Characteristics and gel properties of gelatin from skin of Asian bullfrog (*Rana tigerina*)

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Abstract

Characteristics and gel properties of gelatin from frog skin as influenced by extraction temperatures (45−75 °C) were investigated. Yield of gelatin increased as the extraction temperature increased ($P < 0.05$). All gelatins contained α- and β-chains as the predominant components and showed a high imino acid content (215 residues/1000 residues). Fourier transform infrared (FTIR) spectra indicated that all gelatin samples had major peaks in amide regions. Gelatin extracted at 55 °C exhibited the highest gel strength ($P < 0.05$), which was similar to that of commercial bovine gelatin ($P > 0.05$). Gelling and melting temperatures of frog skin gelatin were 23.47−24.87 and 33.22−34.66 °C, respectively. Gels became more yellowish with increasing extraction temperatures ($P < 0.05$). All gelatin gels were sponge or coral-like in structure but varied in patterns as visualized by scanning electron microscopy (SEM). Gelatin from frog skin could be used as a replacement for land animal counterpart.

Keywords: Asian bullfrog, *Rana tigerina*, Gelatin, Extraction, Gelation

Introduction

Gelatin is a fibrous protein obtained from thermal denaturation of collagen. It has a wide range of applications in food and non-food industries [1]. Gelatin is traditionally produced from skins and bones of certain mammalian species, particularly bovine and porcine [2]. However, outbreaks of bovine spongiform encephalopathy (BSE; mad cow disease) have raised concerns for consumers [3]. Fish gelatin has gained popular as the safe and acceptable ingredient, regardless of religions. Nevertheless, fish gelatin still has low gel strength, thereby limiting its applications. As a consequence, alternative sources for gelatin production have gained increasing attention.

Conversion of collagen into soluble gelatin is due to the cleavage of a number of intra- and intermolecular cross-linking bonds in collagen via heat treatment. As a result, the gelatin obtained generally has molecular weights lower than native collagen and constitutes a mixture of fragments with molecular weights in the range of 15–400 kDa [4]. The degree of conversion of collagen into gelatin and its properties depend on the raw material, pretreatment and processing parameters including temperature, time, and pH [5]. High extraction temperature resulted in the increasing yield but lowered gel strength of resulting gelatin from splendid squid skin [6]. Sinthusamran, et al. [7] also reported that gelatin from seabass skin extracted at a higher temperature (55 °C) had the highest extraction yield, but exhibited the poorer gel properties than those extracted at lower temperature.

Asian bullfrog (*Rana tigerina*) is amphibian species commonly farmed in many parts of Thailand for domestic consumption and export [8]. Frog farming has expanded throughout Thailand due to the productive culture and market demand. The frog production of an approximately 10 tons/day is available for both local and oversea markets, particularly Hong Kong, Singapore and Taiwan [9]. During processing or dressing of frog, skins are generated and considered as byproducts. Frog skins can be used for gelatin production due to their abundance and low cost. Additionally, skins pose no threat of BSE and can be considered as a safe gelatin source. Collagen from skins of various frog species such as *R. tigerina* [10] and bullfrog [11, 12], have been extracted and characterized. However, no information regarding the extraction and characteristics of gelatin from Asian bullfrog skin exists. The aim of the present study was to examine the characteristics and gelling properties, including gel strength, gelling and melting temperatures, of gelatins from the skin of Asian bullfrog (*R. tigerina*) as affected by extraction temperatures.

Materials and Methods

Chemicals

Sodium dodecyl sulphate (SDS), Coomassie Blue R-250, and *N*,*N*,*N'*,*N'*-tetramethylethylenediamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). L-leucine and bovine serum albumin (BSA) were procured from Sigma Chemical Co. (St. Louis, MO, USA). High molecular weight markers including myosin (220 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa) and glutamic dehydrogenase (53 kDa), were obtained from GE Healthcare UK Limited (Buckinghamshire, UK). Food grade bovine bone gelatin was purchased from Halagel (Thailand) Co., Ltd. (Bangkok, Thailand). Fish gelatin produced from tilapia skin was procured from Lapi Gelatine S.p.a. (Empoli, Italy). All chemicals were of analytical grade.

Collection of Frog Skins

Skins of Asian bullfrog (*Rana tigerina*) with a weight of 200–300 g/frog were obtained from a farm in Hat Yai, Songkhla, Thailand. Skins were kept in a polystyrene box containing ice using a skin/ice ratio 1:2 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai, within 1 h. Upon arrival, the skins were washed with iced tap water (1–3 °C). The skins were pooled as a composite sample, placed in polyethylene bags and stored at –20 °C until used. The storage time was less than 2 months. Prior to gelatin extraction, frozen skins were thawed with running water (25–26 °C) for 30 min and cut into small pieces ($1.0 \times 1.0\ cm^2$) using scissors.

Pretreatment of Frog Skins

Removal of Non-Collagenous Proteins

The prepared skins were soaked in 0.3 M NaOH with a skin/alkali solution ratio of 1:10 (w/v) to remove non-collagenous proteins. The mixture was stirred for 6 h at room temperature (28–30 °C) using an overhead stirrer model RW20.n (IKA®-Werke GmbH & Co. KG, Staufen, Germany) at a speed of 300 rpm. The alkaline solution was changed every 2 h at 2nd and 4th hour (totally 2 times). Alkali-treated skin was washed with tap water until a neutral or slightly basic pH (7.0−7.5) of wash water was obtained.

