



Title	Purity and properties of gelatins extracted from the head tissue of the hybrid kalamtra sturgeon
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Highlights

- Sturgeon head shows high potential as a new source of fish gelatin.
- Gelatin purity was determined by relative gelatin content and gelatin-band intensity.
- Type A gelatin had higher purity and gel strength with higher gelling temperature.
- Type B gelatin had higher emulsifying and forming activities.
- Type A gelatin can be used in biomaterials or drugs and type B in foods or cosmetics.

1 **Purity and properties of gelatins extracted from the head tissue of the hybrid kalamtra**
2 **sturgeon**

3

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21

22 **Declaration of Interest:** None

23 **Abstract**

24

25 The head is a major by-product (17.1% of body weight) of sturgeon aquaculture
26 farming and remains unutilized. In this study, the sturgeon head was separated into the skull
27 cartilage and mixed tissue: skin, scales, fins, muscles, bones, gills, and small cartilage pieces.
28 Type A and B gelatins were extracted from the mixed tissue. The type B yield was higher, at
29 5.8% gelatin dry weight per tissue wet weight. The relative gelatin content in the sample
30 weight, estimated from the sample's hydroxyproline content relative to that of the purified
31 collagen, was higher in type A (60.3%) than in type B (39.7%). Type A gelatin showed
32 higher intensities of the α - and β -bands of gelatin in SDS-PAGE, indicating that gelatin purity
33 was higher in type A. The breaking force-strain curve showed a larger breaking force and
34 lower breaking strain in type A (2.0 N, 12.1%), indicating that this gelatin is stronger than
35 type B (0.7 N, 15.8%). Type B gelatin featured higher emulsion activity and stability and
36 higher foam expansion and stability. In conclusion, type A and B gelatins with distinct
37 proximate composition and functional properties were successfully extracted from sturgeon
38 heads, which could act as a promising source of gelatins for industrial applications.

39

40 **Abbreviations**

41

42 BV Bovine bone gelatin

43 DF Dilution factor

44 DW Distilled water

45 EAI Emulsion activity index

46 ESI Emulsion stability index

47 FBC Fat-binding capacity

48 FE Foaming expansion

49 FS Foaming stability

50 GC_p Relative gelatin content in the protein fraction of the sample

51 GC_s Relative gelatin content in the sample weight

52 Hyp Hydroxyproline

53 LMM Low molecular mass

54 NGC Non-gelatinous content

55 WAC Water-absorbing capacity

56 **1. Introduction**

57

58 The denatured form of collagen is termed gelatin (Ranasinghe et al., 2020).
59 Hydrolyzed collagen with a molecular mass less than 30 kDa is not considered gelatin
60 because it lacks gel-forming ability (Boran & Regenstein, 2009). Gelatin is commonly
61 extracted from collagen-rich tissues such as skin by hydrothermal extraction. When the raw
62 materials are pretreated with acidic or alkaline solutions, the extracted gelatin is referred to as
63 type A or type B, respectively (Ahmad et al., 2017). These gelatins, generally obtained from
64 mammalian sources, are used in foods, pharmaceuticals, and tissue engineering industries;
65 however, some consumers have raised concerns of zoonotic disease risks (Karim & Bhat,
66 2009; Ranasinghe et al., 2020). Gelatins derived from aquatic sources are potential
67 alternatives to mammalian gelatins (Gómez-Guillén, Gimenez, Lopez-Caballero, & Montero,
68 2011) and intensive studies have characterized their properties and functionalities
69 (Ranasinghe et al., 2020; Zhang et al., 2020a, 2020b).

