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Research Paper

Preparation and physicochemical characterization of films prepared with salmon skin gelatin extracted by a trypsin-aided process

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ABSTRACT

The recovery of gelatins from Atlantic salmon (*Salmo salar*) skin for film formation and characterization was studied. Fish skins pre-treated with trypsin (250 U/g) produced the highest hydroxyproline content (7.41 ± 0.49 mg hydroxyproline/g treated skin) and yield (53.05 ± 4.38%) of gelatin, as compared to the use of saline solution. Pre-treatment with a lower concentration of trypsin (1 U/g) at a shorter pre-treatment time successfully reduced the degradation of gelatin with co-production of high molecular weight α-chains. Gelatin was further extracted by a trypsin-aided process for film formation and characterization. Films with increasing protein concentration (from 1 to 5%, w/v) exhibited higher thickness, tensile strength, and elongation at break (EAB), but a marked decrease in EAB for films with 6 and 7% (w/v). Films with 5% proteins showed higher thickness, lower tensile strength and higher EAB with increasing concentrations of glycerol (from 10 to 50% of proteins, w/w). All films exhibited high water uptake, decrease in light transmission and an increase in opacity as the protein and glycerol contents increased. Electrophoretic studies showed that the increase in the mechanical properties of the films was correlated with the increase in protein concentration, owing to the increased content of high molecular weight chain fractions. Furthermore, Fourier transform infrared spectroscopy (FTIR) and scanning electron microscopy (SEM) revealed the interaction between the proteins and glycerol for all films. This study demonstrated the viability of the trypsin supplementation process to obtain salmon skin gelatin for film formation.

1. Introduction

Gelatin has attracted much attention for the development of edible films for food packaging due to its film forming ability and safety for food use (Bigi et al., 2000). Films made from fish gelatin have been studied over the years, including gelatin from the skins of bigeye red snapper and brownstripe red snapper (Jongjareonrak et al., 2006), tuna (Gómez-Guillén et al., 2007), Atlantic halibut (Carvalho et al., 2008), blue-shark (Limpisophon et al., 2009), as well as red snapper and grouper (Elango et al., 2014). A comparison study was conducted among mammalian, warm- and cold-water fish gelatins, and significant differences in physical and chemical properties of resulting films were reported (Avena-Bustillos et al., 2006). This comparative study showed that cold-water fish gelatin films exhibited lower water vapor permeability, suggesting its potential applicability as a biopolymer for encapsulating drugs or for packaging frozen food systems. Atlantic salmon (*Salmo salar*) is an economically important cold-water fish that is in high demand for fillet production, thus contributing a large quantity of by-products that can serve as a rich source of gelatin.

The physical and structural properties of gelatin films are affected by the gelatin's amino acid composition which is species-specific, and its molecular weight distribution which depends on the extraction conditions (Carvalho et al., 2008; Gómez-Guillén et al., 2009). Generally, milder processing conditions induce minimal degradation, and favor the production of gelatin with a high content of high molecular weight polypeptide fractions. This could contribute towards the formation of gelatin network that produce films with improved mechanical and light barrier properties (Gómez-Guillén et al., 2002; Jongjareonrak et al.,

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2006; Limpisophon et al., 2009). The formation of the gelatin network relies on the presence of amino acids, specifically hydroxyproline (Hyp), due to its ability to form intra- and intermolecular hydrogen bonds (Brinckmann, 2005). Moreover, hydrophobic and ionic interactions between the high-molecular weight fractions (α-chains) improve the network stability (Galea et al., 2000).

Various studies have been conducted to evaluate the influence of different pre-treatment methods on the physicochemical properties of extracted gelatin. By using an effective pre-treatment method, high quality gelatin can be produced using lower extraction temperatures (Johnston-Bank, 1990). Generally, the selection of a pre-treatment agent depends on the source of the materials, and the collagen type (Benjakul et al., 2012; Gómez-Guillén et al., 2009). Milder pre-treatment using acids (e.g., 0.05 M acetic acid or 0.2 M–1.0 M diluted sulfuric or hydrochloric acid) are found to be more suitable for solubilizing less crosslinked collagen from fish or pig skins. In contrast, an alkaline pre-treatment (e.g., 0.025N sodium hydroxide solution with supersaturated solution of calcium hydroxide) is usually used for highly crosslinked collagen of bovine origins (Benjakul et al., 2012; Schrieber and Gareis, 2007). In some fish gelatin extraction studies, acetic acid showed a superior effect in extracting gelatin regarding yields, viscoelastic properties and gel strength as compared to citric, lactic, propionic, malic, and tartaric acids (Giménez et al., 2005a; Gómez-Guillén et al., 2001; Khiari et al., 2015). Meanwhile, in other studies where an alkali pre-treatment was used, higher alkali concentration facilitated the extraction of fish gelatin having high purity but a lower yield (Yang et al., 2007). Moreover, the fish gelatin was more viscous as compared to alkali-acid mixture (Yoshimura et al., 2000), and the gel strength and the product was higher using strong and weak alkali mixture (Kaewdang et al., 2016). Saline solution pre-treatments are capable of solubilizing collagen structure effectively by interacting with its structurally bound water molecules. This leads to an improved yield of fish skin gelatin extracted while preserving the high molecular weight of the protein chains (Giménez et al., 2005b). In comparison to chemical pre-treatments, proteases were found to specifically cleave the inter-chain cross-links of collagen but not its domain structure, resulting in the improved collagen solubilization (Galea et al., 2000). Studies have reported that pepsin-aided pre-treatment yielded approximately two-fold higher amounts of gelatin, as compared to those without pepsin (Chomarat et al., 1994; Nalinanon et al., 2008). Trypsin is found to be more effective than pepsin in assisting the extraction of gelatin from wastes from leather industry, due to the narrower specificity of trypsin (Cabeza et al., 1997). Therefore, a trypsin-aided extraction process could be studied to produce gelatin from fish skins for film-formation.

Gelatin chains tend to interact via crosslinks to form a three-dimensional network with zones of intermolecular microcrystalline junctions in a polymeric system (Arvanitoyannis, 2002; Slade and Levine, 1987). However, extensive intermolecular interactions together with dehydration of this system may produce brittle films (Vanin et al., 2005). To overcome the brittleness of films, relatively small molecular weight plasticizers are often added to the formulation. Plasticizers compete for hydrogen bonding and electrostatic interactions with protein polymeric chains and increase the free-volume or intermolecular spacing, resulting in an increased molecular mobility and improved flexibility and extensibility (Limpisophon et al., 2009; Sothornvit et al., 2002). The plasticizing effect on films is associated with the plasticizer's ability to attract water, which also acts as a plasticizer. This is influenced by the composition, size and shape of the plasticizer as well as its compatibility with the polymer (Sothornvit and Krochta, 2001). Among different plasticizers that can be added, glycerol and sorbitol are mainly used in gelatin-based films (Arvanitoyannis and Biliaderis, 1998; Carvalho and Grosso, 2004; Menegalli et al., 1999; Sakanaka et al., 2001; Sobral et al., 2001). However, sorbitol can crystallize in the films when stored at low and intermediate relative humidity conditions, affecting its plasticizing effect (Sakanaka et al., 2001).

Few studies have investigated the effects of protein and plasticizer concentrations on the properties of fish gelatin films, especially gelatin from cold water fish skin such as Atlantic salmon. In addition, only few studies have reported on the physical properties of films using spectroscopic methods and morphological analyses. Thus, the aim of this study was to investigate the effect of different extraction methods using saline, saline in combination with alkaline, and trypsin-aided pre-treatments on producing Atlantic salmon skin gelatin for film formation. The physical properties of gelatin films formed at different protein and glycerol concentrations were evaluated, and their mechanical properties were further correlated with their protein patterns via electrophoretic analysis, molecular interactions using FT-IR spectroscopy and morphological analyses using scanning electron microscopy (SEM).

2. Materials and methods

2.1. Materials

Atlantic salmon was obtained from a local fish market, Montreal, Canada. Trypsin from porcine pancreas (EC 3.4.21.4; powdered; 90.97 U/mg) was obtained from ICN Biomedicals Inc. (Ohio, USA); glycerol, isopropanol, methanol, potassium carbonate, sodium dodecyl sulfate (SDS) and Tris base were purchased from Fisher Scientific (Fair Lawn, NJ, USA); bicinchoninic acid (BCA) protein assay reagents and bovine serum albumin (BSA) standard were purchased from Pierce (Rockford, Illinois, USA); glacial acetic acid and hydrochloric acid (HCl) were purchased from Fisher Scientific (Nepean, Ontario, Canada); sodium hydroxide (NaOH) was purchased from Merck (KGaA, Darmstadt, Germany); sodium chloride (NaCl) was purchased from BDH Inc. (Toronto, Ontario, Canada); 2-mercaptoethanol (2-ME), activated charcoal, bromophenol blue, chloramine-T hydrate, Coomassie brilliant blue R-250, Ehrlich's reagent solution and *trans*-4-hydroxy-L-proline (Hyp) were purchased from Sigma Chemical Co. (St. Louis, MO, USA); Laemmli sample buffer was purchased from Bio-Rad Laboratories (Hercules, CA, USA); high-molecular weight markers (53 kDa–220 kDa) were purchased from GE Healthcare (Buckinghamshire, UK). All chemicals and reagents used were of analytical grade.

