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Instructions for use

1 **Molecular characteristics and properties of gelatin**

2 **from skin of seabass with different sizes**

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26   **ABSTRACT**

27   Gelatin was extracted from the skin of seabass (*Lates calcarifer*) with different

28   average sizes (2, 4 and 6 kg/fish), termed G2, G4 and G6, respectively and their

29   characteristics and functional properties were determined. Yields of G2, G4 and G6

30   were 38.22, 40.50 and 43.48% (based on dry weight), respectively. G2 contained α-

31   chains as dominant component, whilst G4 and G6 comprised α-, β- and γ-chains with

32   a larger content of high MW cross-links. All gelatins had the similar imino acid

33   (hydroxyproline and proline) content. Net charge of G2, G4 and G6 became zero at

34   pHs of 6.73, 6.41 and 7.12, respectively. Amongst all gelatin samples, G6 exhibited

35   the highest gel strength (321.5 g) ($p<0.05$), but had the lowest turbidity ($p<0.05$). Gels

36   of G6 sample had the lower L^*-value but higher a^*-, b^*- and ΔE^*-value, compared with

37   others. Gelling and melting temperatures of all gelatins were 17.09-19.01 and 26.92-

38   28.85 °C, respectively. Furthermore, all gelatins were able to set at room temperature,

39   regardless of size of seabass used. G6 had the shorter setting time at room temperature

40   than others. Therefore, size of seabass, in which skin was used for gelatin extraction,

41   had the impact on yield, composition and properties of resulting gelatin.

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

45

46   **1. Introduction**

47   Gelatin is the denatured or partially hydrolysed form of collagen [1]. It

48   represents a major biopolymer with several applications in food, materials, pharmacy

49   and photography industries [2]. Due to its gelling property and surface behaviour (e.g.,

50   formation and stabilisations of foams and emulsions), gelatin has been widely used to

51   enhance the elasticity, consistency and stability of food products [3-5].

52   In general, the main sources of gelatin are skins and bones of pig and cow

53   obtained from processing by-products. Gelatin from those sources can be a problem

54   for certain consumers, e.g., Muslims and Jews, in which porcine gelatin is prohibited.

55   Occurrence of bovine spongiform encephalopathy (BSE) has led to awareness for

56   consumption of bovine gelatin [3]. Nowadays, an increasing interest has been paid to

57   alternative sources of gelatin, especially from the skins and bones from fish

58   processing by-products [6, 7]. Fish gelatin can be extracted from skin of several fish

59   species including skipjack tuna, dog shark [8], cobia [9], farmed Amur sturgeon [10],

60   seabass [6], and brownbanded bamboo and blacktip shark [7]. It has been known that

61   the extraction conditions including temperature, time as well as pretreatment affect

62   the functional properties of gelatin from fish processing by-products [6, 11, 12].

63   Additionally, characteristics and properties of gelatin vary with species and age of raw

64   material [13, 14]. Muyonga, Cole and Duodu [15] reported that gelatin from adult

65   Nile perch skin exhibited the better gel properties than young Nile perch skin when

66   the same extraction condition was used.

67   Seabass is one of economically important fish in Thailand. A number of

68   seabass farms are located in the south of Thailand, especially in the lake of Songkhla.

69   Due to its delicacy, a large amount of seabass has been exported as well as

70   domestically consumed. During processing, particularly fillet production, skin is

71 generated as byproduct. Skins from seabass has been used as raw material for

72 collagen and gelatin extraction with higher yield [16]. Gelatin from seabass skin had

73 higher gel strength than bovine gelatin and could be set at room temperature within 30

74 min [6]. Size or age of seabass, in which skin is used for gelatin extraction, can have

75 the impact on composition and properties of gelatin. Nevertheless, no information

76 regarding gelatin extracted from skin of seabass with different sizes has been reported.

77 Therefore, the aims of this investigation were to extract and determine the chemical

78 characteristics and functional properties of gelatin from the seabass skin as affected

79 by sizes of fish.

80

81 **2. Materials and methods**

82 2.1 Chemicals

83   All chemicals were of analytical grade. Sodium dodecyl sulphate (SDS),

84 Coomassie blue R-250 and *N,N,N',N'*-tetramethylethylenediamine (TEMED) were

85 procured from Bio-Rad Laboratories (Hercules, CA, USA). High-molecular-weight

86 markers were purchased from GE Healthcare UK Limited (Buckinghamshire, UK).

87 Fish gelatin produced from tilapia skin (~240 bloom) was obtained from Lapi

88 Gelatine S.p.A (Empoli, Italy).

89

90 2.2 Fish skin preparation

91   Fresh seabass (*Lates calcarifer*) with different sizes of 1.7-2.3, 3.7-4.3 and

92 5.7-6.2 kg/fish, equivalent to average size of 2, 4 and 6 kg/fish, respectively, were

93 obtained from a farm in Koyo Island, Songkhla, Thailand. The fish were kept in ice

94 with a fish/ice ratio 1/3 (w/w) and transported to the Department of Food Technology,

95 Prince of Songkla University, Hat Yai within 1 h after capture. Fish were washed

96   using cold tap water. Skins were then removed, descaled, and cut into small pieces

97   (0.5 cm x 0.5 cm) using a scissor. The skin was placed in polyethylene bags and

98   stored at -20 °C until used, but not longer than 2 months.

99

100   2.3 Extraction of gelatin from the skin of seabass

101   Gelatin was extracted from seabass skin according to the method of

102   Sinthusamran, Benjakul and Kishimura [6]. Before gelatin extraction, skin was soaked

103   in 0.1 M NaOH with a skin/solution ratio of 1/10 (w/v) to remove non-collagenous

104   proteins. The mixture was stirred for 3 h at room temperature (28-30 °C) using an

105   overhead stirrer model W20.n (IKA®-Werke GmbH & CO.KG, Stanfen, Germany).

