



Title	Characteristics and Gel Properties of Gelatin from Skin of Asian Bullfrog (<i>Rana tigerina</i>)
Author(s)	Karnjanapratum, Supatra; Sinthusamran, Sittichoke; Sae-leaw, Thanasak; Benjakul, Soottawat; Kishimura, Hideki
Citation	Food biophysics, 12(3), 289-298 https://doi.org/10.1007/s11483-017-9485-4
Issue Date	2017-09
Doc URL	http://hdl.handle.net/2115/71423
Rights	The final publication is available at www.springerlink.com via http://dx.doi.org/10.1007/s11483-017-9485-4
Type	article (author version)
File Information	Kishimura.pdf



[Instructions for use](#)

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

2

3

4

5

6

To be submitted to Food Biophysics

7

8

9

10

11 Supatra Karnjanapratum^a, Sittichoke Sinthusamran^a, Thanasak Sae-leaw^a,

12 Sootawat Benjakul^{a,*} and Hideki Kishimura^b

13

14

15

16

17 ^a Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University,

18 Hat Yai, Songkhla, 90112, Thailand

19 ^b Laboratory of Marine Products and Food Science, Research Faculty of Fisheries Sciences,

20 Hokkaido University, Hakodate, Hokkaido 041-8611, Japan

21

22

23

24

*To whom correspondence should be addressed.

25 Tel: +66 7428 6334; Fax: +66 7455 8866, E-mail: soottawat.b@psu.ac.th (S. Benjakul)

26
27
28
29
30
31
32
33
34
35
36
37
38
39
40
41
42
43
44
45
46
47
48
49
50

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

51 **Introduction**

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

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

70 Asian bullfrog (*Rana tigerina*) is amphibian species commonly farmed in many parts
71 of Thailand for domestic consumption and export [8]. Frog farming has expanded throughout
72 Thailand due to the productive culture and market demand. The frog production of an
73 approximately 10 tons/day is available for both local and oversea markets, particularly Hong
74 Kong, Singapore and Taiwan [9]. During processing or dressing of frog, skins are generated
75 and considered as byproducts. Frog skins can be used for gelatin production due to their

76 abundance and low cost. Additionally, skins pose no threat of BSE and can be considered as a
77 safe gelatin source. Collagen from skins of various frog species such as *R. tigerina* [10] and
78 bullfrog [11, 12], have been extracted and characterized. However, no information regarding
79 the extraction and characteristics of gelatin from Asian bullfrog skin exists. The aim of the
80 present study was to examine the characteristics and gelling properties, including gel
81 strength, gelling and melting temperatures, of gelatins from the skin of Asian bullfrog (*R.*
82 *tigerina*) as affected by extraction temperatures.

83

84 **Materials and Methods**

85 **Chemicals**

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

95

96 **Collection of Frog Skins**

97 Skins of Asian bullfrog (*Rana tigerina*) with a weight of 200–300 g/frog were
98 obtained from a farm in Hat Yai, Songkhla, Thailand. Skins were kept in a polystyrene box
99 containing ice using a skin/ice ratio 1:2 (w/w) and transported to the Department of Food
100 Technology, Prince of Songkla University, Hat Yai, within 1 h. Upon arrival, the skins were

101 washed with iced tap water (1–3 °C). The skins were pooled as a composite sample, placed in
102 polyethylene bags and stored at –20 °C until used. The storage time was less than 2 months.
103 Prior to gelatin extraction, frozen skins were thawed with running water (25–26 °C) for 30
104 min and cut into small pieces (1.0 × 1.0 cm²) using scissors.

105

106 **Pretreatment of Frog Skins**

107 **Removal of Non-Collagenous Proteins**

108 The prepared skins were soaked in 0.3 M NaOH with a skin/alkali solution ratio of
109 1:10 (w/v) to remove non-collagenous proteins. The mixture was stirred for 6 h at room
110 temperature (28–30 °C) using an overhead stirrer model RW20.n (IKA[®]-Werke GmbH & Co.
111 KG, Staufen, Germany) at a speed of 300 rpm. The alkaline solution was changed every 2 h
112 at 2nd and 4th hour (totally 2 times). Alkali-treated skin was washed with tap water until a
113 neutral or slightly basic pH (7.0–7.5) of wash water was obtained.

114

115 **Acid Pretreatment**

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

120

121 **Extraction of Gelatin from Frog Skins**

122 To extract gelatin, the pretreated skins were mixed with distilled water at a ratio of
123 1:10 (w/v) at 45, 55, 65 and 75 °C in a water bath (W350, Memmert, Schwabach, Germany).
124 The mixtures were stirred continuously for 12 h using an overhead stirrer (RW 20.n, IKA[®]-
125 Werke GmbH & Co. KG, Staufen, Germany) at a speed of 150 rpm. The mixtures were then

126 filtered using a Buchner funnel with a Whatman No. 4 filter paper (Whatman International,
127 Ltd., Maidstone, England). Thereafter, the filtrates were frozen at $-40\text{ }^{\circ}\text{C}$ for 12 h and then
128 lyophilized using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lyngø, Denmark) at $-50\text{ }^{\circ}\text{C}$ for
129 72 h. Gelatins obtained from frog skins extracted at 45, 55, 65 and $75\text{ }^{\circ}\text{C}$ were referred to as
130 'G45', 'G55' and 'G65' and 'G75', respectively. Lyophilized gelatin samples were
131 subsequently subjected to analyses.