2.2. Fish skins handling

Fish skins were manually removed at the fish market, packed in polyethylene bags and kept in ice with a skin/ice ratio of 1:2 (w/w) in a polystyrene box. Fish skins were transported to the laboratory within 1 h, and any residual meat was removed manually from the skin. The skins were then cut into small pieces (*ca* 1.5 × 1.5 cm^2) with scissors and washed with tap water, then placed in polyethylene bags and stored at −20 °C. The frozen skins were thawed overnight in a refrigerator before use.

2.3. Studies of gelatin extraction methods

Among previous studies on gelatin extraction, three gelatin extraction methods that involved the use of saline solution with or without added alkaline solution, and trypsin-supplementation were chosen. These methods were performed to compare the Hyp contents and yields of gelatin. Gelatin yield was calculated based on the Hyp content of the lyophilized gelatin as compared to the Hyp content of the wet fish skins, by using the following equation:

$$Yield\ of\ gelatin = \frac{Hyp\ content\ of\ gelatin\ \left(\frac{mg}{g}\right)\ x\ weight\ of\ gelatin\ obtained\ (g)}{Hyp\ content\ of\ fish\ skin\ \left(\frac{mg}{g}\right)\ x\ weight\ of\ fish\ skin\ used\ (g)}\ x\ 100\ \% \quad (1)$$

2.3.1. Extraction method with saline solution pre-treatment

The method described by Kołodziejska et al. (2008) was used with slight modifications. Fish skins were pre-treated by gently stirring in 0.45 M NaCl (1:6, w/v) at 4 °C for 3 min, then rinsed five times with distilled water (1:6, w/v), and the gelatin extraction was conducted using distilled water (1:6, w/v) at 45 °C for 60 min. The protein solution was then centrifuged using a laboratory centrifuge (rotor No. 875, Model B-22M, IEC, MA, USA) at 10,000×*g* for 30 min at 15 °C with 50 ml conical-bottom centrifuge tubes (Corning#430290, Corning, New York, USA). The supernatant obtained was lyophilized using a freeze dryer (Modulyod-115, ThermoSavant, Holbrook, NY, USA) at 120 mBar for 48 h at −50 °C. The lyophilized proteins were referred as 'gelatin powder' and stored at −20 °C until further analysis.

2.3.2. Extraction with saline and alkaline solution ns pre-treatment

The method followed by Rahman et al. (2008) was used with slight modifications. Fish skins were washed with distilled water and pre-treated with 0.45 M NaCl (1:6, w/v) at 4 °C for 3 min. The samples were then soaked in 0.1 M NaOH (1:6, w/v) at room temperature (22–25 °C) for 40 min and washed five times with distilled water (1:6, w/v). The extraction was performed using distilled water (1:6, w/v) at 50 °C for 18 h. The protein solution was centrifuged, and the supernatant was lyophilized and stored as described above.

2.3.3. Extraction method with trypsin solution pre-treatment

The method of Cabeza et al. (1997) was used with slight modifications. Fish skins were pre-treated with trypsin at 250 U/g (of fish skin) in Tris-HCl buffer (pH 8.0; 1:6, w/v) for 8 h at room temperature (22–25 °C). The samples were then filtered and rinsed five times with distilled water (1:6, w/v), and extracted with distilled water (1:6, w/v) at 50 °C for 3 h. The protein solution was centrifuged, and the supernatant was lyophilized and stored as described above.

The studies showed that salmon skins pre-treated with trypsin solution produced the highest gelatin yield and Hyp content, but also had a greater effect on the degradation of the major protein chains of gelatin (data not shown). A further investigation was conducted on fish skins incubated at a shorter time with a lower trypsin concentration of 1 U/g for 4 h, and extracted at 50 °C for 3 h. Gelatin obtained in this investigation showed the presence of major polypeptide chains through electrophoretic profile analysis (SDS-PAGE). Thus, a lower trypsin concentration was used to assist the gelatin extraction as described by Fan et al. (2017). The skins were washed with distilled water and pre-treated with 0.45 M NaCl (1:6, w/v) at 4 °C for 3 min (Rahman et al., 2008). The skins were then soaked in 50 mM Tris-HCl buffer (pH 8.0) in the presence of trypsin at 1.5 U/g, and stirred continuously at room temperature (22–25 °C) for 5 h, then filtered and rinsed five times with distilled water (1:6, w/v). Gelatin was extracted by gently stirring the mixture of pretreated skins and distilled water (1:6, w/v) at 45 °C for 6 h 15 min. The protein solution was centrifuged, and the supernatant was lyophilized and stored as described above for film formation.

2.3.4. Analyses

2.3.4.1. Hydroxyproline content. The Hyp content of gelatin was determined according to the method of Nalinanon et al. (2008) with slight modifications. In a typical experiment, a gelatin sample (1.0 g) was hydrolyzed with 6 M HCl (8.0 ml) in an oven at 105 °C for 24 h. The hydrolysate was then clarified with activated charcoal (200 mg) and filtered using a Whatman No. 4 filter paper. The filtrate was neutralized to pH 6.0–6.5 with 10.0 M, 1.0 M and 0.1 M NaOH. The neutralized sample (0.1 ml) was transferred into an amber tube and isopropanol (0.2 ml) was added and mixed well. To the mixture, 0.1 ml of an oxidant solution (a mixture of 7% (w/v) chloroamine T (w/v) and acetate/citrate buffer, pH 6, at a ratio of 1:4 (v/v)) were added and mixed thoroughly. Subsequently, 1.3 ml of Ehrlich's reagent solution (a mixture of 2 g 4-dimethylamino-benzaldehyde in 98 ml of 8% (v/v) hydrochloric acid) and isopropanol at a ratio of 3:13 (v/v)) were added. The mixture was mixed and heated in a shaking water bath at 60 °C for 25 min and cooled in running tap water for 2–3 min. The solution was diluted to 5 ml with isopropanol (99.9%). The absorbance was measured within 30 min at $A_{558\ nm}$ using an UV/Vis spectrophotometer (model DU 800, Beckman Coulter, USA). A Hyp standard curve was prepared using absorbance readings from standard solutions with concentrations ranging from 10 to 60 ppm. Distilled water was used as the blank. Hyp content was calculated and expressed as mg/g sample.

2.3.4.2. Protein electrophoretic profile analysis. SDS–PAGE was performed to determine the gelatin electrophoretic profile according to the method of Laemmli (1970) with minor modifications. The gelatin samples (0.01 g) were dissolved completely in distilled water (1.0 ml). Solubilized samples were mixed at a 1:1 (v/v) ratio with Laemmli sample buffer (containing 62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS and 0.01% bromophenol blue) in the presence of 10% 2-ME, and heated at 100 °C for 10 min. Fifteen micrograms of protein of each sample were loaded onto each well (15 μg/well) of pre-cast gradient polyacrylamide gels of thickness of 1.0 mm x 10 wells (Bio-Rad Mini-PROTEAN® TGX™, a 4–15% polyacrylamide gel, USA). Electrophoresis was conducted using a Mini Protein II unit (Bio-Rad, USA) at constant voltage of 100 V for approximately 95 min of total running time. The gel was stained with 1 g Coomassie brilliant blue R-250 in a 4.5:4.5:1 solution of methanol-water-acetic acid and de-stained several times by gentle shaking with a 8:1:1 solution of water-methanol-acetic acid. High-molecular weight markers ranging from 53 kDa–220 kDa were used to estimate the molecular weight of the protein fractions.

2.4. Preparation of gelatin films

In the first set of experiment, gelatin film forming solutions (FFS) (20 ml) were prepared by mixing freeze dried gelatin powder in distilled water to obtain protein concentrations of 1, 2, 3, 4, 5, 6, 7% (w/v). Glycerol was added as plasticizer into FFS at concentration of 30% (w/w) of protein. The FFS was stirred gently for 30 min at room temperature (22–25 °C), filtered with a Whatman No. 1 filter paper and cast onto rimmed silicone plates (55 × 120 mm). The plates were placed on a leveled surface in a fume hood to evaporate the solvent for a period of 48 h at room temperature (22–25 °C). The dried films were manually peeled off for characterization.

In the second set of experiment, gelatin films of 5% protein concentration were used to evaluate the effect of glycerol concentration on gelatin films. Gelatin FFS with glycerol concentrations of 10, 20, 30, 40 and 50% (w/w) of protein were prepared. The FFS were then cast and dried as previously described.

