Molecular characteristics and properties of gelatin from skin of seabass with different sizes

To be submitted to International Journal of Biological Macromolecules

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ABSTRACT

Gelatin was extracted from the skin of seabass (*Lates calcarifer*) with different average sizes (2, 4 and 6 kg/fish), termed G2, G4 and G6, respectively and their characteristics and functional properties were determined. Yields of G2, G4 and G6 were 38.22, 40.50 and 43.48% (based on dry weight), respectively. G2 contained α-chains as dominant component, whilst G4 and G6 comprised α-, β- and γ-chains with a larger content of high MW cross-links. All gelatins had the similar imino acid (hydroxyproline and proline) content. Net charge of G2, G4 and G6 became zero at pHs of 6.73, 6.41 and 7.12, respectively. Amongst all gelatin samples, G6 exhibited the highest gel strength (321.5 g) ($p<0.05$), but had the lowest turbidity ($p<0.05$). Gels of G6 sample had the lower L^*-value but higher a^*-, b^*- and ΔE^*-value, compared with others. Gelling and melting temperatures of all gelatins were 17.09-19.01 and 26.92-28.85 °C, respectively. Furthermore, all gelatins were able to set at room temperature, regardless of size of seabass used. G6 had the shorter setting time at room temperature than others. Therefore, size of seabass, in which skin was used for gelatin extraction, had the impact on yield, composition and properties of resulting gelatin.

Keywords: Seabass, Gelatin, Size, Gel strength, Setting time, Gelling temperature

1. Introduction

Gelatin is the denatured or partially hydrolysed form of collagen [1]. It represents a major biopolymer with several applications in food, materials, pharmacy and photography industries [2]. Due to its gelling property and surface behaviour (e.g., formation and stabilisations of foams and emulsions), gelatin has been widely used to enhance the elasticity, consistency and stability of food products [3-5].

In general, the main sources of gelatin are skins and bones of pig and cow obtained from processing by-products. Gelatin from those sources can be a problem for certain consumers, e.g., Muslims and Jews, in which porcine gelatin is prohibited. Occurrence of bovine spongiform encephalopathy (BSE) has led to awareness for consumption of bovine gelatin [3]. Nowadays, an increasing interest has been paid to alternative sources of gelatin, especially from the skins and bones from fish processing by-products [6, 7]. Fish gelatin can be extracted from skin of several fish species including skipjack tuna, dog shark [8], cobia [9], farmed Amur sturgeon [10], seabass [6], and brownbanded bamboo and blacktip shark [7]. It has been known that the extraction conditions including temperature, time as well as pretreatment affect the functional properties of gelatin from fish processing by-products [6, 11, 12]. Additionally, characteristics and properties of gelatin vary with species and age of raw material [13, 14]. Muyonga, Cole and Duodu [15] reported that gelatin from adult Nile perch skin exhibited the better gel properties than young Nile perch skin when the same extraction condition was used.

Seabass is one of economically important fish in Thailand. A number of seabass farms are located in the south of Thailand, especially in the lake of Songkhla. Due to its delicacy, a large amount of seabass has been exported as well as domestically consumed. During processing, particularly fillet production, skin is

generated as byproduct. Skins from seabass has been used as raw material for collagen and gelatin extraction with higher yield [16]. Gelatin from seabass skin had higher gel strength than bovine gelatin and could be set at room temperature within 30 min [6]. Size or age of seabass, in which skin is used for gelatin extraction, can have the impact on composition and properties of gelatin. Nevertheless, no information regarding gelatin extracted from skin of seabass with different sizes has been reported. Therefore, the aims of this investigation were to extract and determine the chemical characteristics and functional properties of gelatin from the seabass skin as affected by sizes of fish.

2. Materials and methods

2.1 Chemicals

All chemicals were of analytical grade. Sodium dodecyl sulphate (SDS), Coomassie blue R-250 and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) were procured from Bio-Rad Laboratories (Hercules, CA, USA). High-molecular-weight markers were purchased from GE Healthcare UK Limited (Buckinghamshire, UK). Fish gelatin produced from tilapia skin (~240 bloom) was obtained from Lapi Gelatine S.p.A (Empoli, Italy).

2.2 Fish skin preparation

Fresh seabass (*Lates calcarifer*) with different sizes of 1.7-2.3, 3.7-4.3 and 5.7-6.2 kg/fish, equivalent to average size of 2, 4 and 6 kg/fish, respectively, were obtained from a farm in Koyo Island, Songkhla, Thailand. The fish were kept in ice with a fish/ice ratio 1/3 (w/w) and transported to the Department of Food Technology, Prince of Songkla University, Hat Yai within 1 h after capture. Fish were washed

using cold tap water. Skins were then removed, descaled, and cut into small pieces (0.5 cm x 0.5 cm) using a scissor. The skin was placed in polyethylene bags and stored at -20 °C until used, but not longer than 2 months.

2.3 Extraction of gelatin from the skin of seabass

Gelatin was extracted from seabass skin according to the method of Sinthusamran, Benjakul and Kishimura [6]. Before gelatin extraction, skin was soaked in 0.1 M NaOH with a skin/solution ratio of 1/10 (w/v) to remove non-collagenous proteins. The mixture was stirred for 3 h at room temperature (28-30 °C) using an overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Stanfen, Germany). The alkaline solution was changed every 1 h for totally 3 times. The pretreated skin was washed with tap water until neutral or faintly basic pH was obtained. Then, the washed skin was mixed with 0.05 M acetic acid at a skin/solution ratio of 1/10 (w/v) to swell collagenous material in the fish skin matrix. The mixture was stirred at room temperature for 2 h. The skin was washed using tap water until neutral or faintly acidic pH of wash water was obtained. Finally, the swollen skin was mixed with distilled water at a ratio of 1/10 (w/v) at 45 °C for 3 h with continuous stirring. The mixtures were filtered using a Buchner funnel with Whatman No.4 filter paper (Whatman International, Ltd., Maidstone, England). Then, the filtrates were freeze-dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The freeze-dried gelatins extracted from seabass skin with an average size of 2, 4 and 6 kg/fish were referred to as ‘G2’, ‘G4’ and ‘G6’, respectively. Gelatin samples were subsequently subjected to analyses.

121 2.4 Analyses

122 2.4.1. Yield

123 The yield of gelatin was calculated based on dry weight of starting material.

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

124

125 2.4.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

126 SDS–PAGE was performed by the method of Laemmli [17]. Gelatin samples
127 were dissolved in 5% SDS solution. The mixtures were then heated at 85 °C for 1 h
128 using a temperature controlled water bath model W350 (Memmert, chwabach,
129 Germany). Solubilised samples were mixed at a 1/1 (v/v) ratio with sample buffer (0.5
130 M Tris–HCl, pH 6.8 containing 5% SDS and 20% glycerol). Samples were loaded
131 onto a polyacrylamide gel made of 7.5% separating gel and 4% stacking gel and
132 subjected to electrophoresis at a constant current of 20 mA/gel. After electrophoresis,
133 gels were stained with 0.05% (w/v) Coomassie blue R-250 in 50% (v/v) methanol and
134 7.5% (v/v) acetic acid for 30 min. Finally, they were destained with the mixture of
135 50% (v/v) methanol and 7.5% (v/v) acetic acid for 30 min and destained again with
136 the mixture of 5% (v/v) methanol and 7.5% (v/v) acetic acid for 1 h. High-molecular-
137 weight protein markers were used to estimate the molecular weight of proteins.

