Doctoral Dissertation

CHARACTERIZATION AND UTILIZATION OF HORSE MACKEREL SCALE AS A RAW MATERIAL OF EDIBLE FILM

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CONTENTS

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博士学位論文内容要旨
Abstract

専　攻 Major	Applied Marine Biosciences	氏　名 Name	Le Thi Minh Thuy
論文題目 Title	Characterization and utilization of fish scale as a raw material of edible film		

Marine fish species and their processed products are very important foodstuffs in Japan. During fish processing, a large amount of byproduct, including skin, scales and bone, which accounts for 50-70% of fish weight, has been discarded (Kittiphattanabawon et al., 2005). Utilization of marine waste, such as scale, by using as a raw material to extract collagen and gelatin is necessary from the viewpoints of both environmental conservation and the development of new industries.

In Chapter 2, acid-soluble collagen (ASC) was successfully extracted from the scales of lizard fish (*Saurida spp.*) and horse mackerel (*Trachurus japonicus*) from Japan and Vietnam and grey mullet (*Mugil cephalus*), flying fish (*Cypselurus melanurus*) and yellow back seabream (*Dentex tumifrons*) from Japan. ASC yields were about 0.43-1.5% (on a dry weight basis), depending on the species. The SDS–PAGE profile showed that the ASCs were type I collagens, and consisted of two different α chains, α1 and α2, as well as a β component. ASC of horse mackerel from Vietnam showed a higher imino acid level than that from Japan did. ASC denaturation temperature (T_d) ranged from 26 to 29°C, depending on fish species and imino acid contents ($p<0.01$). Maximal solubility of individual collagens was observed at pHs 1-3. Collagen solubility decreased sharply at NaCl concentrations > 0.4 M, regardless of fish type.

In chapter 3, the optimal conditions for extracting gelatin and preparing gelatin film from horse mackerel scale, such as extraction temperature and time, as well as the protein concentration of film-forming solutions (FFS) were investigated. Yields of extracted gelatin at 70, 80, and 90°C for 15 min to 3 h were 1.08-3.45%, depending on the extraction conditions. Among the various extraction times and temperatures, the film from gelatin extracted at 70°C for 1 h showed the highest tensile strength

(TS) and elongation at break (EAB). Water vapor permeability (WVP) of the gelatin film was lower than that of other fishes and mammalian species. Gelatin films showed excellent UV barrier properties at 200 nm, regardless of the preparation conditions. From the results, it is suggested that gelatin film from horse mackerel scale extracted at 70°C for 1 h can be applied to food packaging due to its lowest WVP value and excellent UV barrier property.

Properties of gelatin film incorporated with various phenolic compounds were characterized in chapter 4. TS of films increased while EAB decreased with increasing phenolic concentration. The increase in phenolic concentration lead to enhance WVP value. Gelatin films at different phenolic concentrations showed the excellent UV barrier properties. The findings of this study showed that the incorporation of phenolic compounds into gelatin films lead to higher DPPH radical scavenging activity of the film than that of the film with no additives. FTIR spectra showed that wavenumber of amide-A band of films decreased with increasing phenolic concentration. This indicates hydrogen bond between –NH group of gelatin and –OH group of phenolic compound lead the enhancement of film TS. From the results, it was elucidated that TS and the antioxidant activity of gelatin film can be improved by phenolic compounds.

Antioxidative properties of gelatin film incorporated with various phenolic compounds were characterized by using gelatin films as a packaging material to cover fresh tuna oil in chapter 5. Both peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) of fish oil increased slightly during initial 2 days (sample without air) and 4 days (sample with infusion of 2 ml air) of storage at 40ºC and relative humidity of 40%, but these values decreased significantly at the later stage of storage. Fish oil covered by flexible pouches of gelatin films incorporated with phenolic compounds always showed the lower PV and TBARS values than the samples covered by gelatin film without phenolic compound during the storage period. This result indicated that gelatin film incorporated with phenolic compounds could effectively prevent the auto-oxidation of fish oil. From the results, the addition of phenolic compounds into gelatin film was demonstrated to improve the antioxidative properties of the film. Thus, it could potentially be applied as a packaging material to prevent the lipid oxidation of food products during storage.

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CHAPTER 1

Literature review

1.1 Collagen

Collagen is the fibrous protein constituent of skin, bone, tendon and other connective tissues in land-based animals. There are 29 genetically distinct collagens present in animal tissues. Among them, type I collagen is most abundant, and has found wide application in various fields, such as food, biomedical and pharmaceutical products, and in the beauty and cosmetic industries (Sobral et al., 2001; Duan et al., 2009).

Type I collagen, the predominant genetic type in the collagen family being the major component of tendons, bones and ligaments, is a copolymer composed of two α1 and one α2 (I) chains. The composition of collagen composed of 20 amino acids (Schrieber & Gareis, 2007). This collagen type contains one-third of Glycine, no Tryptophan or Cystein, and is very low in Tyrosine and Histidine (Muyonga et al., 2004). Although some differences in amino acid composition are apparent across collagens derived from different sources, there are certain features that are common to and uniquely characteristic of all collagens. It is the only mammalian protein to contain large amounts of hydroxyproline and hydroxylysine, and the total imino acid (proline and hydroxyproline) content is higher (Balian & Bowes, 1977) than fish collagen.

Collagen molecules, consisted of three α -chains intertwined, this is the main reason to call it is collagen triple-helix. The triple-helix is approximately 300 nm in length, 1.5 nm in

diameter and the chain has a molecular weight of approximately 10^5 kDa (Papon, Leblon, & Meijer, 2007). The triple helices are stabilized by inter-chain hydrogen bonds.

1.1.1 Manufacture of collagen

The main sources of commercial collagen have been skin and bone of cows and pigs. However, concerns regarding bovine and porcine health, such as the emergence of bovine spongiform encephalopathy and foot and mouth disease, have led to a declining supplying of mammalian collagen in recent years (Kittiphattanabawon et al., 2005). Therefore, fish collagen is a realistic alternative to mammalian collagen (Nomura et al., 1996; Nagai et al., 2002).

The manufacturing procedure consists of cleaning, pretreatment, extraction of collagen, and drying.

1.1.2 Production and characterization of fish collagen

Fish collagen has received considerable attention for its potential as an ingredient in processed functional food manufacturing as well as for cosmetic, biomedical and pharmaceutical applications. Thus, extraction and functional characterization of acid- and/or pepsin-soluble collagen has also been reported for different fish species, such as trout and hake (Montero & Borderías, 1991), plaice (Montero, Alvarez, Martí & Borderías, 1995), squid (Ruiz -Capillas, Moral, Morales, & Montero, 2002), deep-sea redfish (Wang, An et al., 2008), threadfin bream (Nalinanon, Benjakul, Visessanguan, & Kishimura, 2008), walleye pollack (Yan et al., 2008), brownstripe redsnapper (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005) or unicorn leatherjacket (Ahmad, Benjakul, & Nalinanon, 2010).

Beside fish skin, scales constitute another important fish industry residue and may account for around 5% of the material contained in fish collagenous waste (Wang & Regenstein, 2009). Utilization of fish scales for collagen or gelatin extraction has been reported for sea bream and red tilapia (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003), blackdrum and sheepshead (Ogawa et al., 2004), sardine (Harada, Kuwata, & Yamamoto, 2007; Nomura, Sakai, Ishii, & Shirai, 1996), grass carp (Li et al., 2008), deep-sea redfish (Wang, An et al., 2008), Asian silver carp (Wang & Regenstein, 2009) and lizardfish (Wangtueai & Noomhorm, 2009). Unlike skins, scales are rich in Ca phosphate compounds such as hydroxyapatite and Ca carbonate. Therefore, the first step to extract collagen from fish scale is removal of Ca to increase the final yield, and purity of the collagen. These authors found that pre-treatment with EDTA solution can remove Ca substrate in fish scale and improve the yield of extracted collagen.

1.2 Gelatin

Gelatin is a protein derived by partial hydrolysis of collagen, which has wide application in food industry such as a gelling agent in cooking, a stabilizer, thickener, or texturizer in foods (Gómez-Guillén et al., 2011; Irwandi, Faridayanti, Mohamed, Hamzah, Torla & Che Man, 2009).

1.2.1 Process

Gelatin is recovered from hydrolysis of collagen. All gelatin manufacturing processes consist of three main steps: pretreatment of raw material, extraction of gelatin, and purification and drying. There are two different types of gelatin, the structure and the composition of which depend on the source of collagen and the pretreatment method used.

1.2.1.1 Pretreatment method

Two processes to pretreatment material for extracted gelatin are introduced by Johnston-Banks, 1990.

The first pretreatment method was obtained in acid pretreatment conditions to produce as type A gelatin (isoelectric point at pH 6–9). Acidic treatment is most suitable for material from pig and fish skin, which the less covalently cross-linked collagens. The raw material is soaked in 2-5% organic acids or inorganic acids such as sulfuric, hydrochloric, phosphoric acid for 10-48 h at 15-20°C. The pretreated material is washed with cold water to remove salts and excess acid. After washing, the material has a pH around 4 and is ready for gelatin extraction.

The second method obtained in alkaline pretreatment conditions to produce as type B gelatin (isoelectric point at approximately pH 5). Alkaline treatment is suitable for the more complex collagens found in bovine hides. The pretreated material is washed in alkaline solution, neutralized by washing with dilute acid (hydrochloric, sulfuric or phosphoric acid) and then washed with water to remove residual acid. The washing process takes from 5 to 48 h. After washing, the material has a pH of 5-8 and is ready for gelatin extraction (Schrieber & Gareis, 2007).

1.2.1.2 Extraction

Pretreatment of raw materials with the main purpose are reduce cross-linkages between collagen components and remove some of the impurities such as fat and salts. After pretreatment, collagen converted into soluble gelatin by extraction under water or acid conditions. All industrial processes are based on neutral conditions by extracting in hot water with the range of temperature from 55-100°C. The extraction temperature

depends on the degree of cross-linking in the raw material, the type of collagen, species, animal age, etc.

1.2.2 Physical and chemical properties of gelatin

The physical properties of gelatin depend on two main factors: (i) the amino acid composition in gelatin and (ii) the molecular weight distribution, which result mainly from processing conditions (Gómez-Guillén et al., 2002).

The imino acid composition (including proline and hydroxyproline) affected on the rheological properties of fish and mammalian gelatins. Haug et al. (2004), conducting a similar comparative study on the rheological properties of fish and mammalian gelatins, found that the main difference between fish and mammalian gelatins is the content of the imino acids, proline and hydroxyproline, which stabilize the ordered conformation when gelatin forms a gel network. The low content of proline and hydroxyproline gives fish gelatin a low gel strength, and low gelling and melting temperatures.

Ferry & Eldridge (1949) studied the influence of molecular weight on the elastic modulus of mammalian gelatin gels and showed that the elastic modulus increased with increasing of molecular weight.

1.2.3 Production and characterization of fish gelatin

The major sources were extracted gelatin from skin, bones, connective tissues, organs and some intestines of mammalian species (Johnston-Banks, 1990). The most abundant sources of gelatin are pig skin (46%), bovine hide (29.4%) and pork and cattle bones (23.1%). However, concerns regarding bovine and porcine health problems, have led to an increasing attention of fish gelatin (Gómez-Guillén, Giménez, López-Caballero & Montero, 2011) in recent years. Fish gelatin accounted for less than 1.5% of total gelatin production

in 2007, but this percentage was double that of the market data for 2002, indicating that gelatin production from alternative non-mammalian species is growing in importance (Gómez-Guillén, Pérez-Mateos et al., 2009). Furthermore, marine waste, including skin, scale and bone, which is a major byproduct of the fish-processing industry, causing waste and pollution, could provide a valuable source of gelatin (Badii & Howell, 2006). Therefore, utilization of marine waste, by using them as a raw material to extract gelatin is potential strategy to reduce environmental pollution by marine waste and increase the value of fish product.

Fish gelatin film from brownstripe red snapper and bigeye snapper skin (Jongjareonral, Benjakul, Visessanguan, Prodpran & Tanaka, 2006), blue shark skin (Limpisophon, Tanaka, Weng, Abe & Osako, 2009), Nile perch skin (Muyonga, Cole & Duodu, 2004), halibut skin (Carvalho, Sobral, Thomazine, Habitante, Giménez, Gómez-Guillén et al., 2007), and tuna skin (Gómez-Guillén, Ihl, Bifani, Silva & Montero, 2007) has been characterized.

The limitation of fish gelatin is lower imino acid content than mamalian gelatin, leads to a reduction in the gelling temperature (Norland, 1990). Marine gelatins have been known to have worse rheological properties than mammalian gelatins, particularly in the case of gelatins from cold -water fish species, such as cod, salmon or Alaska Pollack. Nevertheless, recent studies have indicated that warm-water fish species like sole, tilapia, and grass carp are well known to have better thermostability and rheological properties than the gelatins obtained from cold-water fish species as cod, salmon, or Alaska pollack (Avena-Bustillos et al., 2006, Gomez-Guillen et al., 2002).

1.2.4 Applications of gelatin

Gelatin has multiple applications in food processing. Schrieber & Gareis, 2007 divided into two application groups according to the functional properties of gelatin. The first application group has gelling ability, for example, gel strength, gelling time, setting and melting temperatures, viscosity, thickening, texturizing, and water binding. The second group relates to the surface behavior of the gelatin, for example, emulsion formation and stabilization, protective colloid function, foam formation and stabilization (such as in marshmallow), film formation, and adhesion/cohesion (Schrieber & Gareis, 2007).

In the first group, gelatin is used as the gelling binder in gummy products, wine gums etc. In the manufacture of these products gelatin is combined with sugar and glucose syrups. Incompatibility between gelatin and glucose syrup can occur (Marrs, 1982) and is a function of the concentration of glucose polymers containing more than 2 glucose units, contained in the syrup. Competition between gelatin and glucose polymers for water in low water content products can result in, at worst, precipitation of the gelatin and at best a marked loss in gelling properties or hardness of the product. It is also known that different gelatin with similar properties in water, can have very different properties in confectionery formulations.

For second group application, gelatin is an efficient foam stabilizer and this property is exploited in the manufacture of marshmallows. Different gelatins have different foam stabilizing properties and gelatin for this use needs to be carefully selected. In marshmallows the gelatin's film forming properties are also used to stabilize the foam on cooling, and because the product is normally not acidified, it has to have a much lower moisture content (>85 % solids) than gummy products (76 % solids) to avoid mould growth in storage (Ledward, 2000)

Furthermore, gelatin uses in food application as a fining application. Gelatin reacts with polyphenols (tannins) and proteins in fruit juices forming a precipitate which settles leaving a supernatant which is stable to further cloud formation with storage time. In wine, usage levels are about 1 to 3 g/hL and excess usage, which would lead to protein instability, needs to be avoided. Traditionally, low Bloom strength gelatins are used but it has been shown that high Bloom strengths are equally effective. However, from the practical point of view, the use of low Bloom Strength gelatin is cheaper and makes it easier to mix the gelatin into the bulk of the cold juice before gelation can occur. In this regard, it has become common practice to treat cold grapes, during the initial crushing process, with gelatin that has been hydrolysed to the extent that it can no longer gel.

One of these important applications of fish gelatin is film-forming ability. Fish gelatin film, which is biodegradable film, has known as important eco-friendly packaging material to reduce the environmental impact of synthetic plastic materials (Nagarajan, Benjakul, Prodpran & Songtipya, 2012). Furthermore, fish gelatin films used as covering foodstuffs have capacity to protect food against drying, light and oxygen (Gómez-Guillén, Pérez-Mateoz, Gómez-Estaca, López-Caballero, Giménez & Montero, 2008).

1.3 Edible film

1.3.1 General edible film

Edible films are thin layer of material which can be consumed and provides a barrier to moisture, oxygen and solute movement for the food. The material can be a complete food coating or can be disposed as a continuous layer between food components (Guilbert, 1986). Edible films have received considerable attention in recent years because of their advantages over synthetic films. The main advantage of edible films over

traditional synthetics is that they can be consumed with the packaged products and lead to the reduction of environmental pollution.

Edible films can be produced from materials with film forming ability. During manufacturing, film materials must be dispersed and dissolved in a solvent such as water, alcohol, mixture of water and alcohol or a mixture of other solvents. Plasticizers, antimicrobial agents, colors or flavors can be added in this process. Adjusting the pH and/or heating the solutions may be done for the specific polymer to facilitate dispersion. Film solution is then cast and dried at a desired temperature and relative humidity to obtain edible film.

1.3.2 Fish gelatin films

Fish gelatin film, which is biodegradable film, has known as important eco-friendly packaging material to reduce the environmental impact of synthetic plastic materials (Nagarajan, Benjakul, Prodpran & Songtipya, 2012). Furthermore, fish gelatin films used as covering foodstuffs have capacity to protect food against drying, light and oxygen (Gómez-Guillén, Pérez-Mateoz, Gómez-Estaca, López-Caballero, Giménez & Montero, 2008).

The physical properties of gelatin films depend on the type of raw materials extracted and on the processing conditions of gelatin manufacturing. They also depend on the physical factors used in film processing, such as temperature and drying time (Menegalli, Sobral, Roques, & Laurent, 1999), and the presence of plasticizers (Lukasik and Ludescher, 2006a, Lukasik and Ludescher, 2006b and Vanin et al., 2005) or cross-linkers (Bigi et al., 2001 and Cao et al., 2007). The plasticizers most commonly used in producing gelatin-based films are sorbitol and glycerol. Glycerol has relative with hydrophilic characteristics, it could be incorporated in the gelatin film network by establishing hydrogen bonds with

amide group and amino acid side chains of protein (Gontard, Guibert, & Cuq, 1993), lead to the reduction of tensile strength of gelatin film.

Jongjareonrak *et al.*, 2006 has been reported the effect of plasticizer type and concentration on fish-gelatin films from bigeye snapper or brownstripe red snapper-skin. The tensile strength of both these fish-skin gelatin films decreased with increasing of glycerol or sorbitol concentration, whereas the elongation at break increased with increasing plasticizer concentration from 25 to 75% of protein content. Gelatin films from the skins of the Nile perch, a warm-water fish species, have been reported to exhibit breaking and elongation values similar to those of bovine-bone gelatin (Muyonga *et al.*, 2004). Similarly, gelatin from channel catfish have also exhibited mechanical and water vapour barrier properties comparable to those of films made from a commercial mammalian gelatin (Zhang *et al.*, 2007).

Fish-gelatin films showed lower water vapor permeability (WVP) values than mammalian gelatin films and films from cold-water fish species had significantly lower of WVP value than that of films from warm-water fish species (Avena-Bustillos *et al.*, 2006). The main reason depend on the amino acid composition, since fish gelatins, especially cold-water fish gelatins, are known to contain higher amounts of hydrophobic amino acids and lower amounts of hydroxyproline. Furthermore, WVP of gelatin films from halibut-skin (Carvalho *et al.*, 2008) and tuna-skin (Gómez-Guillén *et al.*, 2007) was also reported to be lower than that of mammalian-gelatin film (Vanin et al., 2005).

1.3.3 Fish gelatin film and antioxidative compounds

To improve mechanical properties and antioxidative activity of gelatin film, some researchers added antioxidative compounds into gelatin film network. Antioxidative packaging by incorporation of antioxidative compounds and gelatin is an innovative

packaging, which has some functions, such as delayed lipid oxidation, inhibited microbial growth and controlled respiration rate (Ahvenainen, 2003).

Jongjareonrak, Benjakul, Visessanguan, and Tanaka (2008) studied about antioxidative properties of gelatin films from bigeye snapper and brownstripe red snapper-skin incorporated with α-tocopherol or BHT (butylated-hydroxy-toluene). The films containing α-tocopherol at concentration of 200 ppm displayed higher radical scavenging capacity (DPPH method) than the films containing BHT at the similar concentration. The films were also used to study their preventive effect on lard oxidation. However, no differences in TBARs values were observed between lard samples covered with gelatin films with and without antioxidatives.

Antioxidative activity of an active film from silver carp skin gelatin incorporated with green tea extract at concentration of 0, 0.3, and 0.7% (w/v) in film-forming solution were determined by measuring total phenolic content, DPPH radical scavenging activity and physical properties (Wu, Chen, Ge, Miao, Li and Zang, 2013)

1.4 Objectives:

Firstly, my purpose was to utilize horse mackerel scale and other marine fish scale as a material for extract acid soluble collagen. Secondly, I studied the effect of preparation conditions such as extraction times and temperature, protein and glycerol concentration on properties of gelatin film from horse mackerel scale. Thirdly, I conducted experiment to improve antioxidative properties of gelatin film from horse mackerel scale by adding various phenolic compounds at different concentration into film forming solution. Finally, I proved antioxidative properties of gelatin film by using these films to cover fresh tuna oil to inhibit the lipid oxidation.

CHAPTER 2

Isolation and characterization of acid-soluble collagen from the scales of marine fishes from Japan and Vietnam

2.1 Introduction:

Collagen is the fibrous protein constituent of skin, bone, tendon and other connective tissues in land-based animals. There are 29 genetically distinct collagens present in animal tissues. Among them, type I collagen is the most abundant, and has found wide application in various fields, such as food, biomedical and pharmaceutical products, and in the beauty and cosmetic industries (Sobral et al., 2001; Duan et al., 2009).

Generally, the main sources of commercial collagen have been cows and pigs. However, concerns regarding bovine and porcine health, such as the emergence of bovine spongiform encephalopathy and foot and mouth disease, have led to a declining supplying of mammalian collagen in recent years (Kittiphattanabawon et al., 2005). Therefore, fish collagen is a realistic alternative to mammalian collagen (Nomura et al., 1996; Nagai et al., 2002).

Marine fish species and their processed products are very important foodstuffs in Japan. Lizard fish, horse mackerel, grey mullet, flying fish and yellowback seabream are the raw materials used to produce *surimi* in Japan (Suyama et al., 1987; Yamanaka et al., 2007). Horse mackerel is also used for preparing raw fresh *sashimi* and *sushi*, which are popular foods in Japan (Yamanaka et al., 2007). In Vietnam, marine fish also plays an important role in the development of seafood as an economic product. Lizard fish and horse mackerel are

the raw materials used to produce fish balls in Vietnam (http://www.ctu.edu.vn/colleges/aquaculture/aquafishdata. Accessed 31.01.13). However, during fish processing, a large amount of byproduct, such as skin, scales and bone, which accounts for 50-70% of fish weight, is discarded (Kittiphattanabawon et al., 2005). Utilization of marine waste, including scales, is necessary from the viewpoints of both environmental conservation and the development of new industries.

Collagen from the scales of carp (*Cyprinus carpio*) (Duan et al., 2009), spotted golden goatfish (*Parupeneus heptacanthus*) (Matmaroh et al., 2011), sardine (*Sardinops melanostictus*), red seabream (*Pagrus major*) and Japanese sea bass (*Lateolabrax japonicus*) (Nagai et al., 2004) and deep-sea redfish (*Sebastes mentella)* (Wang et al., 2008) has been isolated and characterized. From the literature, it can be predicted that collagen from different fish species differs in molecular composition and functional properties. However, no information exists on scale collagen of commercially important species in Japan and Vietnam, such as horse mackerel and lizard fish. Moreover, sea water temperature is also presumed to affect fish scale collagen properties. Jongjareonrak et al. (2005) reported the effects of environmental and body temperatures on the collagen properties of bigeye snapper skin. However, comparative studies of the effect of habitat temperature on the collagen properties of fish scales are lacking.

Thus, the objective of this study was to characterize acid-soluble collagen (ASC) from the scales of lizard fish and horse mackerel caught in Japan and Vietnam and grey mullet, flying fish, and yellowback seabream from Japan. The effects of environmental temperature on fish scale properties were also studied by analyzing differences between lizard fish and horse mackerel caught in Japan and Vietnam.

2.2 Materials and methods:

2.2.1. Preparation of fish scales

Scales of lizard fish *(Saurida spp.*), grey mullet (*M. cephalis*) and yellowback seabream (*D. tumifrons*) were collected on November, 2011 in Miyazaki Prefecture, Japan. Horse mackerel (*T. japonicus*) and flying fish (*C. melanurus*) were collected on January, 2012 in Nagasaki Prefecture and Chiba Prefecture, Japan, respectively. Scales from lizard fish and horse mackerel were also collected from a frozen seafood company on January, 2012 in Nha Trang City and Kien Giang Province, Vietnam, respectively. The scales obtained in Japan were transported to our laboratory under iced condition, and the samples in Vietnam were frozen before airfreighted under frozen condition. The scales were washed with chilled distilled water before being cut into small pieces with scissors, placed in polyethylene bags and then stored at –20°C until use. The storage time was 3 months or less.