106   The alkaline solution was changed every 1 h for totally 3 times. The pretreated skin

107   was washed with tap water until neutral or faintly basic pH was obtained. Then, the

108   washed skin was mixed with 0.05 M acetic acid at a skin/solution ratio of 1/10 (w/v)

109   to swell collagenous material in the fish skin matrix. The mixture was stirred at room

110   temperature for 2 h. The skin was washed using tap water until neutral or faintly

111   acidic pH of wash water was obtained. Finally, the swollen skin was mixed with

112   distilled water at a ratio of 1/10 (w/v) at 45 °C for 3 h with continuous stirring. The

113   mixtures were filtered using a Buchner funnel with Whatman No.4 filter paper

114   (Whatman International, Ltd., Maidstone, England). Then, the filtrates were freeze-

115   dried using a freeze-dryer (CoolSafe 55, ScanLaf A/S, Lynge, Denmark). The freeze-

116   dried gelatins extracted from seabass skin with an average size of 2, 4 and 6 kg/fish

117   were referred to as 'G2', 'G4' and 'G6', respectively. Gelatin samples were

118   subsequently subjected to analyses.

119

120

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$\pm$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

217   plate and the gap was set to 1.0 mm. The measurement was performed at a scan rate

218   of 0.5 °C/min, a frequency of 1 Hz, oscillating applied stress of 3 Pa during cooling

219   from 35 to 5 °C and heating from 5 to 35 °C. The gelling and melting temperatures

220   were calculated, where tan δ became 1 or δ was 45°.

221

222   2.4.11 Microstructure analysis of gelatin gel

223   Microstructure of gelatin gel was visualised using scanning electron

224   microscopy (SEM). Gelatin gels having a thickness of 2-3 mm were fixed with 2.5%

225   (v/v) glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 12 h. The samples were

226   then rinsed with distilled water for 1 h and dehydrated in ethanol with a serial

227   concentration of 50, 70, 80, 90 and 100 % (v/v). Dried samples were mounted on a

228   bronze stub and sputter-coated with gold (Sputter coater SPI-Module, West Chester,

229   PA, USA). The specimens were observed with a scanning electron microscope (JEOL

230   JSM-5800 LV, Tokyo, Japan) at an acceleration voltage of 20 kV.

231

232   2.5 Statistical analysis

233   All experiments were run in triplicate using three different lots of skin samples.

234   Data were subjected to analysis of variance (ANOVA) and mean comparisons were

235   carried out by using Ducan's multiple range test [20]. Statistical analysis was

236   performed using the statistical Package for Social Sciences (SPSS for windows: SPSS

237   Inc., Chicago, IL, USA).

238

239

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241

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

acid (22.57-23.33 g/100 g) and had a relatively high content of alanine (11.17-11.38

g/100 g). The amino acid composition of all gelatins showed very low contents of

cysteine (0.09-0.10 g/100 g), tyrosine (0.57-0.60 g/100 g), histidine (0.74-0.75 g/100

g) and hydroxylysine (0.79-0.81 g/100 g). In general, cysteine and tryptophan are not

found in collagen and gelatin [4, 6, 7]. For imino acids, all gelatins contained proline

and hydroxyproline contents of 11.95-13.60 and 9.57-9.76 g/100 g, respectively. The

imino acid content of G2 (23.36 g/100 g) and G4 (23.12 g/100 g) was higher than that

of G6 (21.57 g/100 g). Commercial fish gelatin had imino acid of 22.89 g/100 g.

Gelatin of skin from young Nile perch (21.55 g/100 g) showed similar imino acid

content to that of skin from adult Nile perch (21.63 g/100 g) [15]. The imino acid

content of seabass skin gelatin was higher than that of farmed Amur sturgeon skin

(18.90 g/100 g) [10], dog shark skin (19.75 g/100 g), rohu skin (18.38 g/100 g), tuna

skin (17.96 g/100 g) [8] and carp skin (19.47 g/100 g) [24]. Mammalian gelatins

generally contain 30% imino acids, which was higher than fish gelatin [8]. The

species, environment living habitat and body temperature of fish are the main factors

governing hydroxyproline and proline content [25]. Moreover, hydroxyproline

content of gelatin was also affected by the extraction conditions [10]. The stability of

triple-helix in collagen molecule correlated with imino acid content [16]. The

difference in imino acid content, especially hydroxyproline, was considered as the

important factor influencing viscoelastic properties and gel formation of gelatin [4, 8].

In the present study, there was similar hydroxyproline content amongst all samples,

but G6 showed the lowest proline content. The difference in amino acid composition

could be due to the differences in cross-links in the starting skin matrix. With

increasing fish age, the cross-linking of collagen in skin can be enhanced [20]. Those

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    $3293 cm^{-1}$, 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].

367   Thus, the differences in pI of all gelatin samples might be caused by the difference in

368   their amino acid compositions and distribution of amino acid residues, which were

369   more likely influenced by size or age of raw material used.