Acid Pretreatment

After being treated with alkaline solution, the skins were swollen using 0.15 M acetic acid at a skin/solution ratio of 1:10 (w/v). The mixture was stirred at a speed of 300 rpm at room temperature for 4 h and the swollen skin was washed using tap water. Washing was continued until the wash water had neutral or slightly acidic in pH (6.5−7.0).

Extraction of Gelatin from Frog Skins

To extract gelatin, the pretreated skins were mixed with distilled water at a ratio of 1:10 (w/v) at 45, 55, 65 and 75 °C in a water bath (W350, Memmert, Schwabach, Germany). The mixtures were stirred continuously for 12 h using an overhead stirrer (RW 20.n, IKA®-Werke GmbH & Co. KG, Staufen, Germany) at a speed of 150 rpm. The mixtures were then filtered using a Buchner funnel with a Whatman No. 4 filter paper (Whatman International, Ltd., Maidstone, England). Thereafter, the filtrates were frozen at −40 °C for 12 h and then lyophilized using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark) at −50 °C for 72 h. Gelatins obtained from frog skins extracted at 45, 55, 65 and 75 °C were referred to as ‘G45’, ‘G55’ and ‘G65’ and ‘G75’, respectively. Lyophilized gelatin samples were subsequently subjected to analyses.

Analyses

Yield

The yield of gelatin was calculated based on initial weight (wet weight) of the starting material using the following equation:

$$\text{Yield (\%)} = \frac{\text{Weight of freeze−dried gelatin (g)}}{\text{Weight of initial skin (g)}} \times 100 \qquad (1)$$

Determination of Hydroxyproline Content

Hydroxyproline content was analyzed according to the method of Bergman, et al. [13]. Hydroxyproline content was calculated and expressed as mg/g sample.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)

Protein patterns were determined using SDS-PAGE according to the method of Laemmli [14]. The gelatin samples (15 mg/mL protein) were dissolved in 5% SDS and the mixtures were incubated at 85 °C for 1 h using a temperature-controlled water bath. Solubilized samples were mixed at 1:1 (v/v) ratio with sample buffer (0.5 M Tris–HCl, pH 6.8 containing 5% SDS and 20% glycerol). Samples (5 µL) were loaded onto a polyacrylamide gel made of 7.5% separating gel and 4% stacking gel and subjected to electrophoresis at a constant current of 15 mA/gel using a Mini Protein III unit (Bio-Rad

Laboratories, Inc., Richmond, CA, USA). After electrophoresis, gels were stained with 0.05% (w/v) Coomassie blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h. Finally, they were destained with a mixture of 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min and destained again with a mixture of 5% (v/v) methanol and 7.5% (v/v) acetic acid for 1 h. High molecular weight protein markers were used for the estimation of molecular weight of interested proteins.

Fourier Transform Infrared (FTIR) Spectroscopy

Attenuated total reflectance Fourier transform infrared spectrometer model Equinox 55 (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR trough plate crystal cell (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology, Inc., Madison, WI, USA) was used. The spectra, in the range of 4000-400 cm^{-1} (mid-IR region) with automatic signal gain, were collected in 32 scans at a resolution of 4 cm^{-1} and ratioed against a background spectrum recorded from the clean and empty cell at 25 °C. Analysis of spectral data was carried out using the OPUS 3.0 data collection software program (Bruker Co, Ettlingen, Germany.).

Gel Strength

Gelatin gels were prepared according to the method of Fernández-Díaz, et al. [15] with a slight modification. Gelatin sample was dissolved in distilled water at 60 °C to obtain a final concentration of 6.67% (w/v). The gelatin solution was then cooled in a refrigerator at 4 °C for 16–18 h for gel maturation. Gel strength of samples (3 cm diameter; 2.5 cm height) was determined at 8–10 °C using a texture analyzer model TA-XT2 (Stable Micro System, Surrey, UK) with a load cell of 5 kN, cross-head speed of 1 mm/s and equipped with a 1.27

cm diameter cylindrical flat-faced Teflon plunger. The maximum force (g) considered as 'gel strength' was recorded when the penetration distance reached 4 mm.

Determination of Gelling and Melting Temperatures

The gelling and melting temperatures of the gelatin samples were measured following the method of Boran, et al. [16] using a controlled stress rheometer (RheoStress RS 75, HAAKE, Karlsruhe, Germany). The gelatin solution (6.67%, w/v) was prepared in the same manner as described previously. The solution was preheated at 35 °C for 30 min. The measuring geometry used was a 3.5 cm parallel plate and the gap was set at 1.0 mm. The measurement was performed at a scan rate of 0.5 °C/min, frequency of 1 Hz, oscillating applied stress of 3 Pa during cooling from 35 to 5 °C and heating from 5 to 35 °C. The gelling and melting temperatures were calculated, where tan δ became 1 or d was 45°.

Color

Color of gelatin gels were measured using a Hunter Lab Colorimeter (Color Flex, Hunter Lab Inc., Reston, VA, USA). L*, a* and b* indicating lightness/brightness, redness/greenness and yellowness/blueness, respectively, were recorded. Total difference in color (ΔE*) was calculated as described by Wrolstad, et al. [17].

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

where ΔL*, Δa* and Δb* were the differences between the corresponding color parameter of the sample and that of white standard.