Protein concentration of the gelatin powder was determined using a standard BCA protein assay. Gelatin solution (1000 μg/ml) was prepared in distilled water. To 0.1 ml of the gelatin solution, 2.0 ml of the BCA working reagents were added and mixed thoroughly. The mixture was incubated at 37 °C for 30 min using a shaking water bath and then cooled to room temperature (22–25 °C). The absorbance was measured within 10 min at A_{562} nm using an UV/Vis spectrophotometer (model DU 800, Beckman Coulter, USA) and distilled water was used as the blank. The protein concentration was determined by referring to a standard curve, which was prepared using absorbance readings obtained from bovine serum albumin (BSA) standard solutions with concentrations ranging from 25 to 2000 μg/ml, and were treated as described above for the gelatin samples.

2.5. Film characterization

2.5.1. Mechanical properties

Prior to the determination of the mechanical properties, the thickness of the films was measured with a hand-held digital micrometer (Marathon Part No. 030025, Marathon Watch Company Ltd., Ontario, Canada) with an accuracy of 0.002 mm. Six measurements were taken at random positions for each film specimen, and the average thickness was used to estimate the cross-sectional area of the specimen. The tensile strength (TS) and elongation at break (EAB) values were determined according to ASTM method D 882-10 (ASTM, 2010) using an Instron Universal Testing Machine (model 4500, Instron Corporation, Canton, MA, USA). The films were conditioned at 23 ± 2 °C in a desiccator containing saturated solutions of potassium carbonate (50 ± 2% relative humidity) for at least 40 h before testing. The films were fixed on the grips of the device with an initial grip separation of 30 mm, and pulled apart at a mechanical crosshead speed of 10 mm/min and preload of 2 N. At least five replicates were tested for each film and the average was taken as the results. TS (MPa) and EAB (%) were calculated by the following equations:

$$TS(MPa) = F\,max / A \tag{2}$$

where F max = maximum load (N) needed at the moment of rupture, A = cross-sectional area (m^2) of the samples.

$$EAB\ (\%) = \left(\frac{E}{30}\right) x\ 100 \tag{3}$$

where E = film elongation (mm) at the moment of rupture, 30 = initial grip length (mm) of samples.

2.5.2. Water solubility

The water solubility of the films was determined according to the method of Shakila et al. (2012). Films of surface area of 4 cm^2 were cut and weighed (±0.0001 g) to determine the initial weight (W_i). Films were immersed separately in 15 ml of distilled water, gently shaken at room temperature (22–25 °C) for 15 h and then filtered through a Whatman No. 1 filter paper. The unsolubilized film residue collected on the filter paper was dried in a hot air oven at 105 °C for 24 h and weighted (W_f). Three replicates were tested for each film and the average values were taken as the result. The solubility of the film was calculated by the following equation:

$$\text{Solubility}\ (\%) = \left(\frac{W_i - W_f}{W_i}\right) x\ 100 \tag{4}$$

where W_i = initial weight of the film specimen, W_f = weight of unsolubilized film residue.

2.5.3. Light transmission and opacity

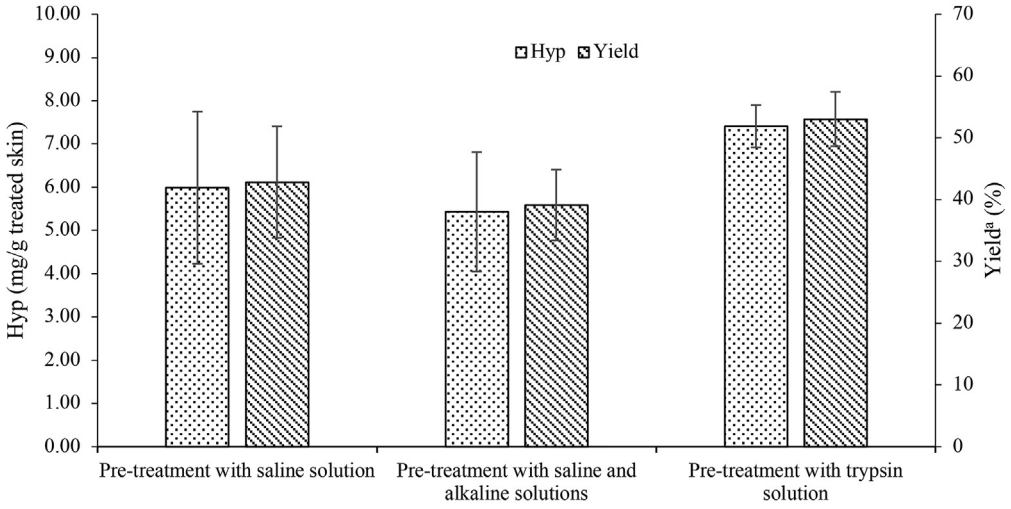
The barrier properties of gelatin films against ultraviolet (UV) and visible light were measured at selected wavelengths (200–800 nm) using an UV/Vis spectrophotometer, according to the method of Fang et al. (2002). The films were cut in rectangular pieces (12 × 43 mm), directly placed into a quartz cuvette and measured. An empty cuvette was used as the blank. The test was performed in triplicate for each film and the averages were taken as the results. Light transmission (T) was recorded using transmittance (%) measured at each wavelength for each film, and the opacity (%) was calculated by the following equation:

$$\text{Opacity}\ (\%) = 100\% - T \tag{5}$$

where T = transmittance (%) at each wavelength.

2.5.4. Electrophoretic analysis

The protein patterns of gelatin films were determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) according to the method of Laemmli (1970) with minor modifications. The gelatin films (0.01 g) were dissolved completely in distilled water



[a]Yield was calculated based on the Hyp content of the lyophilized gelatin compared to the Hyp content of the wet fish skin.

Fig. 1. Hydroxyproline (Hyp) content and yield of gelatin extracted from salmon skin pretreated with different pre-treatments. [a]Yield was calculated based on the Hyp content of the lyophilized gelatin compared to the Hyp content of the wet fish skin.

(1.0 ml). Solubilized samples were mixed at a 1:1 (v/v) ratio with Laemmli sample buffer (containing 62.5 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS and 0.01% bromophenol blue) in the presence of 5% 2-ME, and heated at 100 °C for 10 min. Ten microliters of each sample were loaded into each well of polyacrylamide gels (1.5 mm thickness) comprised of 5% stacking gel and 10% resolving gel. Electrophoresis was conducted using a Mini Protein II unit (Bio-Rad, USA) at constant voltage of 80 V for stacking gel and 120 V for resolving gel for approximately 90 min of total running time. The gels were stained with 1 g Coomassie brilliant blue R-250 in a 4.5:4.5:1 solution of methanol-water-acetic acid, and de-stained several times by gentle shaking with an 8:1:1 solution of water-methanol-acetic acid. High-molecular weight markers ranging from 53 kDa–220 kDa (GE Healthcare UK) were used to estimate the molecular weight of the protein fractions.

2.5.5. Fourier transform infrared (FT-IR) spectra analysis

The differences in frequencies of functional groups in gelatin films prepared with different protein and glycerol concentrations were determined using a Nicolet iS5 FT-IR spectrometer (Thermo, Madison, WI, USA). Films were placed onto the crystal cell and the cell was clamped into the mount of a FT-IR spectrometer. The spectra were collected in 32 scans with a resolution of 4 cm^{-1} over the range of 4000–400 cm^{-1}, and the data were rationed against a background spectrum recorded from the clean empty cell at 25 °C. The spectra were analyzed using the OMNIC 8.2 software package (Thermo Fisher Scientific Inc., USA).

2.5.6. Scanning electron microscopy (SEM)

The morphology of the upper surface of the film samples was studied using a field emission gun scanning electron microscope (FEG-SEM) (JSM-7600TFE, JEOL, Tokyo, Japan). The samples were mounted on specimen stubs using double sided adhesive tape, and made conductive by sputter-coating with gold-palladium. This step was repeated twice for 15 s using a sputter-coater under vacuum for 30 s under a current of 15 mA. After coating, the samples were observed at an accelerating voltage of 2 kV using a LEI (low secondary electron image) detector at low current.

2.6. Statistical analysis

Data were statistically analyzed using the General Linear Models procedure of SAS (Release 9.4, SAS Institute Inc., Cary, NC, USA) software. Mean comparisons were carried out by Duncan's multiple range test ($p < 0.05$) (Steel and Torrie, 1980).