138

139 2.4.3 Amino acid analysis

140 Amino acid composition of gelatin samples was analysed using an amino acid
141 analyser. The samples were hydrolysed under reduced pressure in 4 M
142 methanesulphonic acid containing 0.2% (v/v) 3-2(2-aminoethyl) indole at 115 °C for
143 24 h. The hydrolysates were neutralised with 3.5 M NaOH and diluted with 0.2 M

144 citrate buffer (pH 2.2). An aliquot of 0.04 ml was applied to an amino acid analyser
145 (MLC-703; Atto Co., Tokyo, Japan).
146
147 2.4.4 Fourier transform infrared (FTIR) spectroscopic analysis
148 FTIR spectra of gelatin samples were obtained using a FTIR spectrometer
149 (EQUINOX 55, Bruker, Ettlingen, Germany) equipped with a deuterated l-alanine tri-
150 glycine sulphate (DLATGS) detector. The horizontal attenuated total reflectance
151 accessory (HATR) was mounted into the sample compartment. The internal reflection
152 crystal (Pike Technologies, Madison, WI, USA), made of zinc selenide, had a 45°
153 angle of incidence to the IR beam. Spectra were acquired at a resolution of 4 cm^{-1} and
154 the measurement range was 4000–650 cm^{-1} (mid-IR region) at room temperature.
155 Automatic signals were collected in 32 scans at a resolution of 4 cm^{-1} and were
156 ratioed against a background spectrum recorded from the clean empty cell at 25 °C.
157 Analysis of spectral data was carried out using the OPUS 3.0 data collection software
158 programme (Bruker, Ettlingen, Germany).
159
160 2.4.5 Measurement of ζ -potential
161 Gelatin samples were dissolved in distilled water at a concentration of 0.5
162 mg/ml. The mixture was stirred at room temperature for 6 h. The Zeta (ζ) potential of
163 each sample (20 ml) was measured using a zeta potential analyser (ZetaPALS,
164 Brookhaven Instruments Co., Holtsville, NY, USA). ζ -Potential of samples adjusted
165 to different pHs with 1.0 M nitric acid or 1.0 M KOH using an autotitrator (BIZTU,
166 Brookhaven Instruments Co., Holtsville, New York, USA) was determined. The pI
167 was estimated from pH rendering ζ-potential of zero.
168

169 2.4.6 Determination of gel strength

170 Gelatin gel was prepared by the method of Kittiphattanabawon, Benjakul,
171 Visessanguan and Shahidi [7]. Gelatin was dissolved in distilled water (60 °C) to
172 obtain a final concentration of 6.67% (w/v). The solution was stirred until gelatin was
173 solubilised completely and transferred to a cylindrical mold with 3 cm diameter and
174 2.5 cm height. The solution was incubated at the refrigerated temperature (4 °C) for
175 18 h prior to analysis.

176 Gel strength was determined at 8–10 °C using a texture analyser (Stable Micro
177 System, Surrey, UK) with a load cell of 5 kg, cross-head speed of 1 mm/s, equipped
178 with a 1.27 cm diameter flat-faced cylindrical Teflon® plunger. The maximum force
179 (grams), taken when the plunger had penetrated 4 mm into the gelatin gels, was
180 recorded.

181

182 2.4.7 Determination of gel colour

183 The colour of gelatin gels (6.67% w/v) was measured by a Hunter lab
184 colourimeter (Colour Flex, Hunter Lab Inc., Reston, VA, USA). L^*, a^* and b^* values
185 indicating lightness/brightness, redness/greenness and yellowness/blueness,
186 respectively, were recorded. The colourimeter was warmed up for 10 min and
187 calibrated with a white standard. Total difference in colour (ΔE^*) was calculated
188 according to the following equation [18]:

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

189 where ΔL^* , Δa^* and Δb^* are the differences between the corresponding colour
190 parameter of the sample and that of white standard (L^* = 90.77, a^* = -1.27 and b^* =
191 0.50).

192 2.4.8 Measurement of turbidity

193 Turbidity of gelatin solution (6.67 %, w/v) was determined as per the method
194 of Fernández-Díaz, Montero and Gómez-Guillén [19] with a slight modification.
195 Gelatin solution was preheated at 40 °C for 15 min. The turbidity of gelatin solutions
196 was measured by reading the absorbance at 360 nm using a double-beam
197 spectrophotometer (model UV-1601, Shimadzu, Kyoto, Japan).

198

199 2.4.9 Measurement of setting time

200 Setting time of gelatin solution was determined at 4 °C and room temperature
201 (26±2 °C) according to the method of Kittiphattanabawon, Benjakul, Visessanguan
202 and Shahidi [7]. Gelatin solution (6.67%, w/v) was prepared in the same manner as
203 described previously. The solution (2 ml) was transferred to thin wall (12 mm x 75
204 mm) test tubes (PYREX®, Corning, NY, USA) and preheated at 60 °C for 10 min,
205 followed by incubation in an ice bath (4 °C) or at room temperature. An aluminium
206 needle with the diameter and length of 0.1 and 25 cm, respectively, was inserted
207 manually in the gelatin solution and raised every 10 s. The time at which the needle
208 could not detach from the gelatin sample was recorded as the setting time. The setting
209 time was expressed as min.

210

211 2.4.10 Determination of gelling and melting temperatures

212 Gelling and melting temperatures of gelatin samples were measured following
213 the method of Sinthusamran, Benjakul and Kishimura [6] using a controlled stress
214 rheometer (RheoStress RS 75, HAAKE, Karlsruhe, Germany). Gelatin solution
215 (6.67%, w/v) was prepared in the same manner as described previously. The solution
216 was preheated at 35 °C for 30 min. The measuring geometry used was 3.5 cm parallel

plate and the gap was set to 1.0 mm. The measurement was performed at a scan rate of 0.5 °C/min, a 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 δ was 45°.

2.4.11 Microstructure analysis of gelatin gel

Microstructure of gelatin gel was visualised using scanning electron microscopy (SEM). 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. The samples were then rinsed with distilled water for 1 h and dehydrated in ethanol with a serial concentration of 50, 70, 80, 90 and 100 % (v/v). 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.

2.5 Statistical analysis

All experiments were run in triplicate using three different lots of skin samples. Data were subjected to analysis of variance (ANOVA) and mean comparisons were carried out by using Ducan's multiple range test [20]. Statistical analysis was performed using the statistical Package for Social Sciences (SPSS for windows: SPSS Inc., Chicago, IL, USA).