Microstructure

The microstructure of gelatin gel was visualized using a scanning electron microscopy (SEM). Gelatin gels were prepared in the same manner as those used for gel strength measurement. Gelatin gels having a thickness of 2–3 mm were fixed with 2.5% (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h, rinsed with distilled water for 1 h, and dehydrated in ethanol using a serial concentration of 50−100% with 10% increment. The samples were then subjected to critical point drying. Dried samples were mounted on a bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA, USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 20 kV.

Amino Acid Analysis

Amino acid compositions of frog skin and gelatin from frog skin extracted at 55 °C were analyzed as described by Sae-leaw, et al. [18]. The samples were hydrolyzed under reduced pressure in 4 M methanesulfonic acid containing 0.2% (v/v) 3-2(2-aminoethyl) indole at 115 ºC for 24 h. The hydrolysates were neutralized with 3.5 M NaOH and diluted with 0.2 M citrate buffer (pH 2.2). An aliquot of 0.04 ml was applied to an amino acid analyzer (MLC-703; Atto Co., Tokyo, Japan).

Statistical Analysis

All experiments were run in triplicate using three different lots of samples. The data were subjected to one-way analysis of variance (ANOVA). Comparison of means was carried out using the Duncan’s multiple range test. Statistical analysis was done using the Statistical Package for Social Science (SPSS 11.0 for windows, SPSS Inc., Chicago, IL, USA). Differences between means at the 5% ($P < 0.05$) level were considered significant.

Results and Discussion

Extraction Yield and Hydroxyproline Content

The yields of gelatin extracted at different temperatures from the skin of Asian bullfrog are shown in Fig. 1A. The yield generally increased as the extraction temperatures increased ($P < 0.05$). Yields of 7.14, 12.41, 13.78 and 15.40% (on wet weight basis) were obtained for G45, G55, G65 and G75, respectively. The result suggested that the bondings between α-chains in the native mother collagen were more destabilized when higher heat was employed. As a consequence, the triple helix structure became amorphous and could be extracted into the medium with ease, leading to the higher yield. Higher extraction temperature effectively destroyed the hydrogen bonds stabilizing the collagen localized in skin matrix [19]. The result was in agreement with Kittiphattanabawon, et al. [20] who reported that the extraction yield of gelatin from the skin of brownbanded bamboo shark and blacktip shark increased when the extraction temperature increased. The yield and characteristics of gelatin are governed by the type of raw material and gelatin extraction process, including the pretreatment, etc. [20, 21].

Hydroxyproline content of gelatin from frog skin extracted at various temperatures is depicted in Fig. 1B. The highest hydroxyproline content (143.50 mg/g gelatin) was observed in G55 ($P < 0.05$). When extraction temperatures of 65 and 75 °C were used, lower hydroxyproline contents were obtained in resulting gelatins (G65 and G75). It was noted that no differences in hydroxyproline contents were observed as the extraction temperatures higher than 55 °C were used. Hydroxyproline is the unique amino acid found in collagenous materials [22]. The hydroxyproline content represented the amount of collagen denatured and converted to the amorphous gelatin, while yield represented the amount of solid released from pretreated skin matrix during extraction. Higher hydroxyproline content in G55 was plausibly due to the higher recovery of collagen from skin matrix. The lower content observed in G65 and G75 might be due to the co-extraction of other proteins present in skin into the medium.

Protein Patterns

Protein patterns of gelatin from frog skin extracted at different temperatures are shown in Fig. 2. All gelatin samples contained α-chain with MW of 126–115 kDa as the major constituent. Gelatin samples also contained β-components (α-chain dimers) and γ-components (α-chain trimers). The protein patterns of frog skin gelatins were similar to those of commercial fish gelatin. It was noted that commercial bovine gelatin had higher molecular weight of all components. Among all samples, G45 and G55 had the higher band intensities of α_1-, α_2-, β- and γ-chains. The band intensities of all constituents in gelatin decreased when the extraction temperature was higher than 55 °C. This might be caused by some degradation induced by the thermal process. Among all gelatins, G65 had the lowest band intensity of all components. This was presumed to be due to the presence of indigenous proteases in frog skin, which were able to cleave α-, β- and γ-chains most effectively at 65 °C. Thus, those proteases more likely contributed to the disintegration of gelatin molecules during the extraction process at 65 °C. Proteolysis induced by heat-activated and heat-stable indigenous proteases associated with skin matrix could contribute to the destabilization as well as disintegration of collagen structure by disrupting the intra- and intermolecular cross-links [23]. Heat-activated serine protease in bigeye snapper skin was associated with the drastic degradation of the α- and β-chains of the gelatin extracted at 60 °C [24]. These enzymes are bound with matrix components such as collagens [25]. Thus, extraction temperature played a profound role in protein pattern or distribution of gelatin from frog skin.

Fourier transform infrared (FTIR) spectra

FTIR spectra of gelatin from frog skin extracted at various temperatures are illustrated in Fig. 3. Generally, all the gelatins showed the similar spectra. The FTIR spectroscopy together with attenuated total reflectance (ATR) has been used to determine functional groups as well as intermolecular cross-linking of collagen and gelatin [26]. All gelatin samples had major peaks in amide regions. The absorption in the amide I region is due to C=O stretching/hydrogen bonding coupled with COO [27]. In the present study, the amide I peak was observed in the wavenumber range of 1630–1632 cm^{-1}. Amide I band with the wavenumber between 1700 and 1600 cm^{-1} was useful for infrared spectroscopic analysis of the secondary structure of proteins [28]. G45, G55, G65 and G75 exhibited the amide I band at the wavenumbers of 1630, 1630, 1632 and 1630 cm^{-1}, respectively. The amide I band of G65 was shifted to a higher wavenumber, compared to the others, indicating the higher loss of triple helix via breaking down of H-bonds between α-chains [26]. Additionally, G65 also showed a higher peak amplitude in amide I region than other samples. The result indicated that G65 had more free functional groups, especially C=O. This might be associated with the higher degradation of protein, thereby favoring the exposure of C=O of peptides or proteins. The change in amide I band of gelatin suggested that extraction temperature might affect the helical coil structure of gelatin, especially via exposure of hidden domains.