3. Results and discussion

3.1. Effect of the different extraction methods on the hydroxyproline content and yield of gelatin

The Hyp content and the yield of gelatin extracted using three different methods are shown in Fig. 1. The method using trypsin pre-treatment produced the highest Hyp content (7.41 ± 0.49 mg Hyp/g treated skin) and the highest yield (53.05 ± 4.38%) of gelatin from salmon skins, as compared to the other two pre-treatments used. The difference in Hyp and yield of gelatin extracted may possibly be due to the effectiveness of the protease in collagen hydrolysis, in contrast to the random hydrolysis of collagen by chemical pre-treatments. In general, saline solution is used to solubilize myofibrillar proteins and the remaining muscle adhered to the skins. The saline solution also randomly disrupts the hydrogen bonds that stabilize the collagen structure to facilitate the extraction of gelatin (Giménez et al., 2005b). The alkaline pre-treatment randomly hydrolyzes peptide bonds and cleaves some inter-chain cross-links of the collagen protein which further disrupts the collagen structure (Galea et al., 2000; Yoshimura et al., 2000). Collagen cross-links are readily cleaved by proteases, and protease-aided processes have been successfully used to enhance collagen solubilization with improved gelatin yields (Chomarat et al., 1994; Nalinanon et al., 2008). Therefore, the higher Hyp content and the higher gelatin yield obtained suggest an increased cleavage of collagen cross-links by trypsin, resulting in a higher degree of collagen solubilization and enhanced gelatin extraction efficiency. This is consistent with the higher yields of gelatin reported from bigeye snapper skins treated with pepsin as compared to skins without pepsin treatment (Nalinanon et al., 2008).

From comparing the pre-treatment conditions, the results showed that the trypsin concentrations used had a marked effect on the molecular weight distribution of the gelatin polypeptide chains (data not shown). Both results of yields and protein patterns of gelatin revealed distinct effects of the trypsin concentrations used in pre-treatment, where higher trypsin concentration produced gelatin with higher yield but also had a greater effect on the degradation of the major protein chains of gelatin. The degradation of the gelatin major protein chains into low molecular weight chains is undesirable in the production of high quality gelatin (Galea et al., 2000), as the functional properties of gelatin are influenced by their molecular weight distribution (Muyonga et al., 2004).

Further investigations were conducted to examine the yield and molecular weight distribution of gelatin extracted from salmon fish skins incubated with a lower trypsin concentration of 1 U/g for 4 h, and extracted at 50 °C for 3 h. Gelatin yield was markedly decreased using these processing conditions (8.05 ± 0.16%). Lower yields were reported when lower pepsin concentration (i.e., 5 units/g treated skin) was used in pre-treating fish skin for gelatin extraction studies (Nalinanon et al., 2008) at the specified extracting times and temperatures (Kolodziejska et al., 2008). Nonetheless, gelatin obtained in this investigation showed the presence of major polypeptide chains

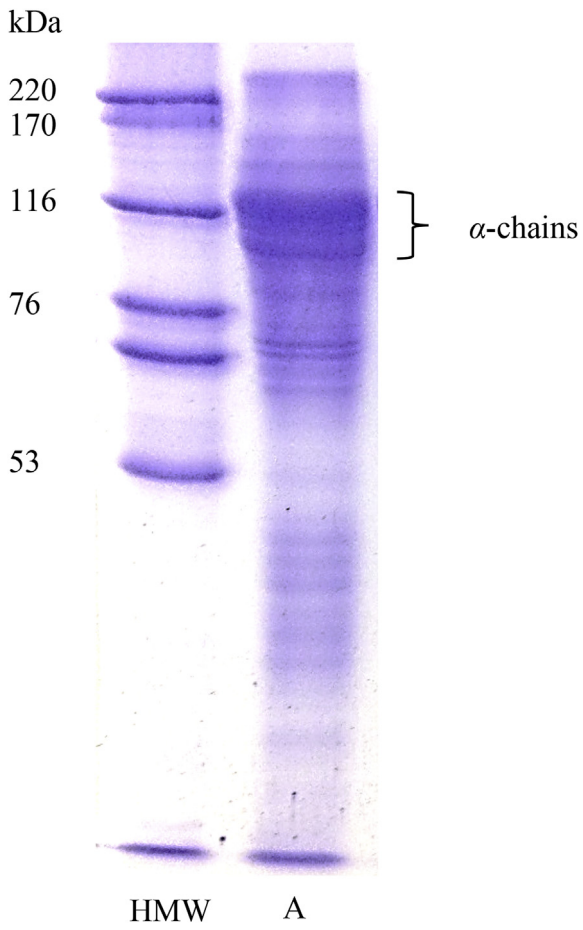


Fig. 2. SDS-PAGE patterns of gelatins extracted from salmon fish skins (A) incubated with 1 U/g trypsin for 4 h and extracted at 50 °C for 3 h. HMW denoted for high molecular weight protein markers.

Table 1
Effect of protein and glycerol concentration on the thickness, mechanical properties and water solubility of salmon skin gelatin films.[a,b].

Protein concentration (% in FFS[c])	Glycerol concentration (% of protein)	Thickness (mm)	TS (MPa)	EAB (%)	Water solubility (%)
1%	30%	0.039 ± 0.006^{e}	6.31 ± 1.80^{c}	2.67 ± 0.39^{b}	94.91 ± 5.01^{a}
2%		0.042 ± 0.005^{e}	11.31 ± 0.63^{bc}	12.31 ± 3.43^{b}	92.10 ± 3.94^{a}
3%		0.059 ± 0.007^{d}	11.77 ± 1.94^{bc}	27.28 ± 5.52^{b}	91.58 ± 6.43^{a}
4%		0.070 ± 0.006^{cd}	11.90 ± 2.78^{bc}	57.66 ± 7.26^{a}	90.14 ± 6.71^{a}
5%		0.079 ± 0.004^{bc}	16.42 ± 3.34^{b}	58.43 ± 4.16^{a}	89.07 ± 1.69^{a}
6%		0.090 ± 0.013^{b}	48.87 ± 8.11^{a}	14.12 ± 3.43^{b}	90.07 ± 0.49^{a}
7%		0.112 ± 0.012^{a}	44.00 ± 4.01^{a}	6.26 ± 1.10^{b}	90.64 ± 1.28^{a}
5%	10%	0.049 ± 0.007^{c}	68.84 ± 12.63^{a}	22.00 ± 1.17^{b}	84.80 ± 1.59^{a}
	20%	0.056 ± 0.008^{c}	56.37 ± 4.29^{a}	31.97 ± 2.40^{b}	85.72 ± 5.30^{a}
	30%	0.079 ± 0.004^{b}	16.42 ± 3.34^{b}	58.43 ± 4.16^{a}	89.07 ± 1.69^{a}
	40%	0.079 ± 0.018^{b}	17.28 ± 3.98^{b}	66.03 ± 4.12^{a}	81.29 ± 7.89^{a}
	50%	0.101 ± 0.016^{a}	1.49 ± 0.47^{c}	78.01 ± 5.52^{a}	84.26 ± 6.32^{a}

[a] Data are expressed as mean ± standard deviation.
[b] Different superscripts in the same column indicate statistical differences ($p < 0.05$).
[c] FFS means film-forming solution.

(α-chains) (Fig. 2). Consequently, an increase in the α-chains content could facilitate an increase in intermolecular interaction that contribute to the formation of gelatin network in film forming. Hence, a lower trypsin concentration was found to successfully minimize the degradation of gelatin extracted from fish skins. This is in agreement with the findings from a study of gelatin extraction conducted by Cabeza et al. (1997), which reported that a very small amount of trypsin was sufficient to produce good quality gelatin as a result of the high efficiency of trypsin in solubilizing collagen.

3.2. Film properties

3.2.1. Mechanical properties

The TS and EAB of films prepared with different protein concentrations are shown in Table 1. The TS of the films increased (from 6.31 to 44.00 MPa) when the protein concentration increased from 1 to 7% (w/v). As shown in Table 1, there was a marked increase in the TS values of the films ($p < 0.05$) when the protein concentration increased from 5 (16.42 MPa) to 6% (48.87 MPa). The TS values of films with 6 and 7% protein concentrations (48.87 and 44.00 MPa, respectively) were significantly higher ($p < 0.05$) than the other films. The increase in the TS values was due to the increase in the number of protein chains per unit surface, resulting in an increase in the potential intermolecular interactions that contribute towards higher TS values (Cuq et al., 1996). Similar effect was also observed for films prepared from bigeye red snapper and brownstripe red snapper skin gelatins (Jongjareonrak et al., 2006), and blue shark skin gelatin (Limpisophon et al., 2009).

Meanwhile, the EAB of the films increased (from 2.67 to 58.43%) with increasing protein concentration of the FFS from 1 to 5% (w/v), where the EAB values of the films at 4 and 5% protein concentration (w/v) were significantly higher ($p < 0.05$) than those for the other films. These higher EAB values indicated that an increase in protein concentration increased the protein chain-to-chain interactions, resulting in an enhanced flexibility of the films (Hoque et al., 2011; Jongjareonrak et al., 2006; Limpisophon et al., 2009). However, decreased EAB values were obtained for films above 5% protein concentration (w/v). For films with 6 and 7% protein (w/v), a remarkable decrease in the EAB values (14.12% and 6.26%, respectively) accompanied with a significant increase in TS values were observed. This could be due to possible extensive protein intermolecular interactions and cross-links formation as a result of the high protein concentrations in the FFS. This led to reduced mobility of the protein chains, resulting in films with high strength but low elasticity. Films with 6 and 7% protein concentration (w/v) were thick, hard and brittle (Table 1).