70 Even if the source tissues are rich in collagen, the extracted gelatin samples can
71 contain traces of non-gelatinous substances such as non-collagenous proteins, lipids,
72 carbohydrates, and ash. Thus, gelatin purity (gelatin content in the sample) is dependent not
73 only on the collagen content of the source tissues but also on the pretreatment and extraction
74 methods. Gelatin purity is a determining factor for the proper applications of aquatic gelatins,
75 as it may significantly affect the properties and functionality of the gelatin sample. One of the
76 gelatin purity indicators, hydroxyproline residue (Hyp), is collagen-specific and is used to
77 measure collagen or gelatin content (Hofman, Hall, Cleaver, & Marshall, 2011; Nelson &
78 Cox, 2005). Although many studies have determined the amino acid composition of aquatic-
79 sourced gelatin samples, only the study by Tümerkan, Cansu, Boran, Regenstein, and Özoğul
80 (2019) addressed the percentage Hyp content of extracted aquatic gelatin relative to the

81 source tissue (skin), and the relationships between gelatin purity and gelatin properties.
82 Another indicator widely used to assess the purity of type I collagen (Capella-Monsonís,
83 Coentro, Graceffa, Wu, & Zeugolis, 2018) is the intensity of gelatin α -, β -, and γ -bands from
84 the extracted sample in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
85 PAGE). The disadvantage of this method is that the band intensity highly depends on the
86 staining condition of the SDS-PAGE gel; thus, only the values of samples on the same gel are
87 comparable. Many studies on fish gelatin have shown SDS-PAGE images; however, few
88 studies have quantitatively studied the relationships between the intensities of the bands and
89 gelatin properties and functionality.

90 In this study, we focused on the head of the sturgeon, as a new source of aquatic
91 gelatin. In 2017, 102 327 tons of sturgeon biomass was produced worldwide on a total of
92 2 329 commercial aquaculture farms (Bronzi et al., 2019). Because the primary commercial
93 product of sturgeon aquaculture is caviar, large quantities of by-products—sometimes
94 including meat—are generated (Bronzi et al., 2019). To utilize these by-products, methods
95 have been developed to extract gelatin from the sturgeon skin (Hao et al., 2009; Nikoo et al.,
96 2011, 2013, 2014) and collagen from the sturgeon skin, scale, muscle, notochord, cartilage,
97 swim bladder, and digestive tract (Liang et al., 2014; Meng et al., 2019; Wang et al., 2014;
98 Zhang et al., 2014). However, there are no reports on the utilization of sturgeon heads for any
99 purpose, including gelatin extraction, although the head tissue is the major by-product,
100 representing approximately 17.1% of total body weight (unpublished observation). This is
101 likely because of low profitability and extraction difficulties arising from the hardness and
102 complexity of the tissue composition.

103 Kalamtra sturgeon (*Huso dauricus* \times *Acipenser schrenckii* \times *Acipenser*
104 *transmontanus*) is a hybrid species developed at the Hokkaido University. Aquaculture
105 production of this species is increasing in Hokkaido, Japan (Islam, Yuhi, Ura, & Takagi,

106 2020). In this study, we extracted type A and type B gelatins from the head of the kalamtra
107 sturgeon and assessed their properties and functionalities against sample gelatin purity, for
108 potential industrial uses. Sample gelatin purities were expressed as the relative gelatin content
109 per sample weight, calculated from the Hyp content of the sample relative to that of purified
110 collagen, and from the intensity of gelatin bands of the sample in SDS-PAGE.

111

112 **2. Materials and methods**

113

114 *2.1 Extraction of gelatins*

115

116 The heads of kalamtra sturgeon were obtained from an aquaculture farm (Bifuka
117 Shinko Kosha, Bifuka, Japan) and stored at -20 °C. The heads (644.4±28.5 g, mean ± SE)
118 were first divided into the skull cartilage and mixed tissue (skin, scales, pectoral fins,
119 muscles, bones, gills, and small cartilage pieces). Gelatin types A and B were extracted from
120 the mixed tissues following our previously optimized conditions (Supplementary Fig. 1). The
121 skull cartilage was stored at -30 °C for future extraction of type II gelatin and chondroitin
122 sulfate. The yield (%) was calculated as the gelatin dry weight per sample wet weight.