370

371   3.6 Gel strength of gelatin

372   Gel strength of gelatin from the skin of seabass with different sizes is shown in

373   Fig. 4A. Gel strength of gelatin increased as the size of fish increased ($p<0.05$). G6

374   had the highest gel strength, compared with others ($p<0.05$). All gelatin gels from

375   seabass skin exhibited higher bloom strength (223-322 g) than that of commercial fish

376   gelatin from tilapia skin (212 g) ($p<0.05$). Muyonga, Cole and Duodu [15] reported

377   that gelatin from young Nile perch skins (222 g) had slightly lower gel strength than

378   adult Nile perch skin gelatin (229 g). The result suggested that gelatin with greater

379   ratio of high-molecular weight components showed the better gelling properties via

380   higher stabilising interactions [34]. G4 and G6 showed higher band intensity of β-

381   chain, γ-chains and high MW cross-links than G2 (Fig. 1). Those large components

382   might serve as the strong strands in gel network. Gelatins with different gel strength

383   were reported for farmed Amur sturgeon skin (141 g) [10], cobia skin (232 g), croaker

384   skin (212 g) [9], dog shark skin (206 g), tuna skin (177 g) [8], tiger-toothed croaker

385   (170 g), pink perch (140 g) [23] and grey triggerfish (168 g) [2]. The difference in gel

386   strength amongst species was mainly due to the different amounts of the β- and α-

387   components and the amino acid composition of gelatin [6]. Furthermore, imino acids,

388   especially hydroxyproline, are involved in gel formation of gelatin via hydrogen

389   bonding through hydroxyl group [5]. However, similar hydroxyproline contents were

390   observed amongst all gelatin samples as shown in Table 2. The result suggested that

391   components of gelatin, particularly cross-links, most likely played an essential role in

392   gel formation and determined gel strength of gelatin.

393

394   3.7 Colour and turbidity of gelatin gel

395   Colours of different gelatin gels from skin of seabass and commercial fish

396   gelatin expressed as L^*, a^*, b^* and ΔE^* are shown in Table 1. Gelatin gel from G2

397   showed the higher L^*-value (lightness) than others (G4 and G6) (p<0.05). The L^*-

398   value of gelatin gels slightly decreased when skin from seabass with larger sizes was

399   used (p<0.05). G2 sample had the lower redness value (a^*-value), compared to

400   commercial fish gelatin (p<0.05). No differences in a^*-value were found amongst

401   gelatins from seabass with different sizes (p>0.05). Additionally, gelatin gels from

402   seabass had higher b^*-value than that from commercial fish gelatin (p<0.05). Slight

403   differences in b^*-value were found amongst gelatin gels from seabass skin with

404   different sizes (p<0.05). For total difference in the colour value (ΔE^*), commercial

405   fish gelatin showed the highest ΔE^*-value (91.27), compared with gelatin gels from

406   seabass skin. ΔE^*-value increased as size of seabass used for gelatin extraction

407   increased. This trend was in agreement with the decrease in L^*-value as the size of

408   fish used increased. Thus, size or age of seabass as raw material had the impact on

409   colour of gelatin and their gels.

410   Turbidity of solutions of gelatin extracted from skin with different sizes

411   expressed as A_{360} is shown in Fig. 4B. Gelatin solution exhibited the lower turbidity

412   when size of seabass used for extraction increased (p<0.05). The highest turbidity was

413   found in solution of G2 sample (p<0.05). Turbidity of gelatin solution from

414   commercial fish gelatin was much lower, compared with all gelatins from seabass

415   skin (p<0.05). The turbidity of gelatin solution is dependent on species of raw

416   material, gelatin extraction process and clarification/filtration process [15, 23]. In this

417   study, only filtration process was implemented, whilst the clarification process has

418   been used for commercial gelatin to remove both light- and heavy-impurities [15].

419   Koli, Basu, Nayak, Patange, Pagarkar and Gudipati [23] reported that inorganic,

420   protein and muco-substance contaminants mainly affected turbidity and dark colour of

421   gelatin solution. Both G4 and G6 samples contained high MW components at higher

422   level than G2 (Fig. 1). Solution of G2 sample with smaller peptides or proteins might

423   prevent transmission of light more effectively, thereby increasing the light scattering.

424   This might contribute to higher turbidity of gelatin solution obtained from skin of fish

425   with smaller sizes. These results showed that the size or age of raw materials had the

426   impact on turbidity of gelatin solution.

427

428   3.8 Setting time for gel formation

429   The setting times required for the gel formation of gelatin extracted from the

430   skin of seabass having different sizes at 4 °C and room temperature (26$\pm$2 °C) are

431   presented in Fig. 5A and 5B, respectively. The setting times at 4 °C of G2, G4 and G6

432   were 2.13, 2.80 and 3.60 min, respectively. The setting time at 4 °C of gelatin

433   increased as seabass size used for gelatin extraction increased ($p < 0.05$). G2 and G4

434   samples had a shorter setting time at 4 °C than the commercial fish gelatin ($p < 0.05$).

435   Due to the higher proportion of high MW peptide chains of G6, the arrangement of

436   those chains formation of gel network plausibly took a longer time as indicated by

437   longer setting time. In addition, the nucleation zone during gel formation required

438   hydroxyproline [35]. With high proportion of cross-links or β- and γ-chains,

439   hydroxyproline residues could be localized inside the molecules. This could lower the

440 accessibility or exposure of hydroxyproline to form H-bond. Thermal degradation of

441 peptides in gelatin during heating extraction affected the setting time of gelatin gel [6].

442 When setting was carried out a room temperature, setting times of 32.0, 28.83

443 and 19.13 min were found for G2, G4 and G6, respectively. Gelatin samples from

444 seabass skin had the longer setting time when small size of seabass was used (p<0.05).

445 All gelatins from seabass were able to undergo setting at room temperature. This

446 result was in agreement with Sinthusamran, Benjakul and Kishimura [6] who reported

447 that gelatin from seabass skin was able to set at room temperature within 30 min.