132

133 **Analyses**

134 **Yield**

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

$$137 \quad \text{Yield (\%)} = \frac{\text{Weight of freeze-dried gelatin (g)}}{\text{Weight of initial skin (g)}} \times 100 \quad (1)$$

138

139 **Determination of Hydroxyproline Content**

140 Hydroxyproline content was analyzed according to the method of Bergman, et al. [13].

141 Hydroxyproline content was calculated and expressed as mg/g sample.

142

143 **SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE)**

144 Protein patterns were determined using SDS-PAGE according to the method of
145 Laemmli [14]. The gelatin samples (15 mg/mL protein) were dissolved in 5% SDS and the
146 mixtures were incubated at $85\text{ }^{\circ}\text{C}$ for 1 h using a temperature-controlled water bath.
147 Solubilized samples were mixed at 1:1 (v/v) ratio with sample buffer (0.5 M Tris–HCl, pH
148 6.8 containing 5% SDS and 20% glycerol). Samples (5 μL) were loaded onto a
149 polyacrylamide gel made of 7.5% separating gel and 4% stacking gel and subjected to
150 electrophoresis at a constant current of 15 mA/gel using a Mini Protein III unit (Bio-Rad

151 Laboratories, Inc., Richmond, CA, USA). After electrophoresis, gels were stained with
152 0.05% (w/v) Coomassie blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid for 3 h.
153 Finally, they were destained with a mixture of 50% (v/v) methanol and 7.5% (v/v) acetic acid
154 for 30 min and destained again with a mixture of 5% (v/v) methanol and 7.5% (v/v) acetic
155 acid for 1 h. High molecular weight protein markers were used for the estimation of
156 molecular weight of interested proteins.

157

158 **Fourier Transform Infrared (FTIR) Spectroscopy**

159 Attenuated total reflectance Fourier transform infrared spectrometer model Equinox
160 55 (Bruker Co., Ettlingen, Germany) equipped with a horizontal ATR trough plate crystal cell
161 (45° ZnSe; 80 mm long, 10 mm wide and 4 mm thick) (PIKE Technology, Inc., Madison, WI,
162 USA) was used. The spectra, in the range of 4000-400 cm^{-1} (mid-IR region) with automatic
163 signal gain, were collected in 32 scans at a resolution of 4 cm^{-1} and ratioed against a
164 background spectrum recorded from the clean and empty cell at 25 °C. Analysis of spectral
165 data was carried out using the OPUS 3.0 data collection software program (Bruker Co,
166 Ettlingen, Germany.).

167

168 **Gel Strength**

169 Gelatin gels were prepared according to the method of Fernández-Díaz, et al. [15]
170 with a slight modification. Gelatin sample was dissolved in distilled water at 60 °C to obtain
171 a final concentration of 6.67% (w/v). The gelatin solution was then cooled in a refrigerator at
172 4 °C for 16–18 h for gel maturation. Gel strength of samples (3 cm diameter; 2.5 cm height)
173 was determined at 8–10 °C using a texture analyzer model TA-XT2 (Stable Micro System,
174 Surrey, UK) with a load cell of 5 kN, cross-head speed of 1 mm/s and equipped with a 1.27

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

177 **Determination of Gelling and Melting Temperatures**

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

186

187 **Color**

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

$$192 \quad \Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (2)$$

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

195

196 **Microstructure**

197 The microstructure of gelatin gel was visualized using a scanning electron microscopy
198 (SEM). Gelatin gels were prepared in the same manner as those used for gel strength
199 measurement. Gelatin gels having a thickness of 2–3 mm were fixed with 2.5% (v/v)

200 glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h, rinsed with distilled water for 1 h,
201 and dehydrated in ethanol using a serial concentration of 50–100% with 10% increment. The
202 samples were then subjected to critical point drying. Dried samples were mounted on a
203 bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester, PA,
204 USA). The specimens were observed with a scanning electron microscope (JEOL JSM-5800
205 LV, Tokyo, Japan) at an acceleration voltage of 20 kV.

206

207 **Amino Acid Analysis**

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

214

215 **Statistical Analysis**

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

221

222 **Results and Discussion**

223 **Extraction Yield and Hydroxyproline Content**

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

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

249

250

251

252 Protein Patterns

253 Protein patterns of gelatin from frog skin extracted at different temperatures are
254 shown in Fig. 2. All gelatin samples contained α -chain with MW of 126–115 kDa as the
255 major constituent. Gelatin samples also contained β -components (α -chain dimers) and γ -
256 components (α -chain trimers). The protein patterns of frog skin gelatins were similar to those
257 of commercial fish gelatin. It was noted that commercial bovine gelatin had higher molecular
258 weight of all components. Among all samples, G45 and G55 had the higher band intensities
259 of α_1 -, α_2 -, β - and γ -chains. The band intensities of all constituents in gelatin decreased when
260 the extraction temperature was higher than 55 °C. This might be caused by some degradation
261 induced by the thermal process. Among all gelatins, G65 had the lowest band intensity of all
262 components. This was presumed to be due to the presence of indigenous proteases in frog
263 skin, which were able to cleave α -, β - and γ -chains most effectively at 65 °C. Thus, those
264 proteases more likely contributed to the disintegration of gelatin molecules during the
265 extraction process at 65 °C. Proteolysis induced by heat-activated and heat-stable indigenous
266 proteases associated with skin matrix could contribute to the destabilization as well as
267 disintegration of collagen structure by disrupting the intra- and intermolecular cross-links
268 [23]. Heat-activated serine protease in bigeye snapper skin was associated with the drastic
269 degradation of the α - and β -chains of the gelatin extracted at 60 °C [24]. These enzymes are
270 bound with matrix components such as collagens [25]. Thus, extraction temperature played a
271 profound role in protein pattern or distribution of gelatin from frog skin.