2.2.2. Determination of protein and ash contents

Moisture, ash and protein contents in the scales of marine fishes were analyzed according to the AOAC methods (2000). A conversion factor of 5.95 was used for calculating protein content (Wang et al., 2008).

2.2.3. Extraction of collagen from fish scales

Collagen was extracted following the method of Nagai and Suzuki (2000a) with slight modifications. Fish scales were removed from the non-collagenous protein with 0.1M NaOH for 6h at a sample/NaOH solution ratio of 1:8 (w/v). The NaOH solution was changed after 3h and washed fully in cold distilled water until a neutral pH was achieved.

Demineralization of the scales was achieved by treating with 0.5M Na_2EDTA (ethylenediaminetetraacetic acid disodium salt) solution (pH 7.5) at a sample/EDTA solution ratio of 1:10 (w/v) for 24h, and then washed with cold distilled water. After pretreatment, scales were extracted with 0.5 M acetic acid at sample/ acid ratio of 1:2.5 (w/v) for 4days. The extract was centrifuged at 20,000 x *g* for 1h using a centrifuge machine (SUPREMA 21; Tomy Seiko Co., Ltd, Tokyo, Japan). The supernatant was salted out by adding NaCl to obtain a final concentration of 2.5M in the presence of 0.05M tris (hydroxymethyl) aminomethane at pH 7.0. The resulting precipitate was collected by centrifugation at 20,000 x *g* for 30min. The resultant pellet was dissolved in 0.5M acetic acid, dialyzed against 0.1M acetic acid and distilled water and then lyophilized. All of the preparation procedures were carried out at below 4°C.

2.2.4. Yield of extracted ASC

The yield of collagen was determined following the method of Wang et al (2008). The hydroxyproline content of extracted solutions and fish scale were determined by HPLC. The extract yield (YD) was calculated using the following equation:

$$\text{YD (\%)} = \frac{\text{Hydroxyproline content in extract (mg/L) x Volume of extract (L)}}{\text{Hydroxyproline content in fish scale (mg/g) x dry weight of fish scale (g)}}$$

2.2.5. Analysis of amino acid composition

Twenty milligrams of ASC was hydrolyzed in 6 M HCl at 110°C for 22h under vacuum. The hydrolysate was neutralized with 6 M and 0.6M NaOH, and filtered through a cellulose membrane filter (0.45μm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate was used for amino acid analysis using an amino acid analysis system (Prominence; Shimadzu, Kyoto,

Japan) equipped with a column (Shim-pack Amino-Li, 100mm × 6.0mm i.d.; column temperature, 39.0°C; Shimadzu) and pre-column (Shim-pack ISC-30/S0504 Li, 150mm × 4.0mm i.d.; Shimadzu). Amino acids were detected using a fluorescence detector (RF-10AXL; Shimadzu).

Tryptophan content of ASC was determined according to the method of María et al (2004) with slight modifications. Ten milligrams of ASC was dissolved in 3ml of 4M sodium hydroxide, sealed in hydrolysis tube under nitrogen, and incubated at 100ºC for 4h. The hydrolysate was neutralized with 12M HCl, diluted to 25ml with 1M sodium borate buffer (pH 9), and filtered through a cellulose membrane filter (0.45μm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate was used for tryptophan analysis using a fluorescence detector (RF-10AXL; Shimadzu).

Sulphur containing amino acid of ASC was analyzed according to the method of Gehrke et al (1987) with slight modifications. One milligram of sample was dissolved in 0.5ml of the oxidation solution (including 88% formic acid and 30% hydrogen peroxide at ratio of 9:1 (v/v), incubated at room temperature for 1h), and kept on ice for 24h. After 24h, the sample was added 0.075 of 48% HBr, dried in vacuum condition, and hydrolyzed in 6M HCl at 110°C for 22h under vacuum condition. The hydrolysate was neutralized with 6M and 0.6M NaOH, and filtered through a cellulose membrane filter (0.45μm; Toyo Roshi Kaisha, Ltd., Tokyo, Japan). The filtrate was used for sulfur containing amino acid analysis using an amino acid analysis system.

2.2.6. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of collagen from scales was determined according to the method of Laemmli (1970) with slight modifications. Collagen samples were dissolved in 0.1M acetic

acid solution and then mixed with sample buffer (0.5M Tris-HCl, pH 6.8, containing 10% (w/v) SDS and 20% (v/v) glycerol) in the presence of 10% (v/v) mercaptoethanol at a collagen/sample buffer ratio of 1:2 (v/v). Each sample (10μg) was loaded onto the polyacrylamide gel (7.5%) and electrophoresed at a constant current of 20mA. After electrophoresis, the gel was fixed with 25% (v/v) methanol and 5% (v/v) acetic acid for 30min, and then stained with 0.1% (w/v) Coomassie blue R-250 in 30% (v/v) methanol and 10% (v/v) acetic acid and destained with 30% (v/v) methanol and 10% (v/v) acetic acid. High molecular weight markers (Sigma Chemical Co., St. Louis, MO, USA) were used to estimate the molecular weight of proteins.

2.2.7. Solubility of collagen

Collagen solubility was measured in 0.1M acetic acid at various NaCl concentrations and pHs according to the method of Montero et al. (1991) with slight modifications. Collagen samples were dissolved in 0.1 M acetic acid with gentle stirring at 4°C to obtain final concentrations of 3 and 6mg/ml.

To determine the effect of NaCl concentration on collagen solubility, 5ml of collagen solution (6 mg/ml) was mixed with 5ml of NaCl in 0.1M acetic acid at various concentrations (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2M). The mixtures were stirred gently for 30min at 4°C and centrifuged at 20,000 x *g* for 30min at 4°C. Protein content in the supernatants was measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the protein standard. Relative solubility of the collagen samples was calculated and compared with the control (without NaCl) using the following equation:

$$\text{Solubility (\%)} = \frac{\text{Protein concentration (mg/ml) of supernatants}}{\text{Protein concentration (mg/ml) of the control (without NaCl)}} \times 100$$

To determine the effect of pH on collagen solubility, 8ml of collagen solution (3 mg/ml) was adjusted with either 6M HCl or 6M NaOH to obtain a final pH range of 1 to 10, and the final volume of the solution was adjusted to 10 ml with distilled water. The mixtures were stirred gently at 4°C for 30min, followed by centrifuging at 20,000 x *g* for 30min at 4°C. Protein content in the supernatants was measured by the Lowry method (Lowry et al., 1951) using bovine serum albumin as the protein standard. Relative solubility was calculated in comparison with that obtained at the pH giving the highest solubility using the following equation:

$$\text{Solubility (\%)} = \frac{\text{Protein concentration (mg/ml) of supernatants}}{\text{Protein concentration (mg/ml) of sample (highest solubility)}} \times 100$$

2.2.8. Determination of collagen denaturation temperature

The denaturation temperature of collagen was analyzed using differential scanning calorimetry (DSC) according to the method of Kittiphattanabawon et al. (2005). ASC was dissolved in 0.1M acetic acid solution at a sample/solution ratio of 1:40 (w/v). The mixtures were allowed to stand for 2days at 4°C. The differential scanning calorimeter was calibrated using indium as the standard. The samples were exactly weighed into aluminum pans and sealed. The aluminum pans were scanned over a range of 20–50°C, with a heating rate of 1°C/min and using N_2 liquid as a cooling medium. An empty sealed pan was used as the reference. The denaturation temperature (T_d, °C) and enthalpy change (ΔH, mJ/mg) were estimated from the peak of the DSC transition curve.

2.2.9. Statistical analysis

All experiments were performed in triplicate and the data were expressed as means ± standard deviation. Differences between variables were evaluated using Duncan's multiple range tests. Analysis was performed by using SPSS software (SPSS 11.5 for Windows).

2.3 Results and discussion:

2.3.1. Proximate composition of scales

The proximate composition of marine fish scales is shown in the Table 2.1. The protein content of horse mackerel scales from Japan (35.5 ± 1.3%) was lower than that from Vietnam (44.8 ± 0.4%). On the other hand, the protein content in lizard fish scales from Japan (46.2 ± 0.1%) was higher than that from Vietnam (44.4 ± 0.5%). However, the ash contents of horse mackerel and lizard fish scales caught in Japan were similar to those from Vietnam. High protein and ash contents were found in scales from grey mullet (50.4 ± 0.4 and 47.9 ± 0.6%, respectively), flying fish (34.7 ± 0.9 and 50.2 ± 0.3%, respectively) and yellowback seabream (38.5 ± 0.9 and 46.7 ± 1.2%, respectively), which was similar to scales from other marine fishes, such as deep sea redfish (56.9 and 39.4%, respectively) (Wang et al., 2008) and spotted golden goatfish (34.5 and 54.7%, respectively) (Matmaroh et al., 2011).

Treatment with 0.5 M Na_2EDTA solution at pH 7.5 for 24 h effectively removed the ash content of marine fish scales (from 42.7-54.7 to 1.4-1.9%).

Table 2.1 Protein and ash contents of fish scales (on a dry weight basis)

Species	Before remove the noncollagenous protein and demineralization		After remove the noncollagenous protein and demineralization	
	Protein (%)	Ash (%)	Protein (%)	Ash (%)
Horse mackerel (Japan)	35.5 ± 1.3^{e}	54.7 ± 0.6^{a}	35.5 ± 1.1^{a}	1.6 ± 0.1^{a}
Horse mackerel (Vietnam)	44.8 ± 0.4^{c}	50.4 ± 0.3^{b}	35.7 ± 1.1^{a}	1.9 ± 0.2^{a}
Lizard fish (Japan)	46.2 ± 0.1^{b}	42.7 ± 0.9^{e}	36.6 ± 1.3^{a}	1.5 ± 0.3^{b}
Lizard fish (Vietnam)	44.4 ± 0.5^{c}	43.5 ± 0.7^{e}	32.2 ± 0.7^{b}	1.4 ± 0.2^{b}
Grey mullet	50.3 ± 0.4^{a}	47.9 ± 0.6^{c}	36.5 ± 1.1^{a}	1.5 ± 0.1^{b}
Flying fish	34.7 ± 0.9^{e}	50.2 ± 0.3^{b}	31.1 ± 1.1^{b}	1.5 ± 0.1^{b}
Yellowback seabream	38.5 ± 0.9^{d}	46.7 ± 1.2^{d}	35.7 ± 0.9^{a}	1.4 ± 0.3^{b}

Data are expressed as mean ± standard deviation.

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

2.3.2. Extraction yield of ASC from marine fish scales

ASC yields from fish scales are presented in Table 2.2. The yield from horse mackerel caught in Japan was the highest (1.51±0.01%) while that from grey mullet was the lowest (0.43 ± 0.02%). The yields from horse mackerel and lizard fish caught in Japan (1.51 ± 0.06 and 0.79 ± 0.01%, respectively) were higher than those from Vietnam (0.64 ± 0.02 and 0.69 ± 0.01%, respectively). The ASC yields in this study (0.43-1.51%) were much smaller than that of deep-sea redfish (6.8%) (Wang et al., 2008). Thus, ASC yield appears to depend on fish type and collagen structure (Duan et al., 2009).

Table 2.2 The extraction yields of acid soluble collagen from marine fish scales

Scale	Extract yield (%)
Horse mackerel (Japan)	1.51 ± 0.06^{a}
Horse mackerel (Vietnam)	0.64 ± 0.02^{f}
Lizard fish (Japan)	0.79 ± 0.01^{c}
Lizard fish (Vietnam)	0.69 ± 0.01^{e}
Grey mullet	0.43 ± 0.02^{f}
Flying fish	0.72 ± 0.06^{d}
Yellowback seabream	0.90 ± 0.01^{b}

Data are expressed as mean ± standard deviation (n=3).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

2.3.3. Amino acid composition

The amino acid composition of ASC is shown in Table 2.3. Glycine, the most abundantly found amino acid in collagen, accounted for from 33.2 to 34.4% of total amino acids, depends on the species. ASCs from sampled fish scales mainly consisted of alanine, proline, glutamic acid and hydroxyproline, similar to the collagen of other fish species (Wang et al., 2008, Bea et al., 2008). No tryptophan could be detected in the all collagen samples. Methionine content (from 31 to 38 residues per 1,000 residues) was somewhat higher than those of other studies (Bea et al., 2008, Wang et al., 2008, Duan et al., 2009). The imino acid contents (proline and hydroxyproline) of ASC (ranged from 165 to 178 residues per 1,000 residues) were somewhat different to those from scales of deep-sea redfish (Wang et al., 2008), spotted golden goatfish (Matmaroh et al., 2011) and carp (Duan et al., 2009), which ranged from 160 to 192 residues per 1,000 residues. The imino acid content is an important factor in estimating the thermal stability of collagen. Kimura et al. (1988) described that the imino acid content is related to the habitat temperature of the fish. ASCs from fish species inhabiting cold environments have a lower imino acid content than those from fishes inhabiting warm environments (Bae et al., 2008). Horse mackerel scale from Japan (Nagasaki Prefecture) and Vietnam (Kien Giang Province) were collected in January, 2012. The water temperature recorded for offshore Nagasaki at this time was about 15-16°C (http://www.data.jma.go.jp. Accessed 20.12.12), while that for offshore Kien Giang Province was 26-28°C (http://www.imh.ac.vn/c_tt_chuyen_nganh/cb_thongbao_khihau. Accessed 20.12.12). Differences in water temperature may be the reason for the low imino acid content of ASC in horse mackerel scale from Japan (165/1000 residues) compared to that from Vietnam (175/1000 residues). Lizard fish scales were collected in November 2011 in Miyazaki Prefecture, Japan and in January 2012 in Nha Trang City, Vietnam. Seawater temperatures differed minimally between the two locations (24-25°C in Miyazaki Prefecture

in November 2011 (http://www.data.jma.go.jp. Accessed 20.12.12) and 25-26°C in Nha Trang City in January 2011 (http://www.imh.ac.vn/c_tt_chuyen_nganh/cb_thongbao_khihau. Accessed 20.12.12). This may be the reason for the almost identical imino acid contents of ASC in lizard fish scales from Japan and Vietnam (170 and 168/1000 residues, respectively).

Marine fish scale collagen in this study contained the greater hydrophobic amino acids (total 662-688 residues per 1000 residues) compared to mammalian (total 622-652 residues per 1000 residues). The greater hydrophobic amino acids content has been put forth as the main reason for the low WVP of the films, as compared to films produced from land-based animal gelatins. Furthermore, horse mackerel (*Trachurus japonicus*) is one of the most important fish species in Japan, with an annual catch of 133,915 tonnes in 2012 (Production figures of fishery and aquaculture industry, Ministry of Agriculture, Forestry and Fisheries, 2012). That is main reason why horse mackerel scale became the main material to extract gelatin and prepare gelatin film in this research.

Table 2.3 Amino acid composition of acid soluble collagen from fish scales

Residues/1000 residues

Amino acid	Horse mackerel (Japan)	Horse mackerel (Vietnam)	Lizard fish (Japan)	Lizard fish (Vietnam)	Grey mullet	Flying fish	Yellowback seabream
Aspartic acid	47 ± 2^{a}	44 ± 1^{ab}	41 ± 2^{b}	40 ± 3^{b}	41 ± 3^{b}	40 ± 4^{b}	42 ± 3^{b}
Threonine	24 ± 4^{a}	21 ± 2^{a}	21 ± 3^{a}	21 ± 2^{a}	22 ± 1^{a}	21 ± 3^{a}	22 ± 3^{a}
Serine	34 ± 3^{c}	41 ± 2^{b}	30 ± 3^{cd}	27 ± 2^{d}	31 ± 4^{cd}	38 ± 3^{b}	47 ± 3^{a}
Glutamic acid	70 ± 4^{a}	71 ± 3^{a}	71 ± 3^{a}	72 ± 4^{a}	71 ± 3^{a}	70 ± 4^{a}	71 ± 2^{a}
Glycine	338 ± 5^{ab}	336 ± 9^{ab}	335 ± 7^{b}	338 ± 8^{ab}	344 ± 5^{a}	332 ± 4^{b}	335 ± 8^{b}
Alanine	130 ± 3^{b}	131 ± 4^{b}	154 ± 4^{a}	156 ± 7^{a}	151 ± 8^{a}	139 ± 8^{b}	138 ± 4^{b}
Valine	21 ± 3^{a}	18 ± 3^{a}	19 ± 2^{a}	19 ± 3^{a}	18 ± 3^{a}	19 ± 3^{a}	18 ± 2^{a}
Cysteine	2 ± 1^{b}	2 ± 1^{b}	4 ± 2^{a}	3 ± 2^{a}	3 ± 1^{a}	2 ± 1^{b}	3 ± 1^{a}
Methionine	31 ± 1^{b}	32 ± 2^{b}	38 ± 2^{a}	34 ± 3^{b}	31 ± 1^{b}	35 ± 3^{b}	34 ± 1^{b}
Tryptophan	ND	ND	ND	ND	ND	ND	ND
Isoleucine	10 ± 2^{a}	8 ± 1^{ab}	4 ± 1^{c}	6 ± 2^{bc}	6 ± 2^{bc}	7 ± 1^{bc}	6 ± 1^{bc}
Leucine	22 ± 3^{a}	20 ± 4^{a}	18 ± 3^{b}	18 ± 3^{b}	17 ± 2^{b}	17 ± 1^{b}	15 ± 3^{c}
Tyrosine	2 ± 1^{b}	2 ± 1^{b}	2 ± 1^{b}	1 ± 1^{b}	2 ± 1^{b}	3 ± 1^{ab}	4 ± 1^{a}
Phenylalanine	13 ± 2^{a}	13 ± 1^{a}	13 ± 2^{a}	12 ± 2^{a}	11 ± 1^{a}	12 ± 1^{a}	12 ± 2^{a}
Hydrolysine	7 ± 1^{a}	6 ± 2^{a}	6 ± 1^{a}	7 ± 1^{a}	6 ± 2^{a}	5 ± 2^{a}	5 ± 1^{a}
Lysine	27 ± 3^{a}	25 ± 2^{a}	24 ± 2^{a}	25 ± 2^{a}	24 ± 1^{a}	24 ± 2^{a}	24 ± 3^{a}
Histidine	6 ± 1^{a}	5 ± 1^{a}	6 ± 1^{a}	5 ± 2^{a}	6 ± 1^{a}	4 ± 2^{ab}	5 ± 1^{a}
Arginine	51 ± 3^{ab}	50 ± 3^{ab}	48 ± 2^{b}	49 ± 3^{b}	45 ± 3^{bc}	54 ± 2^{a}	50 ± 3^{ab}
Hydroxyproline	64 ± 3^{c}	71 ± 4^{ab}	65 ± 2^{c}	68 ± 3^{abc}	67 ± 4^{bc}	73 ± 3^{a}	65 ± 4^{c}
Proline	101 ± 3^{b}	104 ± 2^{ab}	107 ± 3^{a}	100 ± 4^{b}	104 ± 4^{ab}	105 ± 4^{ab}	104 ± 3^{ab}
Imino acids*	165 ± 5^{c}	175 ± 3^{ab}	170 ± 3^{bc}	168 ± 3^{bc}	171 ± 6^{bc}	178 ± 3^{a}	170 ± 2^{bc}
Hydrophobic acid**	666	664	688	683	682	666	662

Data are expressed as mean ± standard deviation (n=3). "ND" means not detected. * indicates total hydroxyproline and proline. Different superscripts in the same row indicate statistical differences ($p < 0.05$).

** indicates total glycine, alanine, valine, methionine, isoleucine, leucine, phenylalanine and proline.

2.3.4. SDS-PAGE analysis of ASC

SDS-PAGE analysis of ASC protein patterns under reducing conditions are shown in Fig. 2.1. The SDS-PAGE pattern showed that all ASCs consisted of two different α chains, α1 and α2, as well as a β component, and there were no differences in patterns among the species. From the electrophoretic pattern and mobility of collagens, it was suggested that all the ASCs were type I collagens (consisted of two different α chains, α1 and α2 and density of α1 is higher than α2). ASCs from scales of other marine species, including sardine, red seabream, Japanese seabass (Nagai et al., 2004), deep-sea redfish (Wang et al., 2008) and spotted golden goatfish (Matmaroh et al., 2011), have also been classified as type I collagen.

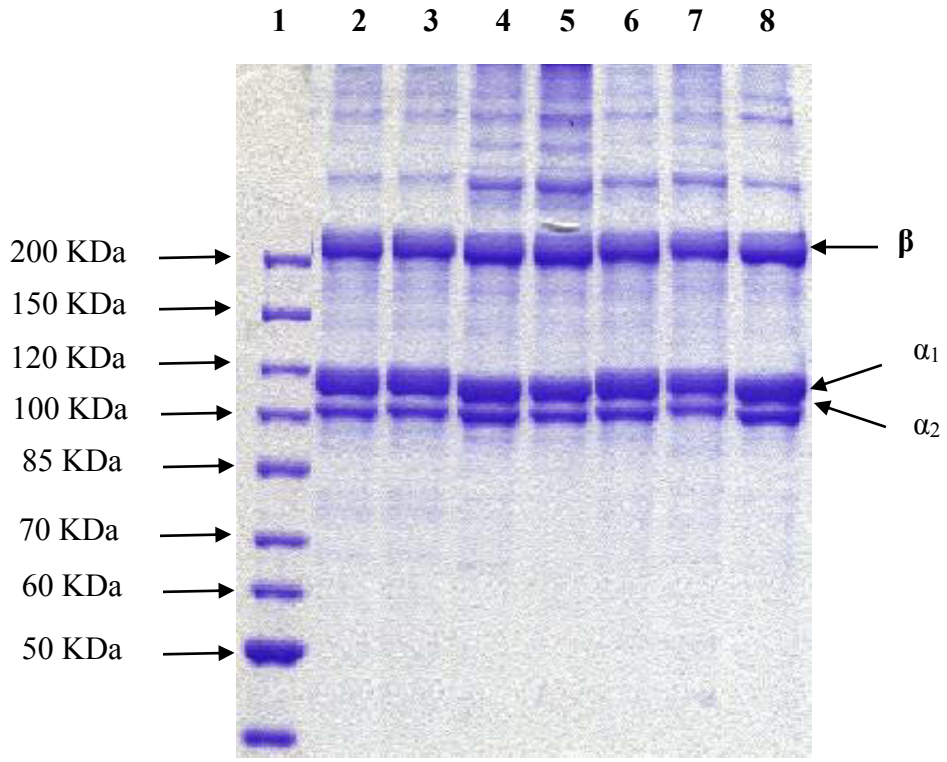


Fig. 2.1 SDS-PAGE pattern of acid soluble collagen from the scale of fishes

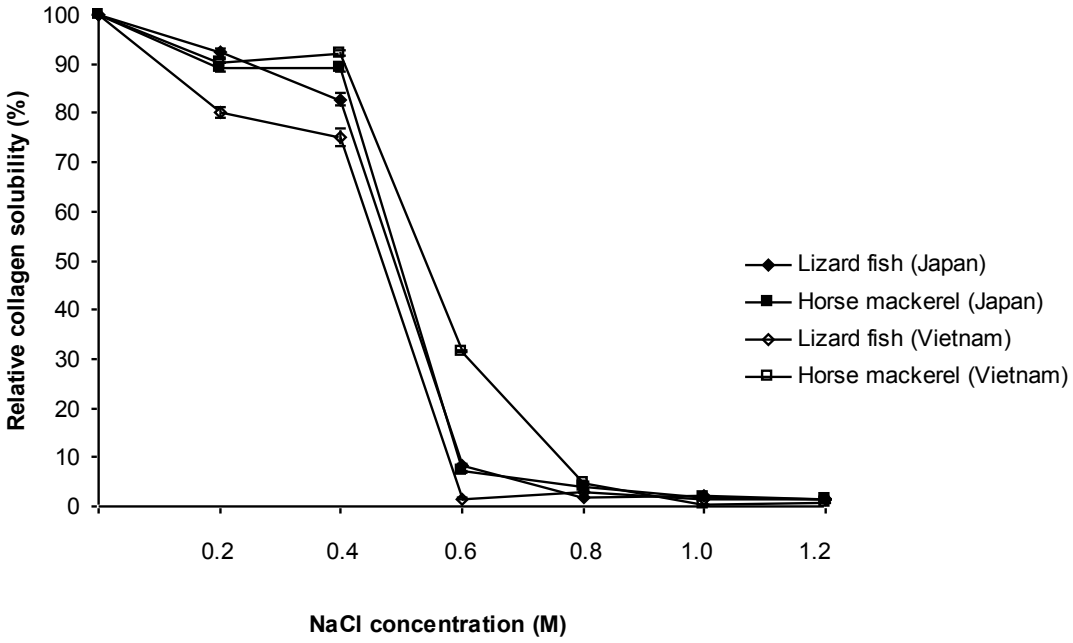
Lane 1, high molecular weight marker; lane 2, lizard fish from Japan; lane 3, lizard fish from Vietnam; lane 4, horse mackerel from Japan; lane 5, horse mackerel from Vietnam; lane 6, grey mullet; lane 7, flying fish; lane 8, yellowback seabream.

