve the properties of protein film, different approaches have been used including chemical (de Carvalho and Grosso, 2004; Nuthong *et al*., 2009), physical (Ouattara *et al*., 2002; Jo *et al*., 2005) and enzymatic (Mariniello *et al.,* 2003) modifications. Additionally, polymer blend technique has become another effective and widely used approach to improve the properties of protein-based films (Cao *et al*., 2007b; Pérez-Mateos *et al*., 2009; Denavi *et al*., 2009; Limpan *et al*., 2010). Polymer blending is a well-used technique to modify the properties of polymer, due to its easy, uncomplicated and low cost procedure (Wang *et al*., 2009). With the proper miscibility or compatibility, polymer blend render new materials with better properties, compared to similar materials made from the respective pure polymers (Tang *et al*., 2003; Li *et al*., 2006; Cao *et al*., 2007b; Pérez-Mateos *et a*l., 2009; Limpan *et al*., 2010). Blending of gelatin with other compatible biopolymers would be an alternative approach to improve the properties of resulting blend films.

Mungbean has served as the important source of plant proteins owing to its abundance and nutritive value. Mungbean protein can be used as film forming materials. Recently, mungbean protein isolate (MPI) has been used for film preparation (Tang *et al*., 2009). Unlike gelatin, MPI is typically more hydrophobic in nature and consists of disulfide bonds. Due to the differences in proteins in terms of structure, amino acid composition and properties between gelatin and mungbean proteins, the blend of both proteins could yield the film with distinctive properties, especially the increased water vapor barrier property. Gelatin based film with increased water vapor barrier property from the mixture of gelatin and soybean proteins have been reported (Denavi *et al*., 2009). However, there is no information on the properties of blend film based on gelatin from cuttlefish skin and mungbean protein isolate. Thus, the objective of this investigation was to characterize the blend film based on cuttlefish skin gelatin and mungbean protein isolate at different ratios.

6.3 Materials and methods

6.3.1 Chemicals

Bovine serum albumin and wide range molecular weight protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sodium hydroxide, glycerol, *p*-dimethylaminobenzaldehyde and tris(hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Hydrogen peroxide was obtained from BDH, VWR International Ltd (Leicestershire, England). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

6.3.2 Collection and preparation of cuttlefish skin

Ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from a dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1 x 1 cm^2), placed in polyethylene bags and stored at -20 ºC until use. Storage time was not longer than 2 months. Prior to gelatin extraction, the frozen skin was thawed using running water (25-26 ºC) until the core temperature reached 0 - 2 ºC.

6.3.3 Extraction of gelatin from cuttlefish skin

Gelatin was extracted from cuttlefish skin according to the method of Hoque *et al*. (2010). Skin was soaked in 0.05 M NaOH with a skin/solution ratio of 1:10 (w/v) with a gentle stirring at room temperature (26–28 ºC). The solution was changed every hour to remove non-collagenous proteins for totally 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching in 5% H_2O_2, using a

sample/solution ratio of 1:10 (w/v) for 48 h at 4 ºC. The skin treated with H_2O_2 was washed three times with 10 volumes of distilled water. Gelatin was extracted from bleached skin using distilled water at 60 ºC for 12 h, with a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously using a paddle stirrer (RW20.n, IKA LABORTECHNIK, Staufen, Germany). The extract was centrifuged at 8,000xg for 30 min at room temperature using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble materials. The supernatant was collected and freeze-dried (Model Duratop™ lP/Dura Dry™ lP, FTS® System, Inc., Stone Ridge, NY, USA). The dry matter was referred to as 'cuttlefish gelatin, CG'. CG contained 89.10% protein as determined by Kjeldhal method (AOAC, 1999).

6.3.4. Collection and preparation of mungbean protein isolate

Peeled mungbean (*Phaseolus aureus*) was purchased from Thai Cereals World Co., Ltd, Bangkok, Thailand. The sample was ground using a blender (Moulinex, Type AY46, Shenzhen, Guangdong, China) to obtain the fine powder. The powder was screened using a mesh 35 with an aperture size of 500 μm, ASTM E11, serial number 5666533 (FRITSCH GMBH, Laborgerätebau, Industriestrasse 8, D-55743 Idar-Oberstein, Germany). Mungbean protein isolate (MPI) was prepared according to the methods of Pastor-Cavada *et al*. (2010) with a slight modification. Mungbean powder was suspended in 10 volumes of 0.2% NaOH solution (pH 12). The mixture was stirred continuously for 2 h at room temperature (28-30 °C) followed by centrifugation at 8000xg for 30 min. The supernatant was collected and pH was adjusted to 4.5 using 6 N HCl. The precipitate formed was recovered by centrifugation at 8000xg for 30 min. The pellet was washed with 10 volume of distilled water (pH 4.5), followed by centrifugation at 8000xg for 30 min. The resulting pellet was freeze-dried. Dried powder obtained was referred to as 'mungbean protein isolate, MPI'. MPI was stored in a dessicator at room temperature (28-30 °C) until use. MPI had the protein content of 87.80% as determined by Kjeldahl method (AOAC, 1999).

6.3.5 Properties of blend films based on CG and MPI

6.3.5.1 Preparation of films with different ratios of CG and MPI

Blend films based on CG and MPI with different CG/MPI ratios (10:0, 8:2, 6:4, 4:6, 2:8 and 0:10, w/w) were prepared. CG and MPI were mixed at different ratios and distilled water was added to obtain the final protein concentration of 3% (w/v). The mixtures were stirred and the pH of mixture was adjusted to 11 using 6 N NaOH to completely solubilize proteins in the mixtures. After pH adjustment, the mixtures were stirred continuously for 30 min to ensure the complete solubilization. Glycerol at a concentration of 50% of total protein was used as a plasticizer and the solution was referred to as 'film forming solution, FFS'. To study the effects of pH on gelatin film, film was also prepared from gelatin at pH 7.0.

To prepare films, FFS (4 ± 0.01 g) was cast onto a rimmed silicone resin plate (5 x 5 cm^2), air-blown for 12 h at room temperature and dried in an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttalingen, Germany) at the temperature of 25 ± 0.5 ºC and 50 ± 5% relative humidity (RH) for 48 h. Dried films were manually peeled-off and subjected to analyses.

6.3.5.2 Analyses

Prior to testing, films were conditioned for 48 h at 50 ± 5% relative humidity (RH) at 25 ± 0.5 ºC. For SEM, ATR-FTIR, DSC and TGA, films were dried in a dessicator containing dried silica gel for 1 week and in dessicator containing P_2O_5 for 2 weeks at room temperature (28-30 ºC) to obtain the most dehydrated films.

6.3.5.2.1 Film thickness

The thickness of film was measured using a digital micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mituyoto Corp., Kawasaki-shi, Japan). Ten random locations around each film sample were used for thickness determination.

6.3.5.2.2 Mechanical properties

Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata *et al*. (2000) using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK). Ten samples (2 x 5 cm^2) with the initial grip length of 3 cm were used for testing. The samples were clamped and deformed under tensile loading using a 100 N load cell 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.

6.3.5.2.3 Water vapor permeability (WVP)

WVP was measured using a modified ASTM (American Society for Testing and Materials, 1989) method as described by Shiku *et al*. (2004). The film was sealed on an aluminum permeation cup containing dried silica gel (0% RH) with silicone vacuum grease and rubber gasket. The cup was placed at 30 ºC in a desiccator containing the distilled water. It was then weighed at 1 h intervals for up to 8 h. Five films were used for WVP testing. WVP of the film was calculated as follows:

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

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

6.3.5.2.4 Film solubility

Film solubility in water was determined according to the method of Gennadios *et al*. (1998) with a slight modification. The conditioned film sample (3x2 cm^2) was weighed and placed in 50 ml-centrifuge tube containing 10 ml of distilled water with 0.1% (w/v) sodium azide. The mixture was shaken continuously at room temperature for 24 h using a shaker (Heidolph UNIMAX 1010, Schwabach, Germany). Undissolved debris film matter was obtained after centrifugation at 3000xg for 10 min at 25 ºC using a centrifuge (Allegra 25R Centrifuge, Beckman Coulter, Krefeld, Germany). The pellet was dried at 105 ºC for 24 h to obtain the dry

unsolubilized film matter. The weight of solubilized dry matter was calculated by subtracting the weight of unsolubilized dry matter from the initial weight of dry matter and expressed as the percentage of total weight.

6.3.5.2.5 Color, light transmission and transparency

Color of film was determined using a CIE colorimeter (Hunter associates laboratory, Inc., Reston, VA, USA). Color of the film was expressed as L^*-, a^*- and b^*-values. Total difference in color (ΔE^*) was calculated according to the following equation (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 corresponding color parameter of the sample and that of white standard (L^*= 93.64, a^*= -0.96 and b^*= 0.45).

Light transmission of films against ultraviolet (UV) and visible light were measured at selected wavelengths between 200 and 600 nm, using a UV–Visible spectrophotometer (model UV-160, Shimadzu, Kyoto, Japan) according to the method of Jongiareonrak *et al*. (2006b).

The transparency value of the film was calculated by the following equation (Han and Floros, 1997):

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

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

6.3.5.2.6 Electrophoretic analysis

Protein patterns of all films were analyzed using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). CG and MPI powders were dissolved in 5% SDS, followed by heating at 85 °C for 30 min. The solutions were allowed to stand at room temperature for 1 h and centrifuged at 3000xg for 5 min at room temperature. For film samples,

they were dissolved according to the method of Jongjareonrak *et al*. (2006) with some modifications. Film samples (200 mg) were mixed with 10 mL of 5% (w/v) SDS. The mixtures were stirred continuously at room temperature for 12 h. Supernatants were obtained after centrifugation at 3000xg for 5 min at room temperature. Proteins contents in the supernatants of all samples were determined using the Biuret method (Robinson and Hodgen, 1940).

All supernatants were mixed with sample buffer (0.5 M Tris–HCl, pH 6.8 containing 4% (w/v) SDS, 20% (v/v) glycerol with and without 10% (v/v) β-ME) at the ratio of 1:1 (v/v). Samples (20 μg protein) were loaded onto the polyacrylamide gel made of 12.5% separating gel and 4% stacking 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 electrophoresis, gels were stained with 0.05% (w/v) Coomassie blue R-250 in 15% (v/v) methanol and 5% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. Wide range molecular weight protein markers (Sigma Chemical Co., St. Louis, MO, USA) were used to estimate the molecular weight of proteins.

6.3.6 Characterization of selected films

CG films prepared at pH 7 and 11 as well as MPI films (pH 11) and selected CG/MPI (6:4) blend films (pH 11) were further subjected to characterization.

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

FTIR spectra of films samples were determined using a Bruker Model Equinox 55 FTIR spectrometer (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR Trough plate crystal cell (45º ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology Inc., Madison, WI, USA) at 25 °C as described by Nuthong *et al*. (2009a). Samples were placed onto the crystal cells and the cells were 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 ºC.

6.3.6.2 Differential scanning calorimetry

Thermal properties of films samples were determined using differential scanning calorimeter (DSC) (Perkin Elmer, Model DSC-7, Norwalk, CT, USA). Temperature calibration was performed using the Indium thermogram. The film samples (2–5 mg) were accurately weighed into aluminum pans, hermetically sealed, and scanned over the temperature range of -50 to 150 ºC with a heating rate of 10 ºC/min. The dry ice was used as a cooling medium and the system was equilibrated at -50 ºC for 5 min prior to the scan. The empty aluminum pan was used as a reference. The maximum transition temperature was estimated from the endothermic peak of DSC thermogram and transition enthalpy was determined from the area under the endothermic peak.

6.3.6.3 Thermo-gravimetric analysis (TGA)

Dried films were scanned using a thermogravimetric analyzer (TG A-7, Perkin Elmer, Norwalk, CT, USA) from 40 to 600 ºC at a rate of 10 ºC/min (Nuthong et al., 2009). Nitrogen was used as the purge gas at a flow rate of 20 mL/min.

6.3.6.4 Microstructure

Microstructure of upper surface and freeze-fractured cross-section of the film samples was visualized using a scanning electron microscope (SEM) (Quanta400, FEI, Tokyo, Japan) at an accelerating voltage of 15 kV. Prior to visualization, the film samples were mounted on brass stub and sputtered with gold in order to make the sample conductive, and photographs were taken at 8000× magnification for surface. For cross-section, freeze-fractured films were mounted around stubs using double sided adhesive tape, coated with gold and observed at the 5000× magnification.

6.3.7 Statistical analysis

Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test. For pair comparison, T-test was used (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

6.4 Results and discussion

6.4.1 Protein content and patterns of CG and MPI

CG contained 89.10% proteins with the yield of 13.65% (wet wt basis). CG had the proteins with molecular weight (MW) ranging from 95 to 200 kDa. Proteins with MW of 116 and 97 kDa were dominant and were likely α_1-chain and α_2-chain, respectively. The result was in accordance with Aewsiri *et al*. (2009) who found that CG consisted of proteins with MW of 117 and 97 kDa as the major proteins. No differences in protein patterns were observed in CG determined under reducing and non-reducing condition, indicating that no or negligible disulfide bonds were present in CG sample. MPI was extracted with the yield of 14.29% (based on mungbean powder). MPI contained protein as the major constituent (87.80%). Tang *et al.* (2009) reported 93.9% protein content in MPI. MPI showed the differences in SDS-PAGE patterns between reducing and non-reducing condition (Figure 23). The result suggested the presence of disulfide bonds in MPI. Under non-reducing condition, MPI contained three major bands with MW of 60, 80 and 90 kDa. However, the band with MW of 80 kDa was disappeared under reducing condition with a coincidental formation of protein band with MW of 24 kDa. The result suggested that disulfide bonds were involved in stabilization of proteins in MPI. It has been reported that most seed proteins are stabilized by disulfide bonds (Deng *et al*., 2011; Tang *et al*., 2009).

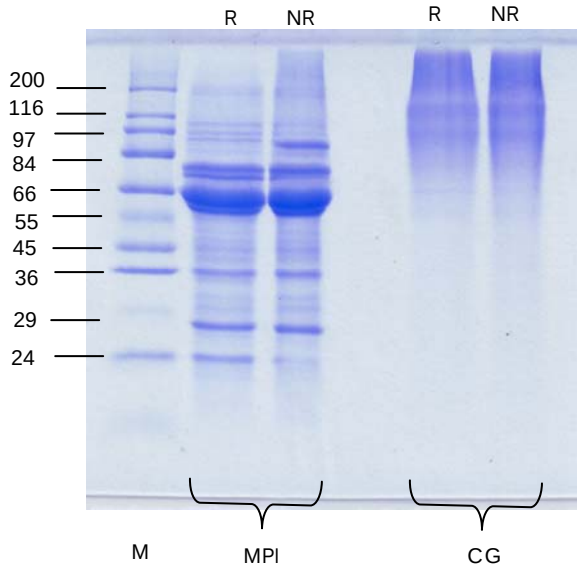


Figure 23. Protein patterns of mugbean protein isolate (MPI) and cuttlefish skin gelatin (CG) under reducing (R) and non-reducing (NR) conditions; M: protein marker.

6.4.2 Properties of blend film based on CG and MPI at different ratios

The blend films prepared using different ratios of CG and MPI showed the different properties and molecular characteristics. Properties of gelatin films prepared at pH 7 were also compared.