123

124 *2.2 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

125

126 SDS-PAGE (7.5% gel) was performed as previously described (Meng et al., 2019)
127 after dissolving the gelatin (w/v 0.2%) in distilled water (DW), heating at 50 °C for 15–20
128 min, and mixing by pipetting. From captured gel images, processed using Image-J software
129 (Version 1.52, National Institutes of Health, Bethesda, MD, USA), the intensities of the
130 gelatin bands (α , β , and γ) and low molecular mass (LMM) bands (<100 kDa) were

131 quantified. Tricine-SDS-PAGE was performed following the method of Schägger (2006).
132 Briefly, 4% stacking gel, 10% spacer gel, and 15.5% separating gel were prepared using gel
133 buffer (3 M Tris-HCl, 0.3% SDS, pH 8.45), acrylamide, bisacrylamide, glycerol, ammonium
134 persulfate, and TEMED. Samples were dissolved in DW and mixed (v/v 1:1) with the sample
135 buffer (0.5 M Tris-HCl, pH 6.8, with 4% SDS and 20% glycerol) containing 10% β -
136 mercaptoethanol. After boiling for 3 min, the samples were loaded onto the gel (25 μ g
137 sample/lane). The protein marker standard (DynaMarker® Protein MultiColor Stable, Low
138 Range, BioDynamics Laboratory Inc., Tokyo, Japan) was used to assess the molecular mass
139 of the samples. A current of 30 volts (14 mA) was applied through the gel for 50–60 min,
140 then 70 volts (24 mA) for 3–3.5 h. Next, the gel was fixed in a mixture of 50% methanol and
141 10% acetic acid for 30 min, stained with 0.1% Coomassie Brilliant Blue R-250 solution for
142 45 min, de-stained with 10% acetic acid for 4–5 h, then imaged.

143

144 *2.3 Amino acid composition and relative gelatin content in the protein fraction of the sample*

145

146 The amino acid composition was quantified according to Meng et al. (2019), with
147 some modifications. Briefly, the gelatin sample was hydrolyzed in 6 M HCl at 110 °C for 24
148 h and then dried by evaporation. The remaining materials were assessed using an automated
149 amino acid analyzer (L-8900, Hitachi High-Technologies Corporation, Tokyo, Japan) after
150 dissolving in a citric acid buffer solution.

151 The relative gelatin content in the protein fraction of the sample (GCp) was calculated
152 using the following formula:

$$153 \quad \text{GCp (\%)} = [(\text{Hyp of the sample})/(\text{Hyp of the purified collagen})] \times 100$$

154 where Hyp is the number of hydroxyproline expressed as Hyp residues/1000 amino acid
155 residues. Purified collagen (Supplementary Fig. 2) was extracted from a mixture of the wet

156 skin of kalamtra and bester sturgeons as described by Meng et al. (2019). Tryptophan (Trp)
157 was not considered for estimating the relative gelatin content in the protein fraction of the
158 sample due to the undetectability of the method. The Trp content is low (0.3%) when
159 estimated from the cDNA sequence of the α 1-chain of type I procollagen in Amur sturgeon
160 (Zhang et al., 2016). Hanani (2016) also reported that gelatin lacked Trp. Thus, the Trp
161 content did not affect the calculation of the amino acid composition and GCp.

162

163 *2.4 Proximate composition*

164

165 The relative gelatin content in the sample weight (GCs) was obtained using the
166 following formula:

$$167 \quad \text{GCs (\%)} = [(\text{Hyp of the sample})/(\text{Hyp of the purified collagen})] \times 100$$

168 where Hyp is the concentration (nmol Hyp/ μ g dry sample) of the gelatin sample or purified
169 collagen.

170 Next, the relative non-gelatinous protein content in the sample (NGCs) was calculated
171 as follows:

$$172 \quad \text{NGCs (\%)} = 100 \text{ GCs/GCp} - \text{GCs}$$

173 where GCs and GCp are the relative gelatin content (%) in the sample weight and protein
174 fraction of the sample, respectively, obtained as described above. The precise methods used
175 to derive this formula are shown in Supplementary Fig. 3.