3. Results and discussion

3.1 Extraction yield

The yields of gelatin extracted from the skin of seabass with different sizes are shown in Table 1. Extraction yields of G2, G4 and G6 were 38.22, 40.50 and 43.48% (based on dry weight), respectively. The highest yield was found for G6 sample extracted from the skin of largest size, in comparison with those of smaller sizes ($p<0.05$). Difference in yield can be associated with varying extraction processes as well as the species or tissue used [13]. The result was in accordance with Muyonga, Cole and Duodu [15] who reported that the lower yield of gelatin was obtained from young Nile perch skins, compared to adult Nile perch skin. The age of animal used as raw material for gelatin extraction has the influences on the connective tissues and protein compositions [21]. The number of cross-links in collagen increased with increasing age of the animal [22]. The result suggested that the lower yield of gelatin from seabass with smaller size might be associated with lower cross-linking in collagen molecule. Leaching of collagen might take place to a higher extent during the swelling process. As a result, the lower yield was gained when skin of seabass with smaller size was used.

On the other hand, the cross-links might be attached tightly in the skin matrix. Thus, the loss during swelling could be lower. The yields of fish skin gelatin varied among species, e.g. dog shark (62.3%), tuna (48.1%), rohu (39.55%) [8], cobia (24.1%), croaker (30.3%) [9], tiger-toothed croaker (36.8%) and pink perch (27.3%) [23]. Therefore, size or age of seabass directly affected the extraction yield of gelatin.

267 3.2 Protein patterns

268 The protein patterns of gelatin extracted from the skin of seabass with
269 different sizes are illustrated in Fig. 1. Gelatins from seabass skin had the typical
270 molecular distribution of collagen type I, in which the ratio of α_1-chain and α_2-chain
271 was approximately 2/1 Apart from α-chain, β- and α-chains with MW of 195 and 125-
272 110 kDa, respectively, were obtained. It was noted that G2 sample had α-chains as the
273 major constituents. Commercial fish gelatin also contained α-chains as major
274 components with some degraded proteins. The band intensity of β-chains, γ-chain and
275 high molecular weight cross-link increased when skin from larger size seabass was
276 used for extraction. The increase in band intensity of proteins larger than α–chains
277 was in agreement with the lower degradation. Generally, G2 samples exhibited more
278 degraded peptides with the range of 100-25 kDa. Cross-links with higher MW found
279 in skin of seabass with the average size of 4 and 6 kg/fish might be more resistant to
280 degradation during extraction at 45 °C, compared with small MW components present
281 in skin of smallest size seabass (2 kg/fish). It was noted that the ratio of α_2/α_1 band
282 intensity of G4 and G6 became lower than that of G2. It was presumed that α_2 might
283 under cross-linking to form a larger MW component such as β-, γ-chains as well as
284 high MW cross-links [7]. The protein components of gelatin as well as degradation of
285 proteins contribute to gelling behaviour of gelatin, especially gel strength, setting time
286 and gelling temperature [7]. Therefore, the components of gelatin from seabass skin
287 were affected by size of seabass used.

288

289 3.3 Amino acid compositions

290 Amino acid compositions of gelatin extracted from the skin of seabass with
291 different sizes are presented in Table 2. All gelatins had glycine as the major amino

292 acid (22.57-23.33 g/100 g) and had a relatively high content of alanine (11.17-11.38
293 g/100 g). The amino acid composition of all gelatins showed very low contents of
294 cysteine (0.09-0.10 g/100 g), tyrosine (0.57-0.60 g/100 g), histidine (0.74-0.75 g/100
295 g) and hydroxylysine (0.79-0.81 g/100 g). In general, cysteine and tryptophan are not
296 found in collagen and gelatin [4, 6, 7]. For imino acids, all gelatins contained proline
297 and hydroxyproline contents of 11.95-13.60 and 9.57-9.76 g/100 g, respectively. The
298 imino acid content of G2 (23.36 g/100 g) and G4 (23.12 g/100 g) was higher than that
299 of G6 (21.57 g/100 g). Commercial fish gelatin had imino acid of 22.89 g/100 g.
300 Gelatin of skin from young Nile perch (21.55 g/100 g) showed similar imino acid
301 content to that of skin from adult Nile perch (21.63 g/100 g) [15]. The imino acid
302 content of seabass skin gelatin was higher than that of farmed Amur sturgeon skin
303 (18.90 g/100 g) [10], dog shark skin (19.75 g/100 g), rohu skin (18.38 g/100 g), tuna
304 skin (17.96 g/100 g) [8] and carp skin (19.47 g/100 g) [24]. Mammalian gelatins
305 generally contain 30% imino acids, which was higher than fish gelatin [8]. The
306 species, environment living habitat and body temperature of fish are the main factors
307 governing hydroxyproline and proline content [25]. Moreover, hydroxyproline
308 content of gelatin was also affected by the extraction conditions [10]. The stability of
309 triple-helix in collagen molecule correlated with imino acid content [16]. The
310 difference in imino acid content, especially hydroxyproline, was considered as the
311 important factor influencing viscoelastic properties and gel formation of gelatin [4, 8].
312 In the present study, there was similar hydroxyproline content amongst all samples,
313 but G6 showed the lowest proline content. The difference in amino acid composition
314 could be due to the differences in cross-links in the starting skin matrix. With
315 increasing fish age, the cross-linking of collagen in skin can be enhanced [20]. Those
316 imino acids might contribute to cross-links. As a consequence, the chains rich in

317 proline were not leached out during extraction as indicated by the lower proline in the
318 extracted gelatin. This result suggested that size or age of fish raw materials might
319 determine the amino acid composition of the resulting gelatin.
320
321 3.4 Fourier transform infrared (FTIR) spectra
322 FTIR spectra of gelatin extracted from the skin of seabass with various sizes
323 are depicted in Fig. 2. FTIR spectroscopy has been used to study the changes in
324 functional groups and the secondary structure of gelatin [10]. All gelatin samples
325 showed similar spectra, which had the major peaks in amide region. G2, G4 and G6
326 samples exhibited the amide I bands at the wavenumber of 1637, 1638 and 1637 cm^{-1},
327 respectively. Normally, amide I band of gelatin appeared at 1700-1600 cm^{-1}, which
328 was associated primarily with a C=O stretching vibration/hydrogen bonding coupled
329 with COO [26]. The absorption in the amide I is probably the most useful for infrared
330 spectroscopic analysis of the secondary structure of protein [7]. In the present study,
331 the amide I peak (1637-1638 cm^{-1}) of all samples was in agreement with Yakimets,
332 Wellner, Smith, Wilson, Farhat and Mitchell [27] who reported that the absorption
333 peak at 1633 cm^{-1} was the characteristic of coiled structure of gelatin. The amide I
334 bands of all gelatin samples were shifted to the higher wavenumber, compared with
335 seabass skin collagen [16]. Loss of triple helix occurred during heating via breaking
336 down hydrogen-bonds between α-chains [15, 28]. The Amide II band of G2, G4 and
337 G6 appeared at 1540, 1536 and 1536 cm^{-1}, respectively. The amide II vibration modes
338 are related to an out-of-phase combination of a CN stretch and in-plane NH
339 deformation modes of the peptide group [26, 29]. In addition, amide III was detected
340 around the wavenumber of 1236, 1235 and 1235 cm^{-1} for G2, G4 and G6, respectively,
341 more likely associated with N–H deformation and C–N stretching vibration as well as

342 the absorptions arising from wagging vibrations of CH_2 groups in the glycine
343 backbone and proline side-chains [22, 30]. It was noted that the amide III band of
344 gelatins extracted from skin of seabass with different sizes showed similar spectra.