6.4.2.1 Thickness

Thickness of blend films having different CG/MPI ratios is shown in Table 26. Regardless of CG/MPI ratio, all films containing MPI had higher thickness, compared with CG films without MPI (p<0.05). Higher thickness was observed in films containing higher proportion of MPI, while MPI film had the highest thickness (p<0.05). This result indicated a higher degree of compactness in CG films, in which those fibrous proteins might align themselves with less protrusion in film matrix. When MPI was incorporated, the rigid globular in MPI might not fully unfolded and the arrangement between MPI molecules or MPI with CG might not occur in the ordered and uniform fashion. This led to protruded film network. However, Denavi *et*

al. (2009) found similar thickness in soy protein isolate (SPI) and gelatin based blend film, regardless of the proportion of SPI was used.

6.4.2.2 Mechanical properties

Mechanical properties of blend films based on CG and MPI at different ratios are shown in Table 26. CG films had higher TS but lower EAB, compared with CG/MPI blend films and MPI films ($p<0.05$). It was noted that CG films prepared at pH 11 showed lower TS but higher EAB than that prepared at pH 7 ($p<0.05$). Alkaline pH might yield the protein with more negative charge, leading to higher repulsion as evidenced by lower TS. Alkaline pH might favor the solubilization and subsequent alignment of extended or stretched fibrous gelatin molecules, in the way which higher inter-junctions with weak bonds were formed. Higher EAB reflected the increased extensibility. The increased EAB of film prepared at alkaline pH was more likely caused by the presence of weaker bonds stabilizing film matrix. Cao *et al*. (2006) also observed high TS of gelatin film when pH of film-forming solution was about 7. With increasing MPI amount incorporated, TS decreased ($p<0.05$). Nevertheless, blend film with CG/MPI ratio of 6:4 and 4:6 had the highest EAB ($p<0.05$), in which EAB increased by 37.9 and 43.8%, compared with CG film (pH 11). CG and MPI at an appropriate ratio might undergo interaction in the way which favored the formation of longer chain length, allowing higher molecular slipage upon tensile deformation. Among all film samples, MPI film had the lowest TS ($p<0.05$), suggesting that proteins in MPI could not form the strong network. This might be governed by rigidity of MPI protein molecules, thereby yielding the film with low interaction between protein molecules. Cao *et al*. (2007b) found the increases in tensile strength with progressively increasing proportions of gelatin in type-B bovine-bone gelatin and SPI based composite films. The deformation of SPI and cod skin gelatin based blend film increased progressively with increasing proportion of gelatin in the mixture (Denavi *et al*., 2009). Denavi *et al*. (2009) also found the higher deformation and a greater breaking force of gelatin films than those of soybean protein/gelatin blend films. The difference in mechanical properties of blend films from different sources or materials might be due to differences in type, nature, molecular weight, amino acid composition of protein as well as type and number of

interaction involved in the film matrix. Stabilization of protein film matrix is influenced by the amino acid composition and molecular weight of the proteins, the type and extent of interactions involved (Denavi *et al*., 2009; Tang *et al*., 2009). Therefore, the mechanical properties of gelatin and MPI blend film based on CG and MPI were largely affected by the type of protein, type of interaction and the ratio of proteins used.

Table 26. Thickness, mechanical properties, water vapor permeability and film solubility of CG, MPI and CG/MPI blend films.

CG : MPI[†]	Thickness (mm)	TS (MPa)	EAB (%)	WVP ($x10^{-10}$ g s^{-1}. m^{-1}.Pa^{-1})	Film solubility (%)
10:0 pH-7	0.031±0.002 A	7.43 ± 0.80 A	119.90 ± 4.84 B	1.22±0.07 AB	95.12±2.67 A
10:0, pH-11	0.030±0.003 d A	5.94 ± 0.59 a B	140.43±7.11 e A	1.29±0.03 a A	85.87±1.19 a B
8:2 , pH-11	0.038±0.002 c	4.50±1.10 bc	177.90±7.62 b	1.27±0.03 a	57.95±3.26 d
6:4 , pH-11	0.041±0.003 bc	3.64±1.03 cd	193.67±6.39 a	1.24±0.05 ab	63.54±4.15 c
4:6, pH-11	0.042±0.002 ab	2.75±1.16 de	201.91±8.38 a	1.20±0.04 bc	67.14±2.83 c
2:8, pH-11	0.044±0.003 ab	1.69±0.41e	165.93±4.52 c	1.16±0.02 cd	77.97±3.03 b
0:10, pH-11	0.045±0.003 a	1.09±0.42 e	156.87±5.54 d	1.12±0.03 d	81.54±1.08 b

Values are given as Mean ± SD (n=3).

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

Different capital letters in the same column indicate the significant differences between gelatin film prepared at pH 7 and pH 11 (p<0.05).

[†]CG: Cuttlefish skin gelatin; MPI: Mungbean protein isolate.

6.4.2.3 Water vapor permeability (WVP)

WVP of blend film based on CG and MPI with different ratios is shown in Table 26. Film with increasing MPI amounts showed the decrease in WVP (p<0.05). Among all films, MPI films had the lowest WVP (p<0.05). Addition of MPI into CG films up to the ratio of 6:4 had no impact on WVP. The result suggested that hydrophilic nature of CG contributed to water binding capacity of resulting film,

while MPI with higher hydrophobicity in nature rendered films with lower WVP. When MPI was incorporated to a higher extent into CG, the lower WVP was obtained in the resulting films. Denavi *et al*. (2009) found the lower WVP for soybean protein/gelatin blend films than for the gelatin one. The presence of soybean proteins in the blend films resulted in a reduction of WVP. Pranoto *et al*. (2007) also observed the significantly reduced WVP of gelatin film added with gellan. Thus, WVP of blend film based on CG and MPI was governed by the nature of protein, especially hydrophilicity and hydrophobicity as well as the ratio of both proteins used.

Similar WVP was observed between CG films prepared at pH 7 and 11 ($p>0.05$). Chinabhark *et al*. (2007) reported that there were no differences in WVP of films from bigeye snapper (*Priacanthus tayenus*) surimi prepared at pH 3 and 11. Furthermore, protein-based films prepared from red tilapia (*Oreochromis niloticus*) washed and unwashed mince solubilized at pH 3 and 11 had no differences in WVP (Tongnuanchan *et al*., 2011a).

6.4.2.4 Film solubility

Solubility of blend films based on CG and MPI at different ratios is presented in Table 26. CG films prepared at pH 7 (95.12%) showed the highest film solubility, followed by CG films prepared at pH 11 (85.87%). Gelatin from cuttlefish skin had high hydrophilic amino acids (Hoque et al. 2010a). Thus, it was soluble with ease in water. The CG/MPI blend films of all ratios exhibited lower film solubility than CG and MPI films did ($p<0.05$). This was more likely due to interaction between these two proteins in the film matrix, resulting in the lower hydrophilic sites available for absorbing water. However, increased film solubility was observed with increasing MPI amount. It was noted that high film solubility was found in MPI films. Among all composite films, that with CG/MPI ratio of 8:2 had the lowest film solubility (57.95%) in water ($p<0.05$). The result suggested that CG/MPI ratios of 8:2 might yield the film matrix, which could trap glycerol efficiently. As a consequence glycerol could not be leached out with ease. High film solubility of MPI films suggested that the poorer interaction was presumed in film network as indicated by poorer TS. As a result, such a weak film matrix could not hold glycerol in their network. Additionally, the non-polymerized proteins were washed out into the water. Denavi *et al*. (2009)

found that the reduction in film solubility was found with increasing ratio of SPI in SPI/gelatin blend film. Therefore, CG/MPI ratios affected the solubility of resulting film.

6.4.2.5 Color of film

Table 27 shows the color of blend films based on CG and MPI with different ratios. CG films prepared at pH 11 had the highest L*-value (lightness) and a*-value (redness/greenness) but lowest b*-value (yellowness) and ΔE* (color difference), compared with CG/MPI blend films as well as MPI film (p<0.05). In general, no differences in color were observed between CG films prepared at both pH, except that L*-value of films prepared at pH 11 was higher than that prepared at pH 7 (p<0.05). It was noticeable that the decreases in L*- and a*- values with coincidental increases in b*- and ΔE*-values were obtained as higher amount of MPI was incorporated. Such changes in color of resulting films were most likely attributed to the coloring components existing in MPI. Denavi *et al*. (2009) also observed the increased yellowish color in film as the proportion of SPI increased in SPI/gelatin blend film. Therefore, CG/MPI ratios had influence on the color of resulting films. However, negligible differences in color of gelatin film were observed when pH of FFS was changed from neutral to alkaline.

Table 27. Color of CG, MPI and CG/MPI blend films

CG: MPI†	Color			
	L*	a*	b*	ΔE*
10:0 pH-7	90.68±0.08 B	-1.31±0.02A	2.62±0.15A	3.48±0.11 A
10:0, pH-11	91.98±0.65aA	-1.39±0.08aA	2.73±0.21fA	2.98±0.54 fA
8:2, pH 11	91.41±0.46 b	-1.66±0.11 b	4.81±0.28 e	4.95±0.44 e
6:4 , pH-11	90.60±0.14 c	-1.85±0.04 c	7.25±0.29 d	7.50±0.31 d
4:6, pH-11	90.56±0.31 c	-2.00±0.10 d	8.95±0.51 c	9.10±0.56 c
2:8, pH-11	89.76±0.53 d	-2.28±0.16 e	10.94±1.16b	11.27±1.26 b
0:10, pH-11	88.87±0.16 e	-2.83±0.12 f	14.19±0.68a	14.67±0.67a

Values are given as Mean ± SD (n=3).

Different capital letters in the same column indicate the significant differences between gelatin film prepared at pH 7 and pH 11 (p<0.05).

†CG: Cuttlefish skin gelatin; MPI: Mungbean protein isolate.

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

6.4.2.6 Light transmission and transparency

Transmission of UV and visible light at wavelength range of 200–800 nm of blend films based on CG and MPI at various ratios is shown in Table 28. The transmission of UV light was very low at 200 and 280 nm for MPI films and all blend films with different CG/MPI ratios, compared with both CG films prepared at pH 7 and 11. Among all films, MPI films had the lowest transmission in both UV and visible range. It was found that film added with higher amount of MPI showed the decreases in light transmission. The result suggested that blend film effectively prevented the UV light. In general, light transmission in visible range (350–600 nm) for all films (prepared at pH 11) was in the range of 45.56 - 86.70%. In visible range, CG film showed higher light transmission as compared with CG/MPI blend films and MPI films. Lowered transmission was observed as MPI amount incorporated increased. The result suggested that MPI, especially at higher amount, with high light

transmission barrier more likely contributed to limited light transmittance of composite films at both UV and visible ranges.

Table 28. Light transmittance and transparency values of CG, MPI and CG/MPI blend films.

CG: MPI[†]	Light transmittance (%)								Transparency values
	200	280	350	400	500	600	700	800	
10:0 pH-7	0.00	13.25	69.15	82.17	87.60	88.81	89.44	89.86	3.50 ± 0.04A
10:0, pH-11	0.01	10.89	57.31	71.95	79.59	84.97	85.42	86.70	3.55 ± 0.06aA
8:2, pH 11	0.02	3.83	53.72	67.91	79.08	82.86	84.81	86.13	3.41 ± 0.04 b
6:4 , pH-11	0.02	0.72	53.17	65.06	78.51	81.30	84.34	86.11	3.39 ± 0.04 bc
4:6, pH-11	0.02	0.56	52.45	64.18	76.48	81.11	83.93	85.84	3.36 ± 0.03 bc
2:8, pH-11	0.01	0.37	48.76	61.34	75.72	79.88	82.38	84.12	3.36 ± 0.05 bc
0:10, pH-11	0.02	0.16	45.56	58.04	71.05	76.47	79.41	81.42	3.34 ± 0.01 c

Values are given as Mean ± SD (n=3).

Different capital letters in the same column indicate the significant differences between gelatin film prepared at pH 7 and pH 11 ($p<0.05$).

[†]CG: Cuttlefish skin gelatin; MPI: Mungbean protein isolate.

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

Higher transparency values were observed in CG films prepared at both pH, compared with other films ($p<0.05$). MPI films showed the lowest transparency values ($p<0.05$) (Table 28). For CG/MPI blend films, the decreases in transparency value were obtained as MPI amount increased ($p<0.05$), indicating the increases in transparency of films. Nevertheless, Denavi *et al*. (2009) reported that transparency of films was not significantly affected by the ratio of soybean protein and gelatin used. Thus, CG/MPI ratio directly affected the color, light transmission and transparency of resulting blend films.

6.4.2.7 Protein patterns

Protein patterns determined under both non-reducing and reducing conditions of blend films based on CG and MPI at different ratios are depicted in Figure 24A and 24B, respectively. Proteins with MW of ~117 and ~97 kDa were found as the major proteins in CG film. CG film had the similar protein pattern under reducing and non-reducing condition. Additionally, the similar protein pattern was observed between CG films and CG (Figures 23 and 24). The result suggested that bondings involved in CG films network were mainly weak bonds, e.g., hydrogen bond, etc. Also, there was no disulfide bond in CG films. Difference in protein pattern was observed in blend films based on CG and MPI with different ratios. In general, films with higher amount of MPI had the pattern more similar to those of MPI films. When protein patterns of CG/MPI blend films and MPI films under reducing and non-reducing condition were compared, slight different patterns were observed. Under non-reducing condition (Figure 24A), MPI films contained major two bands of MW about 65 and 80 kDa. Moreover, protein bands with MW of 90, 38, and 26 were also found in blend films and MPI films. When determined under reducing condition (Figure 24B), protein band with MW of 24 kDa with the concomitant decrease in the intensity of protein band with MW of 90 kDa were observed. The result suggested the presence of disulfide bonds in MPI films as well as CG/MPI blend films. Deniver *et al*. (2009) found the gelatin fractions became hardly visible, while the SPI ratio increased as the result of a dilution effect. Thus, disulfide bonds might be involved in blend film network stabilization to some degree.