448 Conversely, commercial fish gelatin was not able to set within 3 h at room

449 temperature. Setting phenomenon of gelatin samples at room temperature was

450 different from that occurred at lower temperature (4 °C). At higher setting

451 temperatures, higher entropy of gelatin molecules was presumed. Those γ- and β-

452 components found in G4 or G6 samples might promote the formation of gel network

453 with rapid gelation [36]. When those high MW cross-links aligned themselves under

454 the high entropy conditions, they could form gel easily as evidenced by lower setting

455 time. Different extraction condition, especially extraction temperature and time also

456 influenced the setting time of gelatin [7, 15]. Furthermore, difference in setting time

457 of fish gelatin might be due to different age or size of fish used as raw material [8].

458 Thus, setting time could be affected by size of seabass used as raw material.

459 Additionally, setting temperature was another factor affecting time employed for gel

460 formation.

461

462 3.9 Gelling and melting temperatures

463 Gelling and melting temperatures of gelatin extracted from the skin of seabass

464 with different sizes are presented in Table 1. Thermal transition of gelatin solution

465   were monitored by changes in phase angle (δ) during cooling (35-5 °C, 0.5 °C/min)

466   and subsequent heating (5-35 °C, 0.5 °C/min). The formation of junction zones in the

467   three-dimensional network of gelatin gel can be monitored in term of changes of

468   phase angle [6]. The gelling temperatures were 17.09, 18.43 and 19.01 °C for G2, G4

469   and G6, respectively, which were higher than commercial fish gelatin (15.84 °C)

470   (p<0.05). The higher gelling temperatures were observed in G4 and G6 samples

471   extracted from the skin of larger size, in comparison with that of smaller size (G2)

472   (p<0.05). The gelling temperature of gelatin tended to increase with increasing

473   amount of cross-links. This result indicated that the molecular weight distribution in

474   gelatin was associated with gelation. Muyonga, Cole and Duodu [15] also reported

475   that chemical compositions, especially the content of γ-, β-, and α-chain components

476   influenced gelling point of gelatin gel. Additionally, different gelling temperature was

477   related to amino acid composition, especially proline and hydroxyproline, which is

478   governed by temperatures of habitat, where fish live [10, 37]. The imino acid content

479   is normally available to form hydrogen bonds with water [13]. In general, warm water

480   fish or mammalian gelatin has higher gelling and melting temperatures than the cold

481   water fish gelatin [10]. Most of fish gelatin had lower gelling temperature than

482   mammalian gelatin [37]. Varying gelling temperatures were reported for gelatin from

483   different fish, e.g. cobia skin (19.9 °C), croaker skin (17.8 °C) [9], dog shark skin

484   (20.8 °C), tuna skin (18.7 °C) [8], farmed Amur sturgeon skin (14.2 °C) [10], red

485   snapper bone (16.0 °C) and grouper bone (16.0 °C) [37].

486   Melting temperatures of G2, G4 and G6 were 26.92, 27.62 and 28.85 °C,

487   respectively. The melting temperatures of gelatin from seabass skin were higher than

488   that of commercial fish gelatin (25.10 °C) (p<0.05). Nevertheless, the melting

489   temperature of gelatins from seabass skin were lower than those of bovine bone

490   (29.7 °C) and porcine skin (32.3 °C) [38]. It was noted that the melting temperatures

491   of all gelatins from seabass skin were higher than those of gelatins from the dog shark

492   skin (25.8 °C), tuna skin (24.2 °C) [8], farmed Amur sturgeon skin (22 °C) [10], tiger-

493   toothed croaker skin (20.36 °C), pink perch skin (19.23 °C) [23] and tilapia skin

494   (22.4 °C) [39]. Since seabass used in the present study was tropical fish (25-32 °C),

495   gelatin gel might be more stable than those from temperate zone (15-25 °C) [40].

496   Thermal stability of gelatin gel is associated with the temperature of habitat of the

497   animals [13]. G4 and G6 samples had higher melting temperature than G2 ($p < 0.05$).

498   High MW components might contribute to the stability and stronger network of gel.

499   In the present study, G6 showed the higher gelling as well as melting

500   temperatures, although it showed the lower imino acid content than G2 and G4. It was

501   noted that G6 had the higher band intensity of β-chains, γ-chain and high molecular

502   weight cross-link, compared with G2 and G4 (Fig. 1). Due to the higher percentage of

503   β- and γ-components, a shorter maturation time might be required since the

504   entanglement of chain took place with ease. Also, those large components might serve

505   as the strong strands in gel network, leading to the increased thermal stability of the

506   gelatin gel [41] as indicated by the higher melting temperature. As a consequence, G6

507   had higher gelling and melting temperatures. Gelatin extracted from tropical fish has

508   higher gelling and melting temperature than that gelatin extracted from temperate fish.

509   Thus, the differences in gelling and melting temperature of seabass gelatin might be

510   governed by the differences in components, especially those with high MW.

511

512   3.10 Microstructures of gelatin gels

513   Microstructures of the gel from commercial fish gelatin and gels from gelatin

514   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

540 commercial fish gelatin. Thus, skin from seabass with different size used as raw

541 material for gelatin extraction had the influence on chemical compositions and

542 properties of gelatin.

543

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551 **References**

552 [1] L. Chen, L. Ma, M. Zhou, Y. Liu, Y. Zhang, Food Hydrocoll. 36 (2014) 316-322.

553 [2] K. Jellouli, R. Balti, A. Bougatef, N. Hmidet, A. Barkia, M. Nasri, LWT-Food Sci.