2.3.5. Solubility of collagen

2.3.5.1. Effect of NaCl concentration on collagen solubility

Solubility of ASC at different NaCl concentrations is shown in Fig. 2.2a and 2.2b. All collagen samples showed similar solubility behavior. All ASCs showed high solubility at NaCl concentrations of 0.2-0.4 M (75.14-100%). The solubility of all samples decreased sharply with NaCl concentrations >0.4 M. This can be explained as follows: at a high NaCl concentration, the solubility declined by enhancing hydrophobic–hydrophobic interactions between protein chains, leading to protein precipitation (Damodaran, 1996).

a



b

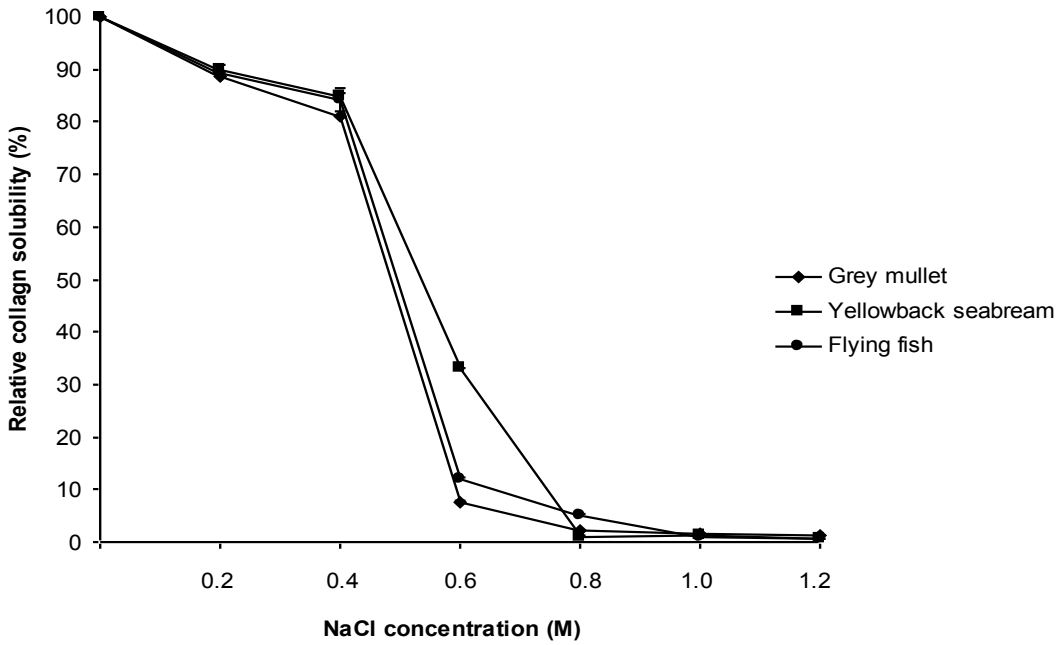


Fig. 2.2 Relative solubility of acid soluble collagen from the scale of fishes at various NaCl concentrations

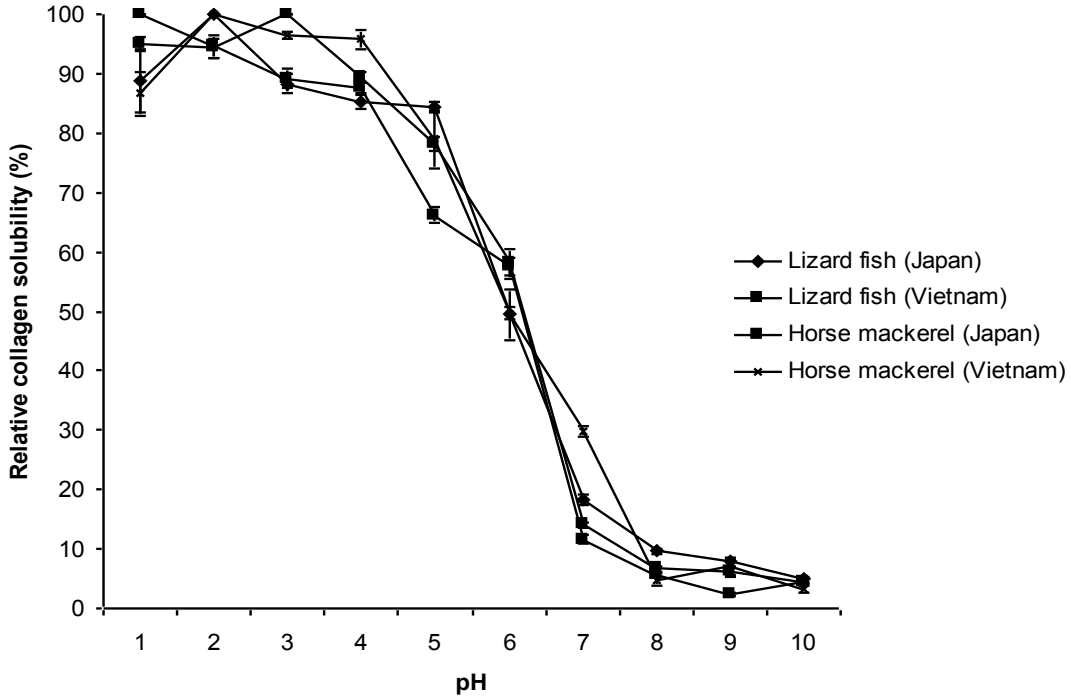
a, Lizard fish and horse mackerel from Japan and Vietnam

b, Grey mullet, yellowback seabream and flying fish

2.3.5.2. Effect of pH on collagen solubility

The effect of pH on the solubility of ASCs in 0.1M acetic acid is shown in Fig. 2.3a and 2.3b. All ASCs showed a similar trend; they showed high solubility at acidic pHs (1-5). However, individual ASCs showed maximal solubility at various pHs depending on the fish species. ASCs of lizard fish from Japan, flying fish, and horse mackerel from Vietnam showed the highest solubility at pH 2, while the highest solubility of horse mackerel ASC from Japan was observed at pH 1. Grey mullet ASC reached maximum solubility at pH 3, similar to that for lizard fish from Vietnam and yellowback seabream. Solubility dramatically decreased in the pH range of 7 to 10, regardless of the species. This indicates that the pH of ASC reached the pI in this range, i.e., the total net charge of ASC, became zero, resulting in protein precipitation (Jongjareonrak et al., 2005). In contrast, the repulsion forces between charged residues of protein molecules are greater than the attracting forces at pHs lower than the pI, resulting in protein solubilization (Damodaran et al., 1996).

a



b

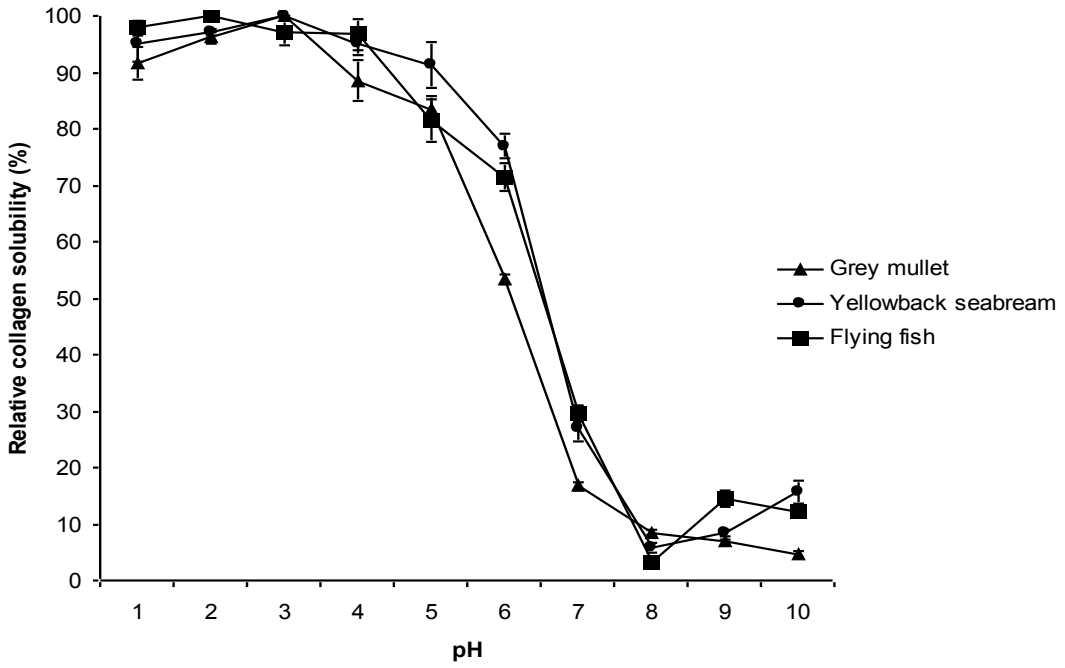


Fig. 2.3 Effect of pH on the solubility of ASC from the scale of fishes

a, Lizard fish and horse mackerel from Japan and Vietnam

b, Grey mullet, yellowback seabream and flying fish

2.3.6. Denaturation temperature of collagen

Thermal transition of ASC from marine fish scales is shown in Fig. 2.4. Denaturation temperature is an important index for evaluating the thermal stability of collagen. By using DSC to measure endothermic heat flow, the denaturation temperature of collagen can be calculated.

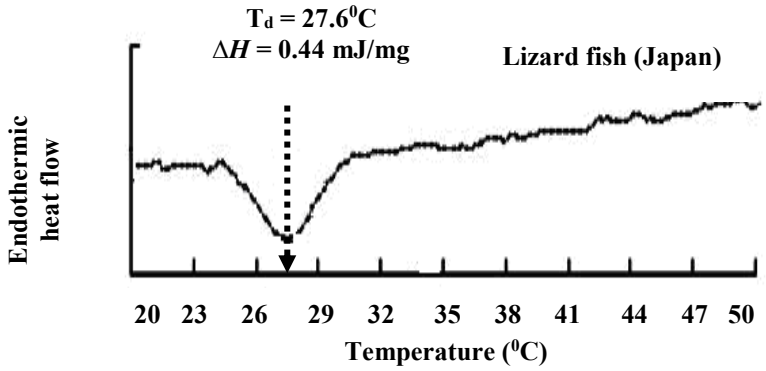
Lizard fishes from Japan and Vietnam showed similar ASC T_d, 27.6°C (ΔH = 0.44 mJ/mg) and 27.4°C (ΔH = 0.42mJ/mg), respectively. Horse mackerel from Japan had a T_d of 26.1°C (ΔH = 0.29mJ/mg), which was lower than that from Vietnam (28.1°C, ΔH = 0.59 mJ/mg). In general, T_d of collagens from marine fish scales were about 26°C to 29°C, indicating that fish collagen is generally less thermally stable than mammalian collagen (T_d about 41°C) (Burjanadze et al., 1992). These results are similar to those reported for collagen from deep-sea redfish scales (17.7°C) (Wang et al., 2008) and carp scales (about 28°C) (Duan et al., 2009).

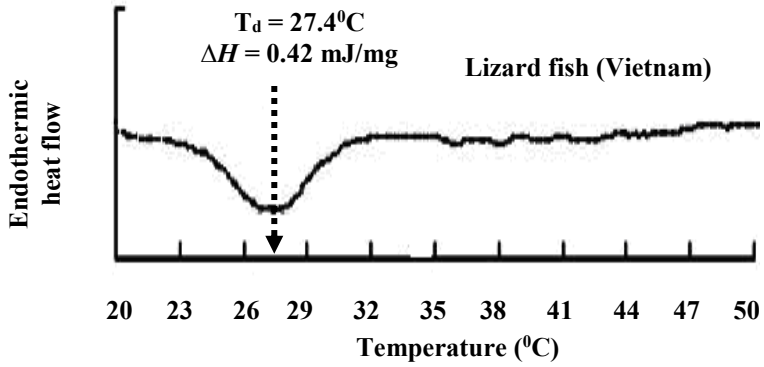
Many reports have focused on the relationship between the thermostability of collagen from marine fish and imino acid (hydroxyproline and proline) content (Bae et al., 2008; Kittiphattanabawon et al., 2005). The heat resistance of collagen is known to increase with imino acid content (Wong, 1989). Our results also showed that a close relationship exists between denaturation temperature and imino acid content of ASC (y=3.9x+62.7, r^2=0.78, p<0.01, n=7), where x is the denaturation temperature (°C) and y is the imino acid content (residues/1000 residues).

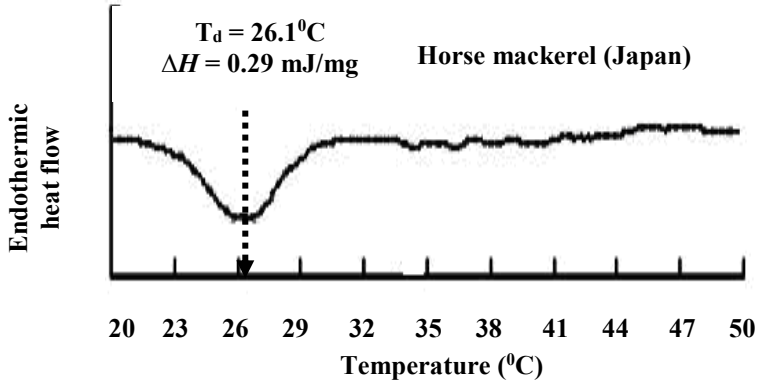
Furthermore, the thermal stability of collagen not only depends on the imino acid content but is also directly correlated with the environmental and body temperatures of fish species (Rigby, 1968).

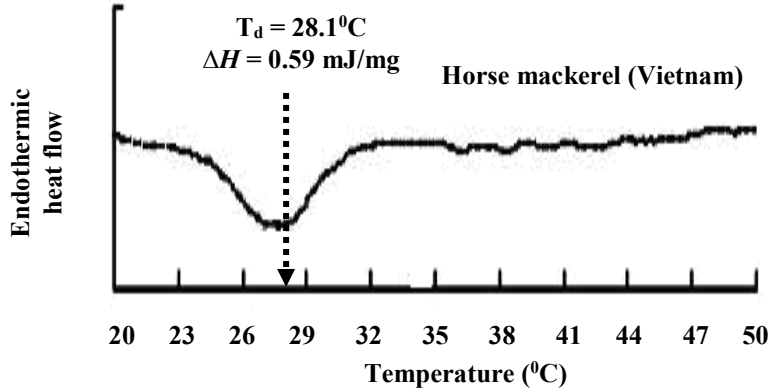
In general, collagen is denatured to gelatin when it is used as foodstuff in food industry. The low denaturation temperature of scale collagen observed in the present study makes it

possible to extract the gelatin at low temperature compared to mammalian gelatin. This may be an economic advantage for using fish scale as a raw material of gelatin.


T_d = 27.6^{0}C
ΔH = 0.44 mJ/mg
Lizard fish (Japan)
Endothermic heat flow
20 23 26 29 32 35 38 41 44 47 50
Temperature (^{0}C)


T_d = 27.4^{0}C
ΔH = 0.42 mJ/mg
Lizard fish (Vietnam)
Endothermic heat flow
20 23 26 29 32 35 38 41 44 47 50
Temperature (^{0}C)


T_d = 26.1^{0}C
ΔH = 0.29 mJ/mg
Horse mackerel (Japan)
Endothermic heat flow
20 23 26 29 32 35 38 41 44 47 50
Temperature (^{0}C)


T_d = 28.1^{0}C
ΔH = 0.59 mJ/mg
Horse mackerel (Vietnam)
Endothermic heat flow
20 23 26 29 32 35 38 41 44 47 50
Temperature (^{0}C)

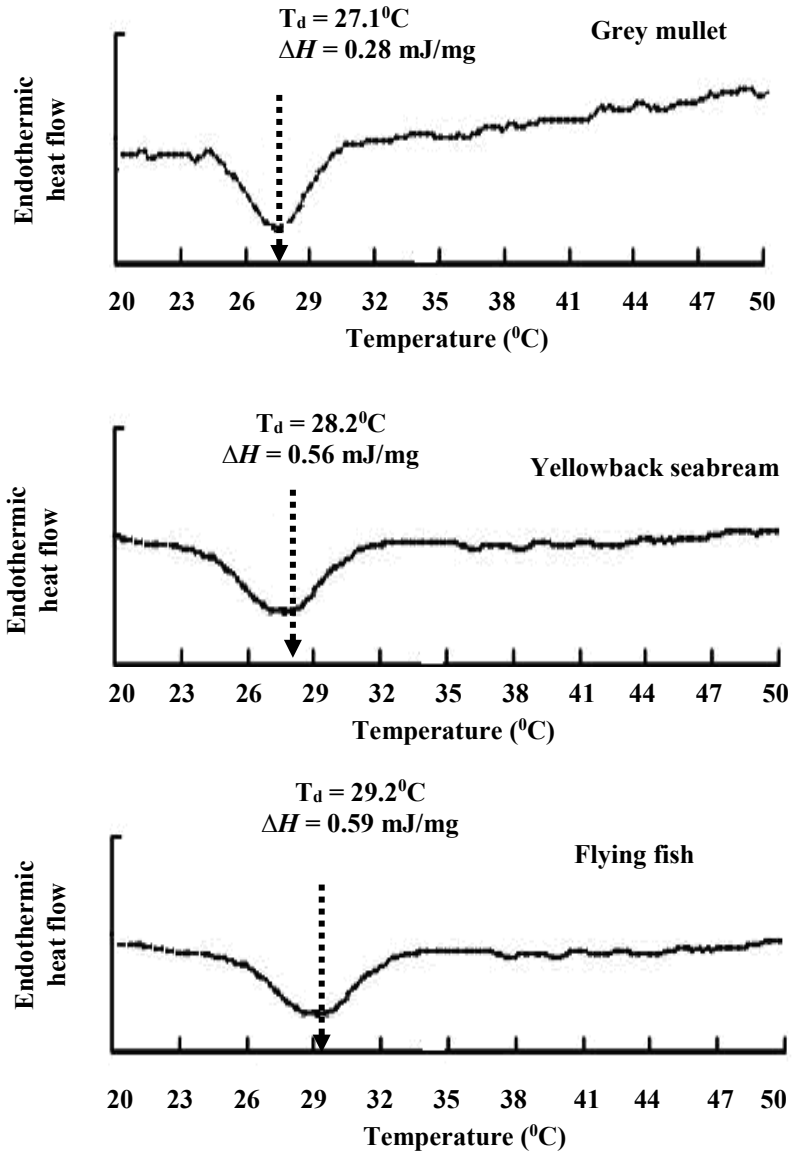


Fig. 2.4 Thermal transition of ASC from marine fish scales

2.4. Summarize of chapter 2:

ASC could be isolated from the scale of marine fishes from Japan and Vietnam. All collagens extracted from marine fish scales in this study were identified as type I collagen. A correlation between the heat resistance of collagen and imino acid content was demonstrated. The imino acid content in ASC from marine fish scales differed according to species and habitat temperature. These results suggest that collagen could be obtained effectively from the processing waste of some commercial marine fishes from Japan and Vietnam, and has potential as a realistic alternative to mammalian collagens.

CHAPTER 3

The effect of preparation conditions on the properties of gelatin film from horse mackerel (*Trachurus japonicus*) scale

3.1 Introduction:

Gelatin is a protein derived by partial hydrolysis of collagen extracted from skin, bones, connective tissues, organs and some intestines of mammalian species (Johnston-Banks, 1990). However, concerns regarding bovine and porcine health, such as the emergence of bovine spongiform encephalopathy and foot and mouth disease, have in recent years led to increased attention on fish gelatin (Gómez-Guillén, Giménez, López-Caballero & Montero, 2011). Gelatin has wide application in the food industry, such as a gelling agent in cooking, a stabilizer, thickener, or texturizer in foods (Gómez-Guillén et al., 2011; Irwandi, Faridayanti, Mohamed, Hamzah, Torla & Che Man, 2009). Another important application of fish gelatin is in the production of film. Fish gelatin film, which is biodegradable, is considered a vital eco-friendly packaging material for reducing the environmental impact of synthetic plastic materials (Nagarajan, Benjakul, Prodpran & Songtipya, 2012). Furthermore, fish gelatin films used as a foodstuff covering have the capacity to protect food against drying, light and oxygen (Gómez-Guillén, Pérez-Mateos, Gómez-Estaca, López-Caballero, Giménez & Montero, 2009).

Horse mackerel (*Trachurus japonicus*) is one of the most important fish species in Japan, with an annual catch of 133,915 tonnes in 2012 (Production figures of fishery and aquaculture industry, Ministry of Agriculture, Forestry and Fisheries, 2012). This fish is used as a raw material of frozen surimi (Shimizu, 1987; Yamanaka & Tanaka, 2007) and in the preparation of raw fresh *sashimi* and *sushi*, which are popular foods in Japan (Yamanaka et al., 2007). However, during fish processing, a large amount of byproduct (accounting for approximately 50-70% of fish weight, and consisting of skin, scales and bone) is produced and discarded, resulting in serious environmental problems (Kittiphattanabawon, Benjakul, Visessanguan, Nagai & Tanaka, 2005). Utilization of marine byproducts, including scales, as raw materials in gelatin extraction represents a potential strategy to reduce the environmental pollution caused by marine waste, as well as increasing the value of the fish product.

Fish gelatin films from brownstripe red snapper and bigeye snapper skin (Jongjareonral, Benjakul, Visessanguan, Prodpran & Tanaka, 2006), blue shark skin (Limpisophon, Tanaka, Weng, Abe & Osako, 2009), Nile perch skin (Muyonga, Cole & Duodu, 2004), halibut skin (Carvalho, Sobral, Thomazine, Habitante, Giménez, Gómez-Guillén et al., 2007), and tuna skin (Gómez-Guillén, Ihl, Bifani, Silva & Montero, 2007) have been characterized. However, little information is available regarding the characteristics of gelatin films from fish scale, with the exception of lizard fish scale (Wangtueai, Noomhorm & Regenstein, 2010). Therefore, the aim of this study was to prepare and characterize the edible film from the gelatin of horse mackerel (*Trachurus japonicus*) scale and to investigate the effect of preparation conditions, i.e., extraction

temperature and time, of gelatin from scale, and the protein concentration of film-forming solutions (FFS) on the properties of horse mackerel scale gelatin film.

3.2 Materials and methods:

3.2.1. Characterization of horse mackerel scale gelatin

3.2.1.1. Fish scale preparation

Horse mackerel scale was collected in Nagasaki Prefecture, Japan. The scale was collected and transported to our laboratory under ice. The fish scale was removed of impurities manually, washed with chilled water, and then placed in plastic bags and stored at –30°C ± 2°C until use.

3.2.1.2. Extraction of gelatin

Gelatin was extracted from horse mackerel scale according to the method of Wangtueai and Noomhorm (2009) with slight modifications. Briefly, frozen scale was thawed and treated with 0.1M NaOH solution for 6h at a 1:8 (w/v) ratio of scale/alkali solution to remove the noncollagenous protein. The alkali solution was changed after 3h and scales were washed thoroughly in cold distilled water to obtain a neutral pH. After the removal of noncollagenous protein, the fish scales were drained using a cheesecloth, squeezed manually and then soaked in 10x distilled water (w/v) at different temperatures

(70, 80, and 90°C) for different times (15min, 30min, 1h, 2h and 3h) with continuous stirring to extract gelatin.

After gelatin extraction, the coarse solids were removed by filtration with two layers of cheesecloth and the liquid was centrifuged at 16,400 x *g* for 30min under 20°C. The extracted gelatin in the supernatant was freeze-dried (DC 401; Yamato Scientific Co., Ltd., Japan).