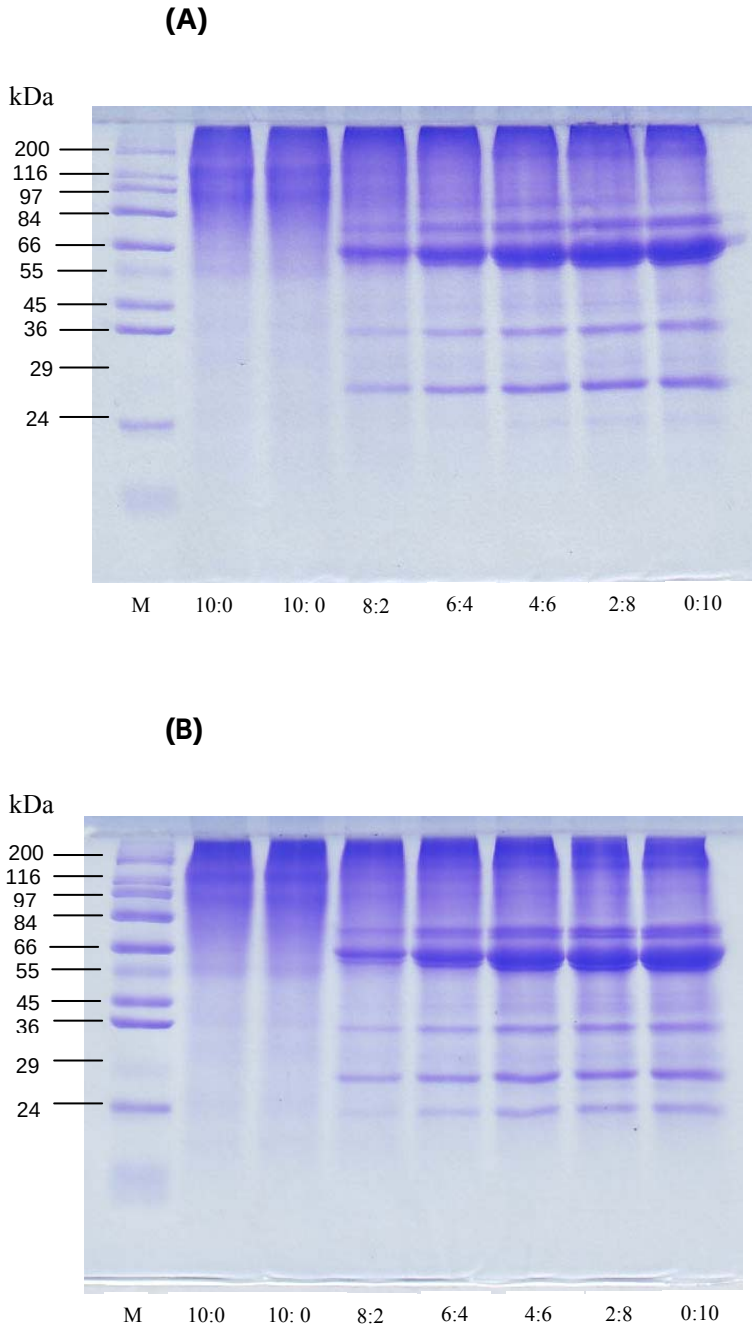


Figure 24. Protein pattern of blend films based on CG and MPI under non-reducing (A) and reducing (B) conditions. M: protein marker. Numbers denote CG/MPI ratios (w/w).

6.4.3 Characteristics of film prepared from selected CG/MPI ratio

Blend film based on CG and MPI at a ratio of 6:4, which showed the high mechanical properties, was characterized, in comparison with CG films prepared at both pH 7 and 11 as well as MPI films.

6.4.3.1 FTIR spectroscopy

FTIR spectra of CG films (prepared at pH 7 and 11), CG/MPI blend film and MPI film are shown in Figure 25. All films exhibited the major bands at 1632 cm^{-1} (amide-I, representing C=O stretching/hydrogen bonding coupled with COO), 1536 cm^{-1} (amide-II, arising from bending vibration of N-H groups and stretching vibrations of C-N groups) and 1236 cm^{-1} (amide-III, representing the vibrations in plane of C-N and N-H groups of bound amide or vibrations of CH_2 groups of glycine) (Aewsiri *et al*., 2009; Hoque *et al*., 2010a, 2010b, 2011; Muyonga, *et al*., 2004a). Pranoto *et al.* (2007) also reported that amide-I, amide-II and amide-III peaks were found in gelatin film at the wavenumbers of 1656, 1550 and 1240 cm^{-1}, respectively. The peak situated around 1032 cm^{-1} might be related to the interactions arising between plasticizer (OH group of glycerol) and film structure (Hoque *et al*., 2011a, 2011b, 2011c; Bergo and Sobral, 2007). Generally, similar spectra were obtained between all gelatin films in the range of 1800-700 cm^{-1}, covering amide-I, amide-II and amide-III. Furthermore, amide-A peak was found at 3277 cm^{-1}, representing NH-stretching coupled with hydrogen bonding. Amide-B peak at 2928 cm^{-1}, representing CH stretching and NH_3^+, was also observed in the spectra (Hoque *et al*., 2011a; Muyonga *et al*., 2004a).

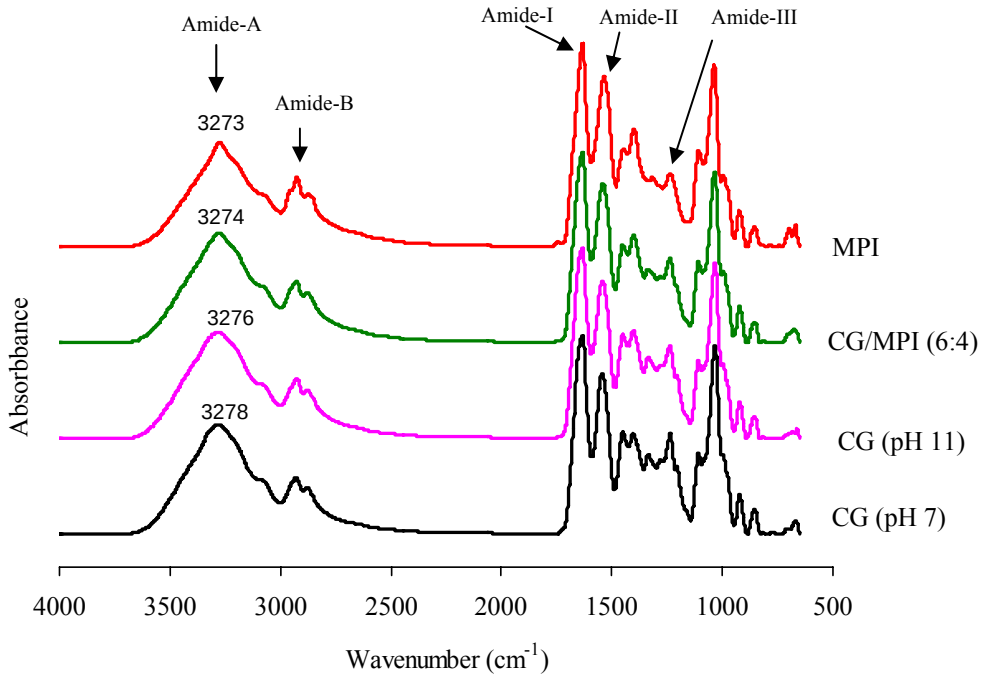


Figure 25. FTIR spectra of films from CG, MPI and CG/MPI (6:4) blend.

CG films prepared at pH 7 and 11 showed slight differences in spectra for amide-A and amide-B. The wavenumbers of amide-A peak shifted from 3278 for CG film (pH 7) to 3276 for CG film (pH 11). The amplitude of amide-A peak at these corresponding wavenumbers was slightly decreased from 0.34 for CG film (pH 7) to 0.32 for CG film (pH 11). Alkaline pH might provoke unfolding of protein, which favored intermolecular protein interaction during film formation. Denavi *et al*. (2009) reported that the extremely high pH (10.5) used for dissolving the SPI proteins for film formation might induce a certain degree of soybean-protein unfolding, thus favoring the interaction between protein-protein and/or plasticizers. On the other hand, for amide-A peak, CG/MPI composite film and MPI film had the lower wavenumber, 3273 and 3271 cm^{-1}, respectively, compared with that of CG film prepared at the same pH (3276 cm^{-1}). The absorbance at these corresponding wavenumbers was also decreased from 0.34 of CG film (pH 11) to 0.31 and 0.29, for CG/MPI (6:4) film and MPI film, respectively. The shift to lower wavenumber and lower amplitude of the amide-A peak were found in CG/MPI blend film, compared with CG film. These results suggested that interaction between gelatin and MPI took place in the blend

film matrix. Typically, the decrease in vibrational wavenumber and broadening of the OH and NH vibration bands could be indicative of a hydrogen bonding interaction between polymer molecules in the film (Xie *et al*., 2006). Cao *et al*. (2007) reported that interactions between the amino and carboxyl groups of SPI and gelatin determined mechanical properties of composite films and hydrogen bonding was mainly responsible for those interactions. Denavi *et al*. (2009) observed stronger interaction between C=O bonds in the blend films prepared from different ratios of SPI and gelatin. Hoque *et al*. (2011) also found the decrease in peak amplitudes in gelatin film incorporated with star anise extracts caused by protein cross-linking. Thus, interaction between proteins of all films can be confirmed by FTIR spectra.

6.4.3.2 Differential scanning calorimetry (DSC)

CG films prepared at pH 7 and 11, CG/MPI (6:4, pH 11) blend film and MPI film after drying under P_2O_5 were subjected to DSC analysis. Thermograms of those film samples and their transition temperatures and enthalpy are shown in Figure 26 and Table 29, respectively. Thermograms of CG films prepared at pH 7 and 11 exhibited glass transition at temperature (T_g) of 45.65 °C and 33.70 °C, respectively, followed by endothermic peak at the peak temperature (T_{max}) of 78.37 and 77.70 °C, respectively. The glass transition is generally associated with molecular segmental motion of disordered (amorphous) structure, while the endothermic peak transition in protein film involves disruption of ordered or aggregated structure, which was stabilized by various protein interactions (Jongiareonrak *et al*., 2006b; Tang *et al.*, 2009). For gelatin, the observed endothermic peak more likely associated with the helix-coil transition of gelatin (Rahman *et al*., 2008). Similar results were reported by Arvanitoyannis *et al*. (1997). The result suggested that gelatin molecules could undergo partial renaturation during film formation process. From the result, CG film at pH 11 had lower T_{max} and enthalpy (ΔH) than did that at pH 7. The lower interaction among the gelatin strands resulted in the lower T_{max} and ΔH of resulting film (Hoque *et al*., 2010). Moreover, the lower ΔH of CG film at pH 11 indicated that lesser portion of ordered structure formed in the film, plausibly owing to the more charged gelatin molecules which could lower the interaction of gelatin. With the lower ordered structure in CG film at pH 11, gelatin molecules in this film could

move with more ease, resulting in lower T_g, thereby lowering TS but yielding higher EAB (Table 26), as compared to the CG film at pH 7.

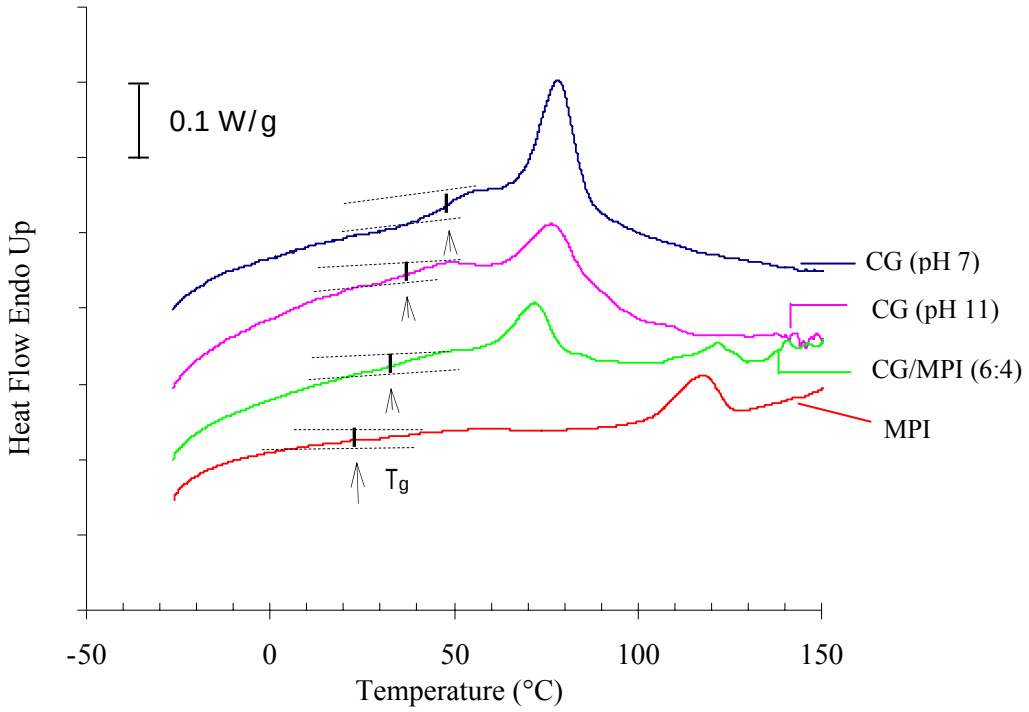


Figure 26. DSC thermograms of films from CG, MPI and CG/MPI (6:4) blend.

For MPI film, its thermogram showed lower and broad T_g at about 22.83 °C and endothermic peak with T_{max} of 117.70 °C and ΔH of 4.18 J/g. The lower T_g of MPI film might be due to the more molecular flexibility and smaller molecular size of MPI, compared to gelatin film. However, MPI film had higher T_{max} than CG film, mostly attributed to the different molecular structure, which resulted in different molecular arrangement and interactions. Tang *et al*. (2009) found an endothermic peak at 135-140 °C for mungbean protein isolate film, caused by the dissociation of globular protein cross-linking in the film matrix. The interaction of globular protein in MPI film would take place with higher amount of hydrophobic interaction between protein molecules, compared to that in gelatin film. Furthermore, disulfide bond was present in MPI films, resulting in higher thermal stability (i.e., higher T_{max}). In case of CG/MPI blend film at pH 11, the thermogram obviously showed two separated

endothermic peaks at the T_{max} of 72.03 and 118.56 °C. This suggested that two different ordered structures coexisted in the blend film matrix, which was mostly related to those separately formed by gelatin for the lower T_{max} and MPI for the higher T_{max}. Moreover, the smaller endothermic peaks in the blend film, compared to those of single protein films, were simply due to the dilution effect. Well separated endothermic peaks observed in CG/MPI blend film suggested partial immiscibility of CG and MPI molecules due to their distinct chemical structures. However, the two components would remain intimately mixed with small phase domain dimensions in the presence of interfacial interaction as evidenced from FTIR result. Thermogram of this blend film showed one broad T_g at approximately 30.08 °C rather than two T_g; this was possibly due to the polydisperse of molecular size of proteins and in part to partial miscibility of CG and MPI in the amorphous phase. Therefore, the mixture of gelatin and MPI showed the pronounced impact on thermal properties (transition) of the resulting films due to the molecular interaction and molecular organization especially in the ordered phase/zone in the film matrix. Based on DSC results, it was postulated that the compatible blend of CG and MPI rendered the solid film matrix, which was stabilized by coexisting two different ordered junction zones as schematically illustrated in Figure 27.