272

273 Fourier transform infrared (FTIR) spectra

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

291 The characteristic absorption bands of gelatin samples in the amide II region were
292 noticeable at the wavenumber range of 1537–1543 cm^{-1} . The amide II vibration mode is
293 attributed to an out-of-phase combination of C–N stretch and inplane N–H deformation
294 modes of the peptide group [27]. It was noted that extraction of gelatin at 65 °C might favor
295 the dissociation and/or degradation of α -chain, as indicated by the shift to higher
296 wavenumber of G65. In addition, the amide III bands of all gelatin samples were detected at
297 the wavenumbers of 1236–1238 cm^{-1} . Amide III represents the combination peaks between
298 C–N stretching vibrations and N–H deformation from amide linkages as well as absorptions

299 arising from wagging vibrations from CH₂ groups from the glycine backbone and proline
300 side chains [29]. G65 had the lowest peak amplitude in the amide III region. This indicated
301 that the greater disorder of molecular structure of native collagen due to transformation of an
302 α -helix to a random coil structure occurred [26]. Moreover, G65 and G75 exhibited the lower
303 wavenumber in the amide III region than those of G45 and G55, suggesting the higher
304 disorder of gelatins extracted at 65 and 75 °C associated with higher degradation. The result
305 was in agreement with the lower band intensities of α -, β - and γ -chains of G65 and G75 (Fig.
306 2).

307 Amide A band, arising from the stretching vibrations of the N–H group coupled with
308 hydrogen bonding [26], appeared at 3292, 3292, 3296 and 3294 cm⁻¹ for G45, G55, G65 and
309 G75, respectively. The position of amide A band shifted to a lower frequency as the NH
310 group of a peptide is involved in hydrogen bonding [6]. The higher wavenumber of G65
311 indicated the higher content of amino groups caused by the enhanced protein degradation.
312 This was coincidental with the lower band intensities of all components (Fig. 2). In addition,
313 the highest amplitude of G65 was probably related to the higher hydrolysis. The amide B
314 band was observed at 3078, 3078, 3076 and 3078 cm⁻¹ for G45, G55, G65 and G75,
315 respectively, corresponding to the asymmetric stretching vibration of =C–H as well as –NH₃⁺
316 [6]. Among all samples, G65 showed the lowest wavenumber for the amide B peak,
317 suggesting interaction of –NH₃ groups between peptide chains. Higher degradation resulted
318 in the release of short peptides, which might undergo reaction to a higher extent, compared
319 with bulky long chains. Therefore, the secondary structure and functional group of gelatins
320 obtained from frog skin were affected by extraction temperatures.

321

322 **Gel Strength**

323 Gel strength of gelatin extracted from frog skin at different temperatures is shown in
324 Table 1. G55 had the highest gel strength, while G65 showed the lowest gel strength ($P <$
325 0.05). The result was in accordance with the highest α - and β -chains of G55 (Fig. 2). The
326 lower gel strength of G65 was more likely associated with the higher degradation of α -, β -
327 and γ -chains as observed in protein pattern (Fig. 2). It was noted that the used of higher
328 extraction temperature, particularly G75, provided the frog skin gelatin with the higher gel
329 strength ($P < 0.05$). This might related with the proteolytic degradation of high molecular
330 weight components caused by indigenous proteases during extraction of gelatin at 65 °C,
331 resulted in adverse effects on gel-forming properties of resulting gelatin [24]. This result was
332 in accordance with protein pattern obtained from SDS-PAGE (Fig. 2). The lower intensity of
333 α -2, β , and γ -chains was observed from G65, compared with others. Gel strength is one of the
334 most important functional properties of gelatins [30]. The differences in gel strength between
335 samples could be due to the differences in intrinsic characteristics, such as molecular weight
336 distribution as well as chain-to-chain interactions determined by the amino acid composition
337 and ratio of α/β chains present in the gelatin [31]. Gelatin structures with large amount of
338 high molecular weight components including α -, β , and γ -chains, have been known to possess
339 the maximal gelation [20]. Hence, the use of an appropriate extraction temperature could be
340 an effective means to obtain the gelatin with the limited or negligible degradation of peptides,
341 while maintaining the protein components in gelatin.