3.2.1.3. Yield of extracted gelatin

The yield of extracted gelatin was evaluated by the method of Limpisophon et al. (2009). Approximately 1g of the noncollagenous fish scale was weighed, and then extracted in 10x distilled water (w/v) as described above. The protein content of the supernatant was determined by the Lowry method (Lowry, Rosbrough, Farr, & Randall, 1951) using bovine serum albumin (BSA) as a protein standard. The yield of extraction (YD) was calculated by the following equation:

$$YD(\%) = \frac{\text{Protein concentration (mg/ml) x 10 times of distilled water (ml) x 100\%}}{\text{Fish scales weight (mg)}}$$

3.2.1.4. SDS – polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of gelatin from horse mackerel scale was conducted according to the method of Laemmli (1970) with slight modifications. The gelatin in the supernatant obtained at each extraction temperature and time was mixed with sample buffer (0.5M

Tris-HCl, pH 6.8, containing 10% (w/v) SDS and 20% (v/v) glycerol) in the presence of 10% (v/v) mercaptoethanol at a gelatin/sample buffer ratio of 1:2 (v/v). The samples (10 μl) were loaded onto a 7.5% polyacrylamide gel and electrophoresed at a constant current of 20 mA. After electrophoresis, the gel was fixed with 25% (v/v) methanol and 5% (v/v) acetic acid for 30minutes, and then stained with 0.1% (w/v) Coomassie Brilliant Blue R-250 in 30% (v/v) methanol and 10% (v/v) acetic acid and distained with 30% (v/v) methanol and 10% (v/v) acetic acid. Molecular weight markers (Sigma Chemical Co., St. Louis, MO, USA) were used to estimate the molecular weight of proteins.

3.2.1.5. Amino acid analysis

Twenty milligrams of gelatin samples from horse mackerel scale were hydrolyzed in 6M HCl at 110°C for 22h *in vacuo*. The hydrolysate was neutralized with 6N and 0.6N NaOH, and filtered through a cellulose membrane filter (0.45μm; Toyo Roshi Kaisha Ltd., Tokyo, Japan). The filtrate was subjected to amino acid analysis using an amino acid analysis system (Prominence; Shimadzu, Kyoto, Japan) equipped with a Shim-pack column (Amino-Li, 100mm × 6.0mm i.d.; column temperature, 39.0°C; Shimadzu) and Shim-pack pre-column (ISC-30/S0504 Li, 150mm × 4.0mm i.d.; Shimadzu). Amino acids were detected using a fluorescence detector (RF-10AXL; Shimadzu).

3.2.2. Effect of extraction temperature and time, and protein concentration of FFS on the gelatin films

3.2.2.1 Preparation of gelatin films

To study the effect of extraction temperature on gelatin films, gelatin powder extracted at various temperatures (70, 80 and 90°C) for 1h were dissolved in distilled water at 60°C for 30min to obtain FFS with a protein concentration of 2% (w/v). The effect of extraction time on gelatin films was investigated by dissolving gelatin powder obtained at different extraction times (15min, 1h and 3h) at 70°C with distilled water at 60°C for 30min to obtain a FFS protein concentration of 2% (w/v). Glycerol was used as a plasticizer, and was added to FFS at a protein concentration of 20% (w/w) with continuous stirring at room temperature for 15min. The air bubbles in FFS were removed using a hybrid mixer (HM-500; Keyence Co., Tokyo, Japan). The prepared FFS (4g) was cast onto a rimmed silicone plate (50 x 50mm) and dried in a ventilated oven (EYELA, KCL-2000A; Tokyo Rikakikai Co., Ltd., Japan) at 25 ± 0.5ºC and 50 ± 5% relative humidity (RH) for 48h. The obtained dried films were manually peeled off.

The effect of FFS protein concentration on fish gelatin film was investigated by dissolved gelatin powder extracted at 70ºC for 1 h in distilled water at 60ºC for 30min to obtain FFS with protein concentrations of 1, 2, and 3% (w/v), determined by Lowry's method (Lowry et al., 1951). Glycerol, used as a plasticizer, was added to FFS at 20% (w/w) protein concentration with continuous stirring at room temperature for 15min. The FFS was cast and dried as previously described.

3.2.2.2. Moisture content of gelatin films

Moisture content of gelatin films was determined following the methods of AOAC (2000).

3.2.2.3. Thickness

Film thickness was measured by using a dial-type thickness gage (Series 7300; Mitsutoyo Co., Kanagawa, Japan). Six random positions around every film sample were used for determination of film thickness.

3.2.2.4. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of film samples were determined according to the ASTM standard method D 882-97 (ASTM, 1999) using a Tensipresser (TTP-50BX II; Taketomo Electric Inc., Tokyo, Japan). Two rectangular strips (20 x 45mm) from each film with an initial grip length of 30mm were used for testing. Gelatin film samples were clamped and strained at a cross-head speed of 1mm/s until the films were disrupted. TS and EAB were calculated by the following equation, respectively.

$$\text{TS (MPa)} = \frac{\text{Maximum load needed to pull the sample apart (N)}}{\text{Cross-sectional area of the samples (m}^2\text{)}}$$

$$\text{EAB (\%)} = \frac{\text{Film elongation at the moment of rupture (mm) x 100}}{\text{Initial grip length of samples (mm)}}$$

3.2.2.5. Water vapor permeability

Water vapor permeability (WVP) of films was measured according to the ASTM standard method (1983) as reported by Gontard et al. (1992). Films were sealed over a glass cup containing silica gel (0% RH) with silicon vacuum grease and a plastic band. The

cups with films were weighed as initial weight. The cups were then placed in a desiccator with saturated water (100% RH) at 30°C. The cups were weighed at 1h intervals over a 9h period. Six samples were determined for each film type. Water vapor permeability of gelatin film was calculated by the following equation (McHugh, Avena-Bustillos, & Krochta, 1993):

$$\text{WVP } (gm^{-1}s^{-1}Pa^{-1}) = w \times x \times A^{-1} \times t^{-1} \times (P_2 - P_1)^{-1}$$

where w = weight gain (g) of the cup, x = film thickness (m), A = area of exposed film (m^2), t = time of gain (s), $(P_2 - P_1)$ = vapor pressure differential (Pa) across the film.

3.2.2.6. Light transmission

Light transmission in ultraviolet (UV) and visible ranges of gelatin films was measured at selected wavelengths between 200 and 800nm using a UV-Visible Recording Spectrophotometer (UV-160; Shimadzu Co., Kyoto, Japan), and according to the method described by Fang, Tung, Britt, Yada and Dalgleish (2002).

3.2.2.7. SDS – polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis of gelatin films from horse mackerel scale was conducted according to the method of Limpisophon et al. (2009) with slight modifications. Film samples (50mg) were dissolved in 5ml of 1% (w/v) SDS solution and shaken continuously at room temperature for 16 h. Supernatants were collected after centrifugation at 5000 x *g* for 5 min at 20°C and then subjected to SDS-PAGE as described in section *3.2.1.4.*

3.2.3. Statistical analysis

All experiments were performed 6 times and the data were expressed as mean ± standard deviation. The differences between variables were evaluated basing on the Duncan's multiple range tests. Analysis was performed by using SPSS software (SPSS 11.5 for Windows).

3.3 Results and discussion:

3.3.1. Characterization of gelatin from horse mackerel scale

3.3.1.1. Extraction yield

Gelatin yields from horse mackerel scale at various extraction times and temperatures are shown in Table 3.1. Yields ranged from 1.08 to 3.45%, depending on the extraction condition. The yield increased with increasing extraction time and temperature. This can be explained by the disruption of hydrogen bonds stabilizing collagen's triple helical structure with increasing extraction time and temperature, leading to helix-to-coil transition and the conversion of collagen into soluble gelatin (Benjakul, Oungbho, Visessanguan, Thiansilakul & Roytrakul, 2009). The results are supported by Limpisophon et al. (2009) and Wangtueai and Noomhorm (2009), who reported that increasing extraction time and temperature were associated with increasing yield of gelatin from blue shark skin and lizard fish scale, respectively.

Table 3.1 Yield of gelatin from horse mackerel scale at various extraction times and temperatures

Extraction time (h)	Temperature (^{0}C)		
	70	80	90
15min	1.08 ± 0.21^{a}	1.81 ± 0.05^{abc}	2.18 ± 0.12^{bcd}
30min	1.37 ± 0.09^{ab}	2.57 ± 0.40^{cdef}	2.73 ± 0.39^{def}
1	2.50 ± 0.54^{cde}	2.90 ± 0.75^{def}	3.08 ± 0.70^{ef}
2	2.59 ± 0.51^{cdef}	2.93 ± 0.56^{def}	3.18 ± 0.72^{ef}
3	2.78 ± 0.57^{def}	3.45 ± 0.99^{f}	3.21 ± 0.37^{ef}

Data are expressed as mean ± standard deviation (n=6).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

3.3.1.2. SDS – PAGE

The SDS-PAGE profile of gelatin extracted at different temperatures and times is presented in Fig 3.1. High molecular weight protein was observed in gelatin from horse mackerel scales, consisting of α and β chains. However, the degradation of α and β chains was observed at extraction temperatures higher than 70°C and extraction times longer than 1h. Limpisophon et al. (2009) observed the degradation of α and β chains in the protein pattern of blue shark skin gelatin at extraction temperatures higher than 80°C. The differences in raw materials (scale and skin) can explain the observed differences in the temperature of protein degradation between horse mackerel scale gelatin (70°C) and blue shark skin gelatin (80°C).

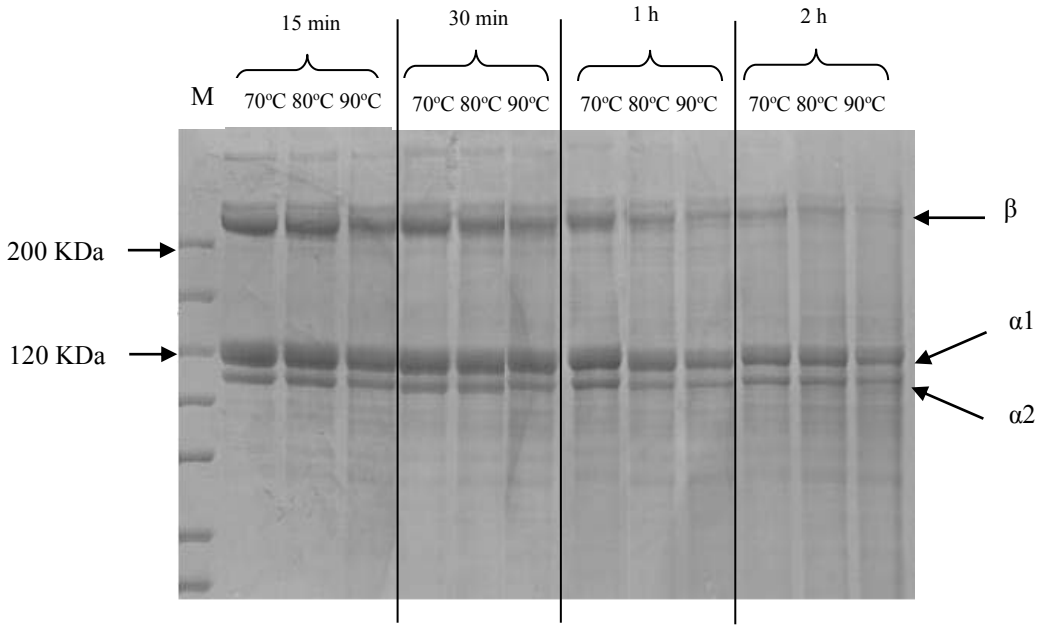


Fig. 3.1 SDS-PAGE patterns of gelatin extracted at various temperatures (70, 80 and 90°C for 1h) and times (at 70°C for 15 min, 30 min, 1 h and 2 h).

"M" means protein marker.

3.3.1.3. Amino acid analysis

The amino acid composition of gelatin from horse mackerel scales extracted at 70ºC for 1h is presented in Table 3.2. Glycine, the most abundant amino acid in horse mackerel scale gelatin, accounted for 30% of total amino acids. Imino acid (proline and hydroxyproline) contents of horse mackerel scale gelatin (178 residues per 1000 residues) were greater than those from grass carp scales (94 residues per 1000 residues) (Zhang, Xu & Wang, 2011) but lower than those of lizardfish scale gelatin (205 residues per 1000 residues) (Wangtueai & Noomhorm, 2009) and mammalian gelatin (216-225 residues per 1000 residues) (Avena-Bustillos, Olson, Chilou, Yee, Bechtel & McHugh, 2006). Imino acids play an important role in the triple helical structure of gelatin and affect the gel strength of gelatin gel (Ward & Courts, 1977; Zhang, Xu & Wang, 2011). Most fish gelatins have lower amino acid content than mammalian gelatins, explaining the poor gelling in comparison with mammalian gelatins. Notably, horse mackerel scale gelatin contained the same or greater hydrophobic amino acids (total 653 residues per 1000 residues) compared to mammalian (total 622-652 residues per 1000 residues), cold-water fish (total 638-654 residues per 1000 residues) and warm-water fish gelatins (total 526-649 residues per 1000 residues) (Avena-Bustillos et al., 2006).

Table 3.2 Amino acid composition of gelatin from horse mackerel scale

Amino acid	Residues/1000 residues
Aspartic acid	49 ± 2
Threonine	25 ± 2
Serine	33 ± 2
Glutamic acid	71 ± 3
Glycine	322 ± 5
Alanine	131 ± 3
Valine	21 ± 2
Methionine	17 ± 2
Isoleucine	10 ± 2
Leucine	23 ± 3
Tyrosine	2 ± 1
Phenylalanine	18 ± 2
Hydrolysine	10 ± 1
Lysine	32 ± 3
Histidine	7 ± 2
Arginine	51 ± 4
Hydroxyproline	67 ± 3
Proline	111 ± 7
Imino acids*	178 ± 9
Hydrophobic amino acids**	653

Data are expressed as mean ± standard deviation (n=3).

* indicates total hydroxyproline and proline.

** indicates total glycine, alanine, valine, methionine, isoleucine, leucine, phenylalanine and proline.

3.3.2. Effect of extraction time and temperature on properties of gelatin film from horse mackerel scale

3.3.2.1 Moisture content of gelatin films

The moisture content of gelatin films ranged from 17.39 to 17.49%, depending on the preparation conditions of films. However, no statistical differences were found among the films.

3.3.2.2 Thickness

The thickness of films prepared from horse mackerel scale gelatin extracted at different temperatures and times differed (Table 3.3). An extraction temperature greater than 70°C and an extraction time longer than 1h resulted in a slightly decreased film thickness. A possible reason may be that increases in extraction temperature and time led to an increased degree of gelatin hydrolysis. Hoque, Benjakul & Prodpran (2010) reported that films prepared from gelatin with a higher degree of hydrolysis showed a slight decrease in thickness. This result is in agreement with Nagarajan et al. (2012), who reported that the thickness of film made from splendid squid (*Loligo formosana*) gelatin decreased with increasing extraction temperature.

Table 3.3 Effect of gelatin-extraction temperature and time on thickness, tensile strength (TS), elongation at break (EAB) and water vapor permeability (WVP) of gelatin film

	Thickness (μm)	TS (MPa)	EAB (%)	WVP ($x10^{-10}gm^{-1}Pa^{-1}s^{-1}$)
Extraction temperature (for 1h)				
70ºC	23.58 ± 0.52^{c}	36.48 ± 3.66^{c}	46.09 ± 7.44^{c}	0.98 ± 0.07^{a}
80ºC	22.44 ± 0.36^{b}	19.95 ± 1.46^{b}	25.56 ± 2.73^{b}	1.07 ± 0.08^{ab}
90ºC	21.57 ± 0.34^{a}	13.14 ± 1.66^{a}	19.74 ± 4.25^{a}	1.11 ± 0.07^{b}
Extraction time (at 70ºC)				
15min	22.75 ± 0.56^{b}	35.62 ± 2.44^{bc}	43.04 ± 6.78^{bc}	1.01 ± 0.02^{a}
1h	23.58 ± 0.52^{c}	36.48 ± 3.66^{b}	46.09 ± 7.44^{b}	0.98 ± 0.07^{a}
3h	22.05 ± 0.4^{a}	28.84 ± 2.19^{a}	33.32 ± 8.79^{a}	1.14 ± 0.07^{b}

Data are expressed as mean ± standard deviation (n=6).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

3.3.2.3. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of gelatin films prepared from gelatin extracted at various temperatures and times are shown in Table 3.3.

Under identical extraction time (1 h), the gelatin film extracted at 70°C had the highest TS (36.48 ± 3.66MPa) and EAB (46.09 ± 7.44%) values when compared with gelatin films extracted at higher temperatures (80 and 90°C); which were 19.95 ± 1.46MPa and 13.14 ± 1.66MPa, respectively, for TS, and 25.56 ± 2.73% and 19.74 ± 4.25%, respectively, for EAB. At an extraction temperature of 70°C, the TS and EAB values of gelatin film extracted for 15 min (35.62 ± 2.44MPa and 43.04 ± 6.78%, respectively) did not significantly differ from those for 1h extraction (36.48 ± 3.66MPa and 46.09 ± 7.44%, respectively). However, both values decreased to 28.84 ± 2.19MPa and 33.32 ± 8.79%, respectively, when heated for 3h. Thus, TS and EAB of gelatin film decreased with increasing extraction temperature and time. It is known that the mechanical properties of gelatin film depend on the triple helical structure of gelatin. Hoque et al. (2010) reported that gelatin films are mainly stabilized by weak forces, including hydrogen bonding and hydrophobic interaction. These weak bonds are likely destroyed by the increasing extraction temperature and time, leading to the disruption of gelatin's triple helical structure and the formation of shorter peptide chains (Nagarajan, Benjakul, Prodpran, Songtipya & Kishimura, 2012), and resulting in the alteration of mechanical properties of gelatin films.

3.3.2.4. WVP of gelatin film

The WVP of gelatin films extracted under various conditions is shown in Table 3.3. Under identical extraction time (1h), WVP increased with increasing extraction temperature; the gelatin film extracted at 70°C showed the lowest WVP value (0.98 ± 0.07 $x10^{-10}gm^{-1}Pa^{-1}s^{-1}$). Increase in extraction time from 15min to 3h at 70°C resulted in increased WVP value (1.01 ± 0.02 $x10^{-10}gm^{-1}Pa^{-1}s^{-1}$ to 1.14 ± 0.07 $x10^{-10}gm^{-1}Pa^{-1}s^{-1}$). WVP appears to be determined by the strength of protein molecule interactions within the network of the film (Tongnuanchan, Benjakul, Prodpran & Songtipya, 2011). The triple helical structure of gelatin may be broken down to small peptide chains with increased extraction time and temperature. These small peptide chains can be easily inserted into the protein network, leading to a decrease in density of intermolecular interactions between gelatin chains, and a subsequent increase in the WVP of gelatin films. The greater hydrophobic amino acids content has been put forth as the main reason for the low WVP of the films, as compared to films produced from other fish or land-based animal gelatins (Limpisophon et al., 2009).

3.3.2.5. Light transmission of gelatin film (%)

UV and visible light transmission of films from horse mackerel scale gelatin prepared at various temperatures and times is presented in Table 3.4. Gelatin films extracted at various temperatures (70°C to 90°C) for 1h showed transmission in the visible range (350-800nm) and UV range (280nm) from 23.68 ± 1.33 to 75.77 ± 2.03% and from 12.15 ± 0.61 to 16.35 ± 2.51%, respectively. Transmission in the visible range and UV range of gelatin film extracted at 70°C for different times was from 29.03 ± 1.91 to 79.08 ± 6.24% and

from 15.97 ± 2.83 to 20.92 ± 0.57%, respectively. However, in the UV range (200nm), the transmission of these films could not be measured, regardless of extraction temperature or time. A possible explanation is that the amino acid composition of gelatin consists of aromatic amino acids with a phenol ring, such as phenylalanine, tryptophan and tyrosine, could prevent UV transmission, similar to that observed for gelatin film from brownstripe red snapper and bigeye snapper skin (Jongjareonrak , Benjakul, Visessanguan, Prodpran, & Tanaka, 2006). Thus, the gelatin film effectively prevented UV light transmission.

Table 3.4 Effect of gelatin-extraction temperature and time on light transmission (%) of gelatin films

	Light transmission at different wavelength (nm)						
	200	280	350	400	500	600	800
Extraction temperature (for 1 h)							
70°C	0.3	16.35 ± 2.51[a]	30.42 ± 2.78[a]	52.70 ± 1.14[a]	62.60 ± 1.59[a]	69.43 ± 1.12[a]	75.77 ± 2.03[a]
80°C	0.3	13.68 ± 0.55[b]	27.30 ± 2.73[b]	45.17 ± 1.74[b]	59.18 ± 0.62[b]	63.58 ± 0.41[b]	68.55 ± 1.74[b]
90°C	0.3	12.15 ± 0.61[b]	23.68 ± 1.33[c]	42.20 ± 0.99[c]	49.82 ± 1.33[c]	57.85 ± 0.91[c]	64.25 ± 2.04[c]
Extraction time (at 70°C)							
15min	0.3	20.92 ± 0.57[a]	35.98 ± 1.53[a]	61.01 ± 2.35[a]	68.77 ± 3.20[a]	75.93 ± 7.97[a]	79.08 ± 6.24[a]
1h	0.3	16.35 ± 2.51[b]	30.42 ± 2.78[b]	52.70 ± 1.14[b]	62.60 ± 1.59[b]	69.43 ± 1.12[b]	75.77 ± 2.03[a]
3h	0.3	15.97 ± 2.83[b]	29.03 ± 1.91[b]	52.35 ± 0.96[b]	59.85 ± 3.28[b]	61.13 ± 2.70[c]	68.88 ± 4.00[b]

Data are expressed as mean ± standard deviation (n=6).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

3.3.2.6. SDS-PAGE

Protein patterns of gelatin films produced under different extraction temperatures and times are shown in Fig 3.2. From the protein pattern, α and β chains were found as major components. Moreover, with increasing extraction temperature and time, the degradation of major components was observed, especially with an extraction temperature higher than 70°C and an extraction time longer than 1h.

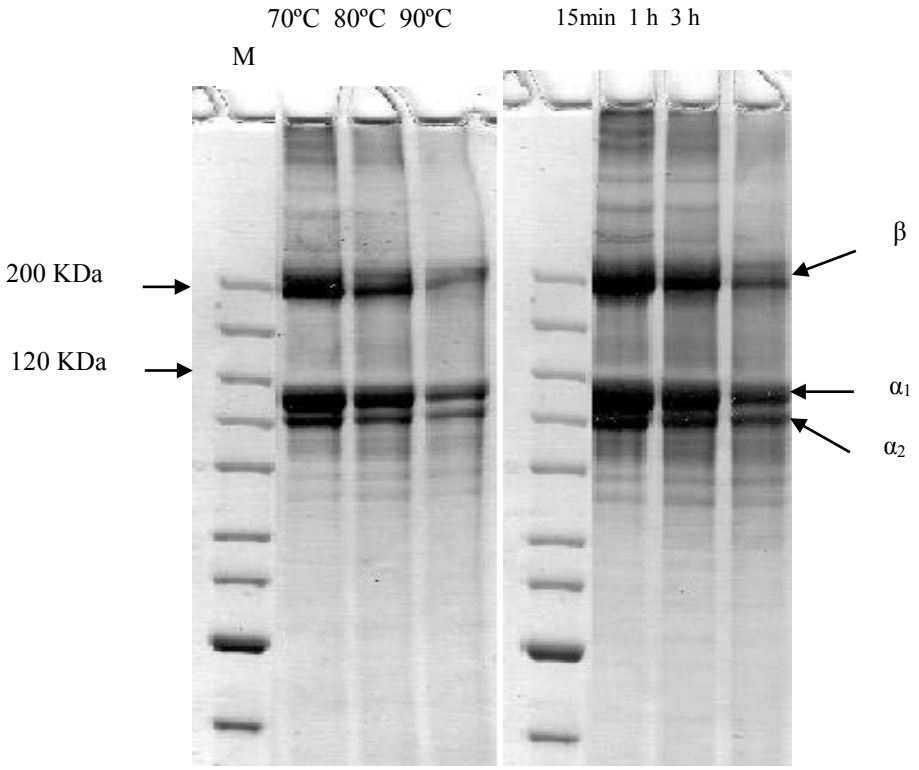


Fig. 3.2 SDS-PAGE patterns of gelatin films at different extraction temperatures (70, 80 and 90ºC for 1h) and times (at 70ºC for 15 min, 1 h and 3 h).

"M" means protein marker.

3.3.3. Effect of protein concentration of FFS on gelatin film

3.3.3.1. Mechanical properties

TS and EAB of gelatin films with various protein concentrations are shown in Table 3.5. TS of gelatin film increased from 19.40 ± 4.75MPa to 36.77 ± 4.13MPa with increasing protein concentration from 1 to 3%. EAB increased from 38.76 ± 9.77% to 48.77 ± 9.23% with increasing FFS protein concentration. The greater FFS protein content might result in increased aggregation of proteins forming the film, resulting in improved flexibility and mechanical properties of films (Jongjareonrak et al., 2006). TS and EAB of gelatin films from horse mackerel scale increased with increasing FFS protein concentration, which shows a similar trend to gelatin films from brownstripe red snapper and bigeye snapper skin (Jongjareonrak et al., 2006).