554 Technol. 44 (2011) 1965-1970.

555 [3] T. Nur Azira, Y.B. Che Man, R.N. Raja Mohd Hafidz, M.A. Aina, I. Amin, Food

556 Chem. 151 (2014) 286-292.

557 [4] S. Benjakul, K. Oungbho, W. Visessanguan, Y. Thiansilakul, S. Roytrakul, Food

558 Chem. 116 (2009) 445-451.

559 [5] S. Benjakul, P. Kittiphattanabawon, J.M. Regenstein, Fish gelatin, in: B.K.

560 Simpson, L.M.L. Nollet, F. Toldrà, S. Benjakul, G. Paliyath, Y.H. Hui (Eds.), Food

561 Biochemistry and Food Processing, John Wiley & Sons, Inc., Ames, 2012, pp. 388-

562 405.

563 [6] S. Sinthusamran, S. Benjakul, H. Kishimura, Food Chem. 152 (2014) 276-284.

564 [7] P. Kittiphattanabawon, S. Benjakul, W. Visessanguan, F. Shahidi, Food Hydrocoll.

565 24 (2010) 164-171.

566 [8] K. Shyni, G.S. Hema, G. Ninan, S. Mathew, C.G. Joshy, P.T. Lakshmanan, Food

567 Hydrocoll. 39 (2014) 68-76.

568 [9] R.S.G. Silva, S.F. Bandeira, L.A.A. Pinto, LWT-Food Sci. Technol. 57 (2014)

569 580-585.

570 [10] M. Nikoo, S. Benjakul, M. Bashari, M. Alekhorshied, A. Idrissa Cissouma, N.

571 Yang, X. Xu, Food Bioscience. 5 (2014) 19-26.

572 [11] M. Nagarajan, S. Benjakul, T. Prodpran, P. Songtipya, H. Kishimura, Food

573 Hydrocoll. 29 (2012) 389-397.

574 [12] P. Kaewruang, S. Benjakul, T. Prodpran, Food Chem. 138 (2013) 1431-1437.

575 [13] A.A. Karim, R. Bhat, Food Hydrocolloid. 23 (2009) 563-576.

576 [14] P. Singh, S. Benjakul, S. Maqsood, H. Kishimura, Food Chem. 124 (2011) 97-

577 105.

578 [15] J.H. Muyonga, C.G.B. Cole, K.G. Duodu, Food Hydrocoll. 18 (2004) 581-592.

579 [16] S. Sinthusamran, S. Benjakul, H. Kishimura, Food Chem. 138 (2013) 2435-2441.

580 [17] U.K. Laemmli, Nature. 227 (1970) 680-685.

581 [18] A. Gennadios, C.L. Weller, M.A. Hanna, G.W. Froning, J. Food Sci. 61 (1996)

582 585-589.

583 [19] M.D. Fernández-Díaz, P. Montero, M.C. Gómez-Guillén, Food Chem. 74 (2001)

584 161-167.

585 [20] R.G.D. Steel, J.H. Torrie, Principles and Procedures of Statistics: A Biometrical

586 Approach., second ed., McGraw-Hill, New York, 1980.

587 [21] E. Foegeding, T.C. Lanier, H.O. Hultin, Characteristics of edible muscle tissue,

588 in: O.R. Fennema (Ed.) Food Chem, Marcel Dekker., New York, 1996, pp. 879-942.

589   [22] J.H. Muyonga, C.G.B. Cole, K.G. Duodu, Food Chem. 85 (2004) 81-89.

590   [23] J.M. Koli, S. Basu, B.B. Nayak, S.B. Patange, A.U. Pagarkar, V. Gudipati, Food

591   Bioprod. Process. 90 (2012) 555-562.

592   [24] L.M. Kasankala, Y. Xue, Y. Weilong, S.D. Hong, Q. He, Biores. Technol. 98

593   (2007) 338-343.

594   [25] J. Zhang, R. Duan, Y. Wang, B. Yan, W. Xue, Food Hydrocoll. 29 (2012) 100-

595   105.

596   [26] J. Bandekar, Biochimica et Biophysica Acta (BBA) - Protein Structure and

597   Molecular Enzymology. 1120 (1992) 123-143.

598   [27] I. Yakimets, N. Wellner, A.C. Smith, R.H. Wilson, I. Farhat, J. Mitchell,

599   Polymer. 46 (2005) 12577-12585.

600   [28] P. Kaewruang, S. Benjakul, T. Prodpran, S. Nalinanon, Food Bioscience. 2

601   (2013) 1-9.

602   [29] F. Lavialle, R.G. Adams, I.W. Levin, Biochemistry. 21 (1982) 2305-2312.

603   [30] M. Jackson, L.P. Choo, P.H. Watson, W.C. Halliday, H.H. Mantsch, Biochimica

604   et Biophysica Acta Molecular Basis of Disease. 1270 (1995) 1-6.

605   [31] B.B. Doyle, E.R. Blout, E.G. Bendit, Biopolymers. 14 (1975) 937-957.

606   [32] P.L.R. Bonner, Protein purification, UK: Taylor & Francis Group, Cornwall,

607   2007.

608   [33] M. Ahmad, S. Benjakul, Food Hydrocoll. 25 (2011) 381-388.

609   [34] H.S. Tabarestani, Y. Maghsoudlou, A. Motamedzadegan, A.R. Sadeghi

610   Mahoonak, Biores. Technol. 101 (2010) 6207-6214.

611   [35] D.A. Ledward, Gelation of gelatin, in: J.R. Mitchell, D.A. Ledward (Eds.),

612   Functional properties of food macromolecules, Elsevier Applied Science., London,

613   1989, pp. 171-201.