The characteristic absorption bands of gelatin samples in the amide II region were noticeable at the wavenumber range of 1537−1543 cm^{-1}. The amide II vibration mode is attributed to an out-of-phase combination of C–N stretch and inplane N–H deformation modes of the peptide group [27]. It was noted that extraction of gelatin at 65 °C might favor the dissociation and/or degradation of α-chain, as indicated by the shift to higher wavenumber of G65. In addition, the amide III bands of all gelatin samples were detected at the wavenumbers of 1236-1238 cm^{-1}. Amide III represents the combination peaks between C–N stretching vibrations and N–H deformation from amide linkages as well as absorptions arising from wagging vibrations from CH_2 groups from the glycine backbone and proline side chains [29]. G65 had the lowest peak amplitude in the amide III region. This indicated that the greater disorder of molecular structure of native collagen due to transformation of an α-helix to a random coil structure occurred [26]. Moreover, G65 and G75 exhibited the lower wavenumber in the amide III region than those of G45 and G55, suggesting the higher disorder of gelatins extracted at 65 and 75 °C associated with higher degradation. The result was in agreement with the lower band intensities of α-, β- and γ-chains of G65 and G75 (Fig. 2).

Amide A band, arising from the stretching vibrations of the N–H group coupled with hydrogen bonding [26], appeared at 3292, 3292, 3296 and 3294 cm^{-1} for G45, G55, G65 and G75, respectively. The position of amide A band shifted to a lower frequency as the NH group of a peptide is involved in hydrogen bonding [6]. The higher wavenumber of G65 indicated the higher content of amino groups caused by the enhanced protein degradation. This was coincidental with the lower band intensities of all components (Fig. 2). In addition, the highest amplitude of G65 was probably related to the higher hydrolysis. The amide B band was observed at 3078, 3078, 3076 and 3078 cm^{-1} for G45, G55, G65 and G75, respectively, corresponding to the asymmetric stretching vibration of =C–H as well as $–NH_3^+$ [6]. Among all samples, G65 showed the lowest wavenumber for the amide B peak, suggesting interaction of $–NH_3$ groups between peptide chains. Higher degradation resulted in the release of short peptides, which might undergo reaction to a higher extent, compared with bulky long chains. Therefore, the secondary structure and functional group of gelatins obtained from frog skin were affected by extraction temperatures.

Gel Strength

Gel strength of gelatin extracted from frog skin at different temperatures is shown in Table 1. G55 had the highest gel strength, while G65 showed the lowest gel strength ($P < 0.05$). The result was in accordance with the highest α- and β-chains of G55 (Fig. 2). The lower gel strength of G65 was more likely associated with the higher degradation of α-, β- and γ-chains as observed in protein pattern (Fig. 2). It was noted that the used of higher extraction temperature, particularly G75, provided the frog skin gelatin with the higher gel strength (P<0.05). This might related with the proteolytic degradation of high molecular weight components caused by indigenous proteases during extraction of gelatin at 65 °C, resulted in adverse effects on gel-forming properties of resulting gelatin [24]. This result was in accordance with protein pattern obtained from SDS-PAGE (Fig. 2). The lower intensity of α-2, β, and γ-chains was observed from G65, compared with others. Gel strength is one of the most important functional properties of gelatins [30]. The differences in gel strength between samples could be due to the differences in intrinsic characteristics, such as molecular weight distribution as well as chain-to-chain interactions determined by the amino acid composition and ratio of α/β chains present in the gelatin [31]. Gelatin structures with large amount of high molecular weight components including α-, β, and γ-chains, have been known to possess the maximal gelation [20]. Hence, the use of an appropriate extraction temperature could be an effective means to obtain the gelatin with the limited or negligible degradation of peptides, while maintaining the protein components in gelatin.

Gelling and Melting Temperatures

The gelling temperatures of gelatins from frog skin extracted at different temperatures were in the range of 24.05–24.87 °C (Table 1). No differences in the gelling temperatures were observed among different gelatins obtained from varying extraction temperatures ($P > 0.05$). It was found that all gelatins from frog skin had the higher gelling temperature than commercial bovine and fish gelatins (*P* < 0.05). Sinthusamran, et al. [7] reported that extraction temperatures affected the physico-chemical properties of gelatin, such as molecular weight distribution, the amount of β- and γ-components as well as gelling temperature. Therefore, the gelling temperature was not much affected by the extraction temperatures used in the present study. The gelling temperatures in this study were much higher than those of gelatins from the skins of bigeye snapper (10.0 °C) [32], yellowfin tuna (18.7 °C) [33], and silver carp (18.7) [16]. Thus, gelatins from frog skin were able to form gel at room temperature, showing the similar characteristic to mammalian gelatin.