Table 3.5 Effect of FFS[*] protein concentration on thickness, tensile strength (TS), elongation at break (EAB) and water vapor permeability (WVP) of gelatin films

Protein concentration	Thickness (μm)	TS (MPa)	EAB (%)	WVP ($x10^{-10}gm^{-1}Pa^{-1}s^{-1}$)
1%	10.65 ± 0.33^{a}	19.40 ± 4.75^{a}	38.76 ± 9.77^{a}	0.46 ± 0.05^{a}
2%	23.58 ± 0.52^{b}	36.48 ± 3.66^{bc}	46.09 ± 7.44^{ab}	0.98 ± 0.07^{b}
3%	38.48 ± 0.71^{c}	36.77 ± 4.13^{b}	48.77 ± 9.23^{b}	1.51 ± 0.07^{c}

[*1] FFS means film-forming solution.

Data are expressed as mean ± standard deviation (n=6).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

3.3.3.2. Water vapor permeability

The effect of various FFS protein concentrations on the WVP values of gelatin films is shown in Table 3.5. WVP of gelatin films increased from 0.46 ± 0.05 to 1.51 ± 0.07 x 10^{-10} $gm^{-1}Pa^{-1}s^{-1}$ with increasing FFS protein concentration. Notably, greater WVP values were observed in gelatin films with increased thickness. WVP values of gelatin films from horse mackerel scale were lower than gelatin films from brownstripe red snapper and bigeye snapper skin (Jongjareonrak et al., 2006) at identical FFS protein concentration (2%). The lower WVP values of horse mackerel gelatin film may be explained by the greater amounts of hydrophobic amino acids. The gelatin film in the present study contained a greater amount of hydrophobic amino acids (total 653 residues per 1000 residues) compared with mammalian (total 622-652 residues per 1000 residues), cold-water fish (total 638-654 residues per 1000 residues) and warm-water fish (total 526-649 residues per 1000 residues) gelatins (Avena-Bustillos et al., 2006).

3.3.3.3. Light transmission of gelatin film (%)

The effect of FFS protein concentration on the light transmission of gelatin films from horse mackerel scale is shown in Table 3.6. Light transmission at each wavelength from 280 to 800nm decreased with increasing FFS protein concentration. A likely explanation is that gelatin films with greater thickness would absorb the light more effectively than those with lower thickness, leading to low light transmission in both the UV and visible ranges.

Table 3.6 Effect of FFS* protein concentration on the light transmission (%) of gelatin films

Protein concentration	Light transmission at different wavelength (nm)						
	200	280	350	400	500	600	800
1%	0.3	23.18 ± 0.89[b]	34.30 ± 1.51[b]	59.40 ± 1.07[b]	69.68 ± 0.88[c]	75.13 ± 0.82[b]	80.62 ± 0.87[b]
2%	0.3	16.35 ± 2.51[a]	30.42 ± 2.78[a]	52.70 ± 1.14[a]	62.60 ± 1.58[b]	69.43 ± 2.12[a]	75.77 ± 2.03[a]
3%	0.3	11.78 ± 1.57[a]	36.43 ± 1.91[a]	47.17 ± 0.96[a]	55.23 ± 1.79[a]	78.63 ± 1.24[c]	79.98 ± 1.43[b]

*1 FFS means film-forming solution.

Data are expressed as mean ± standard deviation (n=6).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

3.3.3.4. SDS-PAGE

Protein patterns of gelatin films prepared with different FFS protein concentrations in Fig. 3.3 showed that all gelatin films consisted of two different α chains, α1 and α2, as well as a β component; furthermore, there were no differences in patterns among the films (in Figure 3.3). Limpisophon et al. (2009) reported no differences in the protein patterns of gelatin films from under-utilized blue shark skin prepared with various FFS protein concentrations.

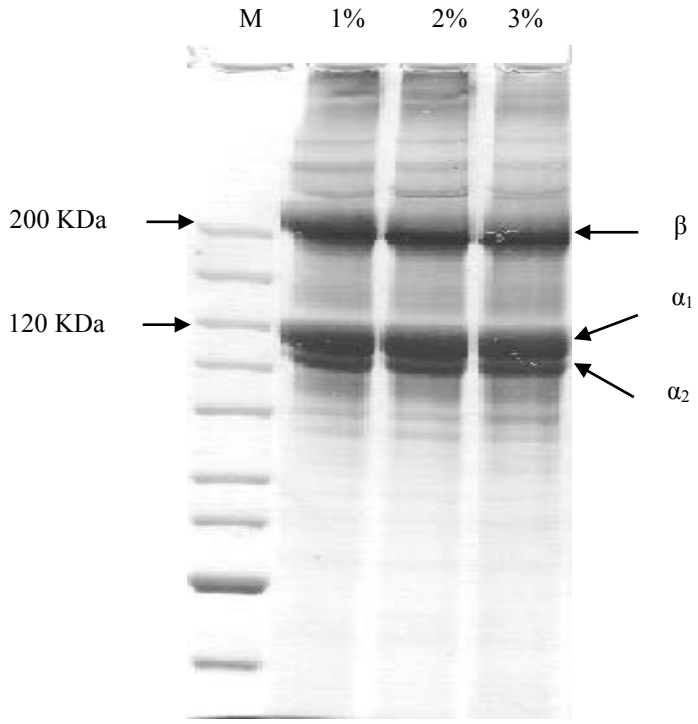


Fig. 3.3 SDS-PAGE patterns of gelatin films at varying protein concentrations (1, 2 and 3 %) in film-forming solution (FFS)

"M" means protein marker

3.3.4 Effect of glycerol concentration on properties of gelatin film

3.3.4.1. Mechanical properties

TS and EAB of gelatin film from a 2% protein in FFS at various glycerol concentrations are shown in Table 3.7. TS decreased from 48.14 ± 1.94 to 29.63 ± 4.12MPa with increasing glycerol concentration from 0 to 25%. These results are similar to the gelatin film from shark skin (Limpisophon et al., 2009) and brownstripe red snapper and bigeye snapper (Jongjareonrak et al., 2006) at various FFS glycerol concentrations. Gelatin film is the hydrophilic film and glycerol, the small molecules, has the relatively with hydrophilic characteristic. When add Glycerol into FFS, it could be incorporated in the gelatin film network by estabilishing hydrogen bonds with amide group and amino acid side chains of protein (Gontard, Guibert, & Cuq, 1993), lead to the reduction of tensile strength of gelatin film.

On the other hand, EAB of gelatin film from horse mackerel scale increased sharply from 4.22 ± 1.01 to 51.15 ± 4.99 %. The result was in agreement with Limpisophon et al (2009) who reported EBA of gelatin film from shark skin increased from 1.57 to 80.40 % when glycerol content increased from 0 to 50%. The presence of plasticizer causes a reduction in intermolecular interaction and also increases the mobility of macromolecules (Gontard et al., 1993). Furthermore, the moisture content of gelatin film increases with increasing the plasticizer concentration because of its high hygroscopic character (Sobral, Menegalli, & Guilbert, 1999).

Table 3.7 Effect of glycerol concentrations in FFS[*1] on thickness, tensile strength (TS), elongation at break (EAB) and water vapor permeability (WVP) of gelatin film

Glycerol concentration (%)	Thickness (μm)	TS (MPa)	EAB (%)	WVP ($x10^{-10}gm^{-1}Pa^{-1}s^{-1}$)
0%	18.93 ± 0.36^{a}	48.14 ± 1.94^{e}	4.22 ± 1.01^{a}	0.74 ± 0.07^{a}
10%	19.68 ± 0.45^{b}	45.08 ± 2.41^{d}	15.11 ± 1.99^{b}	0.79 ± 0.06^{a}
15%	21.56 ± 0.38^{c}	39.92 ± 3.12^{c}	28.09 ± 2.59^{c}	0.89 ± 0.03^{b}
20%	23.58 ± 0.52^{d}	36.48 ± 3.66^{b}	46.09 ± 7.44^{d}	0.98 ± 0.07^{c}
25%	24.42 ± 0.33^{e}	29.63 ± 4.12^{a}	51.15 ± 4.99^{e}	1.08 ± 0.11^{d}

[*1] FFS means film-forming solution.

Data are expressed as mean ± standard deviation (n=6).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

3.3.4.2. Water vapor permeability

WVP of films prepared from gelatin of horse mackerel scale at different glycerol contents is presented in Table 3.7. WVP values of films with 10% glycerol and that without glycerol were no significant difference ($p > 0.05$). WVP of films increased with increasing glycerol level ($p < 0.05$). Glycerol, a hygroscopic plasticier includes three hydroxyl group (-OH), was able to attract water to the gelatin film system, lead to increasing WVP of the film (Sorbral et al., 1999; Limpisophon et al., 2009). Furthermore, the increase in the WVP rates of gelatin films with increasing glycerol level was possibly caused by an increase in free volume of system which enhanced the mobility of the polymeric chains, due to the insertion of glycerol between protein molecules. Therefore, the network structure of films became less dense and more permeable (Gontard et al., 1993). The increase of WVP of gelatin film with increasing glycerol concentration was observed in gelatin films from shark skin (Limpisophon et al., 2009), brownstripe red snapper and big snapper skin (Jongjareonrak et al., 2006) and cuttlefish skin (Hoque et al., 2011). In the contrary, the reduction of WVP was observed in wheat starch edible film with increasing glycerol concentration from 0-20% (Farahnaky et al., 2013). It may be explained due to the difference in material to form the edible film (gelatin film and wheat starch film).

3.3.4.3. Light transmission

Transmission of UV and visible light at selected wavelength in the range of 200-800nm to gelatin film from scale of horse mackerel at different glycerol contents is shown in Table 3.8. Transmission in visible range (350-800nm) of gelatin films was from 28.05 ±

1.12 to 79.62 ± 1.15 %. The transmission of UV light at 280 nm was in the range of 13.75 ± 0.53 - 17 ± 1.41 %. Very low transmission (0.3 %) was found at 200nm. Consequently, gelatin film from horse mackerel scale effectively prevented the UV light when compare with synthetic film except polyester film. The similar results were also observed for gelatin film from shark skin (Limpisophon et al., 2009), brownstripe red snapper and big snapper skin (Jongjareonrak et al., 2006).

Table 3.8 Effect of glycerol concentrations in FFS[*1] on light transmission (%) of gelatin films

Glycerol content	Light transmission at different wavelength (nm)						
	200	280	350	400	500	600	800
0%	0.3	13.75 ± 0.53^{c}	28.05 ± 1.12^{a}	48.21 ± 0.62^{c}	64.16 ± 5.03^{a}	74.32 ± 1.97^{a}	79.62 ± 1.15^{a}
10%	0.3	14.40 ± 1.86^{bc}	29.62 ± 1.60^{a}	49.44 ± 3.00^{bc}	63.85 ± 3.05^{a}	72.16 ± 0.42^{b}	78.11 ± 0.50^{ab}
15%	0.3	15.82 ± 1.31^{abc}	30.33 ± 4.32^{a}	51.12 ± 1.31^{abc}	63.52 ± 0.59^{a}	70.02 ± 0.52^{a}	76.63 ± 0.82^{bc}
20%	0.3	16.35 ± 2.51^{ab}	30.42 ± 2.78^{a}	52.70 ± 1.14^{ab}	62.60 ± 1.58^{a}	69.43 ± 2.12^{c}	75.77 ± 2.03^{c}
25%	0.3	17.00 ± 1.41^{a}	30.78 ± 0.66^{a}	53.48 ± 5.56^{a}	60.18 ± 6.38^{a}	69.02 ± 1.80^{c}	75.12 ± 1.37^{c}

[*1] FFS means film-forming solution.

Data are expressed as mean ± standard deviation (n=6).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

3.3.4.4. SDS-PAGE

SDS – PAGE patterns of gelatin films from horse mackerel scale at different glycerol concentrations are presented in Fig 3.4. The SDS-PAGE patterns showed that all gelatin films at all glycerol concentrations consisted of two different α chains, α1 and α2, as well as a β component, and there were no differences in patterns among the glycerol content. Limpisophon et al (2009) observed no differences in the protein patterns of gelatin film from under-utilized blue shark skin at various glycerol concentrations. It can be explained that film of all gelatin samples were most likely stabilized by weak bond, especially hydrogen bond. These bonds were destroyed in the presence of SDS as well as mercaptoethanol used for electrophoresis, lead to no differences in protein pattern of gelatin film at different glycerol levels.

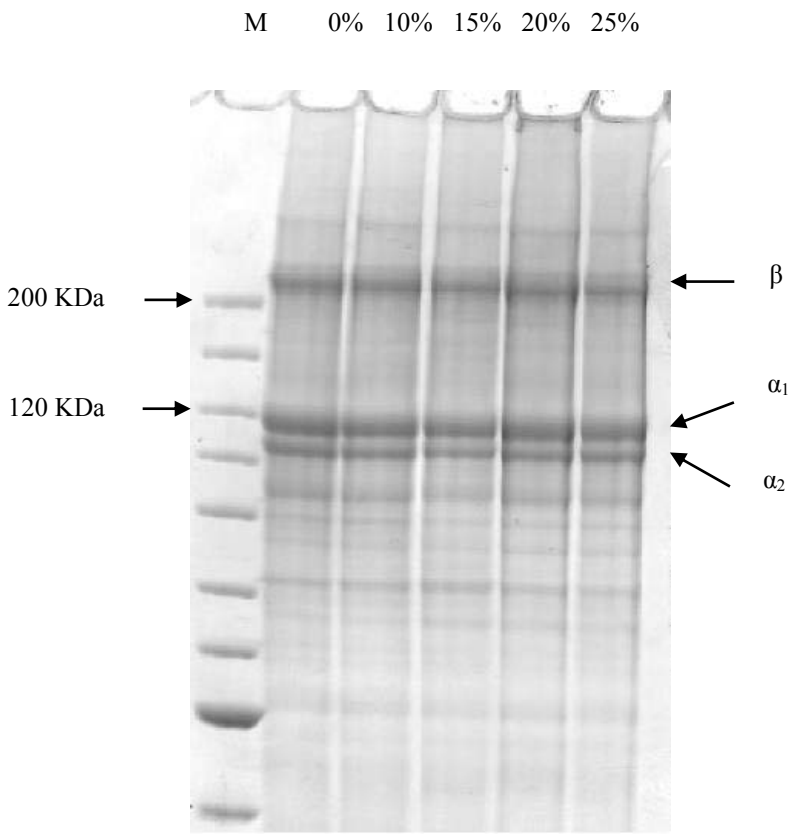


Fig. 3.4 SDS-PAGE patterns of gelatin films at different glycerol concentrations (0, 10, 15, 20 and 25 %) in film-forming solution (FFS)

"M" means protein marker

3.4 Summary of chapter 3:

The properties of gelatin film from horse mackerel scales were affected by gelatin extraction temperature and time, as well as FFS protein concentration. An extraction temperature higher than 70ºC and an extraction time longer than 1h resulted in a deterioration of mechanical properties and an increase in WVP of the gelatin film. Gelatin films from horse mackerel scale exhibited lower WVP values than gelatin films from the skin of other fishes or mammals. Thus, it was demonstrated that film can be produced from horse mackerel scale gelatin, and that the film properties can be modified according to the intended purpose as packaging material in food industry.

CHAPTER 4

Properties of gelatin film from horse mackerel (*Trachurus japonicus*) scale incorporated with various phenolic compounds

4.1 Introduction:

Polyethylene material was used as a traditional method to package food products. However, this material caused a serious global environmental problem by increasing large volumes of non-biodegradable waste (Kirwan & Strawbridge, 2003). Thus, gelatin film was expected to become new biodegradable film to solve this problem of polyethylene film. Gelatin is a protein derived by partial hydrolysis of collagen extracted from skin, bones, connective tissues, organs and some intestines of mammalian species, which is widely used in the manufacture of edible film (Johnston-Banks, 1990). However, concerns regarding bovine and porcine health, such as the emergence of bovine spongiform encephalopathy and foot and mouth disease, have led to an increasing attention of fish gelatin (Gómez-Guillén, Giménez, López-Caballero & Montero, 2011) in recent years. Fish gelatin film, which is biodegradable film, has known as important eco-friendly packaging material to reduce the environmental impact of synthetic plastic materials (Nagarajan, Benjakul, Prodpran & Songtipya, 2012). Furthermore, fish gelatin films incorporated with antioxidative additives used as covering foodstuffs have capacity to protect food against chemical changes of food composition during storage (Jongjareonrak, Benjakul, Visessanguan & Tanaka, 2008). Some authors reported antioxidative activity of fish gelatin film by adding antioxidative compounds, such as gelatin film from silver carp with green tea extract (Wu, Chen, Ge,

Miao, Li & Zhang, 2013), bigeye snapper and brown-stripe red snapper gelatin film with BHT and α-tocopherol (Jongjareonrak et al., 2008) tuna skin gelatin with oregano and rosemary extract (Gómez-Estaca, Bravo, Gomez-Guillén, Alemán & Montero, 2009).

Therefore, the aim of this study was to investigate the effect of phenolic concentration on the properties and antioxidative activity of horse mackerel scale gelatin film.

4.2 Materials and methods:

4.2.1. Preparation of fish scales

Horse mackerel scale was collected in Nagasaki Prefecture, Japan. The scale was collected and transported to our laboratory under ice condition. The fish scale was removed of impurities manually, washed with chilled water, and then placed in plastic bags and stored at – 30°C ± 2°C until use.

4.2.2. Extraction of gelatin

Gelatin was extracted from horse mackerel scale according to the method of Wangtueai and Noomhorm (2009) with slight modifications. Briefly, frozen scale was thawed and treated with 0.1M NaOH solution to remove the noncollagenous protein. The alkali solution was changed after 3 h and scales were washed thoroughly in cold distilled water to obtain a neutral pH. After the removal of noncollagenous protein, the fish scales were drained using cheesecloth, squeezed manually and then soaked in 10x distilled water (w/v) at 70°C for 1h with continuous stirring to extract gelatin.

4.2.3. Preparation of gelatin films with various phenolic compounds

Gelatin powder was dissolved in distilled water at 60ºC for 30min to obtain the film-forming solution (FFS) with the protein concentration of 2% (w/v). Glycerol was used as a plasticizer by adding into FFS at the concentration of 20% (w/w) of protein. Then the phenolic compounds including caffeic acid, catechin, gallic acid, ferulic acid, rutin and tannic acid were added into FFS at the concentration of 0, 1, 3, 5 and 10% (w/w) of protein with continuous stirring at room temperature for 1h. The air bubbles in FFS were removed by a hybrid mixer (HM – 500; Keyence Co., Tokyo, Japan). The prepared FFS (4g) was cast onto a rimmed silicone plate (50 x 50mm) and dried at a ventilated oven (EYELA, KCL – 2000A, Tokyo Rikakikai Co., Ltd., Japan) at 25 ± 0.5ºC and 50 ± 5% relative humidity (RH) for 48h. Dried films obtained were manually peeled off.

4.2.4. Properties of gelatin films

4.2.4.1. Moisture content of gelatin films

Moisture content of gelatin films was determined following the methods of AOAC (2000).

4.2.4.2. Thickness

Film thickness was measured as described in the previous study (chapter 3) by using a dial-type thickness gage (Series 7300; Mitsutoyo Co., Kanagawa, Japan).

4.2.4.3. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of film samples were determined according to the ASTM standard method D 882 – 97 (ASTM, 1999) as described in chapter 3.

4.2.4.4. Water vapor permeability

Water vapor permeability (WVP) of film was measured according to the ASTM standard method (1983) as reported by Gontard, Guilbert, and Cuq (1992).

4.2.4.5. Light transmission and transparency

Light transmission in ultraviolet (UV) and visible ranges of gelatin films was measured at selected wavelengths between 200 and 800nm using a UV-Visible Recording Spectrophotometer (UV-160, Shimazu Co., Kyoto, Japan) according to the method of Fang, Tung, Britt, Yada & Dalgleish (2002) as described in chapter 3.

4.2.4.6. SDS – polyacrylamide gel electrophoresis (SDS-PAGE)

SDS – PAGE of gelatin films from horse mackerel scale was analyzed according to the method of Limpisophon, Tanaka, Weng, Abe & Osako (2009) with slight modifications as described in chapter 3.

4.2.5. Total phenolic content and antioxidative activity of films

Gelatin films at various concentrations of phenolic compounds (10mg) were dissolved in 10ml distilled water at 45ºC, and then centrifuged at 10,000 x *g* for 10min under 20ºC. The film supernatants were collected and used to determiner the total phenolic content and antioxidative activity of films.

4.2.5.1 Total phenolic content

Total phenolic compound of gelatin film incorporated with various phenolic compounds was evaluated according to the method of Singleton, Orthofer & Lamuela-Raventos (1999) with Folin-Ciocalteu reagent by using gallic acid as a standard. The film supernatant (0.5 ml) mixed with 0.1ml of Folin-Ciocalteu reagent (two-fold diluted with ion-exchange water). After 3min, 1.5ml of 2% Na_2CO_3 was added. The mixture was allowed to stand at room temperature for 30min. The absorbance was measured at 760nm by using a spectrophotometer (V-630 Bio spectrophotometer, Jasco Co., Japan). The concentration of total phenolic compounds in the gelatin film samples was determined from a standard curve generated with gallic acid (0.01 – 0.1mg/ml) and expressed as mg of gallic acid equivalent (GAE)/g film samples. The concentration of total phenolic compounds was calculated by the following equation that was obtained from the standard graph ($R^2 = 0.99$)

Abs760 = 18.34 mg gallic acid + 0.04

4.2.5.2. DPPH radical scavenging activity

The antioxidative activity of gelatin films at different concentrations of phenolic compounds was determined by using DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging according to method of Blois (1958) with slight modifications. Firstly, 0.2ml of film supernatant was mixed with 2ml of 0.2mM ethanol solution of DPPH. The mixture was incubated in the dark at room temperature for 30min. Then the mixture was centrifuged at 8,000 x *g* for 5min and the absorbance was read against a blank at 517nm. The DPPH radical scavenging activity was calculated by the following equation.

$$\text{Scavenging activity (\%)} = \frac{(Abs_{blank} - Abs_{sample}) \times 100\%}{Abs_{blank}}$$

where Abs_{blank} and Abs_{sample} are the absorbance of control and sample, respectively.

4.2.6. Fourier transform infrared spectroscopy (FTIR) of gelatin films

FTIR spectra of gelatin films incorporated with various phenolic compounds at different concentrations were analyzed using Omnic 6.0 sofware (Thermo-Nicolet, Madison, Wisconsin). The spectra were recorded using infrared spectrophometer (Nicolet 200SXV) from 4000 to 500cm^{-1} at a resolution of 2cm^{-1} per point at room temperature.

4.2.7. Statistical analysis

All experiments for properties of films were performed 6 times and for total phenolic content and DPPH free radical scavenging were performed triplicate, and the data were expressed as mean ± standard deviation. The differences between variables were evaluated basing on the Duncan's multiple range tests. Analysis was performed by using SPSS software (SPSS 11.5 for Windows).

4.3 Results and discussion:

4.3.1. Characterization of gelatin film from horse mackerel scale incorporated with various phenolic compounds

4.3.1.1. Moisture content

Moisture content of gelatin films were from 17.41 to 17.48%, depending on the type of phenolic compound added in gelatin film. However, no statistical differences were found among the films.

4.3.1.2. Mechanical properties

Tensile strength (TS) and elongation at break (EAB) of gelatin films incorporated with various phenolic compounds at different concentrations are shown in Table 4.1 and 4.2.

TS of gelatin films incorporated with ferulic acid, tannic acid, caffeic acid, gallic acid, catechin and rutin increased from 27.53 ± 1.40MPa (without phenolic content) to 31.88 ± 2.59, 31.49 ± 1.72, 30.13 ± 3.03, 31.36 ± 4.04 and 35.38 ± 3.94MPa, respectively with increasing phenolic concentration. On the other hand, EAB of all gelatin film decreased from 46.09 ± 7.43 (without phenolic content) to 25.35 ± 3.16, 24.36 ± 3.52, 24.99 ± 3.54, 25.82 ± 3.27, 26.80 ± 2.73 and 17.60 ± 2.82%, respectively with the addition of phenolic compound. Increasing of TS and decreasing of EAB of gelatin films when increasing phenolic concentration were the similar tendency of gelatin film incorporated with green tea extract (Wu et al., 2013) and gelatin film with ferulic acid and tannin acid (Cao, Fu & He, 2007). It can be explained that phenolic compounds contain many hydrophobic groups, which can form hydrophobic interaction with hydrophobic region of gelatin molecules (Wu et al., 2013). Furthermore, Gomez-Guillén et at., 2009 reported that in structure of phenolic compounds contain many hydrogen groups, which was able to combine with hydrogen acceptor in gelatin molecules by hydrogen bond, lead to development of a stronger and stiffer film structure.