342

343 **Gelling and Melting Temperatures**

344 The gelling temperatures of gelatins from frog skin extracted at different temperatures
345 were in the range of 24.05–24.87 °C (Table 1). No differences in the gelling temperatures
346 were observed among different gelatins obtained from varying extraction temperatures ($P >$
347 0.05). It was found that all gelatins from frog skin had the higher gelling temperature than

348 commercial bovine and fish gelatins ($P < 0.05$). Sinthusamran, et al. [7] reported that
349 extraction temperatures affected the physico-chemical properties of gelatin, such as
350 molecular weight distribution, the amount of β - and γ -components as well as gelling
351 temperature. Therefore, the gelling temperature was not much affected by the extraction
352 temperatures used in the present study. The gelling temperatures in this study were much
353 higher than those of gelatins from the skins of bigeye snapper (10.0 °C) [32], yellowfin tuna
354 (18.7 °C) [33], and silver carp (18.7) [16]. Thus, gelatins from frog skin were able to form gel
355 at room temperature, showing the similar characteristic to mammalian gelatin.

356 The melting temperatures of gelatin gels from frog skin extracted at various
357 temperatures were in the range of 33.22–34.66 °C (Table 1). No differences in melting
358 temperatures between G45, G55, G65 and commercial bovine gelatin were observed ($P >$
359 0.05). It was noticed that the high extraction temperature (75 °C) resulted in the decreases in
360 melting temperature of gelatin (G75) ($P < 0.05$). Eysturskarð, et al. [34] reported that gelling
361 properties of gelatin from saithe skins was more or less unaffected by the extraction
362 temperature in the range of 22–45 °C, while a drop in gelling properties was found by
363 increasing extraction temperature to 65 °C. This was in accordance with the decreased of
364 melting temperature of frog skin gelatin extracted at 75 °C. The melting temperature related
365 to the number of chemical bonding formed in gel network [...]. The frog skin gel obtained
366 from G75 might be formed with the weak bond or the less crosslink density, resulting the
367 decreased melting temperature, compared with others tested. Varying melting temperatures
368 were reported for gelatin from the skins of bigeye snapper (16.8 °C) [32], clown featherback
369 (15.53–24.71 °C) [35], seabass (26.3–27.0°C) [7] and silver carp (27.1 °C) [16]. The gelling
370 and melting temperatures depend on the species used as raw material, which may have
371 different living environments and habitat temperatures [36]. Proline-rich regions in gelatin
372 molecules of cold water fish were lower than those of warm blooded animals. This was

373 directly correlated with the thermal stability of gelatin gel as indicated by lower gelling
374 temperature of the former [36]. With a higher melting temperature, the gel could be
375 maintained for a longer time, thereby providing a better mouth feel when consumed.

376

377 **Color of Gelatin Gel**

378 The color of gelatin gel from frog skin extracted at various temperatures is shown in
379 Table 1. The color of gel was expressed as the lightness (L^*), redness (a^*) and yellowness
380 (b^*). No differences in L^* - and a^* -values were observed between G45, G55 and G65 ($P >$
381 0.05). G75 exhibited the higher L^* - and a^* -values than the others ($P < 0.05$). Generally, the
382 increase in b^* -value of gelatin gel was observed when the extraction temperatures increased
383 ($P < 0.05$). Among all samples, G75 showed the highest ΔE^* (total color difference). This
384 was related with the highest b^* -value ($P < 0.05$). During gelatin extraction at high
385 temperature, protein and lipid oxidation could be occurred [37] (Duconseille et al., 2017).
386 Cross-links could be formed by oxidation reactions between the aldehyde functions of
387 oxidized lipids, proteins and sugars, and the amine functions of amino acids (Duconseille et
388 al., 2017). Those reactive products could contribute to the formation of yellow pigments via
389 the Maillard reaction [38]. When comparing the color of gel with those of the commercial
390 fish skin and bovine bone gelatins, there were some differences in L^* -, a^* - and b^* -values.
391 However, the much higher yellowness was noticeable in gelatin gel from bovine bone. Bone
392 had more complex structure than the skin, in which the harsher extraction condition was
393 required to obtain the higher yield, leading to the formation of coloring components mediated
394 by several reactions [35]. The result indicated that extraction temperature directly affected the
395 color of gelatin from frog skin.

396

397 **Microstructures of Gelatin Gels**

398 The microstructures of gelatin gels from frog skin with different extraction
399 temperatures are illustrated in Fig. 4. All gelatin gels were sponge or coral-like in structure.
400 G55 exhibited the finest gel network with very small voids. The coarser gel network observed
401 in G45 and G65 gels was in accordance with the lower gel strength (Table 1). In general, the
402 conformation and association of protein molecules in gel matrices directly contribute to gel
403 strength of gelatin [39]. It has been known that the microstructure of gel network was related
404 to the physical properties of gelatin gel [40]. The coarser network had less inter-connected
405 protein chains than the finer counterpart, resulting in weaker gel strength. Sinthusamran, et al.
406 [7] also found that gelatin from seabass skin with finer gel network had higher gel strength
407 than those possessing the coarser network. The result revealed that extraction temperature
408 had a profound impact on the arrangement and association of gelatin molecules in gel matrix.