As shown in Table 1, the TS values decreased (from 68.84 to 1.49 MPa) and the EAB values increased (from 22.00 to 78.01%) when the glycerol concentration increased from 10 to 50% (w/w, of protein) for the same protein concentration (5%, w/v). Glycerol is a relatively small molecule that migrates through the protein chains and form hydrogen bonds with the amide groups and the amino acid side chains of

Table 2
Effects of protein concentration on the light transmission and opacity of salmon skin gelatin films.[a,b].

Protein concentration of FFS[c] (%)	200 nm	280 nm	350 nm	400 nm	500 nm	600 nm	700 nm	800 nm
Light transmission (%*T*) at different wavelength								
1%	0.3 ± 0.0^{a}	7.2 ± 0.1^{a}	75.6 ± 0.1^{a}	82.2 ± 0.1^{a}	87.1 ± 0.1^{a}	88.1 ± 0.1^{a}	89.0 ± 0.0^{a}	89.8 ± 0.0^{a}
2%	0.3 ± 0.0^{a}	4.1 ± 0.1^{b}	76.0 ± 0.1^{a}	80.7 ± 0.1^{b}	84.5 ± 0.0^{b}	85.7 ± 0.0^{b}	86.7 ± 0.0^{b}	87.6 ± 0.0^{b}
3%	0.2 ± 0.1^{a}	1.1 ± 0.1^{c}	59.4 ± 0.8^{b}	69.6 ± 1.4^{c}	78.1 ± 2.6^{c}	81.2 ± 2.7^{c}	83.3 ± 2.8^{c}	84.7 ± 3.1^{c}
4%	0.1 ± 0.0^{b}	0.1 ± 0.0^{d}	45.8 ± 0.1^{e}	55.6 ± 0.1^{f}	68.5 ± 0.1^{d}	72.4 ± 0.1^{d}	75.7 ± 0.0^{d}	78.4 ± 0.0^{d}
5%	0.1 ± 0.0^{b}	0.2 ± 0.0^{d}	50.4 ± 0.0^{c}	58.0 ± 0.0^{d}	67.0 ± 0.0^{d}	69.9 ± 0.0^{e}	72.4 ± 0.0^{e}	74.5 ± 0.0^{e}
6%	0.1 ± 0.0^{b}	0.1 ± 0.0^{d}	47.7 ± 0.0^{d}	56.5 ± 0.1^{e}	65.2 ± 0.1^{e}	68.6 ± 0.0^{e}	70.8 ± 0.0^{e}	72.9 ± 0.1^{e}
7%	0.1 ± 0.1^{b}	0.1 ± 0.0^{d}	26.2 ± 0.1^{f}	38.2 ± 0.1^{g}	51.9 ± 0.0^{f}	57.6 ± 0.1^{f}	61.4 ± 0.1^{f}	64.4 ± 0.1^{f}
Opacity[d] (%) at different wavelength								
1%	99.7 ± 0.0^{b}	92.8 ± 0.1^{d}	24.4 ± 0.1^{f}	17.8 ± 0.1^{g}	12.9 ± 0.1^{f}	11.9 ± 0.1^{f}	11.0 ± 0.0^{f}	10.2 ± 0.0^{f}
2%	99.7 ± 0.0^{b}	95.9 ± 0.1^{c}	24.0 ± 0.1^{f}	19.3 ± 0.1^{f}	15.5 ± 0.0^{e}	14.3 ± 0.0^{e}	13.3 ± 0.0^{e}	12.4 ± 0.0^{e}
3%	99.8 ± 0.1^{b}	98.9 ± 0.1^{b}	40.6 ± 0.8^{e}	30.4 ± 1.4^{e}	21.9 ± 2.6^{d}	18.8 ± 2.7^{d}	16.7 ± 2.8^{d}	15.3 ± 3.1^{d}
4%	99.9 ± 0.0^{a}	99.9 ± 0.0^{a}	54.2 ± 0.1^{b}	44.4 ± 0.1^{b}	31.5 ± 0.1^{c}	27.6 ± 0.1^{c}	24.3 ± 0.0^{c}	21.6 ± 0.0^{c}
5%	99.9 ± 0.0^{a}	99.8 ± 0.0^{a}	49.6 ± 0.0^{d}	42.0 ± 0.0^{d}	33.0 ± 0.0^{c}	34.8 ± 0.1^{b}	27.6 ± 0.0^{b}	25.5 ± 0.0^{b}
6%	99.9 ± 0.0^{a}	99.9 ± 0.0^{a}	52.3 ± 0.0^{c}	43.5 ± 0.1^{c}	34.8 ± 0.1^{b}	31.4 ± 0.0^{b}	29.2 ± 0.0^{b}	27.1 ± 0.1^{b}
7%	99.9 ± 0.1^{a}	99.9 ± 0.0^{a}	73.8 ± 0.1^{a}	61.8 ± 0.1^{a}	48.1 ± 0.0^{a}	42.4 ± 0.1^{a}	38.6 ± 0.1^{a}	35.6 ± 0.1^{a}

[a] Data are expressed as mean ± standard deviation.
[b] Different superscripts in the same column indicate statistical differences ($p < 0.05$).
[c] FFS means film-forming solution.
[d] Opacity (%) = 100% - *T* (*T*, transmittance (%) at each wavelength).

Table 3
Effects of glycerol concentration on the light transmission and opacity of salmon skin gelatin films with 5% protein. [a,b].

Glycerol concentration in FFS[c] (%)	200 nm	280 nm	350 nm	400 nm	500 nm	600 nm	700 nm	800 nm
Light transmission (%*T*) at different wavelength								
10%	0.2 ± 0.0[a]	5.5 ± 0.1[a]	80.1 ± 0.0[a]	84.8 ± 0.0[a]	88.0 ± 0.0[a]	89.0 ± 0.0[a]	89.7 ± 0.0[a]	90.3 ± 0.0[a]
20%	0.2 ± 0.1[a]	2.4 ± 1.8[b]	69.3 ± 8.0[b]	77.0 ± 6.1[b]	83.8 ± 4.6[a]	85.7 ± 2.9[a]	82.4 ± 1.8[b]	88.4 ± 2.2[a]
30%	0.1 ± 0.0[a]	0.2 ± 0.0[c]	50.4 ± 0.0[c]	58.0 ± 0.0[c]	67.0 ± 0.0[b]	69.9 ± 0.0[b]	72.4 ± 0.0[c]	74.5 ± 0.0[b]
40%	0.1 ± 0.0[a]	0.1 ± 0.0[c]	44.5 ± 0.1[cd]	57.0 ± 0.1[c]	67.5 ± 0.1[b]	68.9 ± 0.0[b]	72.9 ± 0.0[c]	74.5 ± 0.0[b]
50%	0.1 ± 0.0[a]	0.1 ± 0.0[c]	40.8 ± 0.0[d]	55.7 ± 0.0[c]	66.9 ± 0.0[b]	68.0 ± 0.0[b]	71.6 ± 0.0[c]	73.0 ± 0.0[b]
Opacity[d] (%) at different wavelength								
10%	99.8 ± 0.0[a]	94.5 ± 0.1[c]	19.9 ± 0.0[d]	15.2 ± 0.0[c]	12.0 ± 0.0[b]	11.0 ± 0.0[b]	10.3 ± 0.0[b]	9.7 ± 0.0[b]
20%	99.8 ± 0.1[a]	97.6 ± 1.8[b]	30.7 ± 8.0[c]	22.8 ± 7.5[b]	16.2 ± 4.6[b]	14.0 ± 3.5[b]	17.6 ± 1.8[a]	11.6 ± 2.1[b]
30%	99.9 ± 0.0[a]	99.8 ± 0.0[a]	49.6 ± 0.0[b]	42.0 ± 0.0[a]	33.0 ± 0.0[a]	30.1 ± 0.0[a]	27.6 ± 0.0[a]	25.5 ± 0.0[a]
40%	99.9 ± 0.0[a]	99.9 ± 0.0[a]	55.5 ± 0.1[a,b]	43.0 ± 0.1[a]	32.5 ± 0.1[a]	31.1 ± 0.0[a]	27.1 ± 0.0[a]	25.5 ± 0.0[a]
50%	99.9 ± 0.0[a]	99.9 ± 0.0[a]	59.2 ± 0.0[a]	44.3 ± 0.0[a]	33.1 ± 0.0[a]	32.0 ± 0.0[a]	28.4 ± 0.0[a]	27.0 ± 0.0[a]

[a] Data are expressed as mean ± standard deviation.
[b] Different superscripts in the same column indicate statistical differences ($p < 0.05$).
[c] FFS means film-forming solution.
[d] Opacity (%) = 100% - *T* (*T*, transmittance (%) at each wavelength).

the proteins. As a result, increasing the glycerol concentration in FFS caused a reduced intermolecular interaction in the protein chains, leading to an increased mobility of the protein chains and elasticity of the films (Gontard et al., 1993). In this study, significant differences ($p < 0.05$) were observed for both TS and EAB values for films at 20 and 30% glycerol concentrations (w/w, of protein).