345 The amide A band of G2, G4 and G6 samples was found at 3294, 3299 and
346 3293cm^{-1}, respectively. A free NH stretching vibration is found in the range of 3400-
347 3440 cm^{-1}. However, when NH group of peptide is involved in hydrogen bond, its
348 position is shifted to lower frequencies, usually 3300 cm^{-1} [31]. The amide B band
349 was observed at 2918, 2922 and 2923 cm^{-1} for G2, G4 and G6, respectively. It
350 represents CH stretching vibrations of the -CH_2 groups [11]. Amplitude of Amide B
351 peak was lower in G6 sample and the peak was shifted to the lower wavenumber,
352 compared with other samples. This suggested that CH_2 groups were more likely
353 interacted via cross-linking. This was in accordance with the higher MW cross-links
354 in G6 sample. Therefore, gelatins from seabass skin showed slight difference in the
355 secondary structure, as affected by size of raw material used.

356

357 3.5 Zeta-potential

358 The ζ-potential of gelatins extracted from the skin of seabass having different
359 sizes tested at different pHs is shown in Fig. 3. Generally, gelatin samples showed
360 positive charge at acidic pH ranges and became negatively charged under alkaline
361 conditions. Zero surface net charge was obtained at pH 6.73, 6.41 and 7.12 for G2, G4
362 and G6, respectively, which were estimate to be their isoelectric points (pI). At pI, the
363 positive charges are balanced out by the negative charges [32]. Gelatin from G6 had
364 higher pI (7.12) than other gelatins extracted from skin of seabass with smaller sizes
365 (G2 and G4). Gelatin from skin of different fish showed varying pIs, e.g 6.65-7.15 for
366 unicorn leatherjacket [33], 8.8 for young Nile perch and 9.4 for adult Nile perch [15].

Thus, the differences in pI of all gelatin samples might be caused by the difference in their amino acid compositions and distribution of amino acid residues, which were more likely influenced by size or age of raw material used.

3.6 Gel strength of gelatin

Gel strength of gelatin from the skin of seabass with different sizes is shown in Fig. 4A. Gel strength of gelatin increased as the size of fish increased ($p<0.05$). G6 had the highest gel strength, compared with others ($p<0.05$). All gelatin gels from seabass skin exhibited higher bloom strength (223-322 g) than that of commercial fish gelatin from tilapia skin (212 g) ($p<0.05$). Muyonga, Cole and Duodu [15] reported that gelatin from young Nile perch skins (222 g) had slightly lower gel strength than adult Nile perch skin gelatin (229 g). The result suggested that gelatin with greater ratio of high-molecular weight components showed the better gelling properties via higher stabilising interactions [34]. G4 and G6 showed higher band intensity of β-chain, γ-chains and high MW cross-links than G2 (Fig. 1). Those large components might serve as the strong strands in gel network. Gelatins with different gel strength were reported for farmed Amur sturgeon skin (141 g) [10], cobia skin (232 g), croaker skin (212 g) [9], dog shark skin (206 g), tuna skin (177 g) [8], tiger-toothed croaker (170 g), pink perch (140 g) [23] and grey triggerfish (168 g) [2]. The difference in gel strength amongst species was mainly due to the different amounts of the β- and α-components and the amino acid composition of gelatin [6]. Furthermore, imino acids, especially hydroxyproline, are involved in gel formation of gelatin via hydrogen bonding through hydroxyl group [5]. However, similar hydroxyproline contents were observed amongst all gelatin samples as shown in Table 2. The result suggested that

components of gelatin, particularly cross-links, most likely played an essential role in gel formation and determined gel strength of gelatin.

3.7 Colour and turbidity of gelatin gel

Colours of different gelatin gels from skin of seabass and commercial fish gelatin expressed as L^*, a^*, b^* and ΔE^* are shown in Table 1. Gelatin gel from G2 showed the higher L^*-value (lightness) than others (G4 and G6) ($p<0.05$). The L^*-value of gelatin gels slightly decreased when skin from seabass with larger sizes was used ($p<0.05$). G2 sample had the lower redness value (a^*-value), compared to commercial fish gelatin ($p<0.05$). No differences in a^*-value were found amongst gelatins from seabass with different sizes ($p>0.05$). Additionally, gelatin gels from seabass had higher b^*-value than that from commercial fish gelatin ($p<0.05$). Slight differences in b^*-value were found amongst gelatin gels from seabass skin with different sizes ($p<0.05$). For total difference in the colour value (ΔE^*), commercial fish gelatin showed the highest ΔE^*-value (91.27), compared with gelatin gels from seabass skin. ΔE^*-value increased as size of seabass used for gelatin extraction increased. This trend was in agreement with the decrease in L^*-value as the size of fish used increased. Thus, size or age of seabass as raw material had the impact on colour of gelatin and their gels.

Turbidity of solutions of gelatin extracted from skin with different sizes expressed as A_{360} is shown in Fig. 4B. Gelatin solution exhibited the lower turbidity when size of seabass used for extraction increased ($p<0.05$). The highest turbidity was found in solution of G2 sample ($p<0.05$). Turbidity of gelatin solution from commercial fish gelatin was much lower, compared with all gelatins from seabass skin ($p<0.05$). The turbidity of gelatin solution is dependent on species of raw

material, gelatin extraction process and clarification/filtration process [15, 23]. In this study, only filtration process was implemented, whilst the clarification process has been used for commercial gelatin to remove both light- and heavy-impurities [15]. Koli, Basu, Nayak, Patange, Pagarkar and Gudipati [23] reported that inorganic, protein and muco-substance contaminants mainly affected turbidity and dark colour of gelatin solution. Both G4 and G6 samples contained high MW components at higher level than G2 (Fig. 1). Solution of G2 sample with smaller peptides or proteins might prevent transmission of light more effectively, thereby increasing the light scattering. This might contribute to higher turbidity of gelatin solution obtained from skin of fish with smaller sizes. These results showed that the size or age of raw materials had the impact on turbidity of gelatin solution.

3.8 Setting time for gel formation

The setting times required for the gel formation of gelatin extracted from the skin of seabass having different sizes at 4 °C and room temperature (26±2 °C) are presented in Fig. 5A and 5B, respectively. The setting times at 4 °C of G2, G4 and G6 were 2.13, 2.80 and 3.60 min, respectively. The setting time at 4 °C of gelatin increased as seabass size used for gelatin extraction increased ($p<0.05$). G2 and G4 samples had a shorter setting time at 4 °C than the commercial fish gelatin ($p<0.05$). Due to the higher proportion of high MW peptide chains of G6, the arrangement of those chains formation of gel network plausibly took a longer time as indicated by longer setting time. In addition, the nucleation zone during gel formation required hydroxyproline [35]. With high proportion of cross-links or β- and γ-chains, hydroxyproline residues could be localized inside the molecules. This could lower the

accessibility or exposure of hydroxyproline to form H-bond. Thermal degradation of peptides in gelatin during heating extraction affected the setting time of gelatin gel [6].