The melting temperatures of gelatin gels from frog skin extracted at various temperatures were in the range of 33.22–34.66 °C (Table 1). No differences in melting temperatures between G45, G55, G65 and commercial bovine gelatin were observed (*P* > 0.05). It was noticed that the high extraction temperature (75 °C) resulted in the decreases in melting temperature of gelatin (G75) (*P* < 0.05). Eysturskarð, et al. [34] reported that gelling properties of gelatin from saithe skins was more or less unaffected by the extraction temperature in the range of 22−45 °C, while a drop in gelling properties was found by increasing extraction temperature to 65 °C. This was in accordance with the decreased of melting temperature of frog skin gelatin extracted at 75 °C. The melting temperature related to the number of chemical bonding formed in gel network [….]. The frog skin gel obtained from G75 might be formed with the weak bond or the less crosslink density, resulting the decreased melting temperature, compared with others tested. Varying melting temperatures were reported for gelatin from the skins of bigeye snapper (16.8 °C) [32], clown featherback (15.53−24.71 °C) [35], seabass (26.3–27.0°C) [7] and silver carp (27.1 °C) [16]. The gelling and melting temperatures depend on the species used as raw material, which may have different living environments and habitat temperatures [36]. Proline-rich regions in gelatin molecules of cold water fish were lower than those of warm blooded animals. This was directly correlated with the thermal stability of gelatin gel as indicated by lower gelling temperature of the former [36]. With a higher melting temperature, the gel could be maintained for a longer time, thereby providing a better mouth feel when consumed.

Color of Gelatin Gel

The color of gelatin gel from frog skin extracted at various temperatures is shown in Table 1. The color of gel was expressed as the lightness (L^*), redness (a^*) and yellowness (b^*). No differences in L^*- and a^*-values were observed between G45, G55 and G65 ($P > 0.05$). G75 exhibited the higher L^*- and a^*-values than the others ($P < 0.05$). Generally, the increase in b^*-value of gelatin gel was observed when the extraction temperatures increased ($P < 0.05$). Among all samples, G75 showed the highest ΔE^* (total color difference). This was related with the highest b^*-value ($P < 0.05$). During gelatin extraction at high temperature, protein and lipid oxidation could be occurred [37] (Duconseille et al., 2017). Cross-links could be formed by oxidation reactions between the aldehyde functions of oxidized lipids, proteins and sugars, and the amine functions of amino acids (Duconseille et al., 2017). Those reactive products could contribute to the formation of yellow pigments via the Maillard reaction [38]. When comparing the color of gel with those of the commercial fish skin and bovine bone gelatins, there were some differences in L^*-, a^*- and b^*-values. However, the much higher yellowness was noticeable in gelatin gel from bovine bone. Bone had more complex structure than the skin, in which the harsher extraction condition was required to obtain the higher yield, leading to the formation of coloring components mediated by several reactions [35]. The result indicated that extraction temperature directly affected the color of gelatin from frog skin.

Microstructures of Gelatin Gels

The microstructures of gelatin gels from frog skin with different extraction temperatures are illustrated in Fig. 4. All gelatin gels were sponge or coral-like in structure. G55 exhibited the finest gel network with very small voids. The coarser gel network observed in G45 and G65 gels was in accordance with the lower gel strength (Table 1). In general, the conformation and association of protein molecules in gel matrices directly contribute to gel strength of gelatin [39]. It has been known that the microstructure of gel network was related to the physical properties of gelatin gel [40]. The coarser network had less inter-connected protein chains than the finer counterpart, resulting in weaker gel strength. Sinthusamran, et al. [7] also found that gelatin from seabass skin with finer gel network had higher gel strength than those possessing the coarser network. The result revealed that extraction temperature had a profound impact on the arrangement and association of gelatin molecules in gel matrix.

Amino Acid Composition

Amino acid compositions of frog skin and G55 are shown in Table 2. Both samples had glycine was the major amino acid (278 and 332 residues/1000 residues), followed by proline (106 and 126 residues/1000 residues) and alanine (101 and 112 residues/1000 residues). No cysteine was found in both samples. Low contents of hydroxylysine (4 and 5 residues/1000 residues), tyrosine (11 and 3 residues/1000 residues) and histidine (10 and 7 residues/1000 residues) were found. Generally, glycine occurs every third position in the α-chain and represents nearly one third of total residues [39]. G55 showed higher glycine content than skin. The glycine content of G55 was around 1/3 of total amino acids. The result confirmed that proteins extracted were gelatin. For skin, the lower glycine content reflected the presence of other non-collagenous proteins in the skin matrix. For imino acids (proline and hydroxyproline), frog skin gelatin (215 residues/1000 residues, respectively) showed a higher content than frog skin (169 residues/1000 residues). This suggested the removal of non-collagenous proteins when G55 was extracted. This coincided with higher contents of other amino acids in frog skin such as aspartic acid/asparagine, glutamic acid/glutamine, isoleucine, leucine, lysine, serine, threonine, tyrosine, valine, etc. The imino acid content of gelatin from frog skin (G55) was higher than that reported in gelatin from seabass skin (195−199 residues/1000 residues) [18], bigeye snapper skin (186–187 residues/1000 residues) [39], and Nile tilapia skin (185 residues/1000 residues) [41]. It was noted that the resulting frog skin gelatin had the higher content of imino acids (215 residues/1000 residues) than bovine (124 residues/1000 residues) [….]. In addition, gel strength of frog skin gelatin was comparable to that of bovine gelatin (Table 1). Imino acid content is an important factor for determining gel strength of gelatin. Benjakul, et al. [3] reported that imino acids, especially hydroxyproline, involve in gel formation by acting as H-donor, in which hydrogen bond can be formed with adjacent chain possessing H-acceptor. Nevertheless, the properties of gelatin are largely influenced not only be the amino acid composition but also their molecular weight distribution [36].