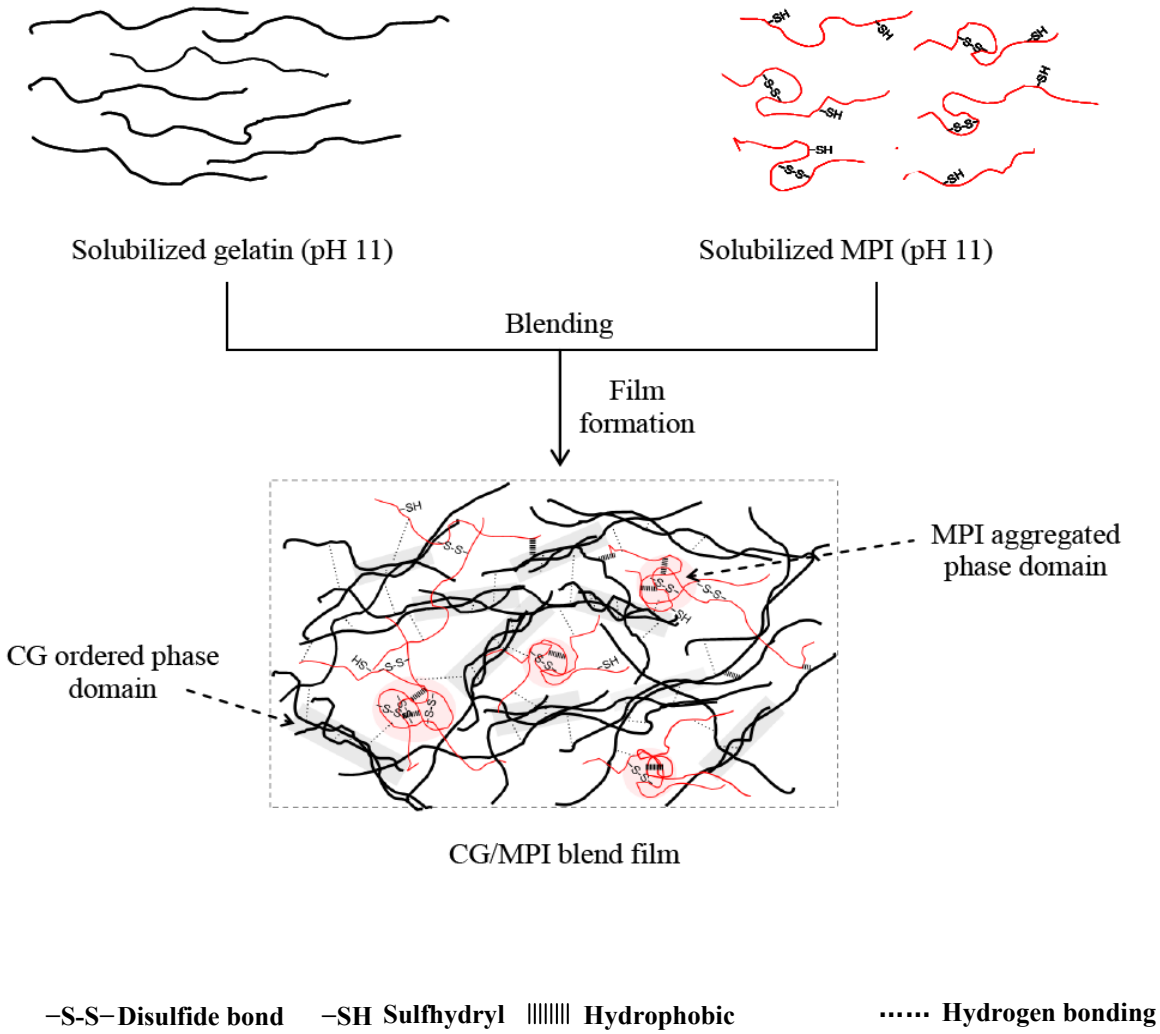


Figure 27. Proposed solid-state morphology of film from CG/MPI blend.

Table 29. Glass transition temperature (T_g), endothermic transition temperature (T_{max}), transition enthalpy (ΔH), thermal degradation temperature (T_d) and weight loss (Δw) of CG, MPI and CG/MPI (6:4) blend films.

CG: MPI †	Endothermic transition			Δ_1		Δ_2		Δ_3		
	T_g (°C)	T_{max} (°C)	ΔH (J/g)	$Td_{1,\ onset}$ (°C)	ΔW_1 (%)	$Td_{2,\ onset}$ (°C)	ΔW_2 (%)	$Td_{3,\ onset}$ (°C)	ΔW_3 (%)	Residue (%)
10:0, pH-7	45.65	78.37	11.34	45.40	2.41	161.85	33.97	301.28	45.39	18.23
10:0, pH-11	33.70	77.70	8.93	47.97	2.81	164.88	38.56	303.40	37.53	21.10
6:4 , pH-11	30.08	72.03	4.56	50.19	1.84	188.79	34.28	310.28	43.92	19.96
		118.56	1.95							
0:10, pH-11	22.83	117.70	4.18	52.94	1.98	174.42	32.63	296.38	44.45	20.94

†CG: cuttlefish skin gelatin; MPI: mungbean protein isolate. Δ_1, Δ_2, and Δ_3 denote the first, second and third stage weight loss, respectively, of film.

6.4.3.3 Thermo-gravimetric analysis (TGA)

TGA thermograms revealing thermal degradation behavior of all films are shown in Figure 28 and their degradation temperatures and weight loss are presented in Table 29. Three main stages of weight loss were observed for all films. The first stage weight loss (Δw_1= 1.98 – 2.81%) was observed over the temperature (Td_1) ranging from 45.40 to 52.94 °C up to approximately 150 °C, possibly associated with the loss of free and bound water adsorbed in the film. The similar result was found in cuttlefish skin gelatin film (Hoque *et al*., 2011a; 2011b, 2011c), porcine plasma protein film added with different cross-linking agents (Nuthong *et al*., 2009) and collagen hydrolysate film plasticizing with glycerol and poly(ethylene glycols) (Langmaier *et al*., 2008). The second stage weight loss (Δw_2= 32.63 – 38.56%) appeared at the onset temperature of 161.85 – 188.79 °C (T_{d2}), depending on the film samples. This was most likely due to the degradation or decomposition of lower molecular weight protein fractions and glycerol compounds. For the third stage of weight loss (Δw_3= 37.53 – 45.39%), T_{d3} of 296.38 - 310.28 °C were observed for all films but varied with the protein used for film formation. This was possibly due to decomposition of higher interacted protein fractions.

The results suggested that CG/MPI blend films and MPI film showed higher heat resistance than CG films, regardless of pH used. CG films prepared at pH 7 and 11 showed almost similar heat stability. However, both CG films had lower T_{d2} (161.85 – 164.88 °C), compared with CG/MPI blend film (T_{d2} = 188.79 °C) and MPI film (T_{d2} = 174.42 °C). Among all films, the CG/MPI blend film had the highest degradation/ decomposition temperature (T_{d2} and T_{d3}). The interaction between CG and MPI as well as the coexisting ordered structures mostly yielded the stronger film network, leading to higher heat resistance of the resulting blend film. Higher amount of hydrophobic interaction between proteins in MPI might contribute to heat resistance of their films. Hoque *et al*. (2011) also observed high heat stability of gelatin film incorporated with star anise extract, where higher amount of bonding between phenolic compounds of star anise extracts and gelatin molecules yielded stronger film network with heat resistance. Additionally, all films had residual mass (representing char content) at 600 °C in the range of 18.23 – 21.10%. Slight difference in char content was most likely due to different compositions of proteins

obtained from different sources and preparation processes. Thus, thermal degradation stability of CG/MPI blend film could be improved in comparison with the film containing single protein.

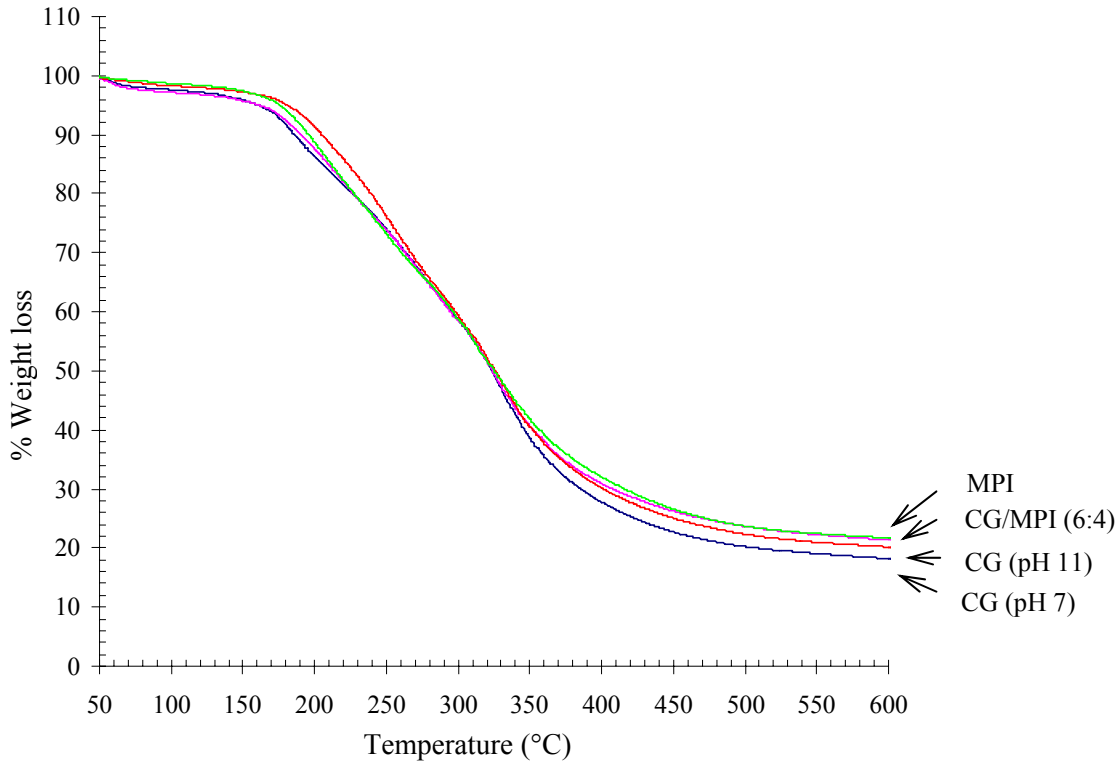


Figure 28. TGA thermogram of films from CG, MPI and CG/MPI (6:4) blend.

6.4.3.4 Microstructure

SEM micrographs of the surface and freeze-fractured cross-section of CG films prepared at pH 7 and 11, CG/MPI (6:4) blend film and MPI film are illustrated in Figure 29. For both CG films at pH 7 and 11, the smooth surface was observed. However, rough surface was obtained in CG/MPI blend film and MPI film. The roughness of surface structure was more pronounced in CG/MPI blend film than that found in MPI film. This was possibly because the arrangements of protein molecules during film formation might take place in different ways when two different types of proteins were blended. This might be associated with the coexisting two different ordered junction zones, presumably formed in the film network. This resulted in more protrusion of film structure as indicated by the increased thickness of resulting films. For cross-section, coarser cross-sectional structure was observed in CG films (gelatin at both pH), compared with those observed in CG/MPI composite film and MPI film. The small voids were found in MPI film. This was more likely related with the less interaction of rigid molecules of MPI. This could be associated with the poorer TS of blend film, compared with other films. Hoque *et al*. (2011) observed more compact cross-sectional structure of gelatin film incorporated with star anise extract, compared with the control. Thus, the microstructures of films were governed by molecular organization in the film network, which depended on types of proteins, the interaction of proteins in film matrix as well as the blend ratio of protein components used for film preparation.

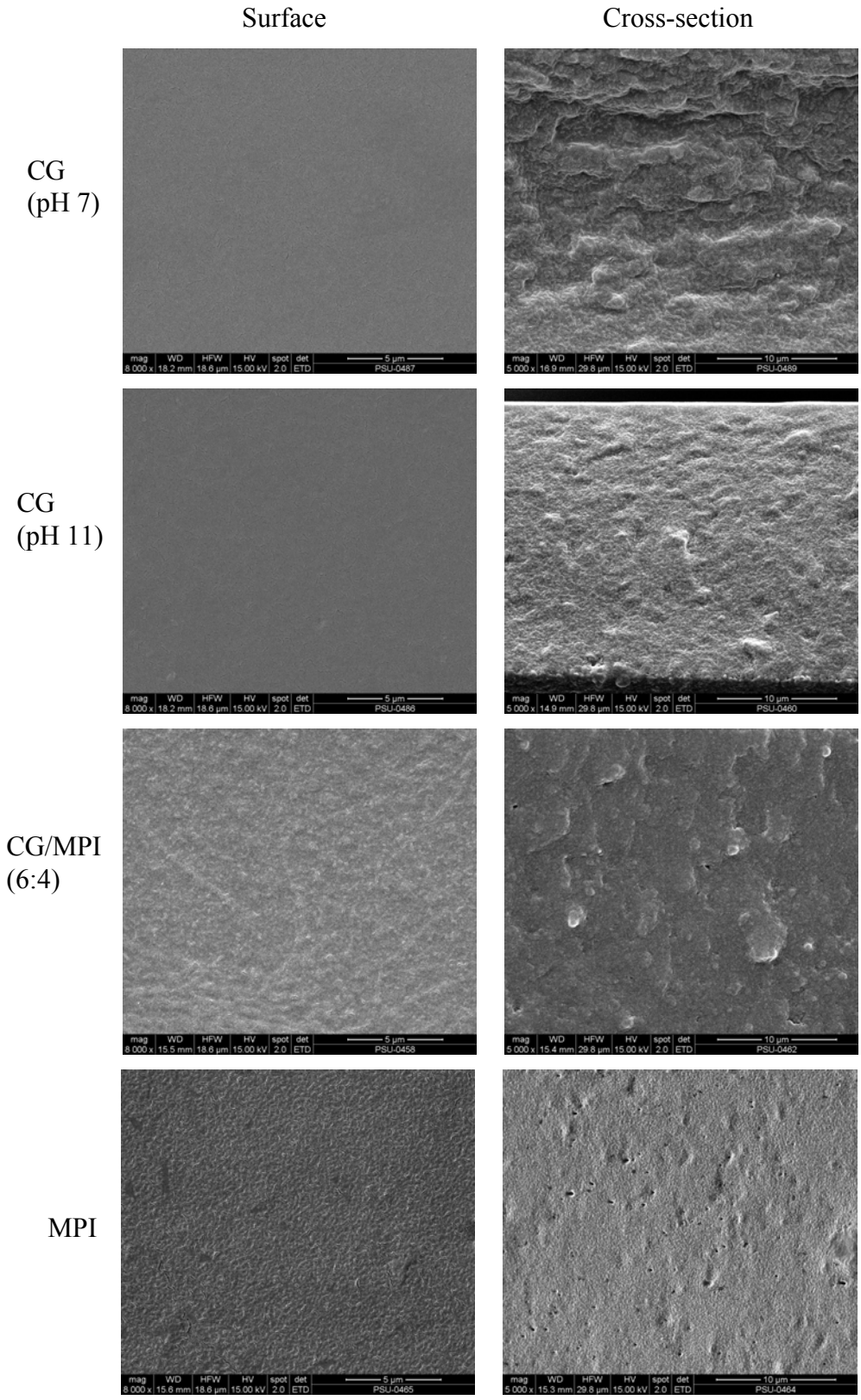


Figure 29. SEM micrographs of surface and freeze-fractured cross-section of films from CG, MPI and CG/MPI (6:4) blend.

6.5 Conclusion

Properties of CG films could be modified by blending with MPI at alkaline pH. The blend between CG and MPI was compatible at the condition studied, due to interaction between these two different proteins via hydrogen bonds, hydrophobic interaction as well as disulfide bonds. The solid film of CG/MPI blend was postulated to consist of amorphous phase and coexisting two different ordered junction zones which stabilized the film network. The CG/MPI blend films had varying properties, depending on CG/MPI ratios. CG films incorporated with MPI at appropriate amount had increased stretchability, water vapor barrier properties, water resistance and thermal stability. The improved properties of film were more likely attributed to the interaction between CG and MPI molecules as well as the specific solid-state morphology, presumably the aggregated ordered structures presented in the blend film matrix.