409

410 **Amino Acid Composition**

411 Amino acid compositions of frog skin and G55 are shown in Table 2. Both samples
412 had glycine was the major amino acid (278 and 332 residues/1000 residues), followed by
413 proline (106 and 126 residues/1000 residues) and alanine (101 and 112 residues/1000
414 residues). No cysteine was found in both samples. Low contents of hydroxylysine (4 and 5
415 residues/1000 residues), tyrosine (11 and 3 residues/1000 residues) and histidine (10 and 7
416 residues/1000 residues) were found. Generally, glycine occurs every third position in the α -
417 chain and represents nearly one third of total residues [39]. G55 showed higher glycine
418 content than skin. The glycine content of G55 was around 1/3 of total amino acids. The result
419 confirmed that proteins extracted were gelatin. For skin, the lower glycine content reflected
420 the presence of other non-collagenous proteins in the skin matrix. For imino acids (proline
421 and hydroxyproline), frog skin gelatin (215 residues/1000 residues, respectively) showed a
422 higher content than frog skin (169 residues/1000 residues). This suggested the removal of

423 non-collagenous proteins when G55 was extracted. This coincided with higher contents of
424 other amino acids in frog skin such as aspartic acid/asparagine, glutamic acid/glutamine,
425 isoleucine, leucine, lysine, serine, threonine, tyrosine, valine, etc. The imino acid content of
426 gelatin from frog skin (G55) was higher than that reported in gelatin from seabass skin
427 (195–199 residues/1000 residues) [18], bigeye snapper skin (186–187 residues/1000
428 residues) [39], and Nile tilapia skin (185 residues/1000 residues) [41]. It was noted that the
429 resulting frog skin gelatin had the higher content of imino acids (215 residues/1000 residues)
430 than bovine (124 residues/1000 residues) [...]. In addition, gel strength of frog skin gelatin
431 was comparable to that of bovine gelatin (Table 1). Imino acid content is an important factor
432 for determining gel strength of gelatin. Benjakul, et al. [3] reported that imino acids,
433 especially hydroxyproline, involve in gel formation by acting as H-donor, in which hydrogen
434 bond can be formed with adjacent chain possessing H-acceptor. Nevertheless, the properties
435 of gelatin are largely influenced not only by the amino acid composition but also their
436 molecular weight distribution [36].

437

438 **Conclusions**

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

448

449 **Acknowledgements**

450 This research was supported by the Postdoctoral Fellowship from Prince of Songkla
451 University, Thailand to Dr. Thanasak Sae-leaw. The Thailand Research Fund (TRF)
452 Distinguished Research Professor Grant was also acknowledged for the financial support.

453

454 **Conflict of Interest**

455 The authors declare that they have no conflict of interest

456

457 **References**

- 458 1. J. Regenstein , P. Zhou, in *Maximising the Value of Marine By-Products*, ed. By F.
459 Shahidi (Woodhead Publishing Limited, Cambridge, 2007), p. 279-303
- 460 2. M. C. Gómez-Guillén, B. Giménez, M. E. López-Caballero , M. P. Montero, *Food*
461 *Hydrocolloid.* **25**(8), 1813-1827 (2011)
- 462 3. S. Benjakul, P. Kittiphattanabawon , J. M. Regenstein, in *Food Biochemistry and Food*
463 *Processing*, ed. By B. K. Simpson, L. M. L. Nollet, F. Toldra, S. Benjakul, G. Paliyath
464 and Y. H. Hui (Wiley-Blackwell, Oxford, 2012), p. 388-405
- 465 4. E. A. Foegeding, T. C. Lanier , H. O. Hultin, in *Food Chemistry*, ed. By O. R. Fennema
466 (Marcel Dekker, New York, 1996), p. 879-942
- 467 5. I. Kołodziejska, E. Skierka, M. Sadowska, W. Kołodziejski , C. Niecikowska, *Food*
468 *Chem.* **107**(2), 700-706 (2008)
- 469 6. M. Nagarajan, S. Benjakul, T. Prodpran, P. Songtipya , H. Kishimura, *Food*
470 *Hydrocolloid.* **29**(2), 389-397 (2012)
- 471 7. S. Sinthusamran, S. Benjakul , H. Kishimura, *Food Chem.* **152**(0), 276-284 (2014)
- 472 8. P. Pariyanonth , V. Daorek, *Infotish International.* 25-28 (1995)