614   [36] D. Fernández, amp, x, M.D. az, P. Montero, M.C. Gómez-Guillén, Food

615   Hydrocoll. 17 (2003) 281-286.

616   [37] R. Jeya Shakila, E. Jeevithan, A. Varatharajakumar, G. Jeyasekaran, D. Sukumar,

617   LWT-Food Sci. Technol. 48 (2012) 30-36.

618   [38] M. Gudmundsson, J. Food Sci. 67 (2002) 2172-2176.

619   [39] S. Zeng, X. Yan, W. Cao, P. Hong, C. Zhang, L. Li, Int. J. Food Sci.Technol. 45

620   (2010) 1807-1813.

621   [40] T.O. Haugen, A.C. Sanger, Am. Fish. Soc. Symp. 76 (2011), 1-24.

622   [41] S. Wangtueai, A. Noomhorm, J.M. Regenstein, J. Food Sci. 75 (2010) 731-739.

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Figure Legends

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

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

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

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

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

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

Molecular characteristics and properties of gelatin

from skin of seabass with different sizes

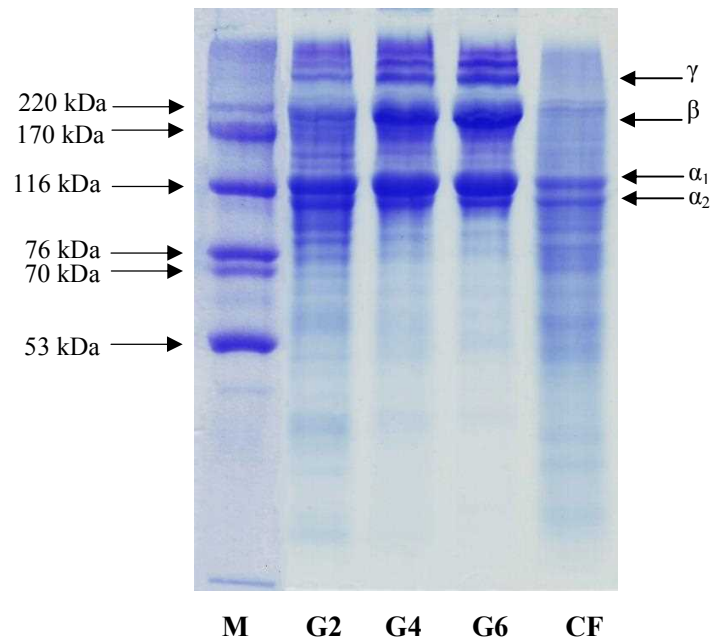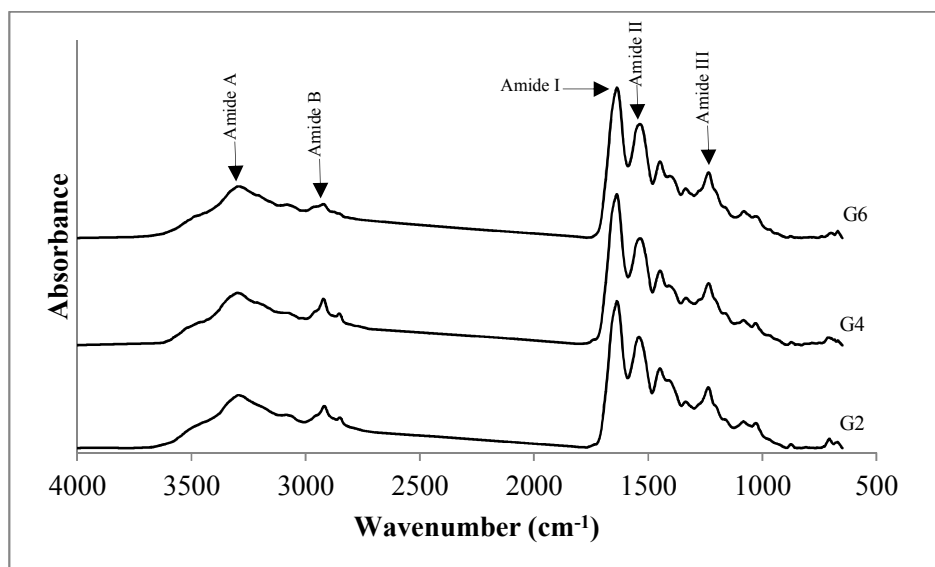Sittichoke Sinthusamran [1] and Soottawat Benjakul [1,*] and Hideki Kishimura [2]
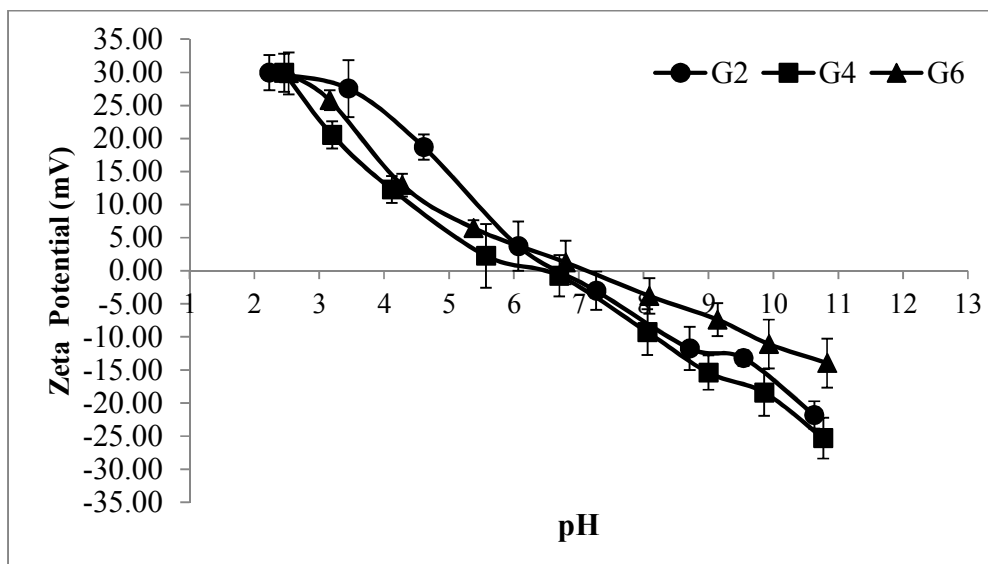
Fig. 1.