CHAPTER 7

CUTTLEFISH (*SEPIA PHARAONIS*) SKIN GELATIN-BASED FILM: STORAGE STABILITY AND ITS EFFECTIVENESS FOR SHELF-LIFE EXTENSION OF CHICKEN MEAT POWDER

7.1 Abstract

Stability of cuttlefish **(***Sepia pharaonis***)** ventral skin gelatin film (CG) and film incorporated with Fenton's reagent (H_2O_2 0.02 M + Fe_2SO_4 0.002 M) (FG) was evaluated after 21 days of storage at 50% relative humidity and 25 °C. No changes in mechanical property were observed for CG film but slight increase in tensile strength (TS) was found for FG film after storage ($p<0.05$). Furthermore, water vapor permeability (WVP) increased for both films ($p<0.05$), while no marked changes in film solubility and transparency values were found ($p>0.05$). DSC and TGA study revealed that molecular reorganization with higher thermal stability were formed in the film matrix during storage. When CG and FG film were used to cover chicken meat powder, the samples covered with both films had lower moisture content, peroxide values (PV) and thiobarbituric acid reactive substances (TBARS), compared with control samples (without cover) ($p<0.05$). Generally, FG film showed more preventive effect than CG film. However, both films were poorer in preventing moisture migration and retarding the color changes of chicken meat powder than low-density polyethylene (LDPE) films. Thus, gelatin-based film, especially modified with Fenton's reagent could be used as a biodegradable packaging material to prevent lipid oxidation in oil enriched foods. Nevertheless, the improvement of its water barrier property is still needed.

7.2 Introduction

Biodegradable films made from renewable biopolymers have become important environmental friendly materials for packaging (Tharanathan, 2003; Prodpran and Benjakul 2005; Hoque *et al.,* 2010). Most synthetic films are non-biodegradable and are associated with environmental pollution and serious ecological problems (Tharanathan, 2003). As a consequence, biodegradable or edible films from biopolymers have paid increasing attention. Among polymers, proteins from different sources have been used to prepare films due to their abundance and the uniqueness in film-forming ability (Ou *et al.,* 2005; Jongiareonrak *et al.,* 2006; Prodpran *et al.,* 2007). Bondings and degree of interactions involved in the stabilization of a protein film matrix are determined by the amino-acid composition and molecular weight of the proteins (Denavi *et al.,* 2009).

Gelatin has been used as a material for preparing biodegradable films with high transparency and excellent barrier characteristics against gas, organic vapor and oil, compared to synthetic films (Jongiareonrak *et al.,* 2006; Jiang *et al.,* 2007). However, gelatin film has poor water barrier property (Hoque *et al.,,* 2011a; 2011b; Jongiareonrak *et al.,* 2006; Jiang *et al.,* 2007; Denavi *et al.,* 2009) and this is the main drawback of gelatin films for their application as a packaging material (McHugh and Krochta, 1994; Gómez-Guillén *et al.,* 2009). Recently, Hoque *et al.,* (2011c) reported that Fenton's reagent (H_2O_2 0.02 M + Fe_2SO_4 0.002 M) could increase the mechanical, barrier properties and thermal stability of cuttlefish skin gelatin-based film. This film could be used as an alternative packaging for prevention of lipid oxidation in foods. However, this protein films might undergo changes during extended storage and its function can be altered.

In general, edible film and coatings from proteins can extend the shelf-life of foods by functioning as solute, gas and vapor barriers (Krochta, 1997). Artharn *et al.* (2009) found the lower thiobarbituric acid reactive substances and yellowness of dried fish powder than control (without cover), when round scad muscle protein-based films were used to cover fish powder stored at room temperature. Thus, the aims of this investigation were to study the storage stability of cuttlefish skin gelatin-based

films without and with Fenton's reagent, and to investigate the use of the films to extend the shelf-life of dried chicken meat powder.

7.3 Materials and methods

7.3.1 Chemicals

Bovine serum albumin and wide range molecular weight protein markers were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Iron (II) sulfate, glycerol, *p*-dimethylaminobenzaldehyde and tris(hydroxymethyl) aminomethane were obtained from Merck (Darmstadt, Germany). Analytical hydrogen peroxide (30%) was obtained from BDH, VWR International Ltd (Leicestershire, England). Sodium dodecyl sulfate (SDS), Coomassie Blue R-250 and *N,N,N',N'*- tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All chemicals were of analytical grade.

7.3.2 Collection and preparation of cuttlefish skin

Ventral skin of cuttlefish (*Sepia pharaonis*) was obtained from a dock in Songkhla, Thailand. Cuttlefish skin was stored in ice with a skin/ice ratio of 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University within 1 h. Upon arrival, cuttlefish skin was washed with tap water and cut into small pieces (1 x 1 cm^2), placed in polyethylene bags and stored at -20 ºC until use. Storage time was not longer than 2 months. Prior to gelatin extraction, the frozen skin was thawed using running water (25-26 ºC) until the core temperature reached 0 - 2 ºC.

7.3.3 Preparation of gelatin from cuttlefish skin

Gelatin was extracted from cuttlefish skin according to the method of Hoque *et al.* (2010). Skin was soaked in 0.05 M NaOH with a skin/solution ratio of 1:10 (w/v) with a gentle stirring at room temperature (26–28 ºC). The solution was

changed every hour to remove non-collagenous proteins for totally 6 h. Alkali treated skin was then washed with distilled water until the neutral pH of wash water was obtained. The prepared skin was subjected to bleaching in 5% H_2O_2, using a sample/solution ratio of 1:10 (w/v) for 48 h at 4 ºC. The skin treated with H_2O_2 was washed three times with 10 volumes of distilled water. Gelatin was extracted from bleached skin using distilled water at 60 ºC for 12 h, with a sample/water ratio of 1:2 (w/v). During extraction, the mixture was stirred continuously using a paddle stirrer (RW20.n, IKA LABORTECHNIK, Staufen, Germany). The extract was centrifuged at 8,000xg for 30 min at room temperature using a refrigerated centrifuge (Beckman Coulter, Avanti J-E Centrifuge, Beckman Coulter, Inc., Palo Alto, CA, USA) to remove insoluble materials. The supernatant was collected and freeze-dried (Model Duratop™ lP/Dura Dry™ lP, FTS® System, Inc., Stone Ridge, NY, USA). The dry matter was referred to as 'gelatin powder'.

7.3.4 Preparation and storage of film from gelatin incorporated without and with Fenton's reagent

Gelatin powder was dissolved in distilled water and heated at 70° C for 30 min (Hoque *et al.,* 2010). Gelatin solutions containing 3% protein were prepared. The solution was then added with glycerol at a level of 20% (based on protein content) and mixed thoroughly. The mixtures were stirred at room temperature for 1 h. The mixtures obtained were referred to as 'film-forming solution; FFS'. To prepare the film added with Fenton's reagent, gelatin solution was added with a mixture of H_2O_2 and $FeSO_4$ to yield the final concentration of 0.02 M H_2O_2 and 0.002 M $FeSO_4$, respectively (Hoque *et al.,* 2011c). Thereafter, glycerol was added and stirred as previously described.

FFS incorporated without and with Fenton's reagent were used for film casting. FFS (4 ± 0.01 g) was cast onto a rimmed silicone resin plate (5 x 5 cm^2), air-blown for 12 h at room temperature and dried in an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttalingen, Germany) at 25 ± 0.5 ºC and 50 ± 5% relative humidity (RH) for 48 h. Dried films were manually peeled-off and subjected to analyses. Films obtained from gelatin (without addition of Fenton's reagent) and

films added with Fenton's reagent were referred to as 'CG' and 'FG' films, respectively.

Both CG and FG films were stored in an environmental chamber (Binder, KBF 115 # 00-19735, D-78532, Tuttalingen, Germany) at 25 ± 0.5 ºC and 50 ± 5% relative humidity (RH). Films samples were taken at 0 and 21 days of storage for analyses.

7.3.5 Analyses

Prior to mechanical properties testing, films were conditioned for 48 h at 50 ± 5% relative humidity (RH) at 25 ± 0.5 ºC. For SEM, DSC and TGA studies, films were conditioned in a dessicator containing dried silica gel for 1 week and 2 weeks in dessicator containing P_2O_5 at room temperature (28-30 ºC) to obtain the most dehydrated films.

7.3.5.1 Film thickness

The thickness of film was measured using a digital micrometer (Mitutoyo, Model ID-C112PM, Serial No. 00320, Mituyoto Corp., Kawasaki-shi, Japan). Ten random locations around each film sample were used for thickness determination.

7.3.5.2 Mechanical properties

Tensile strength (TS) and elongation at break (EAB) were determined as described by Iwata *et al.* (2000) using the Universal Testing Machine (Lloyd Instrument, Hampshire, UK). Ten samples (2 x 5 cm^2) with the initial grip length of 3 cm were used for testing. The samples were clamped and deformed under tensile loading using a 100 N load cell 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.

7.3.5.3 Water vapor permeability (WVP)

WVP was measured using a modified ASTM (American Society for Testing and Materials 1989) method as described by Shiku *et al.* (2004). The film was sealed on an aluminum permeation cup containing dried silica gel (0% RH) with silicone vacuum grease. The cup was placed at 30 ºC in a desiccator containing the distilled water. It was then weighed at 1 h intervals for up to 8 h. Five films were used for WVP testing. WVP of the film was calculated as follows:

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

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

7.3.5.4 Film solubility

Film solubility in water was determined according to the method of Gennadios *et al.* (1998) with a slight modification. The conditioned film sample (3x2 cm^2) was weighed and placed in 50 ml-centrifuge tube containing 10 ml of distilled water with 0.1% (w/v) sodium azide. The mixture was shaken continuously at room temperature for 24 h using a shaker (Heidolph UNIMAX 1010, Schwabach, Germany). Undissolved debris film matter was determined after centrifugation at 3000xg for 10 min at 25 ºC using a centrifuge (Allegra 25R Centrifuge, Beckman Coulter, Krefeld, Germany) and drying them at 105 ºC for 24 h to obtain the dry unsolubilized film matter. The weight of solubilized dry matter was calculated by subtracting the weight of unsolubilized dry matter from the initial weight of dry matter and expressed as the percentage of total weight.

7.3.5.5 Transparency value of film

The transparency value of the film was calculated by the following equation (Han and Floros, 1997):

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

where T_{600} is the fractional transmittance at 600 nm as measured by UV–Visible spectrophotometer (model UV-160, Shimadzu, Kyoto, Japan) and x is the film thickness (mm). The greater transparency value represents the lower transparency of the films.

7.3.5.6 Differential scanning calorimetry

Thermal properties of films samples were determined using differential scanning calorimeter (DSC) (Perkin Elmer, Model DSC-7, Norwalk, CT, USA). Temperature calibration was performed using the Indium thermogram. The film samples (2–5 mg) were accurately weighed into aluminum pans, sealed, and scanned over the temperature range of -30 to 120 ºC with a heating rate of 10 ºC/min. The dry ice was used as a cooling medium and the system was equilibrated at -30 ºC for 5 min prior to the scan. The empty aluminum pan was used as a reference. The second scan was also performed in the same manner followed the quench cooling of the sample after completing the first scanning.

7.3.5.7 Thermo-gravimetric analysis (TGA)

Conditioned films were scanned using a thermogravimetric analyzer (TG A-7, Perkin Elmer, Norwalk, CT, USA) from 50 to 600 ºC at a rate of 10 ºC/min (Nuthong *et al.,* 2009). Nitrogen was used as the purge gas at a flow rate of 20 mL/min.

7.3.5.8 Microstructure

Microstructure of upper surface and freeze-fractured cross-section of the film samples were visualized using a scanning electron microscope (SEM) (Quanta400, FEI, Tokyo, Japan) at an accelerating voltage of 15 kV. Prior to visualization, the film samples were mounted on brass stub and sputtered with gold in order to make the sample conductive, and photographs were taken at 8000× magnification for surface. For cross-section, freeze-fractured films were mounted around stubs using double sided adhesive tape, coated with gold and observed at the 5000× magnification.

7.3.6 Effect of cuttlefish skin gelatin film on storage stability of dried chicken meat powder

7.3.6.1 Preparation of dried chicken meat powder

Fresh chicken meat was purchased from a local market in Hat Yai, Songkhla, Thailand. Meat was washed with cold water. The meat was then steamed for 20 min with an electric steamer (Jixing, CS-032, Guangdong, China). After cooling in air, the steamed chicken was shredded manually. Prepared sample was subjected to drying using a hot-air oven with an air velocity of 1.5 m/s at 60 °C until moisture content was less than 5%. The dried sample was powderized using a blender (Moulinex, Type AY46, Shenzhen, Guangdong, China). The chicken meat powder was screened using a mesh 35 with an aperture size of 500 μm, ASTM E11, serial number 5666533 (FRITSCH GMBH, Laborgerätebau, Industriestrasse 8, D-55743 Idar-Oberstein, Germany).

7.3.6.2 Quality changes of dried chicken meat powder covered with cuttlefish skin gelatin films during storage

Chicken meat powder (15 g) was transferred to a cylindrical glass cup with a diameter of 25 mm. The cup containing chicken meat powder was covered with gelatin-based films from cuttlefish skin without and with incorporation of Fenton's reagent and sealed with an O-ring. LDPE (CO_2, N_2 and O_2: 1.7 $x10^{-10}$, 0.1 $x10^{-10}$ and 0.4 $x10^{-10}$ m^3 mm/cm^2 s cmHg at 25 °C, 1 atm pressure, respectively) films with a thickness of 0.038 ± 0.003 mm were also used to cover the samples. Sample without film covering was used as the control. The samples were stored at 28–30 °C and were taken every 3 days for 21 days for analyses of moisture content (AOAC, 1999), peroxide value, TBARS and color.

7.3.6.2.1 Peroxide value

Peroxide value (PV) was determined as per the method of Richards and Hultin (2002) with a slight modification. Chicken meat powder (1 g) was homogenized at a speed of 13,500 rpm for 2 min in 11 ml of chloroform/methanol (2:1, v/v) using an IKA homogenizer (Selangor, Malaysia). Homogenate was then

filtered using Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England). Two milliliters of 0.5% NaCl were then added to 7 ml of the filtrate. The mixture was vortexed at a moderate speed for 30 s and then centrifuged at 3000x*g* for 3 min to separate the sample into two phases. Two milliliters of cold chloroform/methanol (2:1) were added to 3 ml of the lower phase. Twenty-five microliters of ammonium thiocyanate and 25 µl of iron (II) chloride were added to the mixture (Shantha and Decker, 1994). Reaction mixture was allowed to stand for 20 min at room temperature prior to reading the absorbance at 500 nm. A standard curve was prepared using cumene hydroperoxide at a concentration range of 0.5–2 ppm.