- 473 9. J. Wongtavatchai, A. Rungsipipat, A. Chumkaeo , W. Surachetpong, presented at the
474 The 11th International Symposium of the World Association of Veterinary Laboratory
475 Diagnosticians and OIE Seminar on Biotechnology, Bangkok, Thailand, 2003
- 476 10. K. P. Sai , M. Babu, *Comp. Biochem. Physiol. B, Biochem. Mol. Biol.* **128**(1), 81-90
477 (2001)
- 478 11. H. Li, B. L. Liu, L. Z. Gao , H. L. Chen, *Food Chem.* **84**(1), 65-69 (2004)
- 479 12. Y. K. Lin , D. C. Liu, *Food Chem.* **99**(2), 244-251 (2006)
- 480 13. I. Bergman , R. Loxley, *Anal. Chem.* **35**(12), 1961-1965 (1963)
- 481 14. U. K. Laemmli, *Nature.* **227**(5259), 680-685 (1970)
- 482 15. M. D. Fernández-Díaz, P. Montero , M. C. Gómez-Guillén, *Food Chem.* **74**(2), 161-167
483 (2001)
- 484 16. G. Boran, S. J. Mulvaney , J. M. Regenstein, *J. Food Sci.* **75**(8), E565-E571 (2010)
- 485 17. R. E. Wrolstad , D. E. Smith, in *Food Analysis*, ed. By S. S. Nielsen (Springer, New
486 York, 2010), p. 573-586
- 487 18. T. Sae-leaw, S. Benjakul, N. M. O'Brien , H. Kishimura, *Int. J. Food Sci. Technol.*
488 **51**(5), 1204-1211 (2016)
- 489 19. P. Kaewruang, S. Benjakul , T. Prodpran, *Food Chem.* **138**(2-3), 1431-1437 (2013)
- 490 20. P. Kittiphattanabawon, S. Benjakul, W. Visessanguan , F. Shahidi, *Food Hydrocolloid.*
491 **24**(2-3), 164-171 (2010)
- 492 21. P. Montero , M. C. Gómez-Guillén, *J. Food Sci.* **65**(3), 434-438 (2000)
- 493 22. D. W. S. Wong, *Mechanism and Theory in Food Chemistry.* (Van Nostrand Reinhold,
494 New York, 1989).
- 495 23. M. Ahmad, S. Benjakul, M. Ovissipour , T. Prodpran, *Food Chem.* **127**(2), 508-515
496 (2011)

- 497 24. R. Intarasirisawat, S. Benjakul, W. Visessanguan, T. Prodpran, M. Tanaka , N. K.
498 Howell, *Food Hydrocolloid*. **21**(4), 537-544 (2007)
- 499 25. J. F. Woessner, *FASEB J.* **5**(8), 2145-2154 (1991)
- 500 26. J. H. Muyonga, C. G. B. Cole , K. G. Duodu, *Food Chem.* **86**(3), 325-332 (2004)
- 501 27. J. Bandekar, *Biochim. Biophys. Acta.* **1120**(2), 123-143 (1992)
- 502 28. G. K. Pal, T. Nidheesh , P. V. Suresh, *Food Res. Int.* **76**, 804-812 (2015)
- 503 29. M. Jackson, L. P. Choo, P. H. Watson, W. C. Halliday , H. H. Mantsch, *Biochim.*
504 *Biophys. Acta.* **1270**(1), 1-6 (1995)
- 505 30. A. A. Karim , R. Bhat, *Food Hydrocolloid.* **23**(3), 563-576 (2009)
- 506 31. F. Badii , N. K. Howell, *Food Hydrocolloid.* **20**(5), 630-640 (2006)
- 507 32. P. K. Binsi, B. A. Shamasundar, A. O. Dileep, F. Badii , N. K. Howell, *Food*
508 *Hydrocolloid.* **23**(1), 132-145 (2009)
- 509 33. S. M. Cho, Y. S. Gu , S. B. Kim, *Food Hydrocolloid.* **19**(2), 221-229 (2005)
- 510 34. J. Eysturskarð, I. J. Haug, N. Elharfaoui, M. Djabourov , K. I. Draget, *Food*
511 *Hydrocolloid.* **23**(7), 1702-1711 (2009)
- 512 35. P. Kittiphattanabawon, S. Benjakul, S. Sinthusamran , H. Kishimura, *LWT - Food Sci.*
513 *Technol.* **66**, 186-192 (2016)
- 514 36. M. C. Gómez-Guillén, J. Turnay, M. D. Fernández-Díaz, N. Ulmo, M. A. Lizarbe , P.
515 Montero, *Food Hydrocolloid.* **16**(1), 25-34 (2002)
- 516 37. E. N. Frankel, in *Lipid Oxidation*, ed. By E. N. Frankel (Woodhead Publishing Limited,
517 Cambridge, 2005), p. 67-98
- 518 38. S. Khantaphant, S. Benjakul , M. R. Ghomi, *LWT - Food Sci. Technol.* **44**(4), 1139-
519 1148 (2011)
- 520 39. S. Benjakul, K. Oungbho, W. Visessanguan, Y. Thiansilakul , S. Roytrakul, *Food*
521 *Chem.* **116**(2), 445-451 (2009)

- 522 40. Z. Pang, H. Deeth, P. Sopade, R. Sharma , N. Bansal, Food Hydrocolloid. **35**, 484-493
523 (2014)
- 524 41. S. Zeng, X. Yan, W. Cao, P. Hong, C. Zhang , L. Li, Int. J. Food Sci. Technol. **45**(9),
525 1807-1813 (2010)

526

527

528

529

530

531

532

533

534

535

536

537 **Figure legends**

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

541

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

546

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

549

550 **Fig. 4** Microstructures of gel of gelatin from frog skin extracted at different temperatures.

551 Magnification: 3,000 times. G45, G55, G65 and G75 represent gelatin from frog skin

552 extracted at 45, 55, 65 and 75 °C, respectively.