Conclusions

Asian bullfrog skin could be a promising source of gelatin having good gelling property. Gelatin extracted at a higher temperature had the higher yield. Gelatin with different extraction temperatures contained α- and β-chains as the major components. Gelatin extracted at 55 °C showed the highest gel strength and had a similar value to commercial bovine gelatin. The gelling temperatures of gelatins from frog skin extracted at different temperatures were in the range of 24.05–24.87 °C, which were higher than commercial fish and bovine gelatins. Melting temperatures of gelatin from frog skin (33.22−34.66 °C) were also higher than that of commercial fish gelatin. Due to superior gelling property, gelatin from frog skin could be used as an alternative to replace bovine or porcine gelatin.

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Conflict of Interest

The authors declare that they have no conflict of interest

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Figure legends

Fig. 1 Extraction yield (A) and hydroxyproline content (B) of gelatins from frog skin extracted at different temperatures. Bars represent the standard deviation (*n* = 3). Different letters on the bars denote the significant differences ($P < 0.05$).

Fig. 2 SDS-PAGE patterns of gelatins from frog skin extracted at different temperatures. M, F and B denote high molecular weight markers, commercial fish gelatin and bovine gelatin, respectively. G45, G55, G65 and G75 represent gelatin from frog skin extracted at 45, 55, 65 and 75 °C, respectively.

Fig. 3 FTIR spectra of gelatins from frog skin extracted at different temperatures. G45, G55, G65 and G75 represent gelatin from frog skin extracted at 45, 55, 65 and 75 °C, respectively.

Fig. 4 Microstructures of gel of gelatin from frog skin extracted at different temperatures. Magnification: 3,000 times. G45, G55, G65 and G75 represent gelatin from frog skin extracted at 45, 55, 65 and 75 °C, respectively.

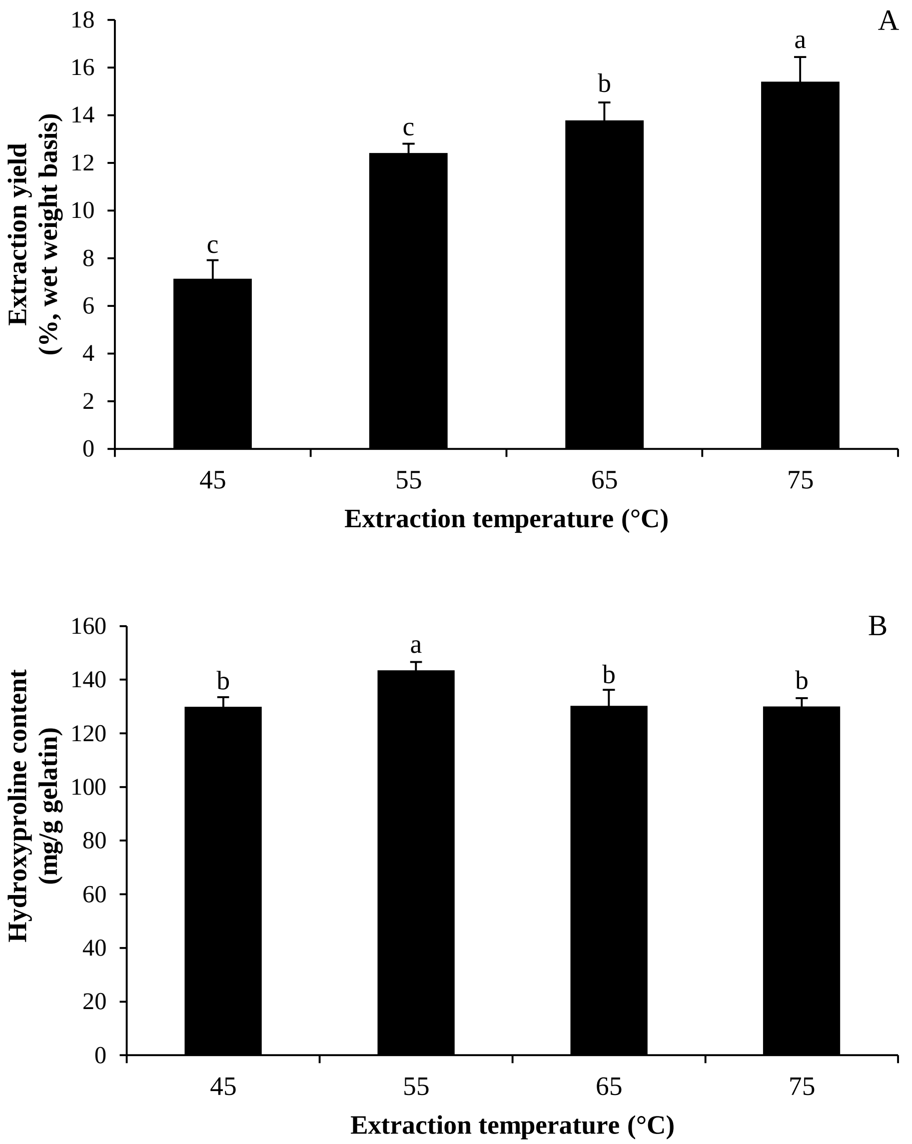
A
Extraction yield
(%, wet weight basis)
0
2
4
6
8
10
12
14
16
18
c
c
b
a
45
55
65
75
Extraction temperature (°C)
B
Hydroxyproline content
(mg/g gelatin)
0
20
40
60
80
100
120
140
160
b
a
b
b
45
55
65
75
Extraction temperature (°C)