Fig. 2.
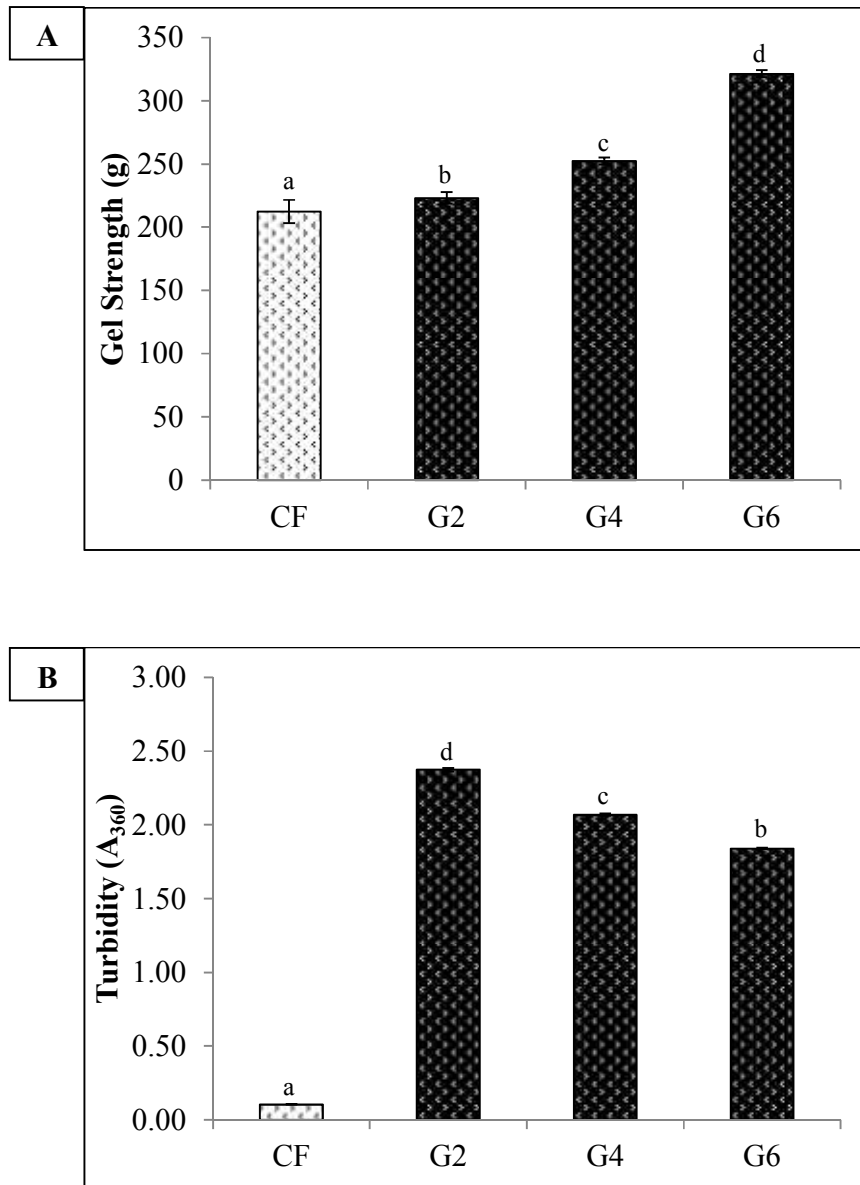

Fig. 3.
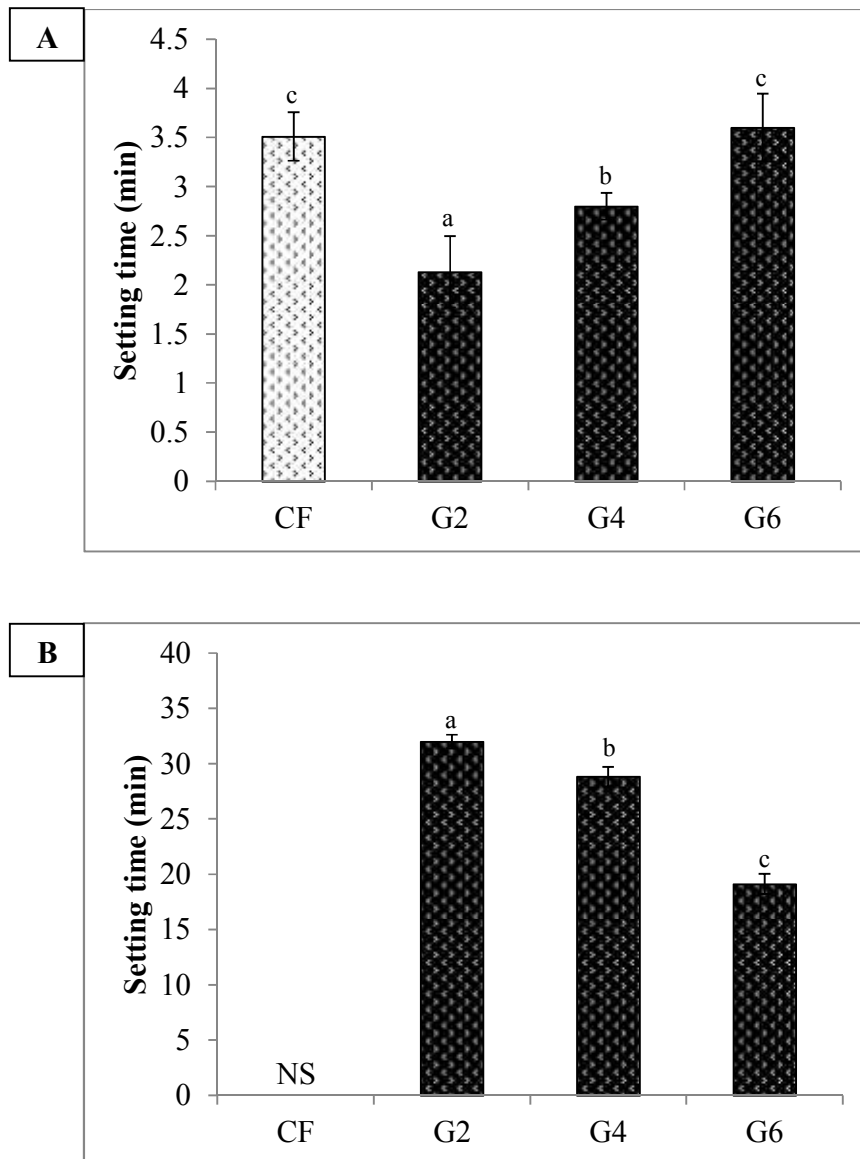