When setting was carried out a room temperature, setting times of 32.0, 28.83 and 19.13 min were found for G2, G4 and G6, respectively. Gelatin samples from seabass skin had the longer setting time when small size of seabass was used ($p<0.05$). All gelatins from seabass were able to undergo setting at room temperature. This result was in agreement with Sinthusamran, Benjakul and Kishimura [6] who reported that gelatin from seabass skin was able to set at room temperature within 30 min. Conversely, commercial fish gelatin was not able to set within 3 h at room temperature. Setting phenomenon of gelatin samples at room temperature was different from that occurred at lower temperature (4 °C). At higher setting temperatures, higher entropy of gelatin molecules was presumed. Those γ- and β-components found in G4 or G6 samples might promote the formation of gel network with rapid gelation [36]. When those high MW cross-links aligned themselves under the high entropy conditions, they could form gel easily as evidenced by lower setting time. Different extraction condition, especially extraction temperature and time also influenced the setting time of gelatin [7, 15]. Furthermore, difference in setting time of fish gelatin might be due to different age or size of fish used as raw material [8]. Thus, setting time could be affected by size of seabass used as raw material. Additionally, setting temperature was another factor affecting time employed for gel formation.

3.9 Gelling and melting temperatures

Gelling and melting temperatures of gelatin extracted from the skin of seabass with different sizes are presented in Table 1. Thermal transition of gelatin solution

were monitored by changes in phase angle (δ) during cooling (35-5 °C, 0.5 °C/min) and subsequent heating (5-35 °C, 0.5 °C/min). The formation of junction zones in the three-dimensional network of gelatin gel can be monitored in term of changes of phase angle [6]. The gelling temperatures were 17.09, 18.43 and 19.01 °C for G2, G4 and G6, respectively, which were higher than commercial fish gelatin (15.84 °C) ($p<0.05$). The higher gelling temperatures were observed in G4 and G6 samples extracted from the skin of larger size, in comparison with that of smaller size (G2) ($p<0.05$). The gelling temperature of gelatin tended to increase with increasing amount of cross-links. This result indicated that the molecular weight distribution in gelatin was associated with gelation. Muyonga, Cole and Duodu [15] also reported that chemical compositions, especially the content of γ-, β-, and α-chain components influenced gelling point of gelatin gel. Additionally, different gelling temperature was related to amino acid composition, especially proline and hydroxyproline, which is governed by temperatures of habitat, where fish live [10, 37]. The imino acid content is normally available to form hydrogen bonds with water [13]. In general, warm water fish or mammalian gelatin has higher gelling and melting temperatures than the cold water fish gelatin [10]. Most of fish gelatin had lower gelling temperature than mammalian gelatin [37]. Varying gelling temperatures were reported for gelatin from different fish, e.g. cobia skin (19.9 °C), croaker skin (17.8 °C) [9], dog shark skin (20.8 °C), tuna skin (18.7 °C) [8], farmed Amur sturgeon skin (14.2 °C) [10], red snapper bone (16.0 °C) and grouper bone (16.0 °C) [37].

Melting temperatures of G2, G4 and G6 were 26.92, 27.62 and 28.85 °C, respectively. The melting temperatures of gelatin from seabass skin were higher than that of commercial fish gelatin (25.10 °C) ($p<0.05$). Nevertheless, the melting temperature of gelatins from seabass skin were lower than those of bovine bone

(29.7 °C) and porcine skin (32.3 °C) [38]. It was noted that the melting temperatures of all gelatins from seabass skin were higher than those of gelatins from the dog shark skin (25.8 °C), tuna skin (24.2 °C) [8], farmed Amur sturgeon skin (22 °C) [10], tiger-toothed croaker skin (20.36 °C), pink perch skin (19.23 °C) [23] and tilapia skin (22.4 °C) [39]. Since seabass used in the present study was tropical fish (25-32 °C), gelatin gel might be more stable than those from temperate zone (15-25 °C) [40]. Thermal stability of gelatin gel is associated with the temperature of habitat of the animals [13]. G4 and G6 samples had higher melting temperature than G2 ($p<0.05$). High MW components might contribute to the stability and stronger network of gel.

In the present study, G6 showed the higher gelling as well as melting temperatures, although it showed the lower imino acid content than G2 and G4. It was noted that G6 had the higher band intensity of β-chains, γ-chain and high molecular weight cross-link, compared with G2 and G4 (Fig. 1). Due to the higher percentage of β- and γ-components, a shorter maturation time might be required since the entanglement of chain took place with ease. Also, those large components might serve as the strong strands in gel network, leading to the increased thermal stability of the gelatin gel [41] as indicated by the higher melting temperature. As a consequence, G6 had higher gelling and melting temperatures. Gelatin extracted from tropical fish has higher gelling and melting temperature than that gelatin extracted from temperate fish. Thus, the differences in gelling and melting temperature of seabass gelatin might be governed by the differences in components, especially those with high MW.

3.10 Microstructures of gelatin gels

Microstructures of the gel from commercial fish gelatin and gels from gelatin extracted from skin of seabass with different sizes are shown in Fig. 6. Generally, all

515 gelatin gels from seabass skin were sponge or coral-like in structure. The gel from G2
516 had a looser network with larger voids and thinner strands, compared with gels from
517 other gelatin samples (G4 and G6). G6 showed thicker strands in gel network,
518 compared with others. The gelation process was governed by cross-linked polypeptide
519 network stabilized mainly by hydrogen-bonded junction zones [5]. Gelatin with high
520 molecular weight distribution could form the junction zones with ordered gel structure,
521 leading to higher gel strength. Sinthusamran, Benjakul and Kishimura [6] reported
522 that gelatin extracted from the seabass skin at milder condition (45 °C for 3 h) had a
523 higher gel strength with the finest structure and smaller voids in gel matrix, compared
524 with gelatin extracted under the harsher conditions. Zhang, Duan, Wang, Yan and
525 Xue [25] reported that the denser strands of gel structure were governed by greater
526 content of high molecular weight peptides (γ- and β-chains) in gelatin, whilst looser
527 strands in gel matrix were found in gelatin sample containing smaller and shorter
528 peptides. Based on protein pattern (Fig. 1), it was found that gelatin extracted from
529 skin of seabass with the larger sizes contained higher content of longer or larger
530 peptides chains. Those chains could build up the strong strands, which could
531 strengthen the network as evidenced by the stronger gel strength.
532

533 **4. Conclusions**

534 Yield and gelling properties of gelatin were influenced by the size of seabass.
535 Gelatin obtained from the skin of seabass with 6 kg/fish had the highest yield and
536 better gelling properties. This was associated with the higher content of high MW
537 components or cross-links. Nevertheless, gels of all gelatins were able to set at room
538 temperature. The gelling and melting temperatures were in the range of 16.49-
539 18.64 °C and 26.92-27.69 °C, respectively, which were higher than that of

commercial fish gelatin. Thus, skin from seabass with different size used as raw material for gelatin extraction had the influence on chemical compositions and properties of gelatin.

Acknowledgements

The authors would like to express their sincere thanks to Thailand Research Fund under the Royal Golden Jubilee Ph.D. Program to Sittichoke Sinthusamran (PHD/0053/2553) and the Grant-in-Aid for dissertation from Graduate School, Prince of Songkla University, Thailand for financial support. The TRF senior research scholar program was also acknowledged.