3.2.2. Water solubility

The water resistance and integrity of a film can be measured by film solubility (Rhim et al., 2000). Gelatin films are known for their low water resistance because of their hydrophilic nature (McHugh and Krochta, 1994). Water solubility of gelatin films is shown in Table 1. No significant differences ($p > 0.05$) were found for the solubility of the films (from 89 to 95%) prepared with protein concentrations varying from 1 to 7% (w/v). The results were consistent with the findings obtained previously in other fish gelatin films (Carvalho et al., 2008; Hoque et al., 2011; Jiang et al., 2010). Meanwhile, the water solubility ranged from 81 to 89% for films having a glycerol concentration ranging from 10 to 50% (w/w, of protein); however, the differences observed were also not significant ($p > 0.05$). Glycerol, is a hydrophilic plasticizer capable of attracting water to the plasticized protein system due to the presence of three hydroxyl groups (Sothornvit and Krochta, 2001). Consequently, the addition of glycerol can increase the hydrophilicity and water solubility of protein films (Cuq, 2002; Nemet et al., 2010). An increase in film solubility was reported for gelatin-based composite films having a glycerol concentration ranging from 0.2 to 0.8%, but the differences were not significant (Nur Hanani et al., 2013a).

3.2.3. Light barrier properties

The data on light transmission (UV and visible), as well as opacity of the films at varying protein concentrations are presented in Table 2. As the protein concentration increased from 1 to 7% (w/v), the light transmission decreased and the opacity increased (wavelength from 200 to 800 nm). The lowest transmission and the highest opacity were recorded for films with the highest protein concentration (7%, w/v). Films with higher protein concentration absorbed much more light than those with lower protein concentration, owing to their greater thickness (Jongjareonrak et al., 2006) and the presence of more peptide bonds in the gelatin chains (Bao et al., 2009). Meanwhile, noticeable low values of light transmission (0.1–0.3%) accompanied by high opacity (92.8–99.9%) were recorded for all films in the UV light range of 200–280 nm (Table 2). Higher UV light barrier capacity was also reported for gelatin films by Jongjareonrak et al. (2006) and Hoque et al. (2011). These results suggested a possible reduction in UV-induced lipid

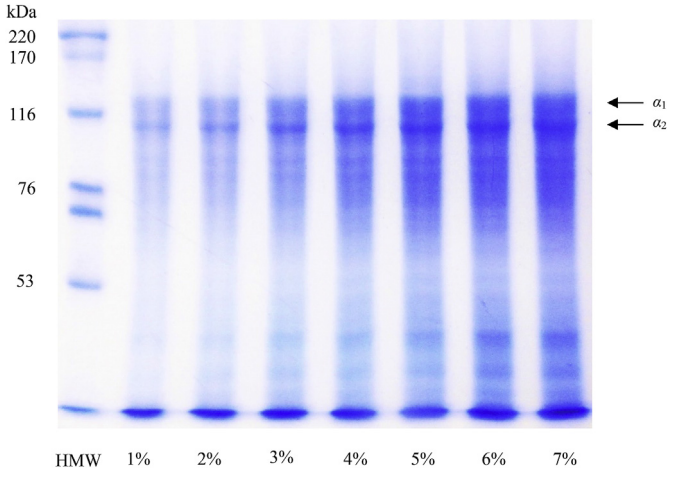


Fig. 3. Electrophoretic profile of gelatin films prepared with different protein concentrations (%, w/v); HMW denoted for high molecular weight protein markers.

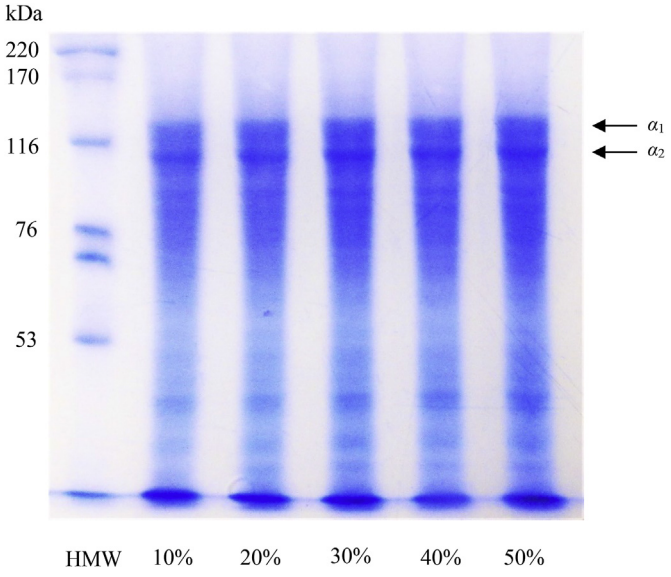


Fig. 4. Electrophoretic profile of gelatin films containing different glycerol concentrations (%, w/w, of protein); HMW denoted for high molecular weight protein markers.

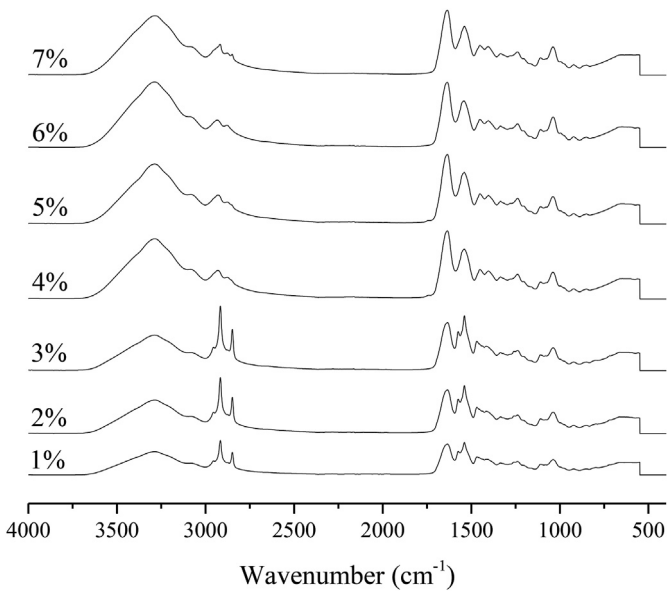


Fig. 5. FT-IR spectra of gelatin films prepared with different protein concentrations (%, w/v).

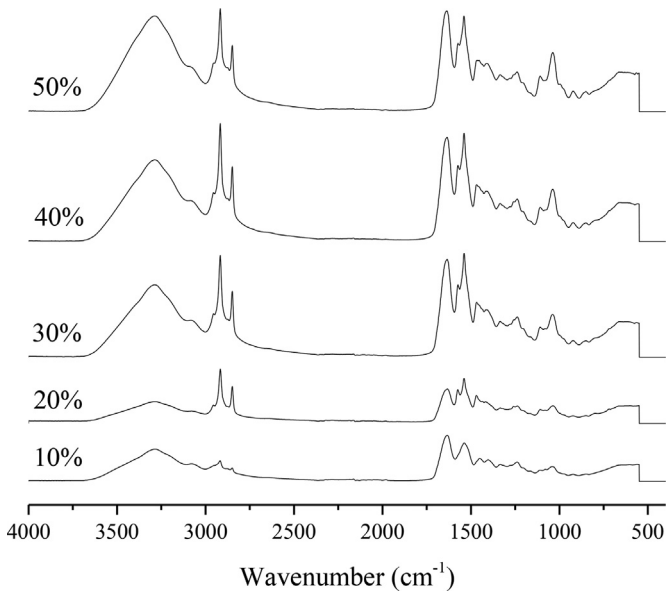


Fig. 6. FT-IR spectra of gelatin films containing glycerol concentrations (%, w/w of protein).

oxidation when applied to food systems (Gómez-Guillén et al., 2007). Similar to increasing protein concentration, the light transmission decreased and the opacity increased as the glycerol concentration increased from 10 to 50% (w/w, of protein) (Table 3). The lowest light transmission with the highest opacity was recorded for films having a glycerol concentration of 50% (w/w). An increase in glycerol concentration was found to the improve light barrier properties of gelatin films. This is possibly due to the different diffractive index between gelatin and glycerol (Limpisophon et al., 2009).