553

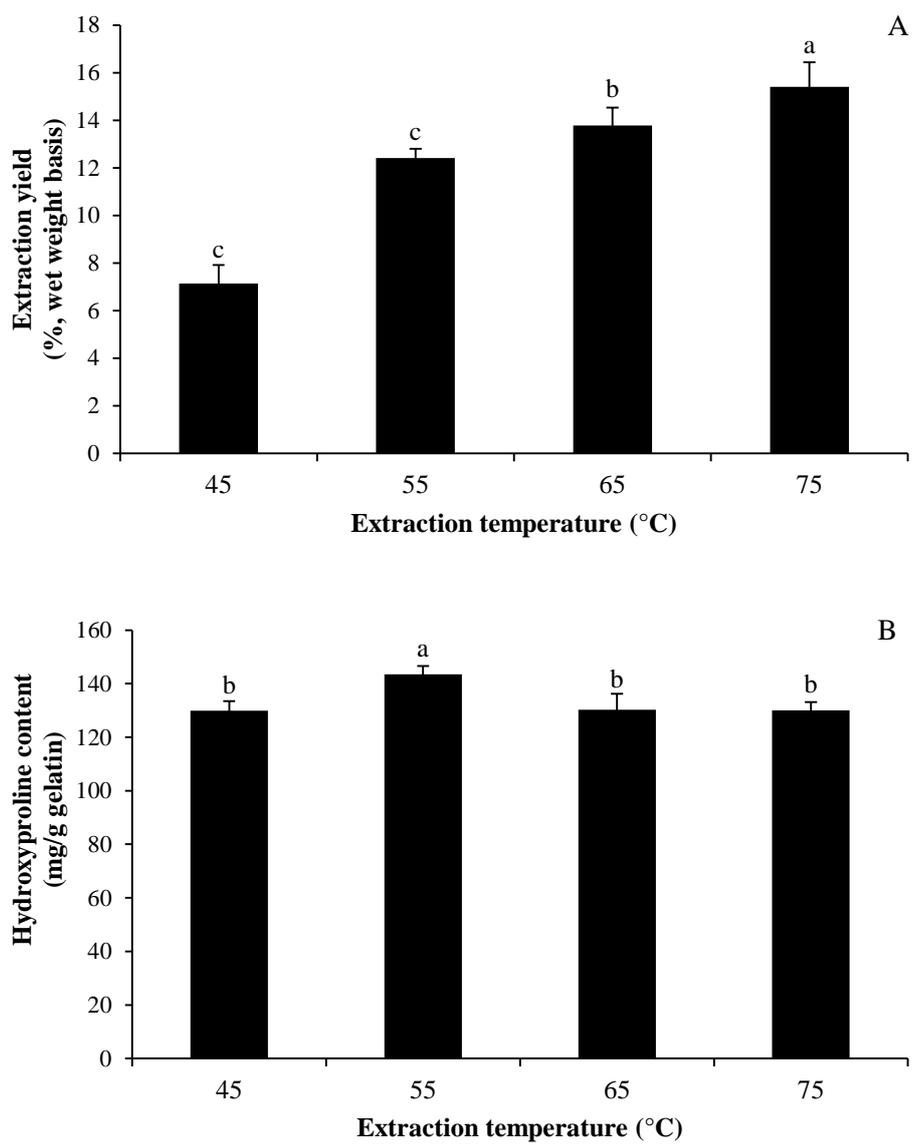


Fig. 1

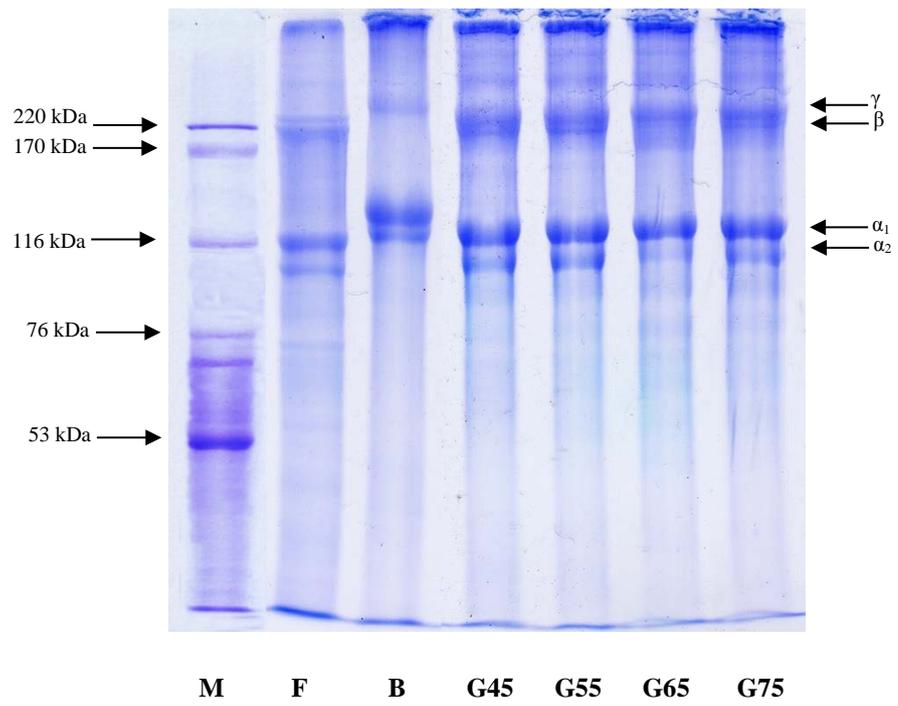


Fig. 2

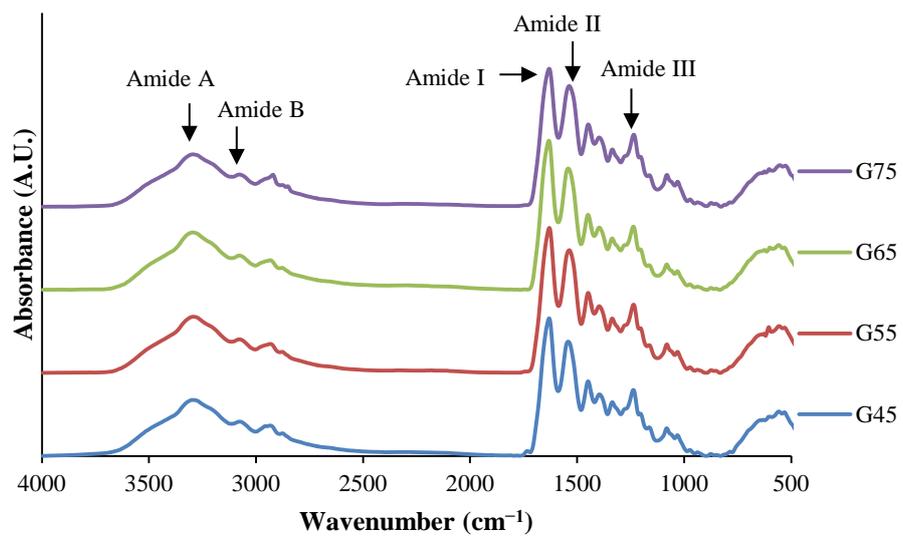


Fig. 3

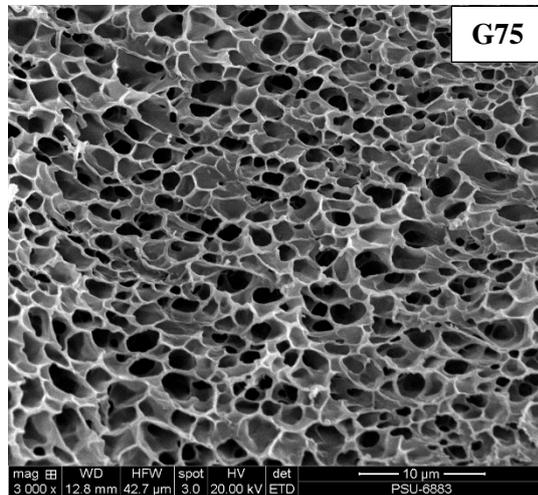
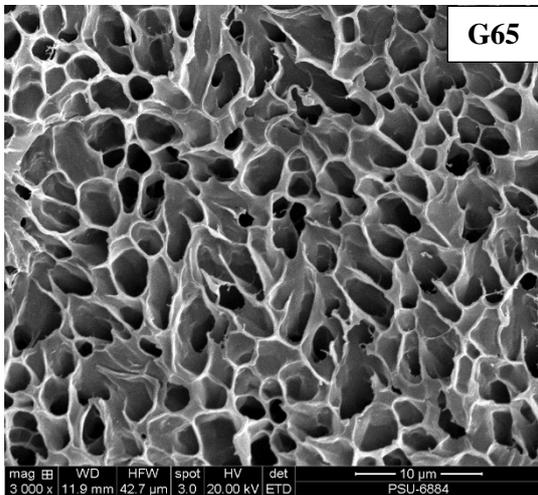