7.3.6.2.2 TBARS

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

7.3.6.2.3 Color

Color of chicken meat powder was determined using a CIE colorimeter (Hunter associates laboratory, Inc., Reston, VA, USA). Color of the chicken meat powder was expressed as L^*-, a^*- and b^*-values. Total difference in color (ΔE*) was calculated according to the following equation (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 corresponding color parameter of the sample and that of white standard (L^*= 93.63, a^*= -0.92 and b^*= 0.42).

7.3.7 Statistical analysis

Experiments were run in triplicate. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by Duncan's multiple range test, T-test was used for pair comparison (Steel and Torrie, 1980). Analysis was performed using the SPSS package (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA).

7.4 Results and discussion

7.4.1 Stability of cuttlefish skin gelatin films

7.4.1.1 Thickness

Thickness of CG and FG films at day 0 and 21of storage is shown in Table 30. The higher thickness was observed for FG film, compared with CG film ($p<0.05$). The result suggested that Fenton's reagent could affect the film matrix via radical mediated protein modification, in which the pretruded film matrix was developed when Fenton's reagent was added. This result confirmed that reported by Hoque *et al.* (2011c). However, no differences in thickness were observed for both films after storage for 21 days ($p>0.05$).

7.4.1.2 Mechanical properties

Mechanical properties of CG and FG films before and after storage for 21 days are shown in Table 30. FG films showed the higher TS but lower EAB, compared with the CG film (without addition of Fenton's reagent) ($p<0.05$). TS of FG film was 35.65% higher than that of CG film. 'Fenton-type' reaction is a metal-catalyzed oxidation system, where the $HO^{\bullet}$ radicals are produced when certain transition metals react with H_2O_2 (Kocha *et al.,* 1997). $HO^{\bullet}$ radical involves abstraction of the alpha-hydrogen atom from amino acid residues to form a carbon-

centered radical derivative. Two different carbon-centered amino acid radicals can react with one another to form –C–C– protein cross-linked products (Stadtman 2001). Hoque *et al.* (2011c) also found the similar results for both gelatin and partially hydrolyzed gelatin films, in which TS increased via radical-mediated protein modification induced by Fenton's reagent.

After 21 days of storage, similar mechanical properties were observed for CG film ($p>0.05$). However, FG film had increased TS and EAB after storage for 21 days ($p<0.05$). The increases in TS and EAB of films during the storage were possibly due to the increased radical-mediated aggregation, which still took place to some extent during storage. Bigi *et al.* (2002) reported that the cumulative release of gelatin from the films was remarkably low at higher degree of cross-linking induced by genipin, after 1 month of storage in physiological solution. However, Pérez-Mateos *et al.* (2009) found the decreased puncture force for the gelatin film without and with oil during storage for 30 days. Increased TS but decreased EAB were observed for fish muscle protein-based film during storage (Tongnuanchan *et al.,* 2011b, 2011c; Artharn *et al.,* 2009). Thus, modification or alteration of film matrix still occurred when Fenton's reagent was incorporated, especially as the storage time increased. Radicals generated in film might be involved in inducing the covalent cross-linking of gelatin, thereby strengthening film matrix.

Table 30. Thickness, mechanical properties, water vapor permeability, solubility and transparency values of films from cuttlefish skin gelatin without and with Fenton's reagent at day 0 and 21 of storage.

Storage time (days)	Film sample	Thickness (mm)	TS (MPa)	EAB (%)	WVP ($x10^{-10}$ g $s^{-1}.m^{-1}.Pa^{-1}$)	Film solubility (%)	Transparency values
0	CG	0.038±0.002 bA	32.45±2.49 bA	5.94±0.49 aA	1.02±0.06 aB	93.36±1.31 aA	3.37±0.03aA
	FG	0.042±0.002 aA	44.02±1.20 aB	5.04±0.20 bB	0.92±0.04 bB	71.59±1.76 bA	3.28±0.02bA
21	CG	0.038±0.002 bA	35.50±2.83 bA	6.18±0.55 aA	1.26±0.07 aA	90.58±1.55 aB	3.36±0.02aA
	FG	0.042±0.002 aA	45.84±1.44 aA	5.60±0.48 aA	1.11±0.04 bA	66.85±1.93 bB	3.29±0.03bA

Values are given as Mean ± SD (n=3).

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

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

CG: control films from gelatin (without addition of Fenton's reagent).

FG: films from gelatin added with Fenton's reagent containing 0.02 M H_2O_2 + 0.002 M $FeSO_4$.

7.4.1.3 Water vapor permeability (WVP)

WVP of films prepared from CG and FG at day 0 and 21 of storage is presented in Table 30. FG film showed the lower WVP, compared with GF film (p<0.05). The result suggested that radical-mediated cross-linking of protein molecules in film matrix might decrease the free volume and mobility of polymeric structure, thereby lowering the diffusion of water as indicated by the lower WVP.

After storage of 21 days, WVP of both films increased (p<0.05). The result suggested that the hydrophilic nature of gelatin favored interaction between gelatin molecules and water during storage. An increased hydrophilicity of film matrix contributed to the decreased water barrier property of film. Increased WVP was also observed for cod skin gelatin film with and without addition of sunflower oil after storage for 30 days (Pérez-Mateos *et al.,* 2009). However, red tilapia muscle protein film had the decrease in WVP after storage for 4 weeks (Tongnuanchan *et al.,* 2011b). Different changes in WVP between films from varying proteins might be governed by the differences in amino acid compositions and molecular weight distribution of materials used for film preparation. Bondings and degree of interactions involved in the stabilization of a protein film matrix are determined by the amino-acid composition and molecular weight of the proteins (Denavi *et al.,* 2009). Increased WVP of both films during storage negatively affected the ability to protect the foods from moisture migration.

7.4.1.4 Film solubility

Films solubility of CG and FG films at day 0 and 21 of storage is shown in Table 30. CG film showed the higher solubility than FG film. Gelatin from cuttlefish skin had high hydrophilic amino acids, thus it was soluble with ease in water (Hoque *et al.,* 2010). However, Fenton's type reaction induces the covalent cross-linking of gelatin via radical generated (Stadtman, 2001), as evidenced by decreased film solubility. Film solubility can be viewed as a measure of the water resistance and integrity of a film (Rhim *et al.,* 2000). Cross-linking markedly reduced the degree of swelling of gelatin film added with genipin (Bigi *et al.,* 2002). It was noted that the decreases in film solubility were observed for both films after storage for 21 days (p<0.05). During storage of film, interaction among the proteins

molecules still occurred to some degree. This might induce the migration of glycerol to the surface. The intermolecular rearrangement of gelatin to form rigid polymeric structure might cause a decreased solubility. The slight decrease in solubility was in accordance with the slight increases in TS and EAB of film. Thus, interaction of gelatin molecules still proceeded in film matrix to some extent during storage.

7.4.1.5 Transparency value

Generally, FG film had the lower transparency values, compared with CG film (p<0.05) (Table 30), indicating the higher transparency in the former. However, no differences in transparency values were observed for both films after the storage for 21 days (p>0.05). Transparency of cod gelatin film remained unchanged over the storage period of 30 days (Pérez-Mateos *et al.,* 2009). The result suggested that the light transmission property of films was not affected by the extended storage time.

7.4.1.6 Differential scanning calorimetry (DSC)

CG and FG films stored for 0 and 21 days were subjected to DSC analysis. DSC thermograms of both films and their transition temperatures are shown in Figure 30 and Table 31, respectively. Thermograms of all film samples showed only glass transition at temperature range of 76.8 - 87.1 °C, depending on film types and storage times. At day 0, FG films had the higher glass transition temperature (T_g) (81.5 °C) than CG film (T_g = 76.8 °C). The higher T_g found in films added with Fenton's reagent might be due to the greater interaction of protein molecules induced by radical-mediated protein modification process, which restricted the molecular mobility of gelatin in the film matrix. T_g is generally the temperature causing the onset of molecular segmental motion. The greater interaction among the gelatin strands resulted in higher T_g (Sobral and Habitante, 2001; Sobral *et al.,* 2001). Thermal stability of films was possibly affected by the presence of intermolecular interaction of proteins, such as hydrogen bonds, ionic interactions, hydrophobic–hydrophobic interactions and covalent bonds, which stabilized the film network (Barreto *et al.,* 2003). In general, the higher T_g was coincidentally attained in the films with the higher stiffness and TS (Table 30).

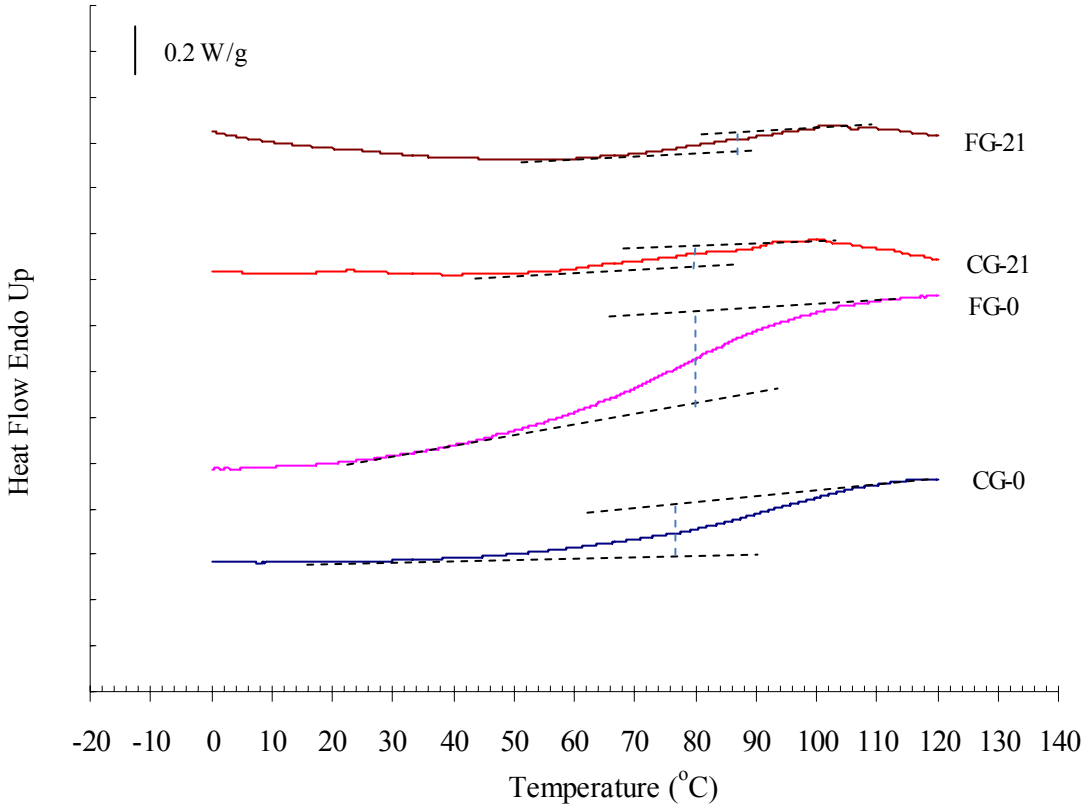


Figure 30. DSC thermogram of films from cuttlefish skin gelatin (CG) and gelatin film added with Fenton's reagent (FG) at day 0 and 21 of storage.

After 21 days of storage, the T_g of both films shifted to higher temperatures and the transition was less pronounced, compared to those of films at day 0 (Table 31). The result suggested that intermolecular rearrangements between gelatin molecules or between gelatin and glycerol might take place to some degree during extended storage time. Gelatin molecules more likely underwent intermolecular interaction to a higher extent during storage, thereby decreasing chain mobility and increasing T_g. This new molecular organization might contribute to the changes in mechanical properties of films after storage for 21 days.

For the second DSC scan, no transition was observed for both films stored for 0 and 21 days (data not shown). It was postulated that the bound water acting as plasticizer might be removed during the first heating scan. As a consequence, the interaction between gelatin molecules could be enhanced and the more rigid film network was obtained. Thus, the transition temperature of the film became too high and could not be detected in the temperature range tested.

7.4.1.7 Thermo-gravimetric analysis (TGA)

Thermal degradation behavior of CG and FG films at 0 and 21 days of storage was studied. TGA traces with their corresponding degradation temperatures (T_d) and weight loss (Δw) are shown in Figure 31 and Table 31, respectively. At day 0, three main stages of weight loss were observed for both films. The first stage weight loss (Δw_1= 4.54 and 7.02%) was observed approximately at temperature (Td_1) of 61.37 and 66.27 °C, for CG and FG films, respectively. This was plausibly associated with the loss of free water adsorbed in the film. The similar results were found in porcine plasma protein film added with different cross-linking agents (Nuthong *et al.,* 2009) and in collagen hydrolysate film plasticized with glycerol and poly(ethylene glycols) (Langmaier *et al.,* 2008). The second stage weight loss (Δw_2= 19.06 and 16.39%) appeared at Td_2 of 230.73 and 245.80 °C for CG and FC films, respectively. This was most likely due to the loss of lower molecular weight protein fractions as well as glycerol. For the third stage weight loss (Δw_3= 50.54 and 53.56%), Td_3 of 318.17 and 326.76 °C were observed for CG and FG films, respectively. The results revealed that FG film showed higher heat resistance than CG film. The incorporation of Fenton's reagent yielded the stronger film network, leading to the higher heat

resistance of the resulting film. Similar thermal degradation of gelatin films has been previously reported. Bigi *et al.* (2002) reported that the genipin induced cross-linking enhanced the thermal stability of gelatin film, as shown by the slight increase in denaturation temperature (T_d). Chiou *et al.* (2009) reported that gelatin from cold-cast fish began to degrade at 270 – 271 °C. Chongjun *et al.* (2010) reported the thermal degradation of cast gelatin film appeared at initial temperature of 263 °C with a mass loss of about 58%, and the degradation temperature of the film increased to 300 °C when the gelatin was cross-linked with cerium (III) nitrate hexahydrate. Hoque *et al.* (2011a) showed that T_d of major protein component in films prepared from cuttlefish gelatin hydrolysate (249.5 – 255.8 °C) was lower than those from gelatin without hydrolysis (271.4 – 290.2 °C).

After storage for 21 days, both CG and FG films had the higher Td_1, Td_2 and Td_3 with the coincidentally lower Δw_1 than those of films at day 0. However, slightly higher Δw_2, Δw_3 and lower residue mass (representing char content) were observed for both films after storage. Upon storage, the higher degradation temperature of resulting film was coincidental with the higher T_g. Higher heat stability of both films was more likely attributed to increased intermolecular interaction during storage. Higher T_d was also observed for tilapia (*Oreochromis niloticus*) muscle protein isolate and unwashed mince films after storage for 40 days (Tongnuanchan *et al.*, 2011c). Therefore, the changes in molecular arrangements of film matrix during storage affected thermal property of film, regardless of Fenton's reagent incorporated.

Table 31. Glass transition temperature (T_g), thermal degradation temperature (T_d) and weight loss (Δw) of films from cuttlefish skin gelatin without and with Fenton's reagent at day 0 and 21 of storage

Storage Time (days)	Film sample	T_g (°C)	Δ_1		Δ_2		Δ_3		Residue (%)
			$Td_{1,onset}$ (°C)	Δw_1 (%)	$Td_{2,onset}$ (°C)	Δw_2 (%)	$Td_{3,onset}$ (°C)	Δw_3 (%)	
0	CG	76.83	61.37	4.54	230.73	19.06	318.17	50.54	25.86
	FG	81.57	66.27	7.02	245.80	16.39	326.76	53.56	23.03
21	CG	81.21	72.01	1.87	238.13	21.18	324.26	53.97	22.98
	FG	87.16	73.65	2.05	244.73	21.05	328.97	52.91	23.99

CG: control films from gelatin (without addition of Fenton's reagent).