3.2.4. Electrophoretic protein patterns

The electrophoretic profiles for all films displayed the presence of α-chains in gelatins but at different intensities, confirming no excessive hydrolysis by trypsin on gelatin molecules. It was observed that increasing the protein concentration (from 1 to 7%, w/v) produced films with increased band intensity for the high molecular weight α-chains (α_1 and α_2-chains) (Fig. 3). The α-chains of gelatin can form inter- and intramolecular crosslinks mainly via hydrogen bonds, producing gelatin networks which are directly involved in film formation (Galea et al., 2000). Hence, the increased content of α-chains in films prepared with high protein concentrations probably caused an increase in the crosslinking density, leading to improved strength and elasticity of the films. This was evidenced by an increase in the TS and EAB values of the films (Table 1). Protein chains with different molecular weights affect the formation of the film network and resulting properties (Hoque et al., 2011). A high content in α-chains improves the functional properties (e.g. viscoelastic properties and gelling strength) of gelatin (Gómez-Guillén et al., 2002). In contrast, a decrease in high molecular weight protein chains and/or an increase in low molecular weight protein chains yield weaker film network (e.g. low TS and EAB) (Hoque et al., 2011; Jongjareonrak et al., 2006).

As shown in Fig. 4, there was no difference in protein pattern observed for all films with increasing glycerol concentration (from 10 to 50% of protein, w/w). High molecular weight proteins (α-chains) with no difference in their band intensities were observed in all gelatin films at varying glycerol concentrations. Similar observation was reported for films prepared from blue shark skin gelatin (Limpisophon et al., 2009) and cuttlefish skin gelatin (Hoque et al., 2011). However, a decrease in TS values and an increase in EAB values were observed for films prepared with increasing glycerol concentration (Table 1). These results are due to a decrease in intermolecular interactions between protein chains (Jongjareonrak et al., 2006).

3.2.5. FT-IR spectroscopy

FT-IR spectra for gelatin films prepared with different protein concentrations (1–7%, w/v) are shown in Fig. 5. Similar spectra were recorded for all films ranging from wavenumbers 1800-600 cm^{-1}, covering the amide-I, II and III bands. All films displayed major absorption bands at around 1634 cm^{-1} (amide-I, representing C═O stretching/hydrogen bonding coupled with COO), 1539 cm^{-1} (amide-II, attributed to the bending vibration of N–H groups and stretching vibrations of C–N groups), and 1239 cm^{-1} (amide-III, attributed to the vibrations in plane of C–N and N–H groups of bound amide or vibrations of CH_2 groups of glycine) (Aewsiri et al., 2009; Muyonga et al., 2004). Arfat et al. (2014) reported similar results for fish gelatin films, where the amide-I, amide-II and amide-III absorption bands were found at wavenumbers 1633, 1536 and 1238 cm^{-1}, respectively. In addition, the shift to a higher wavenumber (from 1633 to 1634 cm^{-1}) of amide-I band (Fig. 5) was coherent with the FT-IR spectra displayed for films prepared with increasing gelatin concentrations (Nur Hanani et al., 2013a). The band corresponding to the glycerol was found at around 1038 cm^{-1} (Fig. 5) (Arfat et al., 2014; Bergo and Sobral, 2007; Hoque et al., 2011).

The FT-IR results showed that the amide-A band at wavenumbers around 3286-3289 cm^{-1}, and the amide-B band at 2916-2930 cm^{-1} were present for all films (Fig. 5). Arfat et al. (2014) reported that amide-A and amide-B bands at wavenumbers of 3270–3280 cm^{-1} and 2926-2928 cm^{-1} respectively, were observed in all yellow stripe trevally skin gelatin films. Moreover, from Fig. 5, as the protein concentration increased from 1 to 7% (w/v), an increase in the amplitude of the amide-A band and a decrease in the amplitude of the amide-B band were observed, with noticeable changes for film made from 4% protein concentration (w/v). The amide-A band represents the stretching vibrations of N–H groups, whilst the amide-B band represents the stretching vibrations of CH and NH_3^+ groups (Ahmad and Benjakul, 2011; Muyonga et al., 2004). The higher amplitude of amide bands indicates the higher availability of amino groups, reflecting the lower interaction between gelatin molecules, and vice versa (Hoque et al., 2011). Meanwhile, the shift of wavenumbers of amide bands to lower frequencies demonstrates the higher involvement of N–H group in a hydrogen bond, indicating a higher interaction between the functional groups of peptide chains

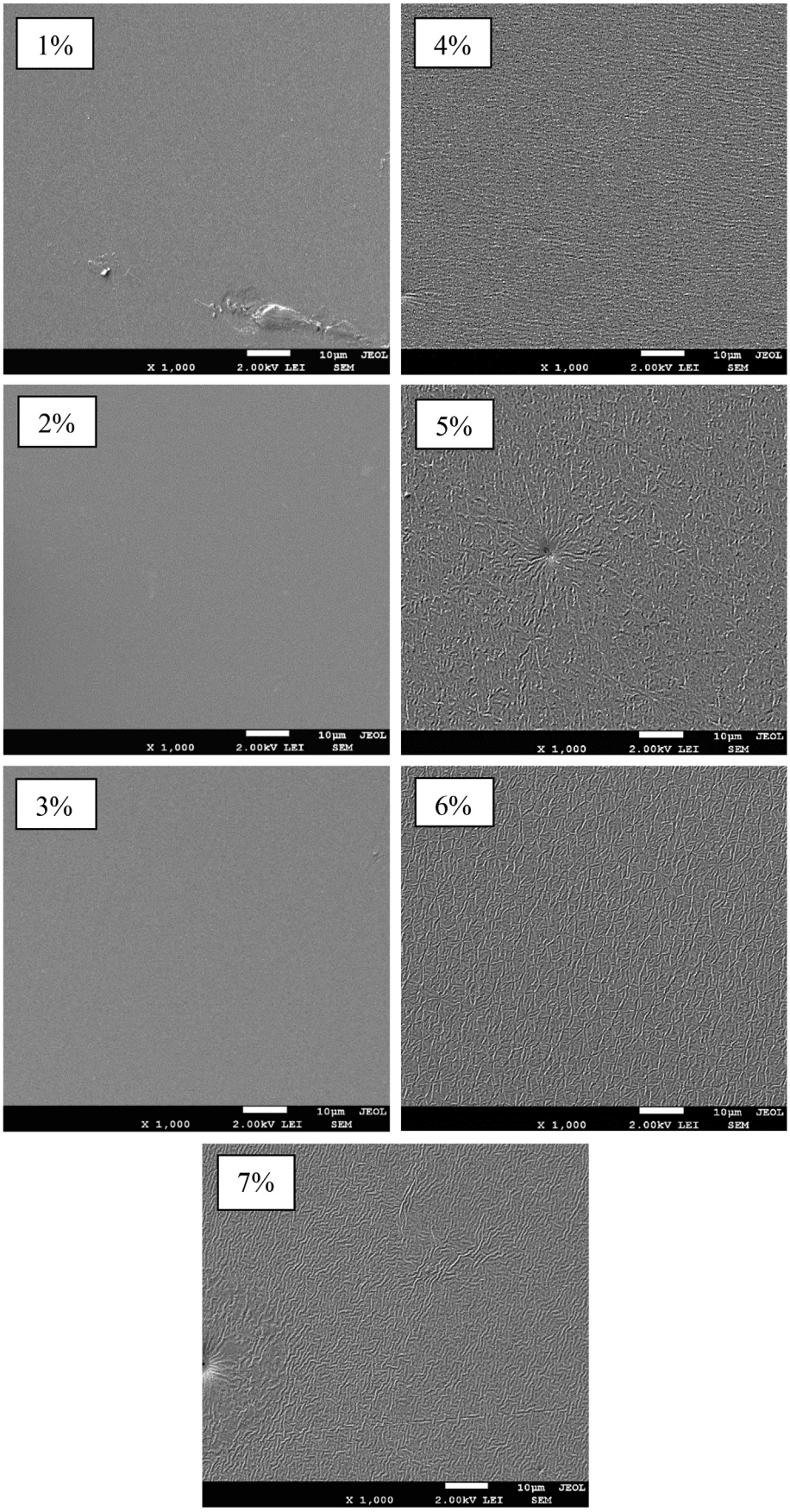


Fig. 7. SEM micrographs (at 1000× magnification) of surface of salmon gelatin films prepared with different protein concentrations (%, w/v).