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639 **Figure Legends**

640 **Fig. 1.** SDS-PAGE patterns of gelatin extracted from the skin of seabass with
641 different sizes. M and CF denote high molecular weight markers and commercial fish
642 gelatin.

643 **Fig. 2.** FTIR spectra of gelatin extracted from the skin of seabass with different sizes.

644 **Fig. 3.** Zeta-potential of gelatins extracted from the skin of seabass with different
645 sizes. Bars represent the standard deviation (n=3).

646 **Fig. 4.** Gel strength (A) and turbidity (B) of gelatin extracted from the skin of seabass
647 with different sizes. CF denotes commercial fish gelatin. Bars represent the standard
648 deviation (n=3). Different lowercase letters on the bars indicate significant differences
649 ($p< 0.05$).

650 **Fig. 5.** Setting time of gelatin extracted from the skin of seabass with different sizes
651 at 4 °C (A) and room temperature (26±2 °C) (B). NS: Gel was not set within 3 h. Bars
652 represent the standard deviation (n=3). Different lowercase letters on the bars indicate
653 significant differences ($p< 0.05$).

654 **Fig. 6.** Microstructures of gel from the commercial fish gelatin (CF), gelatin extracted
655 from the skin of seabass with the average size of 2 kg/fish (G2), gelatin from the skin
656 of seabass with the average size of 4 kg/fish (G4) and gelatin from the skin of seabass
657 with the average size of 6 kg/fish (G6). Magnification: 3000x.

658

659

Molecular characteristics and properties of gelatin

from skin of seabass with different sizes

Sittichoke Sinthusamran [1] and Soottawat Benjakul [1,*] and Hideki Kishimura [2]

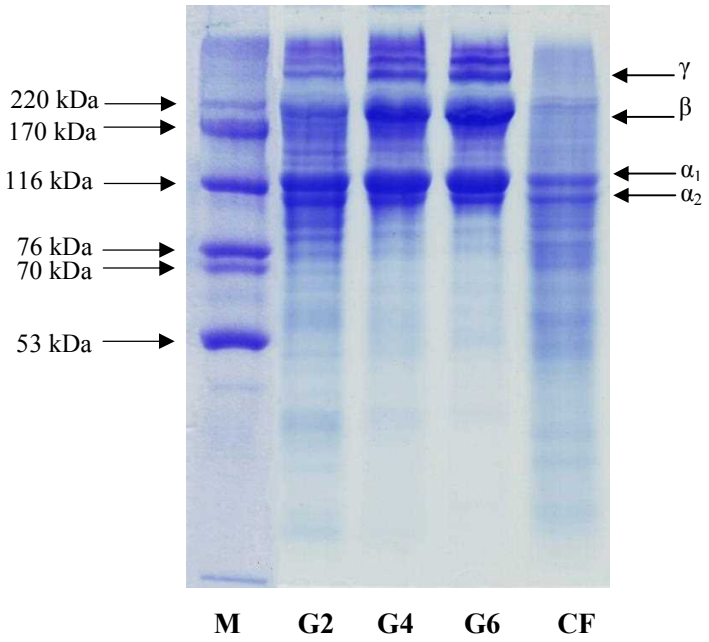

Fig. 1.

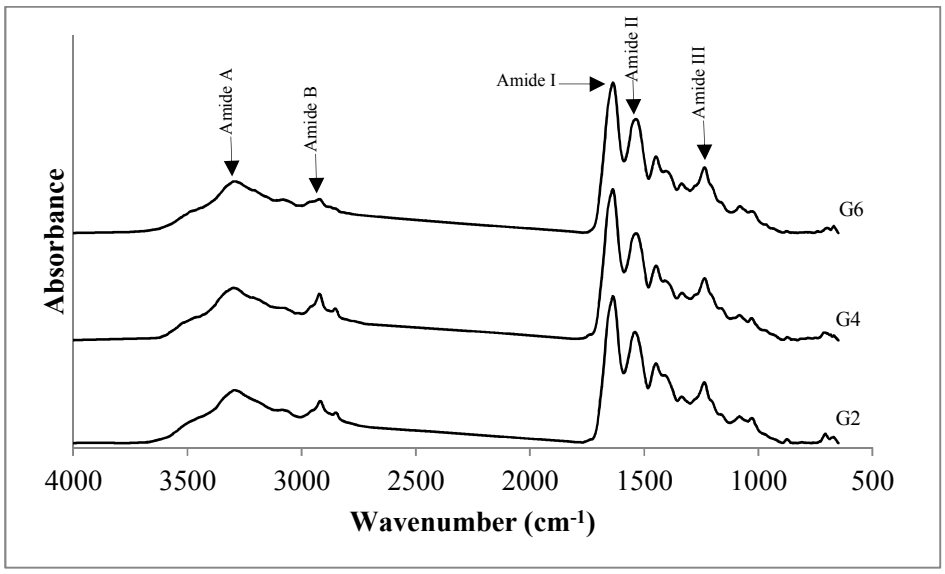
Amide A
Amide B
Amide I
Amide II
Amide III
G6
G4
G2
Absorbance
4000
3500
3000
2500
2000
1500
1000
500
Wavenumber (cm-1)

Fig. 2.

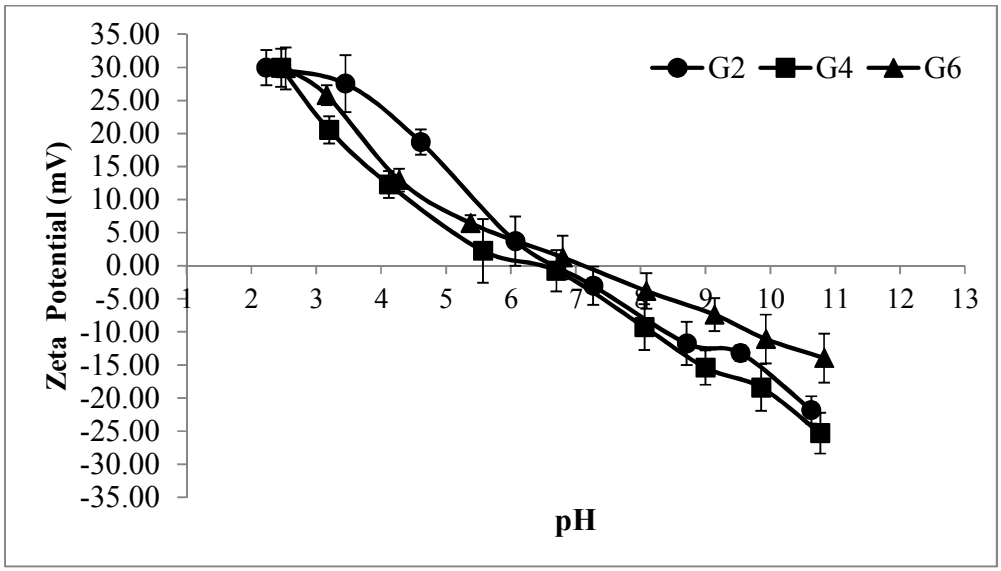
G2
G4
G6
Zeta Potential (mV)
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
-5.00
-10.00
-15.00
-20.00
-25.00
-30.00
-35.00
1
2
3
4
5
6
7
8
9
10
11
12
13
pH

Fig. 3.