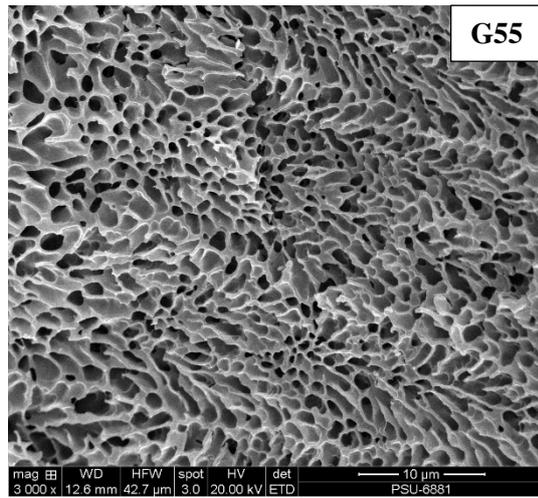
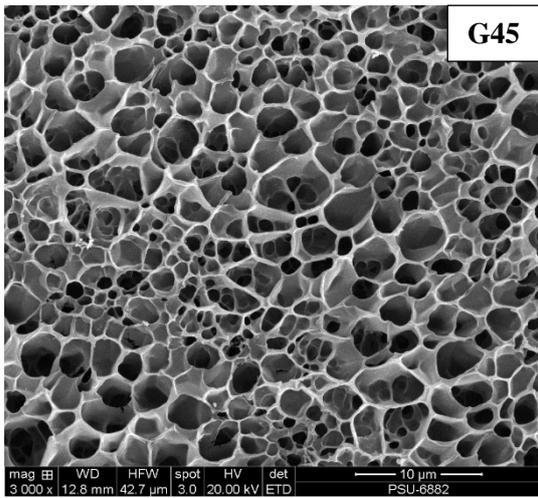


Fig. 4

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±0.08a	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