Fig. 1

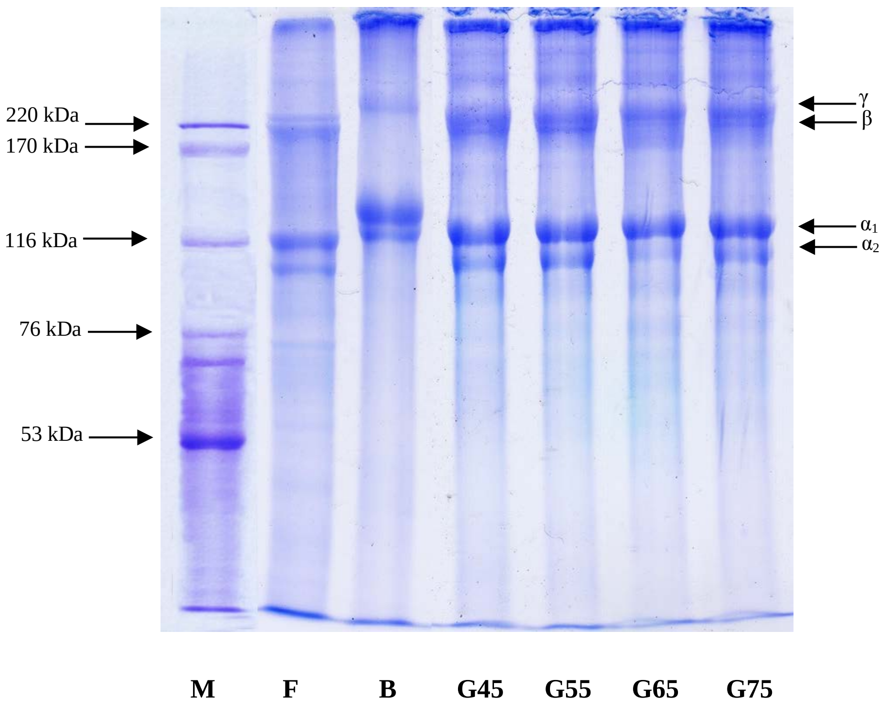

Fig. 2

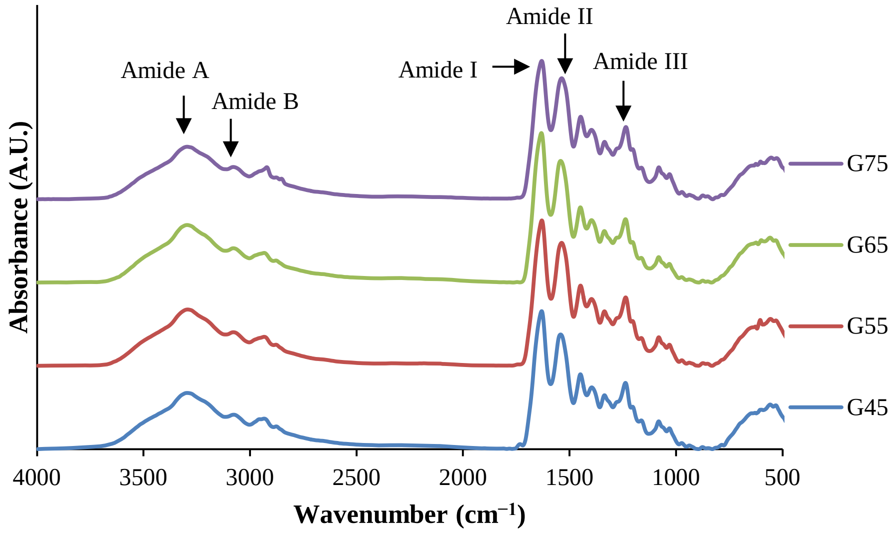
Amide II
Amide A
Amide I
Amide III
Amide B
G75
G65
G55
G45
Absorbance (A.U.)
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm⁻¹)