Table 4.1 Tensile strength (MPa) of gelatin film incorporated with various phenolic compounds

Film type	Phenolic concentration				
	0%	1%	3%	5%	10%
Ferulic acid	27.53 ± 1.40^{a}	30.70 ± 2.54^{b}	31.60 ± 4.44^{b}	31.88 ± 2.59^{b}	28.42 ± 1.57^{a}
Tannic acid	27.53 ± 1.40^{a}	28.08 ± 0.93^{a}	30.73 ± 1.38^{b}	31.49 ± 1.72^{b}	27.24 ± 2.00^{a}
Caffeic acid	27.53 ± 1.40^{a}	28.39 ± 6.38^{a}	28.97 ± 2.29^{a}	29.31 ± 1.97^{a}	27.19 ± 1.60^{a}
Gallic acid	27.53 ± 1.40^{a}	28.54 ± 3.42^{a}	29.54 ± 1.15^{a}	30.13 ± 3.03^{b}	27.56 ± 1.31^{a}
Catechin	27.53 ± 1.40^{a}	28.70 ± 1.51^{a}	30.84 ± 3.21^{b}	31.36 ± 4.04^{b}	27.75 ± 4.39^{a}
Rutin	27.53 ± 1.40^{a}	34.83 ± 3.58^{b}	35.02 ± 3.04^{b}	35.38 ± 3.94^{b}	33.53 ± 5.83^{b}

Data are expressed as mean ± standard deviation (n=6).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

Table 4.2 Elongation at break (%) of gelatin film incorporated with various phenolic compounds

Film type	Phenolic concentration				
	0%	1%	3%	5%	10%
Ferulic acid	46.09 ± 7.43^{a}	46.58 ± 8.02^{a}	45.35 ± 6.44^{a}	39.48 ± 8.21^{b}	25.35 ± 3.16^{c}
Tannic acid	46.09 ± 7.43^{a}	44.58 ± 8.20^{a}	41.57 ± 5.36^{a}	35.66 ± 3.61^{b}	24.36 ± 3.52^{c}
Caffeic acid	46.09 ± 7.43^{a}	45.21 ± 5.21^{a}	40.88 ± 4.31^{b}	34.96 ± 3.31^{c}	24.99 ± 3.54^{d}
Gallic acid	46.09 ± 7.43^{a}	46.23 ± 4.64^{a}	42.73 ± 6.89^{ab}	38.48 ± 6.09^{c}	25.82 ± 3.27^{d}
Catechin	46.09 ± 7.43^{a}	46.66 ± 4.03^{a}	44.89 ± 4.43^{a}	38.35 ± 2.61^{b}	26.80 ± 2.73^{c}
Rutin	46.09 ± 7.43^{a}	32.18 ± 3.69^{b}	28.05 ± 4.40^{c}	21.67 ± 4.57^{d}	17.60 ± 2.82^{e}

Data are expressed as mean ± standard deviation (n=6).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

4.3.1.3. WVP of gelatin film

The water vapor permeability of film from gelatin with the addition of phenolic compounds at different levels is shown in Table 4.3. WVP of gelatin-phenolic films increased with increasing phenolic concentration from 0 to 10%. This might be gelatin and phenolic compounds contained the hightly hydroscopic leading to an increase in WVP values of film with increasing phenolic concentration. Ahmad, Benjakul, Prodpran & Agustini (2012) reported that WVP of gelatin-bergamot oil tended to increase with higher amount of bergamot oil. However, WVP of wheat gluten film incorporated with gallic acid and tannic acid decreased with increasing phenolic concentration (Hager, Vallons & Arendt., 2012). This can be explained that the differences in the structure of gelatin film and wheat gluten film leading to the differences of WVP value.

Table 4.3 Water vapor permeability ($x10^{-10}gm^{-1}Pa^{-1}s^{-1}$) of gelatin film incorporated with various phenolic compounds

Film type	Phenolic concentration				
	0%	1%	3%	5%	10%
Ferulic acid	0.99 ± 0.07^{a}	0.99 ± 0.06^{a}	1.00 ± 0.09^{a}	1.04 ± 0.02^{b}	1.10 ± 0.01^{c}
Tannic acid	0.99 ± 0.07^{a}	1.13 ± 0.02^{b}	1.18 ± 0.04^{c}	1.19 ± 0.01^{c}	1.20 ± 0.03^{c}
Caffeic acid	0.99 ± 0.07^{a}	1.04 ± 0.04^{b}	1.15 ± 0.05^{c}	1.17 ± 0.01^{c}	1.24 ± 0.06^{d}
Gallic acid	0.99 ± 0.07^{a}	1.04 ± 0.03^{b}	1.10 ± 0.03^{c}	1.14 ± 0.02^{cd}	1.17 ± 0.02^{d}
Catechin	0.99 ± 0.07^{a}	1.05 ± 0.02^{b}	1.15 ± 0.05^{c}	1.16 ± 0.02^{c}	1.18 ± 0.02^{c}
Rutin	0.99 ± 0.07^{a}	1.02 ± 0.02^{a}	1.11 ± 0.03^{b}	1.15 ± 0.01^{bc}	1.17 ± 0.02^{c}

Data are expressed as mean ± standard deviation (n=6).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

4.3.1.4. Light transmission of gelatin film (%)

The transmission of gelatin film prepared from gelatin of horse mackerel scales incorporated with phenolic compounds at various concentrations in UV range could not be measured, regardless of type and concentration of phenolic compounds added in the gelatin films (data shown in table 4.4). It can be explained that the amino acid composition of gelatin consists of aromatic amino acid with phenol ring, such as phenylalanine, tryptophan and tyrosine could prevent UV transmission. Furthermore, phenolic compounds with phenol ring in the structure, lead to an increase in the ability prevent UV transmission when incorporated with gelatin film. In the visible range, gelatin films had lower transmission with increasing the concentration of phenolic compounds, indicating that the addition of phenolic compounds could improve the light barrier properties (Wu et al., 2013). Wu et al. (2013) and Prodpran et al. (2012) also reported that the light barrier property was improved when increasing the concentration of green tea powder and phenolic compounds consist of catechin, caffeic acid, ferulic acid and tannic acid of gelatin film and fish myofibrillar protein film, respectively.

Table 4.4 Effect of phenolic concentration on light transmission (%) of gelatin films

	Light transmission at different wavelength (nm)						
	200	280	350	400	500	600	800
Caffeic acid							
0%	0.3	16.35 ± 2.51	30.42 ± 2.78	52.70 ± 1.14	62.60 ± 1.58	69.43 ± 2.12	75.77 ± 2.03
1%	0.3	0.30 ± 0.00	0.30 ± 0.00	20.05 ± 1.23	56.38 ± 1.52	69.87 ± 1.25	75.20 ± 2.22
3%	0.3	0.30 ± 0.00	0.30 ± 0.00	16.83 ± 1.11	52.10 ± 1.52	67.18 ± 1.44	74.20 ± 1.92
5%	0.3	0.30 ± 0.00	0.30 ± 0.00	10.43 ± 0.61	46.63 ± 2.29	60.35 ± 2.25	64.20 ± 1.92
10%	0.3	0.30 ± 0.00	0.30 ± 0.00	6.93 ± 0.53	38.43 ± 1.62	46.18 ± 1.63	55.40 ± 1.86
Catechin							
0%	0.3	16.35 ± 2.51	30.42 ± 2.78	52.70 ± 1.14	62.60 ± 1.58	69.43 ± 2.12	75.77 ± 2.03
1%	0.3	0.30 ± 0.00	0.30 ± 0.00	45.97 ± 1.08	63.03 ± 1.17	67.73 ± 1.22	75.50 ± 2.86
3%	0.3	0.30 ± 0.00	0.30 ± 0.00	41.67 ± 1.41	60.45 ± 1.36	65.40 ± 1.34	71.52 ± 1.04
5%	0.3	0.30 ± 0.00	0.30 ± 0.00	46.60 ± 2.10	57.90 ± 2.13	62.93 ± 1.89	68.27 ± 0.77
10%	0.3	0.30 ± 0.00	0.30 ± 0.00	39.02 ± 2.46	47.56 ± 1.44	58.50 ± 5.39	64.62 ± 3.39
Ferulic acid							
0%	0.3	16.35 ± 2.51	30.42 ± 2.78	52.70 ± 1.14	62.60 ± 1.58	69.43 ± 2.12	75.77 ± 2.03
1%	0.3	0.30 ± 0.00	0.30 ± 0.00	40.17 ± 2.42	62.23 ± 2.58	71.40 ± 2.49	80.62 ± 0.87
3%	0.3	0.30 ± 0.00	0.30 ± 0.00	19.67 ± 1.77	55.60 ± 3.58	66.25 ± 3.25	76.88 ± 2.55
5%	0.3	0.30 ± 0.00	0.30 ± 0.00	14.13 ± 2.13	34.57 ± 2.11	42.20 ± 1.61	51.47 ± 0.67
10%	0.3	0.30 ± 0.00	0.30 ± 0.00	14.13 ± 2.13	34.57 ± 2.11	42.20 ± 1.61	51.47 ± 0.67
Gallic acid							
0%	0.3	16.35 ± 2.51	30.42 ± 2.78	52.70 ± 1.14	62.60 ± 1.58	69.43 ± 2.12	75.77 ± 2.03
1%	0.3	0.30 ± 0.00	0.30 ± 0.00	27.12 ± 4.13	54.58 ± 0.22	69.18 ± 2.47	74.02 ± 4.77
3%	0.3	0.30 ± 0.00	0.30 ± 0.00	14.02 ± 1.09	52.00 ± 5.76	65.62 ± 6.24	65.48 ± 2.52
5%	0.3	0.30 ± 0.00	0.30 ± 0.00	11.18 ± 0.22	28.88 ± 2.94	54.52 ± 3.14	58.10 ± 3.29
10%	0.3	0.30 ± 0.00	0.30 ± 0.00	7.85 ± 2.19	20.17 ± 3.84	46.87 ± 4.58	56.90 ± 1.29
Rutin							
0%	0.3	16.35 ± 2.51	30.42 ± 2.78	52.70 ± 1.14	62.60 ± 1.58	69.43 ± 2.12	75.77 ± 2.03

1%	0.3	0.30 ± 0.00	0.30 ± 0.00	13.21 ± 3.75	50.07 ± 1.98	55.85 ± 2.12	61.72 ± 2.29
3%	0.3	0.30 ± 0.00	0.30 ± 0.00	9.13 ± 0.38	43.73 ± 2.26	49.68 ± 2.21	57.88 ± 2.77
5%	0.3	0.30 ± 0.00	0.30 ± 0.00	6.12 ± 0.45	40.07 ± 2.17	45.85 ± 2.34	55.88 ± 2.10
10%	0.3	0.30 ± 0.00	0.30 ± 0.00	3.07 ± 0.31	37.48 ± 0.48	41.38 ± 0.41	50.10 ± 1.71
Tannic acid							
0%	0.3	16.35 ± 2.51	30.42 ± 2.78	52.70 ± 1.14	62.60 ± 1.58	69.43 ± 2.12	75.77 ± 2.03
1%	0.3	0.30 ± 0.00	0.30 ± 0.00	33.72 ± 2.01	60.40 ± 1.95	70.87 ± 1.56	79.30 ± 1.12
3%	0.3	0.30 ± 0.00	0.30 ± 0.00	15.93 ± 1.14	57.10 ± 1.99	69.70 ± 1.42	78.67 ± 0.90
5%	0.3	0.30 ± 0.00	0.30 ± 0.00	9.83 ± 1.18	51.77 ± 1.42	67.18 ± 1.07	78.03 ± 0.84
10%	0.3	0.30 ± 0.00	0.30 ± 0.00	4.35 ± 0.63	26.90 ± 1.79	43.43 ± 2.35	56.48 ± 2.22

Data are expressed as mean ± standard deviation (n=6).

4.3.1.5. SDS-PAGE

Protein patterns of gelatin film from the scale of horse mackerel extracted incorporated with various phenolic compounds is shown in Fig. 4.1. From the protein pattern, α chain and β chain were found as the major components, and there were no differences in patterns among the phenolic concentration. Wu et al (2013) observed no differences in the protein patterns of gelatin film from silver carp skin incorporated with green tea extract. This may be because gelatin and phenolic compounds interacted through weak bond in the film forming solution, such as hydrogen bond and hydrophobic interaction. These bonds may have been destroyed when the films were diluted in SDS solution.

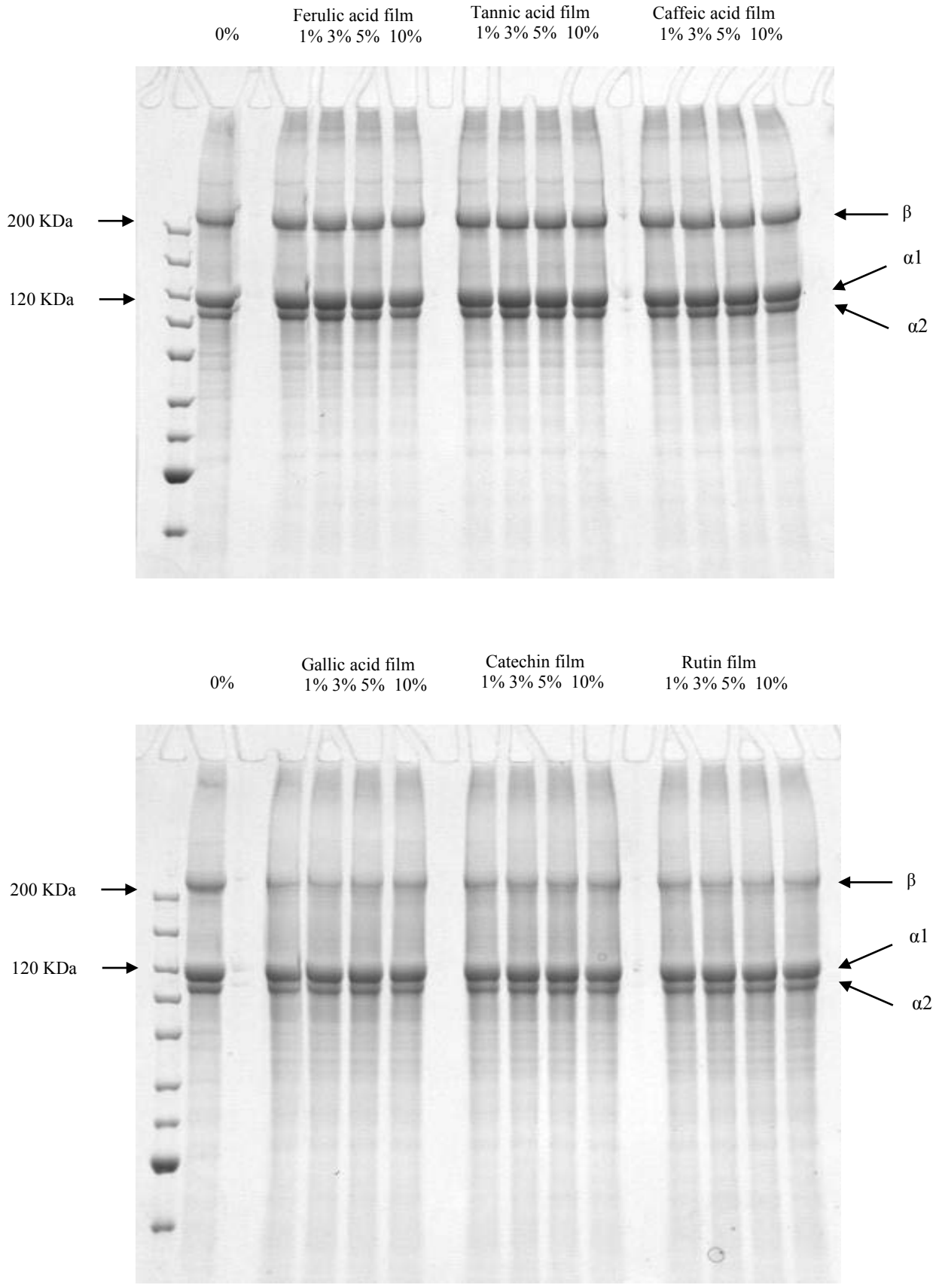


Fig 4.1 SDS-PAGE of gelatin film incorporated with various phenolic compounds

4.3.2. Total phenolic content and antioxidative activity of gelatin-phenolic compound films

The total phenolic content of the films was shown in Fig. 4.2. The total phenolic content of gelatin film without phenolic compound was 4.51mg/ml. The total phenolic content increased with increasing phenolic compound concentrations.

DPPH radical scavenging activity of gelatin film incorporated with various phenolic compounds are presented in Fig. 4.2. DPPH radical scavenging activity was used to indicate the antioxidative activity of the films. The gelatin film without phenolic was showed little scavenging activity on DPPH free radical. DPPH radical scavenging activity of the films incorporated with phenolic compounds significantly increased with increasing phenolic concentration from 1 to 10%. The reason might be the increasing the total phenolic content in gelatin-phenolic films.

The data of DPPH radical scavenging activity is commonly reported as IC50, which the concentration of antioxidative required of 50% scavenging of DPPH radical in the specified time period (Pokorný, 2001). The half inhibitory concentration (IC50) of each gelatin film is different, depend on the type of phenolic compound. Gelatin without phenolic had higher IC50 at 37.97mg/ml as compared to gelatin film incorporated with ferulic acid, caffeic acid, catechin, gallic acid, rutin and tannic acid (13.64, 14.27, 14.79, 14.97, 14.69 and 14.86mg/ml, respectively). However, the gelatin film including phenolic acid did not play more activity of radical scavenging than ascorbic acid, since ascorbic acid with low concentration (0.025 – 0.15mg/ml) could give similar scavenging activity of gelatin-phenolic film with higher content.

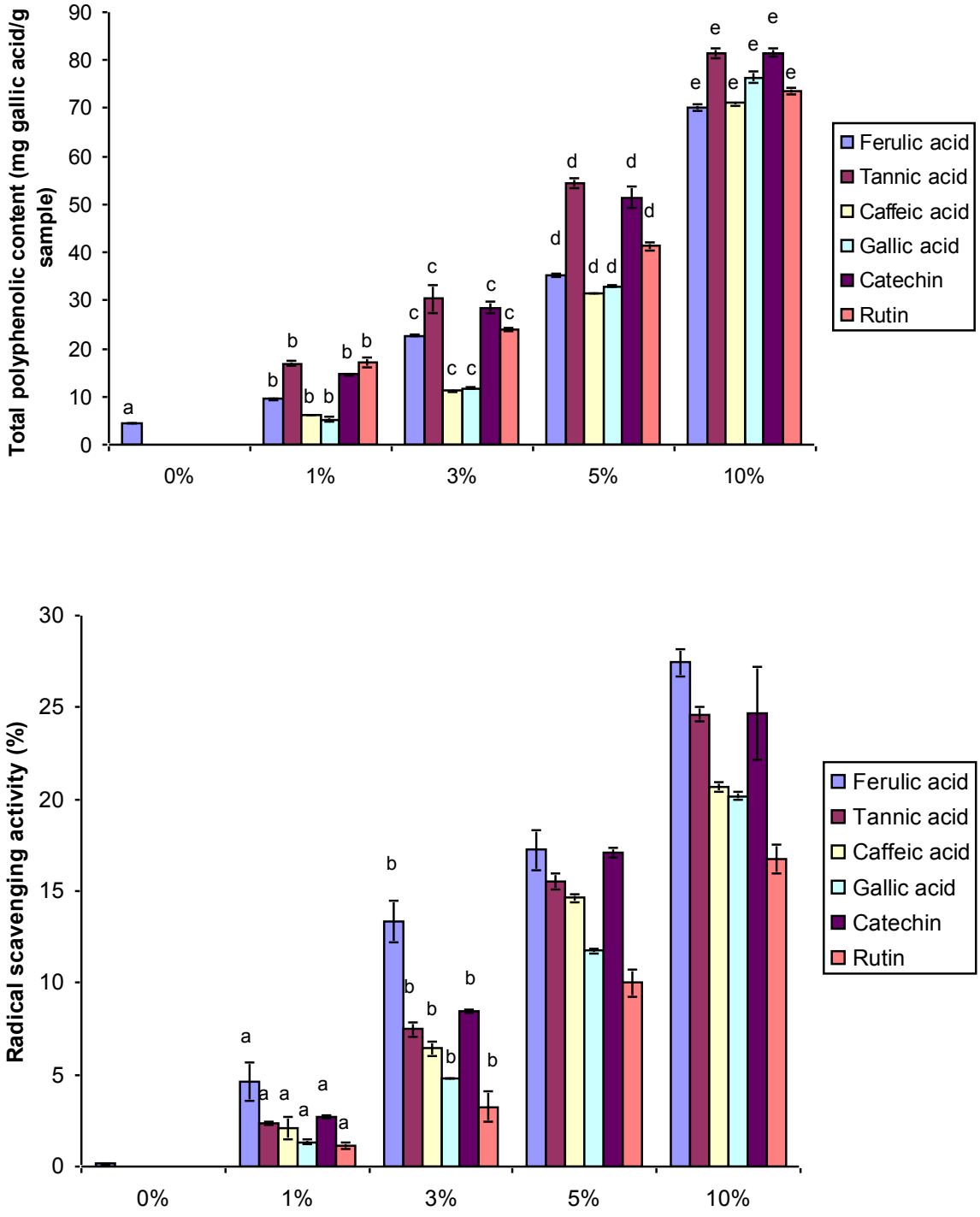
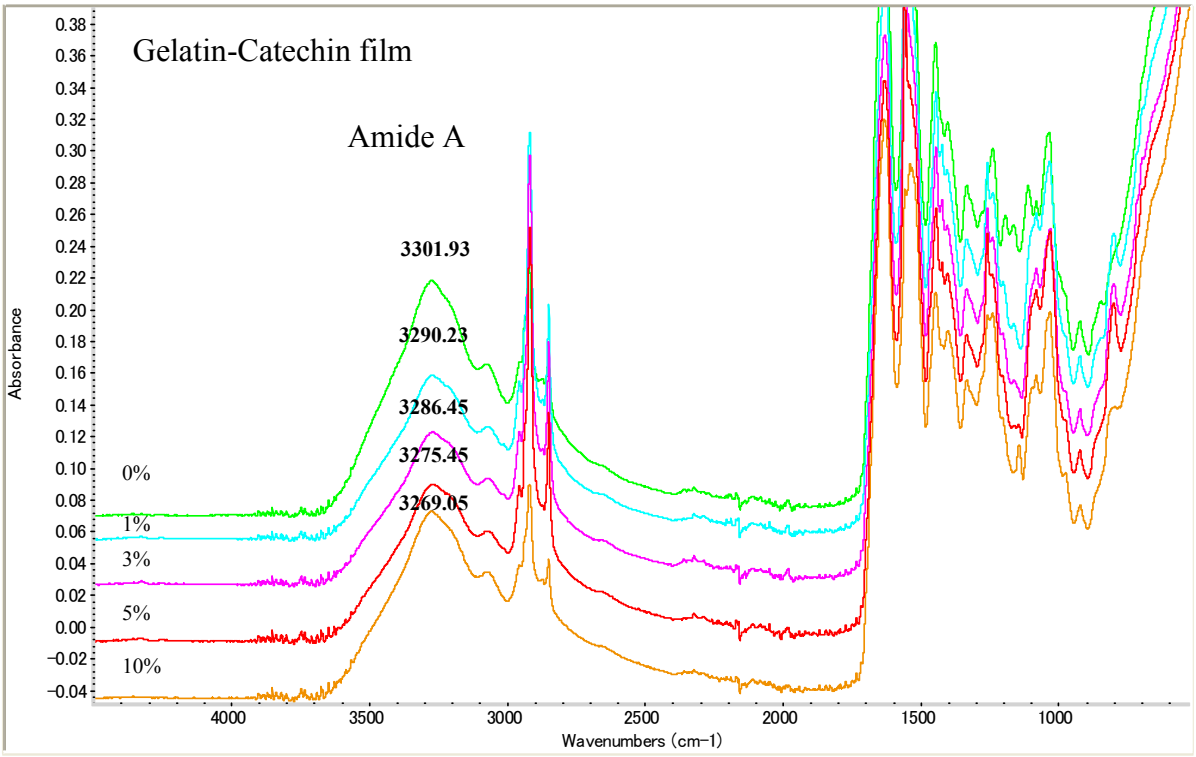
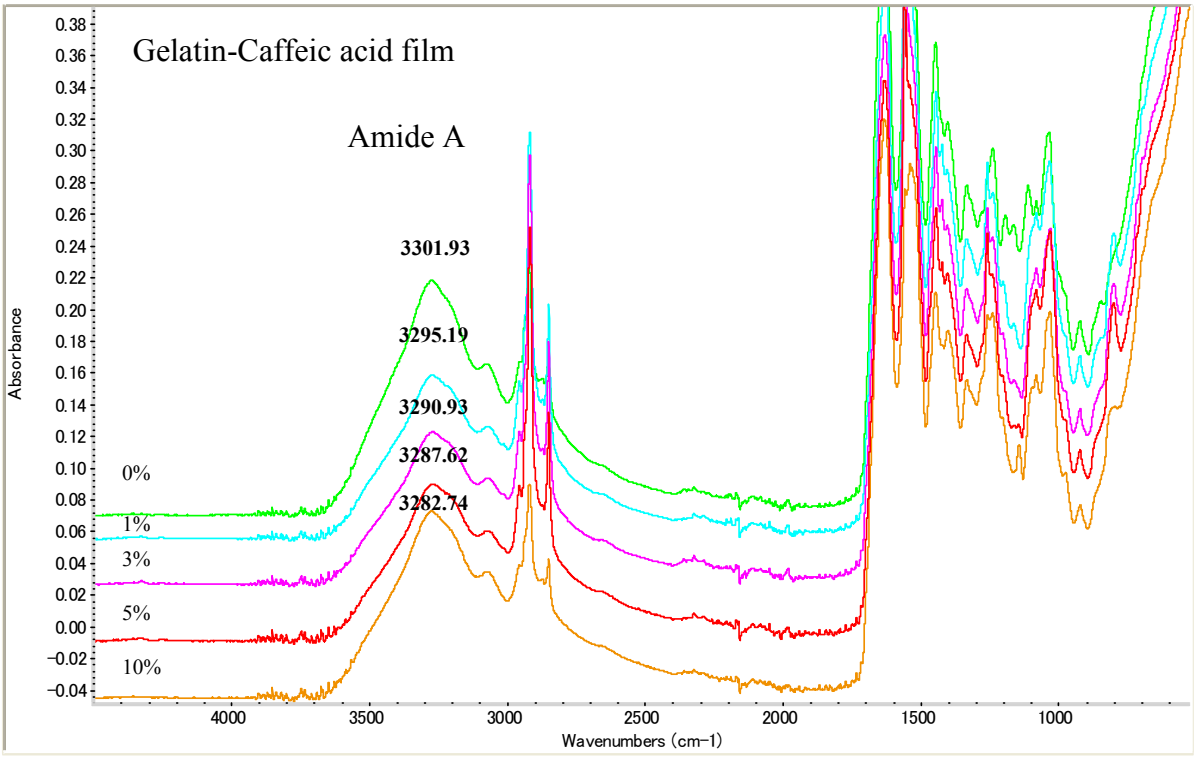