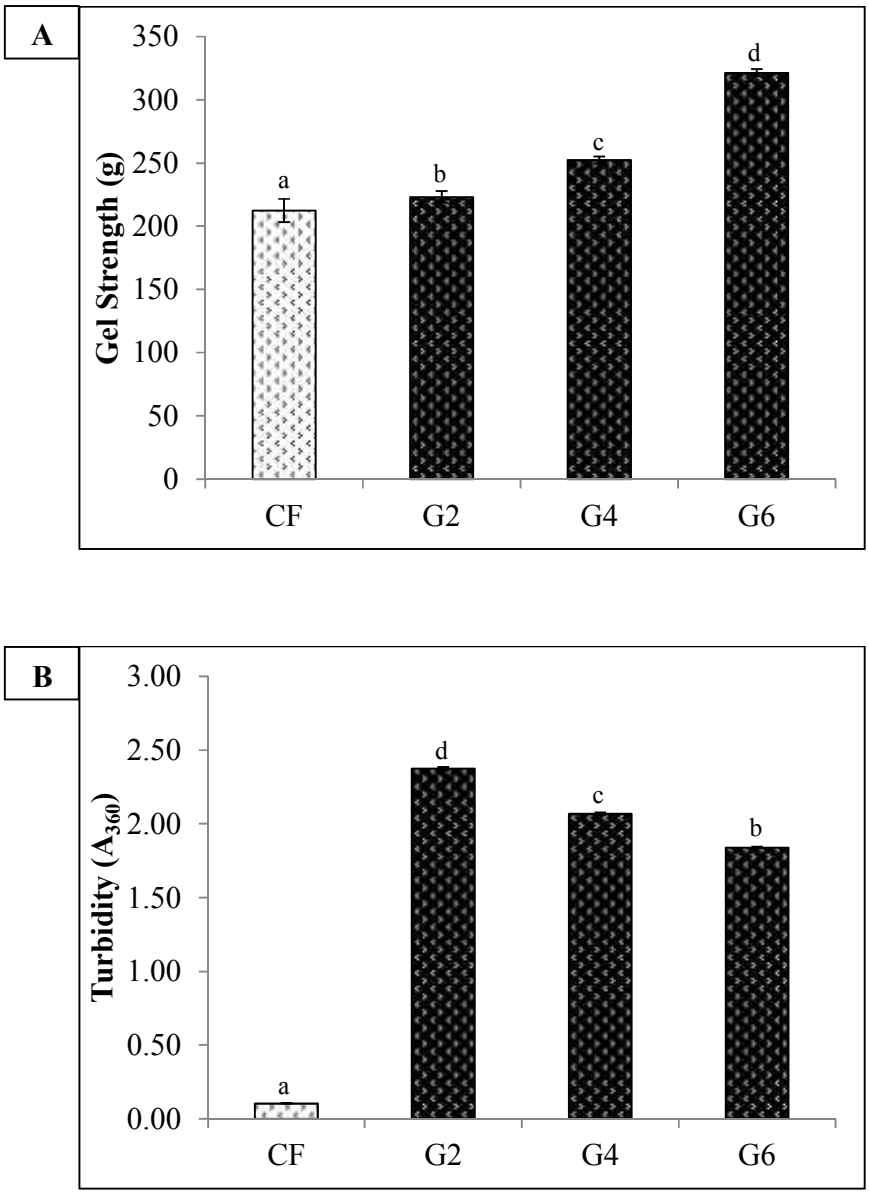
A
Gel Strength (g)
350
300
250
200
150
100
50
0
a
b
c
d
CF
G2
G4
G6
B
Turbidity (A_{360})
3.00
2.50
2.00
1.50
1.00
0.50
0.00
a
d
c
b
CF
G2
G4
G6

Fig. 4.

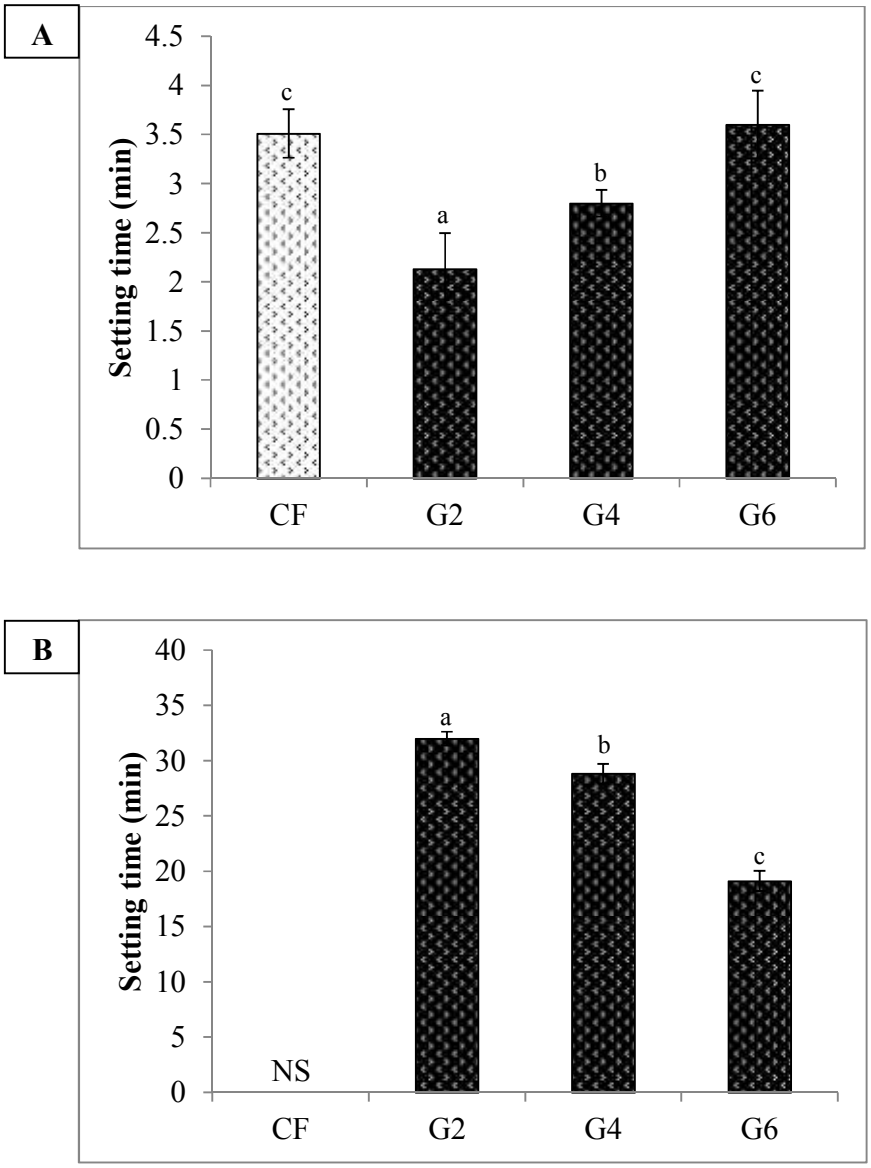
A
Setting time (min)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
c
a
b
c
CF
G2
G4
G6
B
Setting time (min)
40
35
30
25
20
15
10
5
0
NS
a
b
c
CF
G2
G4
G6

Fig. 5.