FG: films from gelatin added with Fenton's reagent containing 0.02 M H_2O_2 + 0.002 M $FeSO_4$.

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

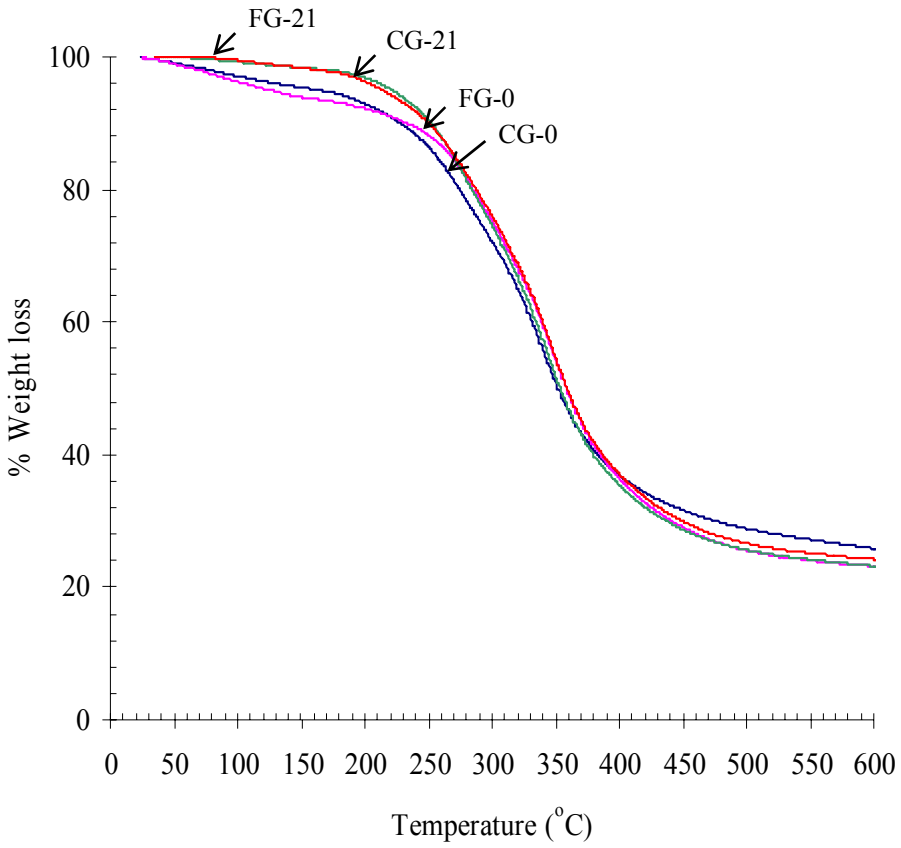


Figure 31. Thermo-gravimetric curves of films from cuttlefish skin gelatin (CG) and gelatin film added with Fenton's reagent (FG) at day 0 and 21 of storage.

7.4.1.8 Microstructure

SEM micrographs of the surface and freeze-fractured cross-section of CG and FG films at 0 and 21 days of storage are illustrated in Figure 32. At day 0, smooth surface was observed for both films. After 21 days of storage, no obvious changes were found on the surface of both films. For cross-section, the rough cross-sectional structure was observed in CG film, whereas FG film samples showed the compact/coarser structure. After storage for 21 days, the crack was formed throughout the film. The fracture was more pronounced in FG films. Those cracks in the film matrix could allow water vapors to migrate through the fracture, as indicated by increased WVP of both films after 21 days of storage. The significant decrease in moisture content and intensive cross-linking between proteins molecules possibly led to the presence of non-uniform shrinkage of the internal network structure. This resulted in the formation of higher micro-crack in the film matrix. Those cracks exhibited the detrimental effect on the water barrier property of gelatin film during storage. The increase in crack with higher gap was also found in red tilapia muscle protein isolate and unwashed mince films after storage of 40 days (Tongnuanchan *et al.*, 2011c).

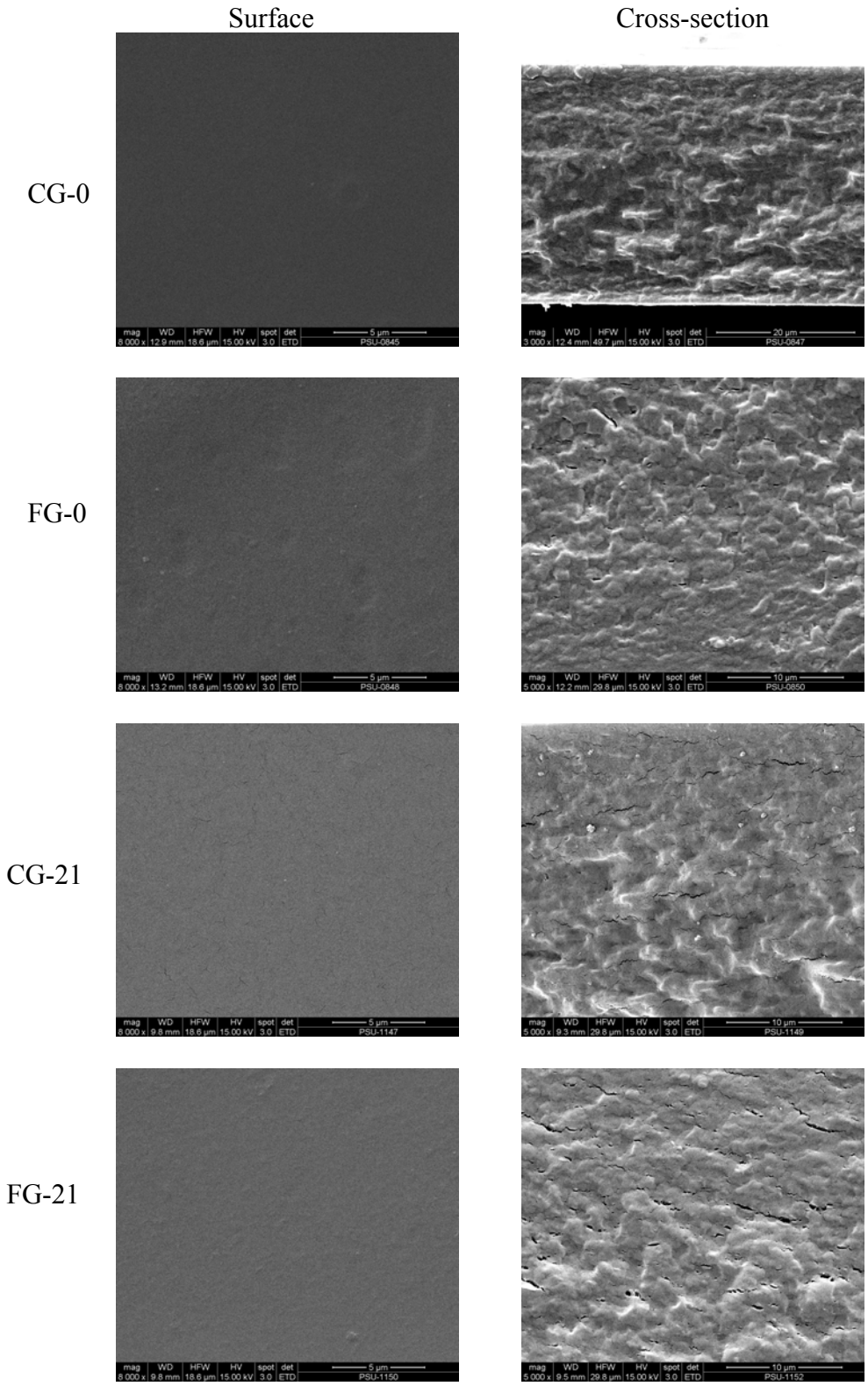


Figure 32. Morphology of films from cuttlefish skin gelatin (CG) and gelatin film added with Fenton's reagent (FG) at day 0 and 21 of storage. Magnification: x 8000 and x 5000 for surface and cross-section, respectively.

7.4.2 Effects of cuttlefish skin gelatin films on quality changes of dried chicken meat powder during storage

7.4.2.1 Moisture content of dried chicken meat powder

Moisture contents of dried chicken meat powder without cover (control) and covered with CG and FG films in comparison with those of samples covered with low density polyethylene (LDPE) films during storage of 21 days at 28-30 ºC are shown in Figure 33. In general, moisture content of dried chicken meat powder uncovered and covered with CG and FG films increased continuously during 21 days of storage ($p< 0.05$). However, the highest increase in moisture content of dried chicken meat powder was observed from the uncovered samples, especially during the first 12 days of storage ($p<0.05$). The sample covered with LDPE films had much lower moisture content than other samples during the storage ($p< 0.05$). Dried chicken meat powder was able to bind water molecules via specific hydrophilic domains, such as carboxylic, amino and hydroxyl residues of proteins (D'Arcy and Watt, 1981). The higher moisture diffusion from the environment through the packaging material increases the moisture content of packed sample. Additionally, the micro-cracks formed in CG and FG films (Figure 32) might favor the migration of water vapor into chicken meat powder. The result suggested that gelatin film possessed poor water barrier property, mainly due to high amount of hydrophilic amino acids with negligible or no sulfur containing amino acids (Hoque *et al.,* 2010; Jongiareonrak *et al.,* 2006; Jiang *et al.,* 2007; Denavi *et al.,* 2009). Artharn *et al.* (2009) also reported that moisture content of dried fish powder packed with round scad protein-based film and chitosan film containing 25 % palm oil was higher than that of those packed with HDPE film ($p<0.05$) during storage of 21 days. Thus, the gelatin and modified gelatin film able to prevent moisture absorption by the products to some extent but their preventive effect was lower than LDPE films.

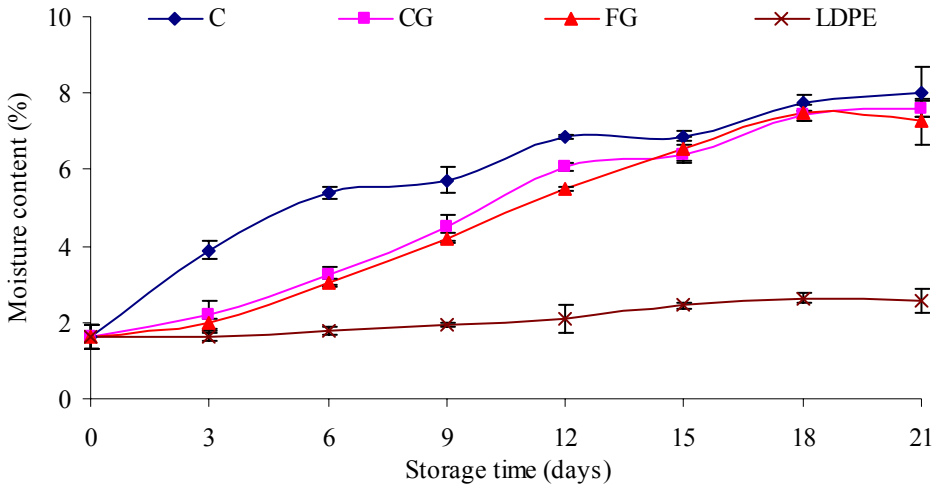


Figure 33. Changes in moisture content of dried chicken meat powder uncovered and covered with different films during storage of 21 days. C: Uncovered; CG: cuttlefish skin gelatin film; FG: gelatin film added with Fenton's reagent; LDPE: low density polyethylene. Bars represent the standard deviation (n=3).

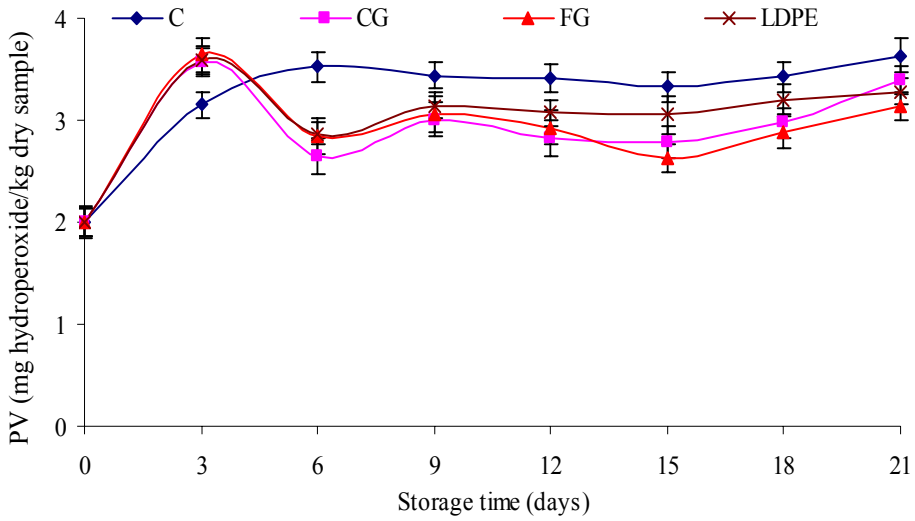
7.4.2.2 Lipid oxidation of dried chicken meat powder

Lipid oxidation of dried chicken meat powder uncovered (control) and covered with CG and FG films in comparison with that of samples covered with LDPE films during storage of 21 days was monitored by measuring PV and TBARS (Figure 34A and 34B, respectively). PV value of chicken meat powder samples uncovered and covered with all films increased at day 3 of storage ($p<0.05$). Thereafter, the decrease was found in all samples at day 6 ($p<0.05$), except the uncovered sample. The decrease in PV was more likely caused by decomposition of hydroperoxide formed. In general, the highest PV was found in uncovered samples during 6-21 days of storage ($p<0.05$). No marked changes in PV were observed for sample covered with all films during storage, but the values were slightly different between samples. Nevertheless, sample covered with FG film tended to have the lowest PV, followed by CG film, suggesting the prevention of oxidation by the FG film. Hydrophilic nature of gelatin can successfully prevent the hydrophobic oxygen gas permeation into the products, thus reducing the oxidation catalytic process.

Jongiareonrak *et al.* 2006) and Jiang *et al.* (2007) also reported that gelatin film has excellent barrier characteristics against gas, compared to synthetic films. Thus, gelatin film, especially gelatin film incorporated with Fenton's reagent, could retard the lipid oxidation of dried chicken meat powder during extended storage time.

TBARS of dried chicken meat powder uncovered (control) and covered with different films during storage of 21 days at 28-30 ºC are presented in Figure 3C. Similar TBARS values of chicken meat powder samples uncovered and covered with all films were observed within the first 6 days of storage ($p>0.05$). Subsequently, the gradual increase in TBARS was observed for all samples up to 21 days of storage ($p<0.05$). The sample without cover showed the highest TBARS value than those covered with all films up to 21 days ($p<0.05$). It was noted that TBARS values of sample covered with FG and LDPE were similar throughout the storage of 21 days. However, sample covered with CG film had the higher TBARS value than others during 15-18 days of storage ($p<0.05$). The result suggested that FG film, a radical induced modified gelatin film, had higher efficiency to retard the lipid oxidation than CG films. Protein-based films have impressive oxygen and carbon dioxide barrier properties in low relative humidity condition compared to synthetic films (Limpan *et al.,* 2010; Shiku *et al.,* 2003). Therefore, the protein-based film can be used as the packaging material to retard rancidity of foods and also can be served as alternative material for chemically synthesized polymeric films.