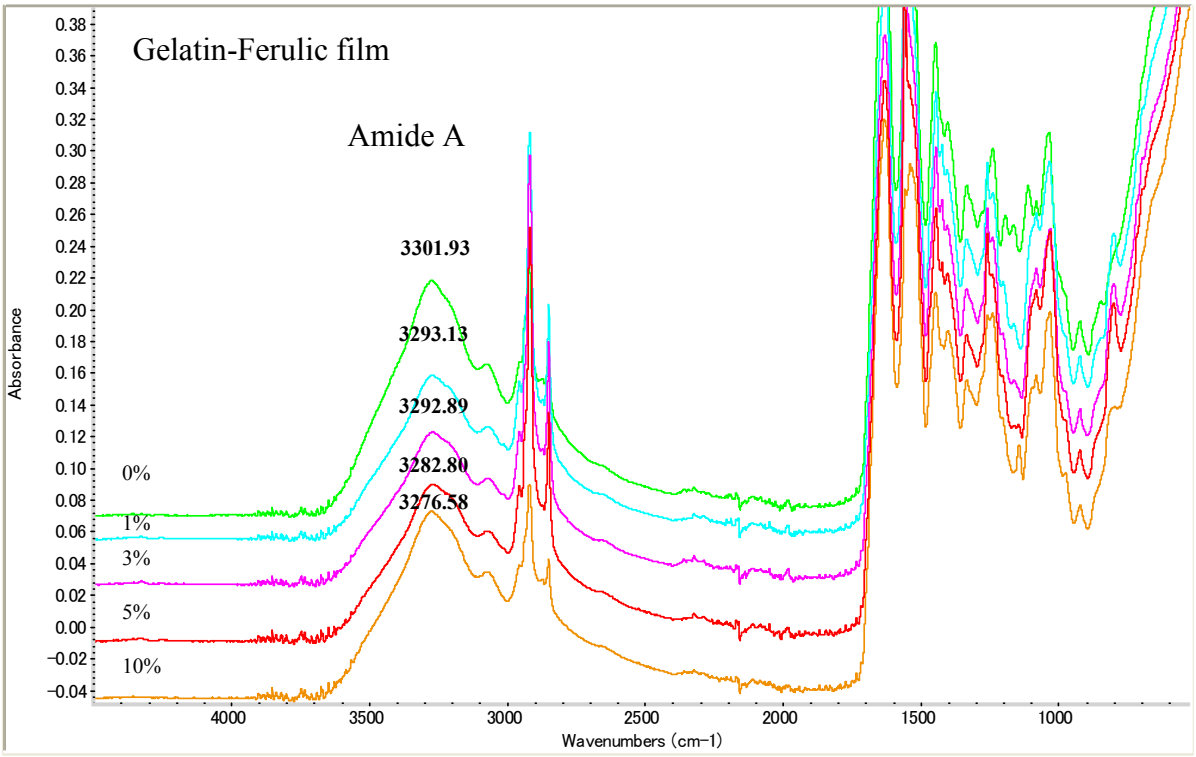
Fig 4.2 Total phenol content and DPPH scavenging activity of gelatin film incorporated with various phenolic compounds

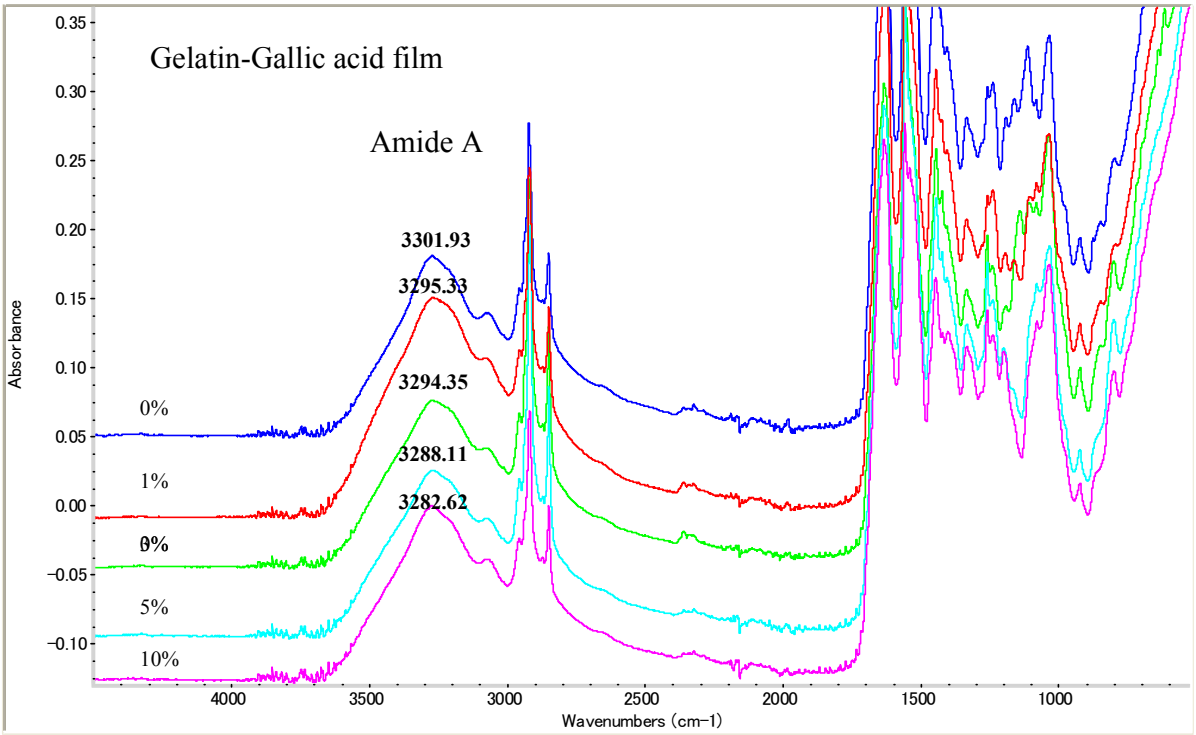
4.3.3 Fourier transforms infrared spectroscopy (FTIR) of gelatin films

FTIR spectra of gelatin film incorporated with various phenolic compounds at different concentrations were presented in Fig 4.3. FTIR spectra have been used to indicate the functional groups and structure changes of gelatin film samples when incorporated with phenolic compounds. In this study, FTIR spectra was used to determine the effect of phenolic compounds on the mechanical properties of gelatin films by establishing hydrogen bond in the amide-A band. Amide-A band, arising from the stretching vibration of N-H, appeared at wavenumber of 3301.93 m^{-1} for the gelatin film without phenolic. Amide-A band of gelatin-ferulic acid films at different concentration from 1-10% showed at wavenumbers of 3293.13, 3292.89, 3282.80 and 3276.58cm^{-1}, respectively. Amide-A band of gelatin-tannic acid films was 3295.64, 3294.85, 3286.68 and 3284.51cm^{-1}, respectively. With gelatin-caffeic acid films, amide A band was 3295.19, 3290.93, 3287.62 and 3282.74cm^{-1}, respectively. Amide-A band of gelatin-gallic acid films was 3295.33, 3294.35, 3288.11 and 3282.62cm^{-1}, respectively. Wavenumber of amide-A of gelatin-catechin films was 3290.23, 3286.45, 3275.45 and 3269.05cm^{-1}, respectively and gelatin-rutin films had wavenumber of 3274.54, 3272.19, 3271.11 and 3267.40cm^{-1}. The differences in the frequencies of gelatin incorporated with phenolic compound depended on the type of phenolic compound. When the N-H group of a peptide is contained in the hydrogen bonds, the position shifted to lower frequencies (Doyle, Blout & Bendit, 1975). This is the evidence to prove that N-H group of gelatin combined with –OH group of phenolic compound via hydrogen bond and leading to the increase of tensile strength of gelatin-phenolic films.


Gelatin-Catechin film
Amide A
3301.93
3290.23
3286.45
3275.45
3269.05
0%
1%
3%
5%
10%
Absorbance
Wavenumbers (cm-1)


Gelatin-Caffeic acid film
Amide A
3301.93
3295.19
3290.93
3287.62
3282.74
0%
1%
3%
5%
10%
Absorbance
Wavenumbers (cm-1)


Gelatin-Ferulic film
Amide A
3301.93
3293.13
3292.89
3282.80
3276.58
0%
1%
3%
5%
10%
Absorbance
Wavenumbers (cm-1)


Gelatin-Gallic acid film
Amide A
3301.93
3295.33
3294.35
3288.11
3282.62
0%
1%
3%
5%
10%
Absorbance
Wavenumbers (cm-1)

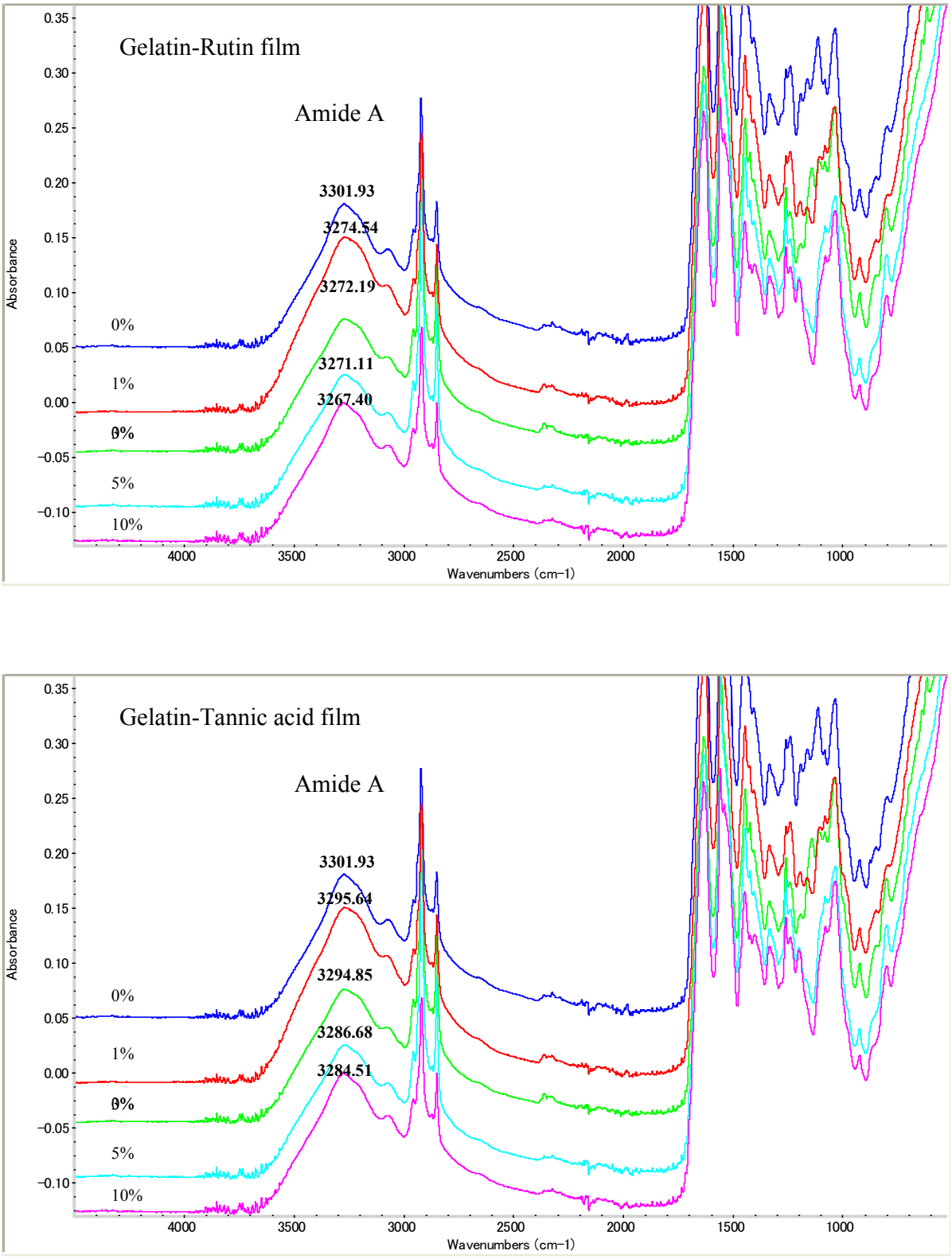


Fig. 4.3 Fourier transforms infrared (FTIR) spectroscopy of gelatin film incorporated with various phenolic compounds

4.4. Summary of chapter 4:

Increasing antioxidative activity of gelatin film from horse mackerel scale was investigated by incorporating phenolic compounds into gelatin film network. Furthermore, the addition of phenolic compounds into gelatin film improved mechanical properties, light transmission of gelatin film from horse mackerel scales in both of UV and visible ranges. Gelatin-phenolic film with antioxidative ability can be used as a biodegradable material for food packaging to prevent lipid oxidation during food storage.

CHAPTER 5

Antioxidative properties of gelatin film from horse mackerel (*Trachurus japonicus*) scale incorporated with various phenolic compounds

5.1 Introduction:

Fish gelatin film was used as a new packaging material to cover foodstuffs because of their capacity to protect food against drying, light and oxygen (Gómez-Guillén, Pérez-Mateos, Gómez-Estaca, López-Caballero, Giménez & Montero, 2009). Futhermore, gelatin film, which is biodegradable film, has known as important eco-friendly packaging material to reduce the environmental impact of synthetic plastic materials (Nagarajan, Benjakul, Prodpran & Songtipya, 2012). In recent years, the development of antioxidative edible films by incorporating with antioxidative compounds has been focused.

The major reason caused the deterioration of foods during storage is lipid oxidation. Lipid oxidation leads to development of off-flavor and off-odors and reduce the nutritive values of food products. In food industry, antioxidative additives are added in food products to prevent lipid oxidation but there are concerns on the potential health risks of such synthetic antioxidatives for humans. Therefore, the development of antioxidative edible films to cover food stuffs is necessary.

In recent years, the development of antioxidative edible films by incorporating with antioxidative compounds to improve antioxidative properties of films has been focused, such as gelatin film from silver carp with green tea extract (Wu, Chen, Ge, Miao, Li &

Zhang, 2013), bigeye snapper and brown-stripe red snapper gelatin film with BHT and α-tocopherol (Jongjareonrak et al., 2008) tuna skin gelatin with oregano and rosemary extract (Gómez-Estaca, Bravo, Gomez-Guillén, Alemán & Montero, 2009). Therefore, this study was investigated the oxidation of fresh tuna oil during storage by using gelatin film incorporated with various phenolic compounds as a packaging material to preserve fresh tuna oil.

5.2 Materials and methods:

5.2.1. Material

Horse mackerel scale was collected in Nagasaki Prefecture, Japan. The scale was collected and transported to our laboratory under iced condition. The fish scale was removed its impurity manually, washed with chilled water, and then placed in plastic bag to store at -30ºC ± 2ºC until use.

Fresh tuna oil was obtained from Yaidu-meal Co., Ltd (Tokyo, Japan) and stored at -30ºC ± 2ºC during the experiment.

5.2.2. Preparation of gelatin films incorporated with various phenolic compounds

Gelatin powder was dissolved in distilled water at 60ºC for 30min to obtain the film-forming solution (FFS) with the protein concentration of 2% (w/v). Glycerol was used as a plasticizer by adding into FFS at the concentration of 20% (w/w) of protein. Then the phenolic compounds including caffeic acid, catechin, gallic acid, ferulic acid, rutin and tannic acid were added into FFS at the concentration of 5% (w/w) of protein with continuous stirring at room temperature for 1h. The air bubbles in FFS were removed by a hybrid mixer (HM – 500; Keyence Co., Tokyo, Japan). The prepared FFS (4g) was cast onto a rimmed silicone plate (50 x 50 mm) and dried at a ventilated oven (EYELA, KCL – 2000A, Tokyo

Rikakikai Co., Ltd., Japan) at 25 ± 0.5ºC and 50 ± 5% relative humidity (RH) for 48 h. Dried films obtained were manually peeled off.

5.2.3. Preparation of samples for lipid oxidation experiment

5.2.3.1. Immerse gelatin films in tuna oil

Fresh tuna oil (2g) with four immersed sheet of gelatin films with and without phenolic compound (20 x 20 mm) in a beaker with diameter 30mm was srored in a ventilated oven (EYELA, KCL – 2000A, Tokyo Rikakikai Co., Ltd., Japan) at 40 ± 0.5ºC and 40 ± 2% relative humidity (RH) for 5days in the dark. Everyday, the oxidation of tuna oil was determined by peroxide value (PV) and thiobarbituric acid reactive substances (TBARS) analysis.

5.2.3.2 Addition of tuna oil in the gelatin pouches with or without phenolic compound

Gelatin films with or without phenolic compound were fabricated into flexible pouches by a heat-sealing machine. These pouches were filled with 1g tuna oil, removed air in the pouches and sealed with heat-sealing machine. After sealing, the pouches were hung in a ventilated oven (EYELA, KCL-2000A, Tokyo Rikakikai Co., Ltd., Japan) at 40 ± 0.5ºC and 40 ± 2% relative humidity for 30days.

In another series of experiment, gelatin pouches filled with 1g tuna oil, infused 2 ml air in the pouches by using a syringe and sealed with heat-sealing machine. After sealing, these pouches were hung in a ventilated oven in the similar conditions with previous experiment. The oxidation of tuna oil was evaluated by PV and TBARS stored in gelatin pouches for 30days.

5.2.4. PV analysis

The PV of tuna oil was analyzed according to method of Takagi, Mitsuno, and Masumura (1978). Tuna oil sample (5-50mg) was mixed with 5ml chloroform and 10ml acetic acid, and then reacted with 1ml of 50% (w/v) potassium iodine solution. The reaction mixture was allowed to stand in the darkness for 5min, then added to 9ml of 2% (w/v) cadmium acetate dehydrate solution, and placed in the darkness again until two phase were clearly separated. The blank sample was prepared in a similar method without fish oil. The absorbance of the upper aqueous phase was measured at 410nm by using a spectrophotometer (V-630 Bio spectrophotometer, Jasco Co., Japan). The PV was calculated as meq per kilogram sample using following equation:

$$\text{PV} = \frac{\{(a - b) \text{ x } 60.14 + 0.69\} \text{ x } 1000}{8 \text{ x } w}$$

where a and b are the absorbance of sample and blank, respectively, and w is the sample weight (mg)

5.2.5. TBARS analysis

The TBARS of tuna oil packed in the gelatin film pouches with or without phenolic compound was determined according to method of Buege and Aust (1978). Tuna oil (5-50mg) was mixed with 5ml stock solution of 0.375% thiobarbituric acid, 15% trichloroacetic acid, and 0.25M HCl. The mixing solution was heated for 10min in a boiling water bath to develop pink color, cooled in running tap water, and centrifuged at 6,400 x *g* for 10min. The absorbance of the supernatant was determined at 532nm with using malondialdehyde (MDA) as a standard.

5.2.6. SDS – polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE of gelatin films from horse mackerel scale incorporated with various phenolic compounds at the concentration of 5% using as a cover of fish oil at 0day, 2days and 30days of storage was analyzed according to the method of Limpisophon, Tanaka, Weng, Abe & Osako (2009) with slight modifications as described in previous study.

5.2.7. Statistical analysis

All experiments for properties of films were performed 4times, and the data were expressed as mean ± standard deviation. The differences between variables were evaluated basing on the Duncan's multiple range tests. Analysis was performed by using SPSS software (SPSS 11.5 for Windows)

5.3 Results and discussion:

5.3.1. Lipid oxidation of tuna oil with immersed gelatin films incorporated with various phenolic compounds

Changes in PV and TBARS values of fresh tuna oil with immersed gelatin film with or without phenolic compounds showed in Table 5.1 and 5.2. PV of tuna oil increased sharply from 13.09 ± 1.90 to 1224.44 ± 14.02meq kg^{-1} during 5 days storage at 40ºC, 40% RH in a ventilated chamber. It can be explained that tuna oil containing omega-3 (*n*-3) long chain polyunsaturated fatty acids, consisting of both docosahexaenoic acid (22:6*n*-3) and eicosapentaenoic acid (22:5*n*-3) are the main reason of lipid oxidation during storage. The fish oil with immersed gelatin film showed lower PV than the fish oil, indicating that the gelatin film could slightly inhibit the initial oxidation of the tuna oil. The gelatin film incorporated with various phenolic compounds showed the higher ability to inhibit the oxidation of tuna oil than the gelatin film without phenolic compound. The gelatin film

incorporated with ferulic acid showed the highest antioxidative property to inhibit lipid oxidation when compared with the gelatin-phenolic film. The antioxidative property of gelatin-phenolic film depends on the DPPH radical scavenging acivity and IC 50 of the film (in previous study). The gelatin film incorporated with ferulic acid showed the highest DPPH radical scavenging acivity and IC 50, leaded to the lowest in PV when compared with the gelatin film incorporated with different types phenolic. A similar tendency of TBARS values was observed in the tuna oil with immersed gelatin-phenolic films in similar conditions of storage.