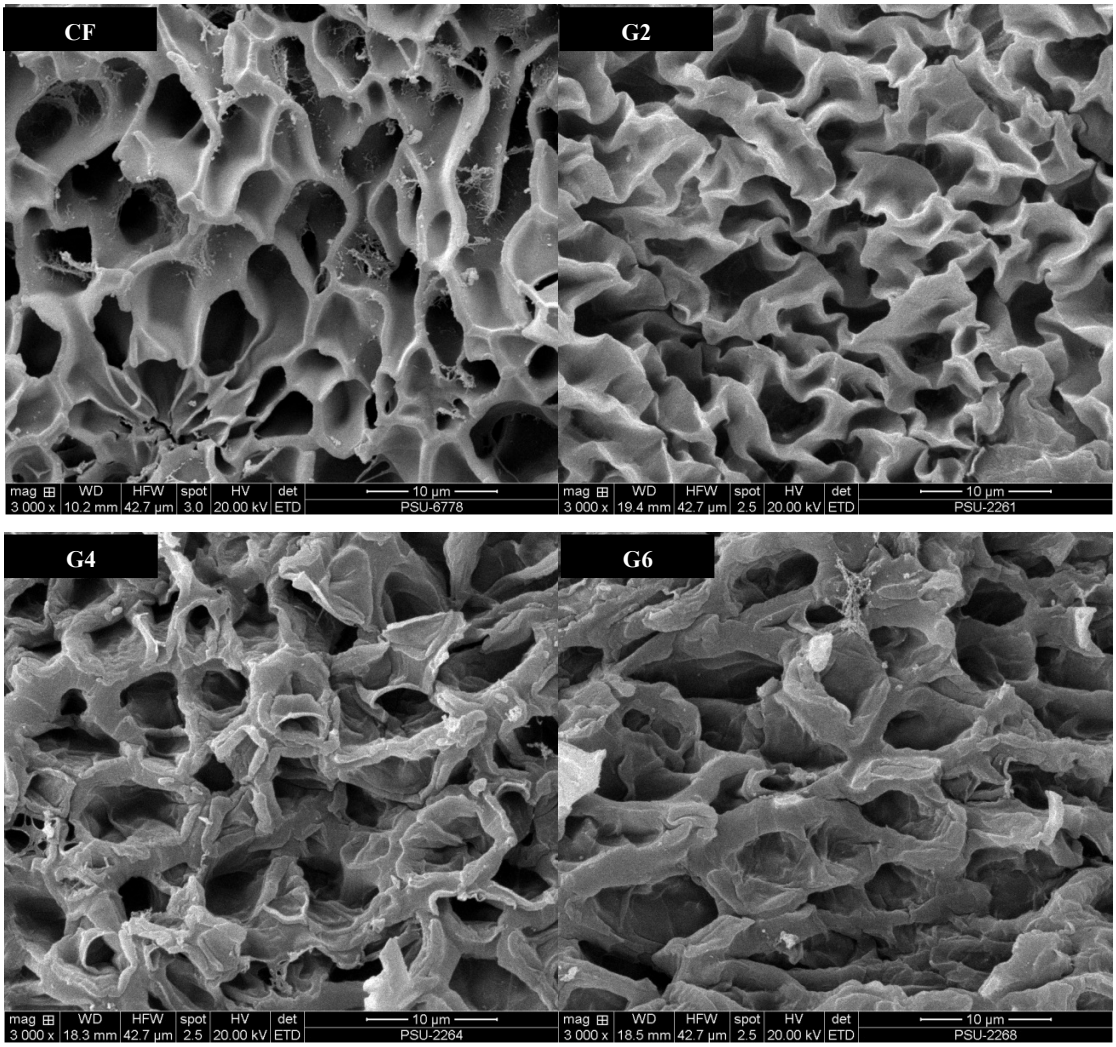
CF
mag WD HFW spot HV det
3 000 x 10.2 mm 42.7 µm 3.0 20.00 kV ETD
10 µm
PSU-6778
G2
mag WD HFW spot HV det
3 000 x 19.4 mm 42.7 µm 2.5 20.00 kV ETD
10 µm
PSU-2261
G4
mag WD HFW spot HV det
3 000 x 18.3 mm 42.7 µm 2.5 20.00 kV ETD
10 µm
PSU-2264
G6
mag WD HFW spot HV det
3 000 x 18.5 mm 42.7 µm 2.5 20.00 kV ETD
10 µm
PSU-2268

Fig. 6.

Table 1. Extraction yield, gel colour, gelling and melting temperatures of gelatin extracted from seabass skin with different sizes

Gelatin samples	Yield (%, dry wt basis)	L^*	a^*	b^*	ΔE^*	Gelling temperatures (°C)	Melting temperatures (°C)
CF	NM	4.17±0.37a	-0.18±0.36b	-3.35±0.40a	91.27±0.25d	15.84±0.28a	25.10±0.57a
G2	38.22±0.21c	17.07±0.49d	-0.47±0.11a	-1.31±0.14b	73.09±0.68a	17.09±0.42b	26.92±0.35b
G4	40.50±0.31b	16.33±0.34c	-0.43±0.07ab	-0.91±0.11c	74.25±0.44b	18.43±0.29c	27.62±0.63bc
G6	43.48±1.27a	14.46±0.04b	-0.33±0.14ab	-1.19±0.16bc	76.86±0.08c	19.01±0.63c	28.85±0.62c

Mean±SD (n = 3).

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

NM: Not mentioned

Table 2. Amino acid compositions of gelatins extracted from seabass skin with different sizes

Amino acids (g/100 g)	CF	G2	G4	G6
Alanine	10.08	11.17	11.18	11.38
Arginine	8.59	8.26	8.19	8.58
Aspartic acid/asparagine	5.25	5.31	5.34	5.36
Cysteine	0.06	0.09	0.10	0.10
Glutamine/glutamic acid	9.68	9.50	9.49	9.60
Glycine	22.90	22.57	22.65	23.33
Histidine	0.64	0.74	0.74	0.75
Isoleucine	1.30	1.11	1.11	1.10
Leucine	2.58	2.14	2.17	2.14
Lysine	3.63	3.56	3.55	3.64
Hydroxylysine	0.80	0.79	0.80	0.81
Methionine	1.31	1.85	1.86	1.88
Phenylalanine	1.84	1.93	1.95	1.94
Hydroxyproline	9.89	9.76	9.57	9.62
Proline	12.99	13.60	13.55	11.95
Serine	3.31	2.55	2.58	2.57
Threonine	2.49	2.39	2.43	2.44
Tyrosine	0.43	0.57	0.58	0.60
Valine	2.26	2.13	2.15	2.21
Imino acid	22.89	23.36	23.12	21.57

Research highlights

- Gelatin from seabass skin had higher gel strength than commercial fish gelatin.
- Gelatin from skin of larger size seabass exhibited the better gelling properties.
- Higher cross-links were found in gelatin from skin of seabass with larger sizes.