A



B

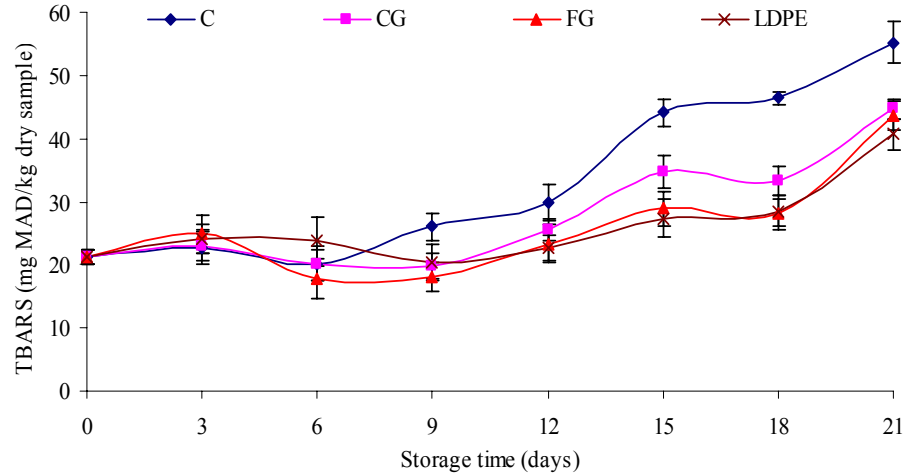


Figure 34. Changes in PV (A) and TBARS (B) of dried chicken meat powder uncovered and covered with different films during storage of 21 days. C: Uncovered; CG: cuttlefish skin gelatin film; FG: gelatin film added with Fenton's reagent; LDPE: low density polyethylene. Bars represent the standard deviation (n=3).

7.4.2.3 Color of dried chicken meat powder

L*, a*, b* and ΔE*-values of dried chicken meat powder uncovered (control) and covered with different films during storage of 21 days are shown in Figure 35. Generally, continuous changes in color values were observed for all samples during storage. The uncovered dried chicken meat powder and powder covered with CG and FG films had the increase in L*-value but decrease in a*-, b*- and ΔE*-values during the extended storage of 21 days ($p< 0.05$). The uncovered sample had the highest L*- value and the lowest a*-, b*- and ΔE*-values during storage time. Highest moisture content in the uncovered sample might contribute to light scattering, leading to increased lightness. When comparing the sample covered with CG and FG films, the former sample had the higher b*- and ΔE*-values than the latter ($p<0.05$). Generally, chicken meat powder covered with LDPE films had the constant values for L*-, a*-, b*- and ΔE*-values during 21 days of storage. The result suggested that the poorer water barrier property of CG and FG films was more likely associated with the induced changes in color of dried chicken meat powder. Higher moisture content might favor the movement of reactants for discoloration reaction, especially the decrease in a*- values (redness) and b*- values (yellowness). Pigment oxidation may catalyze lipid oxidation, and free radicals produced during oxidation may oxidize the iron atoms or denature the myoglobin molecules, negatively changing the color of the products (Selani *et al.,* 2011). Thus, lipid oxidation products, especially in the control, in the presence of high moisture content, might destruct heme pigments. This resulted in decreased a*- and b*-values during the storage. Seydim *et al*. 2006) reported that the decreased redness in ground ostrich meat was due to myoglobin oxidation. Furthermore, Artharn *et al.* (2009) reported the changes in color of dried fish powder during storage due to Maillard reaction effect. Although CG and FG films could retard lipid oxidation of chicken meat powder to some degree, they were not able to maintain the color of the chicken powder during storage.

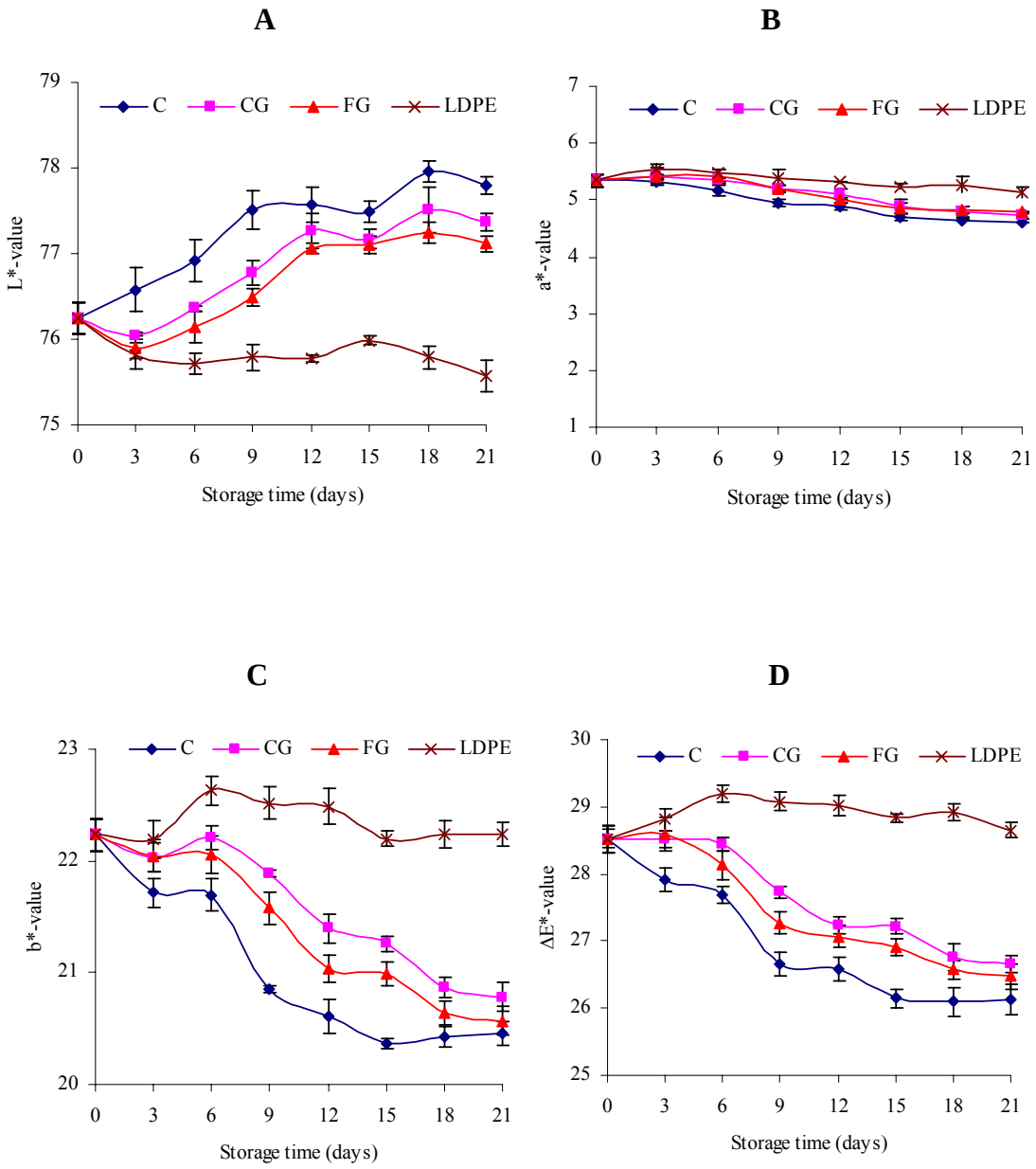


Figure 35. Changes in L*-value (A), a*-value (B), b*-value (C) and ΔE*-value (D) of dried chicken meat powder uncovered and covered with different films during storage of 21 days. C: Uncovered; CG: cuttlefish skin gelatin film; FG: gelatin film added with Fenton's reagent; LDPE: low density polyethylene. Bars represent the standard deviation (n=3).

7.5 Conclusion

Both CG and FG films underwent the molecular changes during storage of 21 days. This was associated with the increased mechanical properties but lowered water vapor barrier property. This change directly determined the protective role of films in dried chicken meat powder. FG film could prevent lipid oxidation of chicken meat powder comparably to LDPE film, but had the poorer water barrier property. Thus, the improvement of water barrier property is still needed to maximize the use of cuttlefish skin gelatin films.

CHAPTER 8

SUMMARY AND FUTURE WORKS

8.1 Summary

1. Gelatin from cuttlefish skin exhibited the good film-forming ability. Properties of gelatin films were affected by the heat treatment of their FFS. Heat treatment at appropriate temperature (70 ºC) brought about the stretching or unfolding of gelatin strands, in which higher inter-chain interaction could be formed via hydrogen bond or hydrophobic interaction and the improved mechanical property was obtained. With the excessive heating, gelatin degradation occurred and the corresponding film showed the increased EAB but lower TS.

2. Shorter gelatin molecules generated by hydrolysis yielded the film with the lower junction zone or shorter strands via weak bonds during film formation. This led to the lower mechanical properties and thermal stability of their resulting films. Increased amount of $–NH_2$ and –COOH group from hydrolysis process and -OH group of glycerol formed H-bond with water molecules, resulting in the increased WVP.

3. Incorporation of herb extracts including cinnamon, clove and star anise extracts into gelatin and partially hydrolyzed gelatin increased the TS and decreased WVP of resulting films. However, those extracts could affect the color of resulting films to some degree. Star anise extract was the most effective in improving the mechanical properties and water barrier property of the gelatin films. Extracts with oxidation showed the greater efficiency in increasing the strength of films than non-oxidized counterpart.

4. Incorporation of H_2O_2 and Fenton's reagent into gelatin and partially hydrolyzed gelatin increased TS and caused the little changes in WVP of resulting films. However, higher concentration of H_2O_2 and Fenton's reagent could affect the color of resulting films to some extent. H_2O_2 (0.02 M) and Fenton's reagent (0.02 M H_2O_2 + 0.002 M $FeSO_4$) were effective in enhancing the molecular interactions,

thereby improving the strength of their resulting films. Fenton's reagent showed greater efficiency in increasing the strength, decreasing film solubility and modifying of film property than did H_2O_2.

5. Properties of cuttlefish gelatin films could be modified by blending with mungbeen protein isolate (MPI) at alkaline pH. Gelatin films incorporated with MPI at appropriate amount had increased stretchability, water vapor barrier properties, water resistance and thermal stability.

6. Both gelatin film and film modified with Fenton's reagent (FG) underwent the molecular changes during storage of 21 days. This was associated with the increased mechanical properties but lowered WVP. FG film could prevent lipid oxidation of chicken meat powder comparably to LDPE films, but had the poorer water barrier property.

8.2 Future works

1. The improvement of mechanical properties of gelatin based film should be further investigated by blending with other proteins, especially those with high hydrophobicity.

2. The improvement of water barrier properties of gelatin based film should be further studied using different approaches.

3. The sealability of gelatin based film should be investigated for application as biodegradable food packaging materials.

4. The practical application of gelatin based film to extend the shelf-life of food should be further studied, particularly via hurdle concept.

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1. Graduate Scholarship granted by the Graduate School, Prince of Songkla University, Hat Yai, Thailand.

List of Publications and Proceedings

Publications

1. Hoque, M. S., Benjakul, S and Prodpran, T. 2010. Effect of heat treatment of film forming solution on the properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin. J. Food Eng. 96: 66-77.

2. Hoque, M. S., Benjakul, S and Prodpran, T. 2011. Effects of partial hydrolysis and plasticizer content on the properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin. Food Hydrocolloids. 25: 82-92.

3. Hoque, M. S., Benjakul, S and Prodpran, T. 2011. Properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin incorporated with cinnamon clove and start anise extract. Food Hydrocolloids. 25: 1085-1097.

4. Hoque, M. S., Benjakul, S and Prodpran, T. 2011. Effects of hydrogen peroxide and Fenton's reagent on the properties of film from cuttlefish (*Sepia*

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5. **Hoque, M. S**., Benjakul, S and Prodpran, T. 2011. Properties of blend film based on cuttlefish (*Sepia pharaonis*) skin gelatin and mungbean prortein isolate. Int. J. Biol. Macromol. (In review).
6. **Hoque, M. S.,** Benjakul, S and Prodpran, T. 2011. Cuttlefish skin (*Sepia pharaonis*) skin gelatin film: storage stability and its effectiveness for shelf-life extension of chicken meat powder. Food Bioprocess. Technol. (In review).

Proceedings

1. **Hoque, M. S**., Benjakul, S and Prodpran, T. 2009. Properties and molecular characteristics of film from cuttlefish skin gelatin as influenced by heat treatment of film forming solution. Food Innovation Asia Conference-2009. The 11th Agro-industrial conference, BITEC, Bangkok, Thailand: 18-19th June 2009. Poster presentation.
2. Prodpran, T., **Hoque, M. S.** and Benjakul, S. 2009. Properties of cuttlefish skin gelatin film as affected by heat treatment. 3rd Joint Trans-Atlantic Fisheries Technology Conference, Copenhagen, Denmark: 15-18th September 2009. Poster presentation.
3. **Hoque, M. S**., Benjakul, S and Prodpran, T. 2010. Properties and molecular characteristics of film from cuttlefish (*Sepia pharaonis*) skin gelatin as affected by partial hydrolysis and glycerol content. Food Innovation Asia Conference-2010. The 12th Agro-industrial Conference, BITEC, Bangkok, Thailand: 17 – 18th June 2010. Poster presentation.
4. **Hoque, M. S**., Benjakul, S and Prodpran, T. 2010. Mechanical and Barrier Properties of Film from Cuttlefish (*Sepia pharaonis*) Skin Gelatin as Influenced by Addition of Cinnamon, Clove and Star anise Extracts. The 7th IMT-GT UNINET and 3rd joint International PSU-UNS Conference, on Bioscience for future, Prince of Songkla University, Hat Yai, Thailand, 7-8th October, 2010. Oral presentation.

5. **Hoque, M. S**., Benjakul, S and Prodpran, T. 2010. Characteristics and Molecular Properties of Cuttlefish (*Sepia pharaonis*) Skin Gelatin Film Incorporated with Star Anise Extracts. International Conference on Agriculture and Agro-Industry 2010 (ICAAI' 2010): Food, Health and Trade: Mae Fah Luang University, Chiang Rai, Thailand, 19-20th November, 2010. Poster presentation.
6. **Hoque, M. S**., Benjakul, S and Prodpran, T. 2010. Effects of hydrogen per oxide and Fenton's reagent on the properties of film from cuttlefish (*Sepia pharaonis*) skin gelatin. "International Conference on Food Research" 2010, Putrajaya, Malaysia: 22-24th November, 2010. Oral presentation.
7. **Hoque, M. S**., Benjakul, S and Prodpran, T. 2011. Properties of cuttlefish skin gelatin/mungbean protein isolate composite film. The 3rd Biochemistry and Molecular Biology International Conference, Chiang Mai, Thailand: 6-8th April, 2011. Poster presentation.