Table 5.1 Changes of peroxide values (PV) of fresh tuna oil with the immersed gelatin film incorporated with the various phenolic compounds during storage at 40ºC and 40%RH

Sample	Storage time (days)					
	0	1	2	3	4	5
Fish oil	13.07±1.90^{a}	109.63±9.28^{d}	264.07±5.79^{d}	422.92±4.80^{d}	785.31±8.36^{d}	1224.44±14.02^{b}
Gelatin film	13.07±1.90^{a}	101.94±6.51^{d}	246.07±2.80^{c}	390.97±9.11^{c}	618.29±16.92^{c}	912.62±12.34^{b}
Caffeic acid film	13.07±1.90^{a}	6.67±8.47^{bc}	187.82±1.50^{ab}	365.12±31.34^{b}	584.67±1.49^{b}	785.77±4.16^{a}
Catechin film	13.07±1.90^{a}	5.88±3.41^{ab}	177.01±4.96^{a}	328.44±10.13^{a}	573.63±8.24^{b}	763.17±11.08^{a}
Gallic acid film	13.07±1.90^{a}	88.08±1.73^{c}	193.80±4.70^{b}	378.22±6.07^{bc}	582.61±13.28^{b}	798.07±10.95^{a}
Ferulic acid film	13.07±1.90^{a}	65.50±6.19^{a}	172.89±12.25^{a}	311.78±13.25^{a}	532.11±9.08^{a}	746.57±28.17^{a}
Rutin film	13.07±1.90^{a}	90.63±7.24^{c}	194.38±16.92^{b}	377.64±4.20^{bc}	590.54±12.56^{b}	799.06±17.57^{a}
Tannic acid film	13.07±1.90^{a}	2.47±1.96^{bc}	186.29±2.59^{ab}	354.74±9.07^{b}	583.13±13.51^{b}	776.89±10.36^{a}

Data are expressed as mean ± standard deviation ($n=4$).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

Table 5.2 Changes of TBARS values of fresh tuna oil with immersed gelatin film incorporated with various phenolic compounds during storage at 40ºC and 40%RH

Sample	Storage time (days)					
	0	1	2	3	4	5
Fish oil	15.78±0.74^{a}	27.51±0.93^{e}	55.11±0.75^{g}	84.30±0.84^{f}	160.23±0.10^{e}	240.76±2.55^{e}
Gelatin film	15.78±0.74^{a}	24.44±0.89^{d}	38.57±1.12^{f}	62.93±0.93^{e}	114.67±5.12^{d}	171.84±7.53^{d}
Caffeic acid film	15.78±0.74^{a}	22.74±1.15^{bc}	31.88±0.73^{c}	53.85±1.38^{c}	92.93±0.98^{b}	143.60±2.01^{b}
Catechin film	15.78±0.74^{a}	21.91±1.42^{b}	29.19±0.25^{b}	48.94±2.19^{b}	88.80±0.70^{a}	134.72±1.81^{a}
Gallic acid film	15.78±0.74^{a}	23.24±1.19^{cd}	35.09±0.63^{d}	56.91±1.72^{d}	95.05±0.96^{b}	149.37±1.22^{c}
Ferulic acid film	15.78±0.74^{a}	16.75±0.91^{a}	25.16±0.64^{a}	40.10±0.83^{a}	87.60±1.48^{a}	132.83±3.14^{a}
Rutin film	15.78±0.74^{a}	24.29±0.45^{d}	36.81±1.01^{e}	56.15±0.84^{d}	98.89±0.66^{c}	151.16±0.72^{c}
Tannic acid film	15.78±0.74^{a}	22.81±1.31^{bc}	31.14±0.84^{c}	52.09±0.75^{c}	92.33±0.69^{b}	138.17±1.05^{ab}

Data are expressed as mean ± standard deviation (n=4).

Different superscripts in the same column indicate statistical differences ($p < 0.05$).

5.3.2. Lipid oxidation of tuna oil in gelatin film-phenolic pouches during storage at 40ºC and 40%RH with or without infusion of 2ml air

The changes of PV and TBARS values of fresh tuna oil in gelatin film-phenolic pouches during storage at 40ºC, 40%RH with or without infusion of 2ml air showed in Table 5.3, 5.4, 5.5 and 5.6. Fish oil in gelatin pouches (without air) showed a sharply increase in PV from 13.27 ± 2.64 to 44.69 ± 1.19meq kg^{-1} during the initial 2 days of storage. It can be explained that the presence of inherent dissolved oxygen in gelatin pouches was the main reason for lipid rancidity. However, after 3days of storage, PV of sample decreased and maintained at 33meq kg^{-1} during 10-30days of storage. PV of tuna oil in gelatin pouches with infusion of 2ml air increased rapidly from 13.27 ± 2.64 to 112.69 ± 6.05meq kg^{-1} during the initial 4days of storage. After 5days of storage, PV of fish oil decreased gradually because of the disappearance of oxygen in gelatin pouches as a result of PV in Table 5.4. Fish oil in gelatin-phenolic pouches with or without infusion of 2ml air showed lower PV than samples pouches in gelatin film. Sample in gelatin-ferulic acid film had lowest PV when compared with sample in gelatin film incorporated with different type phenolic. It was evident that gelatin film incorporated with various phenolic compounds could prevent oxidation of tuna oil due to their DPPH radical scanvenging activity and IC 50 of films.

Table 5.5 and 5.6 showed the alteration of TBARS values of fish oil in gelatin fim incorporated with various phenolic compounds with or without infusion of 2ml air. TBARS value of fish oil in gelatin pouches (without air) showed a slight increase from 16.14 ± 0.55 to 33.79 ± 0.46mg MDA kg^{-1} during the initial 1 days of storage. Sample with infusion of 2ml air in gelatin pounches reached 47.20 ± 0.52mg MDA kg^{-1} at the beginning of storage, but unexpectedly decreased to 23mg MDA kg^{-1} after 30 days of storage. It is known that thiobarbituric acid, which is used to determine TBARS values, responds not only with malondialdehyde but also with other aldehyde formed during lipid oxidation (Janero, 1990).

Futhermore, those aldehydes are also known to crosslink protein molecules (Laguerre, Lecomte, & Villeneuve., 2007). From the results, it can be supposed that the secondary products of fresh tuna oil oxidation reacted with protein of gelatin film, leading to reduction of TBARS values during storage.

Table 5.3 Changes of peroxide values (PV) of fresh tuna oil in gelatin film-phenolic pouches during storage at 40ºC and 40%RH

Storage time (days)	Sample						
	Gelatin film	Caffeic acid film	Catechin film	Gallic acid film	Ferulic acid film	Rutin film	Tannic acid film
0	13.27±2.64^{a}	13.27±2.64^{a}	13.27±2.64^{a}	13.27±2.64^{a}	13.27±2.64^{a}	13.27±2.64^{a}	13.27±2.64^{a}
1	41.12±2.27^{c}	33.98±1.09^{abc}	30.38±3.16^{ab}	36.11±0.59^{abc}	28.91±5.82^{a}	37.70±4.01^{bc}	30.66±3.41^{ab}
2	44.69±1.19^{c}	36.13±2.94^{ab}	32.85±4.52^{a}	39.85±1.52^{abc}	31.48±5.18^{a}	42.79±2.91^{bc}	34.80±3.78^{ab}
3	38.46±5.91^{a}	34.51±2.73^{a}	29.77±3.88^{a}	35.06±2.65^{a}	29.67±3.87^{a}	36.24±4.12^{a}	31.39±2.12^{a}
4	37.63±4.60^{b}	32.12±3.41^{ab}	28.27±2.19^{ab}	33.41±3.85^{ab}	26.87±5.70^{a}	34.36±2.70^{ab}	31.01±4.48^{ab}
5	35.67±1.01^{b}	29.01±4.21^{ab}	26.93±5.23^{a}	31.02±1.36^{ab}	24.26±2.44^{a}	31.75±3.67^{ab}	28.50±1.59^{ab}
10	33.90±2.29^{b}	27.86±1.96^{ab}	25.03±3.45^{a}	29.48±4.05^{ab}	22.27±2.07^{a}	29.94±3.02^{ab}	27.29±4.25^{ab}
20	33.69±5.69^{b}	28.37±3.96^{ab}	26.46±2.87^{ab}	30.57±2.72^{ab}	23.36±3.98^{a}	30.20±3.15^{ab}	26.68±2.52^{ab}
30	33.35±4.11^{b}	27.87±3.84^{ab}	26.36±2.43^{ab}	29.77±4.31^{ab}	22.25±1.95^{a}	29.73±5.34^{ab}	26.23±1.22^{ab}

Data are expressed as mean ± standard deviation ($n=4$).

Different superscripts in the same row indicate statistical differences ($p < 0.05$).

Table 5.4 Changes of peroxide values (PV) of fresh tuna oil with infusion of 2ml air in gelatin film-phenolic pouches during storage at 40ºC and 40%RH

Storage time (days)	Sample						
	Gelatin film	Caffeic acid film	Catechin film	Gallic acid film	Ferulic acid film	Rutin film	Tannic acid film
0	13.27±2.64^{a}	13.27±2.64^{a}	13.27±2.64^{a}	13.27±2.64^{a}	13.27±2.64^{a}	13.27±2.64^{a}	13.27±2.64^{a}
1	99.55±0.88e	93.21±2.97^{cd}	80.59±5.76^{ab}	95.72±2.64^{cd}e	77.62±5.34^{a}	99.61±1.36^{d}e	88.82±1.95^{bc}
2	103.00±1.17^{c}	97.45±2.75^{bc}	91.14±4.25^{ab}	100.01±3.84^{bc}	87.06±2.13^{a}	101.59±1.67^{c}	95.08±7.03^{abc}
3	108.81±1.75^{c}	103.43±4.07^{abc}	97.27±5.21^{ab}	104.08±6.33^{abc}	95.33±6.15^{a}	107.13±2.04^{bc}	99.02±2.85^{abc}
4	112.69±6.05^{b}	109.83±4.81^{ab}	100.79±4.53^{ab}	110.51±6.93^{b}	97.72±3.20^{a}	111.88±5.68^{b}	103.86±0.90^{ab}
5	97.10±2.22^{a}	96.34±3.13^{a}	93.04±6.12^{a}	96.66±4.85^{a}	90.02±4.76^{a}	96.79±6.80^{a}	95.78±6.70^{a}
10	89.71±4.79^{a}	87.69±5.15^{a}	86.21±3.96^{a}	88.26±5.36^{a}	84.30±7.12^{a}	88.68±8.28^{a}	87.02±2.90^{a}
20	79.60±2.73^{a}	76.14±3.40^{a}	73.59±3.78^{a}	77.79±1.99^{a}	71.74±3.27^{a}	78.32±4.26^{a}	75.87±2.21^{a}
30	47.08±2.12^{c}	41.41±3.53^{abc}	36.50±2.94^{ab}	43.14±3.38^{abc}	34.52±4.45^{a}	44.85±2.97^{bc}	38.93±5.81^{abc}

Data are expressed as mean ± standard deviation ($n=4$).

Different superscripts in the same row indicate statistical differences ($p < 0.05$).

Table 5.5 Changes of TBARS of fresh tuna oil in gelatin film-phenolic pouches during storage at 40ºC and 40%RH

Storage time (days)	Sample						
	Gelatin film	Caffeic acid film	Catechin film	Gallic acid film	Ferulic acid film	Rutin film	Tannic acid film
0	16.14±0.55^{a}	16.14±0.55^{a}	16.14±0.55^{a}	16.14±0.55^{a}	16.14±0.55^{a}	16.14±0.55^{a}	16.14±0.55^{a}
1	33.79±0.46^{c}	30.99±0.85^{b}	29.53±0.57^{b}	31.20±1.16^{b}	26.86±1.33^{a}	31.54±1.50^{bc}	30.16±0.47^{b}
2	29.93±0.89^{b}	28.38±1.83^{b}	26.67±2.98^{b}	28.75±1.75^{b}	23.89±0.77^{a}	29.82±4.06^{b}	27.81±3.89^{a}
3	23.07±2.68^{b}	22.02±1.38^{ab}	21.06±2.11^{ab}	22.43±1.58^{ab}	18.22±1.06^{a}	22.90±1.91^{ab}	21.57±1.94^{ab}
4	22.26±2.09^{c}	20.08±0.69^{bc}	18.95±0.41^{ab}	21.03±0.27^{bc}	16.94±1.69^{a}	20.55±0.66^{bc}	19.76±0.83^{bc}
5	21.96±0.72^{b}	19.01±2.38^{ab}	17.56±1.71^{ab}	19.28±1.43^{ab}	16.05±1.50^{a}	19.44±2.65^{ab}	18.90±0.69^{ab}
10	19.53±1.60^{b}	17.71±1.52^{ab}	16.38±2.79^{ab}	17.75±1.74^{ab}	15.89±2.63^{a}	17.82±1.72^{ab}	17.57±1.41^{ab}
20	19.17±2.42^{b}	16.98±1.55^{ab}	16.49±1.61^{ab}	17.05±1.49^{ab}	16.21±1.72^{a}	17.11±1.97^{ab}	16.89±1.60^{ab}
30	19.50±1.64^{b}	16.57±0.78^{a}	16.28±0.89^{a}	16.73±1.03^{a}	16.18±0.68^{a}	16.94±0.84^{ab}	16.48±0.77^{a}

Data are expressed as mean ± standard deviation ($n=4$).

Different superscripts in the same row indicate statistical differences ($p < 0.05$).

Table 5.6 Changes of TBARS of fresh tuna oil with infusion of 2ml air in gelatin film-phenolic pouches during storage at 40ºC and 40%RH

Storage time (days)	Sample						
	Gelatin film	Caffeic acid film	Catechin film	Gallic acid film	Ferulic acid film	Rutin film	Tannic acid film
0	16.14±0.55^{a}	16.14±0.55^{a}	16.14±0.55^{a}	16.14±0.55^{a}	16.14±0.55^{a}	16.14±0.55^{a}	16.14±0.55^{a}
1	41.74±0.29^{d}	37.93±0.66^{c}	35.79±0.90^{ab}	39.24±1.18^{c}	34.26±0.50^{a}	41.09±0.13^{d}	37.49±0.90^{bc}
2	47.20±0.52^{b}	40.68±3.47^{a}	37.63±1.85^{ab}	41.05±3.26^{ab}	36.19±1.52^{a}	42.12±3.30^{ab}	39.84±3.52^{a}
3	34.24±0.75^{b}	31.25±2.75^{ab}	29.67±1.39^{ab}	32.16±4.01^{ab}	27.17±2.29^{a}	32.62±2.27^{ab}	30.17±1.37^{ab}
4	29.27±1.00^{c}	25.93±1.26^{abc}	24.93±0.81^{ab}	26.71±1.42^{abc}	23.63±2.47^{a}	28.22±1.22^{bc}	25.08±1.65^{ab}
5	24.59±0.89^{b}	23.15±1.28^{ab}	21.74±0.96^{ab}	23.83±1.05^{ab}	20.88±1.17^{a}	24.13±2.03^{ab}	22.50±1.25^{ab}
10	23.41±1.16^{b}	21.09±1.24^{ab}	20.21±1.52^{a}	21.40±0.66^{ab}	19.63±1.26^{a}	22.13±1.19^{ab}	20.57±1.77^{ab}
20	23.93±0.81^{b}	21.60±0.99^{ab}	20.56±1.97^{a}	21.62±1.79^{ab}	20.56±1.08^{a}	22.61±0.92^{ab}	20.99±1.85^{ab}
30	23.57±0.60^{b}	21.33±1.33^{ab}	20.95±1.38^{a}	21.79±1.60^{ab}	20.64±1.60^{a}	22.28±1.32^{ab}	20.80±0.70^{ab}

Data are expressed as mean ± standard deviation ($n=4$).

Different superscripts in the same row indicate statistical differences ($p < 0.05$).

5.3.2. SDS – polyacrylamide gel electrophoresis (SDS-PAGE)

SDS – PAGE of gelatin film pouches with fresh tuna oil is shown in Fig 5.1. From the result, the intensity of α chain and β chain gradually reduced with the concomitant appearance of high molecular weight fractions that are too large to enter the polyacrylamide gel, and α chain and β chain disappeared after 30days of storage. It can be explained that the secondary products of tuna oil oxidation such as aldehydes reacted with protein in gelatin films, leading to the polymerization of gelatin proteins.

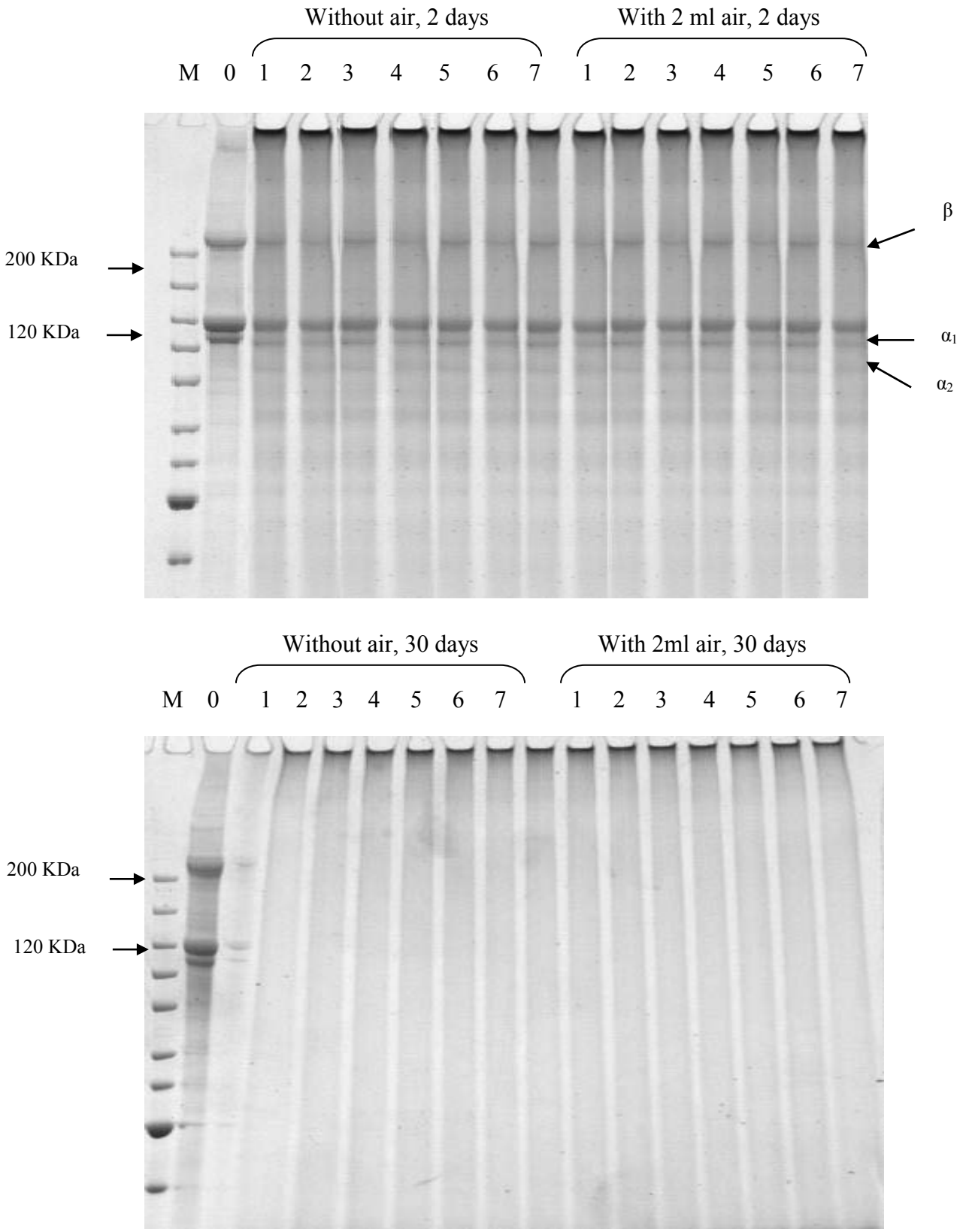


Fig 5.1 SDS – PAGE patterns of gelatin-phenolic films pounches used to store tuna oil

0 Control 1 Gelatin film 2 Caffeic acid film 3 Catechin film

4 Gallic acid film 5 Ferulic acid film 6 Rutin film 7 Tannic acid film

5.4. Summary of chapter 5:

The antioxidative properties of gelatin film were improved by incorporating with various phenolic compounds. Gelatin-phenolic film showed the higher antioxidative properties than gelatin film by preventing lipid rancidity of fresh tuna oil during storage. Gelatin film incorporated with ferulic acid with highest in the DPPH radical scavenging activity and the lowest in IC 50 values when compared with other gelatin-phenolic films, showed the ability to prevent lipid oxidation better than other samples. From the results, gelatin-phenolic film with antioxidative properties could potentially be used as biodegradable materials for food packaging to prevent lipid rancidity during food storage.

CHAPTER 6

General conclusions

Utilization of marine fish scale was started from acid soluble collagen (ASC) extraction study. First step, scales of lizard fish (*Saurida spp.*) and horse mackerel (*Trachurus japonicus*) from Japan and Vietnam and grey mullet (*Mugil cephalis*), flying fish (*Cypselurus melanurus*) and yellowback seabream (*Dentex tumifrons*) was soak in 0.1 M NaOH at 4ºC for 6h and 0.5 M Na_2EDTA (ethylenediaminetetraacetic acid disodium salt) solution (pH 7.5) at 4ºC for 24h to remove non-collagenous protein and demineralization, respectively. Second step, after pretreatment, scales were extracted with 0.5 M acetic acid for 4 days, gave extraction yields from 0.43-1.5% (on a dry weight basis), depending on the species. The SDS–PAGE profile showed that the ASCs were type I collagens, and consisted of two different α chains, α1 and α2, as well as β component, similar to another fish ASC. Maximal solubility of individual collagens was observed at pHs 1-3. Collagen solubility decreased sharply at NaCl concentrations > 0.4 M, regardless of fish type. ASC denaturation temperature (T_d) ranged from 26 to 29°C, depending on fish species and imino acid content ($p<0.01$). ASC with higher imino acid content had higher denaturation temperature. However, fish collagen is generally less thermally stable than mammalian collagen (T_d about 41°C). The low denaturation temperature of scale collagen observed in the present study makes it possible to extract the gelatin at low temperature compared to mammalian gelatin. This may be an economic advantage for using fish scale as a raw material of gelatin.

Firstly, gelatin extracted from horse mackerel scale in Japan at various temperatures (70, 80, and 90 °C) and times (15 min, 30 min, 1 h, 2 h and 3 h). The yields of gelatin

extraction were 1.08-3.45 %, depending on the extraction conditions. Extraction yield increased with increasing extraction time and temperature. However, the degradation of α chain and β chain in SDS-PAGE profile was shown when the extraction temperature was higher than 70°C and extraction time was longer than 1h.

Secondly, gelatin was utilized by production of biodegradable film. Effect of preparation conditions such as extraction temperature and time, protein concentration of film-forming solution (FFS) and glycerol concentration were investigated. Among various extraction times and temperatures, the film from gelatin extracted at 70°C for 1 h showed the highest tensile strength and elongation at break. Mechanical properties of gelatin increased with increasing protein concentration in FFS. The addition glycerol (0-25 %) as a plasticizer in FFS leads to the increasing in elongation at break and water vapor permeability of film. However, WVP of gelatin film was lower than gelatin film from other fishes and mammalian species. Gelatin films from different preparation conditions showed the excellent UV barrier properties at 200 nm. It is suggested the film can be produced from horse mackerel scale gelatin, and that the properties of the film can be modified according to the intended purpose.

The main purpose of this study is using gelatin film as a packaging material in food application. The main reason of changes in food during storage time is the rancidity of lipid. Thus, next step to improve antioxidant activity of gelatin film by adding phenolic compounds in gelatin film network. In this experiment, properties of gelatin film incorporated with various phenolic compounds such as ferulic acid, cafeic acid, catechin, gallic acid, rutin and tannic acid were characterized. Tensile strength (TS) of films increased while elongation at break decreased with increasing phenolic concentration. It can be explained that phenolic compounds contain many hydrophobic groups, which can

form hydrophobic interaction with hydrophobic region of gelatin molecules by hydrogen bond. FTIR spectra showed that wavenumber of amide-A band of films decreased with increasing phenolic concentration. This indicated N-H group of gelatin combined with -OH group of phenolic compound via hydrogen bond leading to enhancing of TS of films. The increase in phenolic concentration leads to enhance water vapor permeability values. Gelatin films at different phenolic concentrations showed the excellent UV barrier properties at UV range. Gelatin films incorporated with phenolic compounds showed higher total phenolic content and DPPH radical scavenging activity than gelatin film without phenolic. This result showed that gelatin film incorporated with phenolic compound lead to improve antioxidant activity of gelatin film.

Finally, gelatin-phenolic film with antioxidant ability could be used as a biodegradable material to cover fresh tuna oil. Gelatin-phenolic film showed the higher antioxidative properties than gelatin film by preventing lipid rancidity of fresh tuna oil during storage. Gelatin-ferulic acid film with the highest in DPPH radical scavenging activity and the lowest in IC 50 values when compared with other gelatin-phenolic films, showed the ability to prevent lipid oxidation better than other samples. From the results, gelatin-phenolic film with antioxidative properties could potentially be used as biodegradable materials for food packaging to prevent lipid rancidity during food storage.

From the results of this thesis, it can be suggested that horse mackerel scale can be utilized in industry. Utilization of horse mackerel scale as a raw material of edible film could be solved the environmental pollution by seafood waste during fish processing. The film property can be modified according to the intended purpose.

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