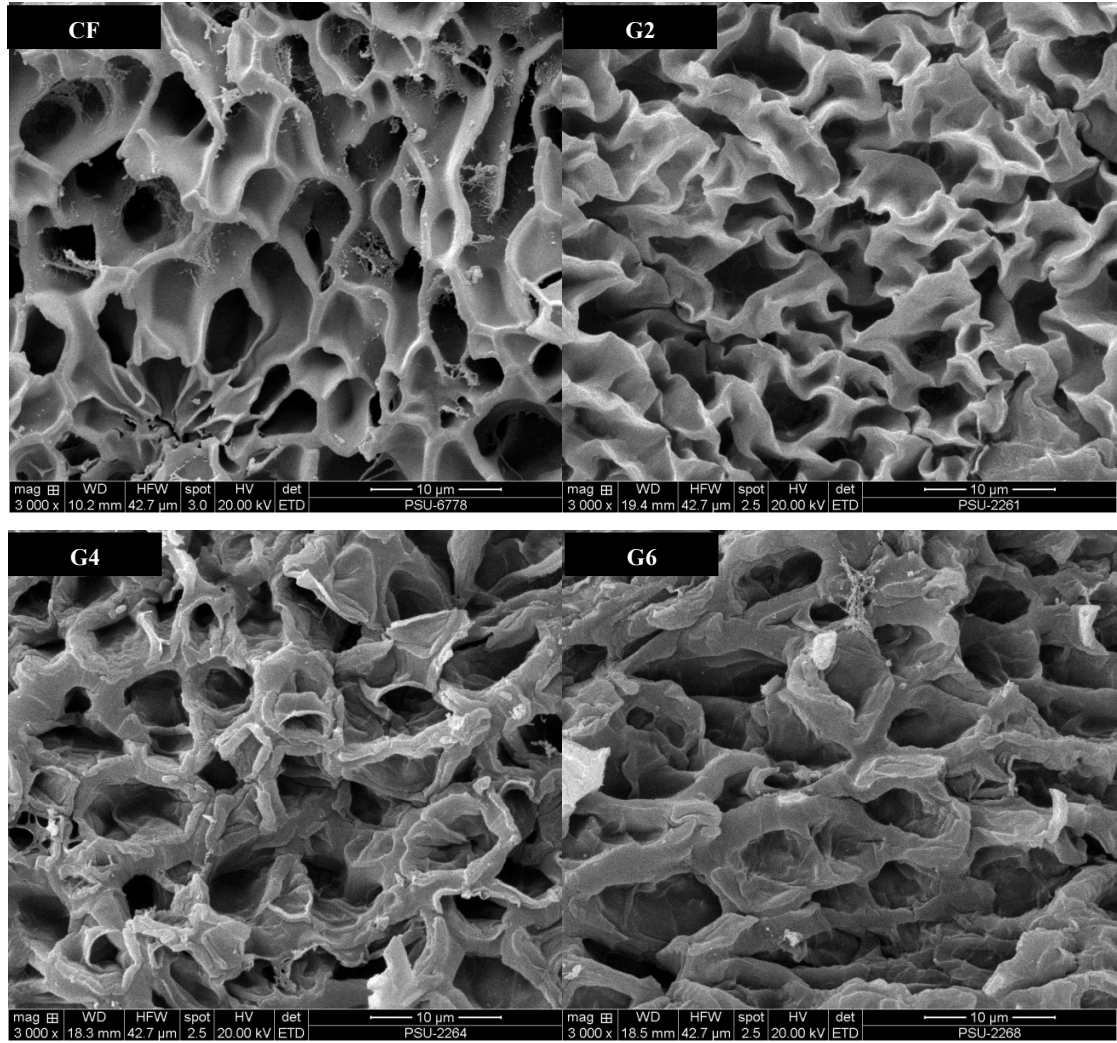
Fig. 4.

Fig. 5.

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.