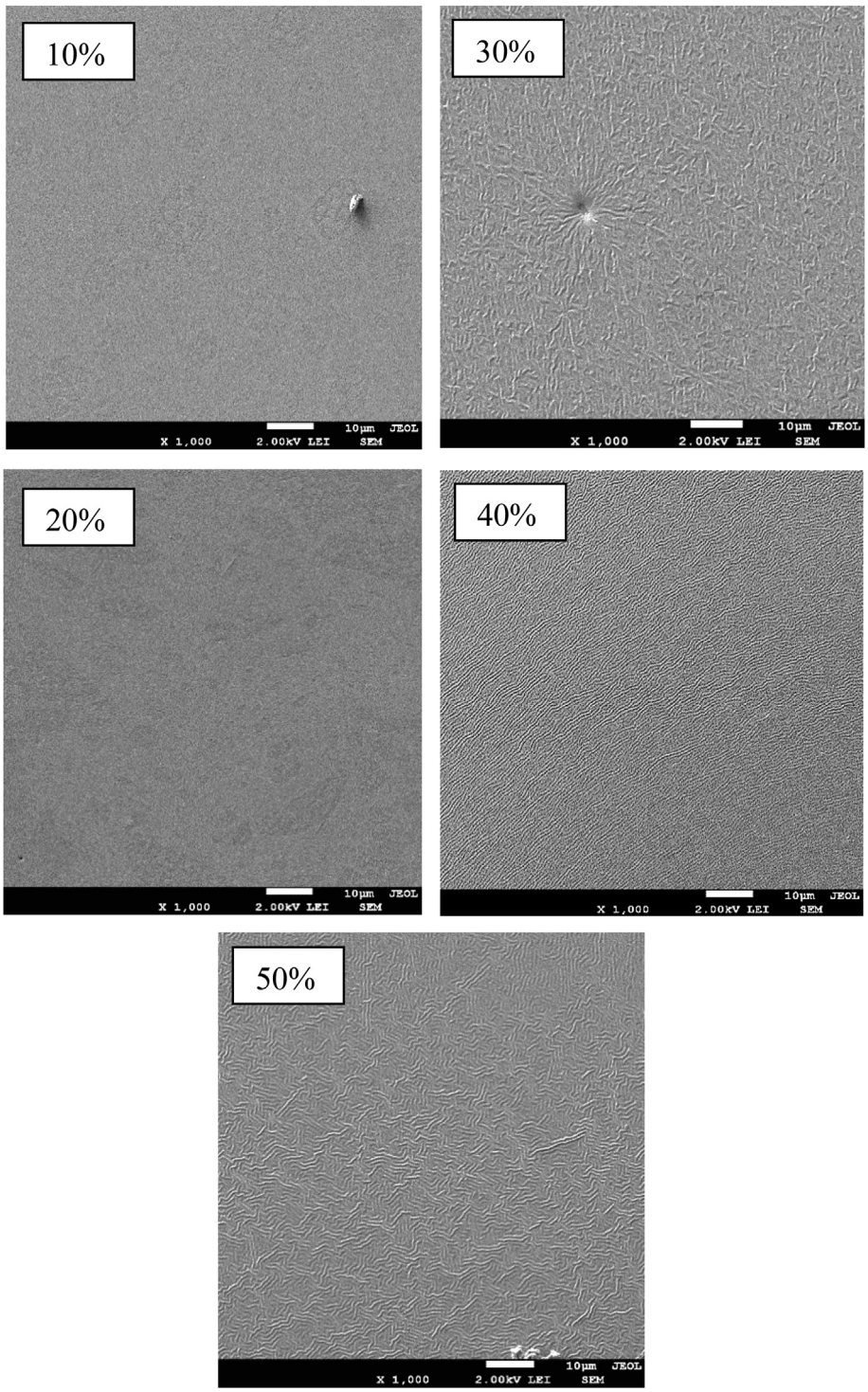


Fig. 8. SEM micrographs (at 1000× magnification) of surface of salmon gelatin films containing different glycerol concentrations (%, w/w, of protein).

(Ahmad et al., 2012; Doyle et al., 1975). Particularly at amide-B region, the shift to lower wavenumber (from 2930 to 2916 cm^{-1}) and lower amplitude of the amide-B band (Fig. 5) were shown for films prepared at increasing protein concentrations from 1 to 7% (w/v), suggesting the increased interaction of $–NH_3$ group between gelatin molecules (Ahmad and Benjakul, 2011; Ahmad et al., 2012).

Thus, the FT-IR results in this study confirmed the influence of protein concentrations in the film network on the mechanical properties of the resulting films. At increasing protein concentrations, the noticeable changes of amide bands' amplitudes and wavenumbers could support the increase in elasticity (EAB) of the films, particularly films prepared with 4 and 5% protein concentration (w/v) (Table 1). Furthermore, the FT-IR spectra of films at higher protein concentrations (6 and 7%, w/v) demonstrated higher changes in amplitudes and wavenumbers of amide bands, suggesting that the excess of a certain threshold amount of protein could lead to the possible extensive protein intermolecular interactions, which was reflected by the significant increase in TS values and a decrease in EAB of the films (Table 1).

The FT-IR spectra of gelatin films containing glycerol concentrations ranging from 10 to 50% (w/w, of protein) are shown in Fig. 6. Similar to

films with increasing protein concentration, major absorption bands of amide-I, II and III were located at wavenumbers 1634 cm^{-1}, 1539 cm^{-1}, and 1239 cm^{-1}, respectively. The amplitude of the band located at around 1038 cm^{-1} increased with increasing glycerol concentration (Bergo and Sobral, 2007; Hoque et al., 2011). This is consistent with the findings observed on the effect of increasing glycerol content on pigskin gelatin films (Bergo and Sobral, 2007) and beef skin gelatin films (Nur Hanani et al., 2013b). In addition, the amplitudes of the amide-A band (located at wavenumbers around 3286–3289 cm^{-1}) and the amide-B band (located at 2916-2918 cm^{-1}) increased as the glycerol concentration increased in films. An increase in amplitudes for both amide peaks formed are attributed to the higher availability of the amino groups, reflecting a decrease in interactions between gelatin chains in the presence of increased concentrations in glycerol (Hoque et al., 2011). On the other hand, an increase in the EAB values accompanied by a decrease in the TS values was observed for films with increasing glycerol concentration (Table 1).

3.2.6. Morphology

SEM micrographs of the surface of gelatin films prepared with different protein concentrations are shown in Fig. 7. Gelatin films prepared with 1–3% protein concentration (w/v) showed smooth surfaces, indicating a homogenous structure of films. Rough surface was noticed for films prepared with 4–7% protein concentrations (w/v), particularly for films with 6 and 7% protein concentrations (w/v). The roughness and compact structure of the films could be attributed to the increased number of interactions between the biopolymer chains via covalent and non-covalent bonding (Hoque et al., 2011; Prodpran et al., 2007). Moreover, the rough surface for films with 6 and 7% protein concentrations (w/v) could be indicative of extensive protein intermolecular interactions and cross-links formation, resulting in films with high mechanical strength and brittleness, as evidenced by their high TS and low EAB values (Table 1).

SEM micrographs of the surface of gelatin films having different glycerol concentrations are shown in Fig. 8. Smooth surface was observed for films having 10 and 20% glycerol concentration (w/w, of protein). Meanwhile, protein chains organization was more pronounced on the surface of films when the glycerol content increased from 30 to 50% (w/w), with a more ordered arrangement for glycerol concentrations of 40 and 50% (w/w). Interactions between small molecular weight compounds and gelatin produced uncoiled and elongated protein chains (Shakila et al., 2012). Consequently, higher glycerol concentrations increased gelatin molecules' elongation and mobility, contributing to an increased elasticity of films as evidenced by lower TS and higher EAB values (Table 1).

4. Conclusion

Extraction of salmon skin gelatin with trypsin supplementation induced a higher collagen solubilization and yield of gelatin as compared to extraction methods using chlorides and alkaline solutions pre-treatments, however, with noticeable degradation of the gelatin's major protein chains. Consequently, salmon gelatins extracted by a very low level of trypsin-aided process displayed higher molecular weight chains and were successfully used for film formation. The TS and EAB values of films increased with an increase in protein concentration from 1 to 5% (w/v). However, the EAB value reduced markedly for films with 6 and 7% protein concentrations (w/v), indicating the possible extensive protein intermolecular interactions and cross-links formation that exceeded a certain threshold amount of protein. Meanwhile, the decrease in the TS coupled with the increase in the EAB values was affected by the increased plasticizing effect as the concentration of glycerol increased. The increasing protein and glycerol concentrations had no effect on the water solubility, but a decrease light transmission accompanied by an increase in opacity for all films. The electrophoretic study showed the presence of α-chains that confirmed no hydrolysis by trypsin on gelatin molecules, and the increased in mechanical properties was attributed to the increased content of high molecular weight chains in gelatin as the concentration of protein increased. Meanwhile, the FT-IR spectra and morphological analysis revealed the interaction behavior between protein chains and with glycerol as they increased in films. This study confirms the feasibility of producing film using fish skin gelatin extracted by a trypsin-aided process.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest with respect to the work in this manuscript.

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