Fig. 3

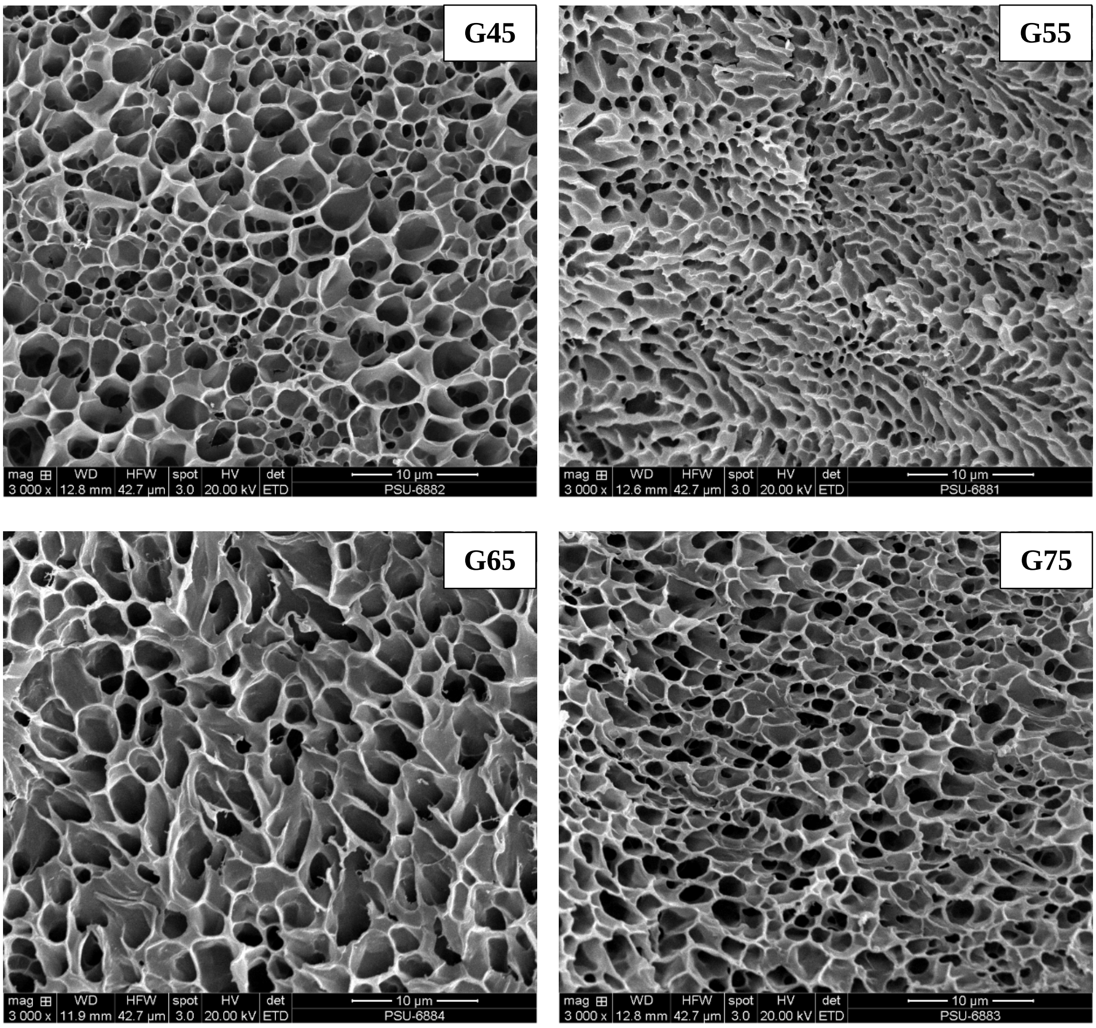
G45
mag ⊞ WD HFW spot HV det 10 µm
3 000 x 12.8 mm 42.7 µm 3.0 20.00 kV ETD PSU-6882
G55
mag ⊞ WD HFW spot HV det 10 µm
3 000 x 12.6 mm 42.7 µm 3.0 20.00 kV ETD PSU-6881
G65
mag ⊞ WD HFW spot HV det 10 µm
3 000 x 11.9 mm 42.7 µm 3.0 20.00 kV ETD PSU-6884
G75
mag ⊞ WD HFW spot HV det 10 µm
3 000 x 12.8 mm 42.7 µm 3.0 20.00 kV ETD PSU-6883

Fig. 4

Table 1 Gel strength, gelling and melting temperatures, and gel color of gelatin from the skin of frog extracted at different temperatures.

Samples	Gel strength (g)	Gelling temperature (°C)	Melting temperature (°C)	Color			
				L^*	a^*	b^*	ΔE^*
F	215.66±2.63b	18.44±0.06b	27.08±0.14c	75.46±0.88a	-2.06±0.04d	13.19±0.15d	26.48±0.39f
B	240.46±9.71a	18.78±0.58b	34.00±0.55a	65.13±0.79b	1.85±0.19c	33.00±1.16a	40.06±0.55d
G45	156.80±6.21d	24.87±0.53a	34.66±0.42a	45.44±0.66d	4.12±0.09b	11.84±0.71e	43.79±0.43e
G55	248.14±8.04a	24.47±0.28a	34.16±0.46a	46.51±0.38d	4.25±0.22b	15.16±0.65c	47.54±0.18c
G65	130.13±2.04e	24.21±0.07a	34.29±0.07a	45.55±0.06d	4.43±0.21b	14.55±0.42c	50.10±0.16b
G75	175.43±3.53c	24.05±0.28a	33.22±0.22b	48.91±0.03c	6.56±008a	24.26±0.56b	55.10±0.20a

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

Different lowercase letters within the same column indicate significant differences ($P < 0.05$).

F and B denote commercial fish gelatin and bovine gelatin, respectively. G45, G55, G65 and G75 represent gelatin from frog skin extracted at 45, 55, 65 and 75 °C, respectively.

Table 2 Amino acid compositions of frog skin and gelatin from frog skin extracted at 55 °C.

Amino acids	Content (residues/1000 residues)	
	Frog skin	Frog skin gelatin
Alanine	101	112
Arginine	50	51
Aspartic acid/asparagine	59	46
Cysteine	0	0
Glutamic acid/glutamine	89	75
Glycine	278	332
Histidine	10	7
Isoleucine	18	9
Leucine	36	19
Lysine	39	28
Hydroxylysine	4	5
Methionine	11	8
Phenylalanine	19	13
Hydroxyproline	63	89
Proline	106	126
Serine	50	40
Threonine	29	19
Tyrosine	11	3
Valine	28	19
Total	1000	1000
Imino acid	169	215