176 Lipid and ash contents were measured according to the methods of Bligh and Dyer
177 (1959) and AOAC (2000, # 920.153), respectively. Finally, the content of other components,
178 mainly carbohydrates, was calculated as the remainder of the sum of gelatin, non-gelatinous
179 protein, lipid, and ash.

180

181 *2.5 Transparency*

182

183 The absorbance of the gelatin solution (w/v 1%) was measured using a
184 spectrophotometer (UH5300, Hitachi, Tokyo, Japan) as per Zhang et al. (2020a) and Kim et
185 al. (2020). The transparency (%) was calculated using the following formula:

186
$$\text{Transparency (\%)} = 10^{-\text{(Absorbance at 600 nm)}} \times 100$$

187

188 *2.6 Rheological properties*

189

190 The gelling and melting temperatures of the samples were measured according to the
191 method of Shakila, Jeevithan, Varatharajakumar, Jeyasekaran, & Sukumar (2012) with slight
192 modifications. Briefly, 2.5 mL of the gelatin solution (w/v 6.67%) was transferred to each test
193 tube (each test was repeated 3 times). The temperatures were lowered or raised at a rate of
194 0.5 °C/min in an incubator (FMU-133I, Fukushima Industries Corp., Osaka, Japan). Bovine
195 bone gelatin (BV; Wako Pure Chemical Industries Ltd., Osaka, Japan) was used as a control.

196 To measure the gel strength, gelatin solution (w/v 6.67%) was poured into a plastic
197 well (2.3 cm diameter × 1.8 cm height) and left undisturbed at 10 °C for 17 h. The breaking
198 force-strain curve of the gel was drawn using a creep meter (RE-3305S, YAMADEN Co.,
199 Ltd., Tokyo, Japan) with a gelatin gel penetration diameter (of the flat-faced cylindrical
200 plunger) of 1.27 cm at 0.5 mm/s crosshead speed. The breaking force at the fracture of the gel
201 was recorded as the gel strength (N). The breaking strain was defined as the percentage of the
202 distance to gel fracture divided by the initial gel height. BV was used as a control.

203 *2.7 Scanning electron microscopy*

204

205 Gelatin gels (w/v 1.50%, 3.00%, 6.67%) were fixed with 2.5% (v/v) glutaraldehyde in
206 a 0.1 M phosphate buffer (pH 7.4) for 1 h at 4 °C and 6 h at room temperature. Gels were
207 washed with phosphate buffer (pH 7.6) and serially dehydrated with 70%, 80%, 90%, and
208 95% (v/v) ethanol. A scalpel was used to make a 2–3 mm thick cross-section of each gel.
209 Samples were dehydrated with 100% ethanol overnight then twice with 100% *t*-butyl alcohol
210 for 1 h. They were frozen at -30 °C, dried with a freeze-drier (JFD-320, JEOL Ltd., Tokyo,
211 Japan), and coated with gold-platinum using an auto fine coater (JFC-1600, JEOL Ltd.). The
212 surface structure of the sample was observed with a scanning electron microscope
213 (JSM6010LA, JEOL Ltd.) at 8 kV.

214

215 *2.8 Functional properties*

216

217 The water-absorbing capacity (WAC) and fat-binding capacity (FBC) of the gelatins
218 were measured following the method of Zhang et al. (2020a) with minor modifications.
219 Briefly, samples were collected in a pre-weighed centrifuge tube, and DW or soybean oil was
220 added to adjust the concentration to 2% (w/v). The tube was kept at room temperature and
221 vortexed 5 times for 5 s at 15-min intervals. Next, the samples were centrifuged at 4 500 ×g
222 for 20 min. The upper phases were discarded, the tubes were drained (tilt angle 45°) on filter
223 paper for 30 min, and the weight of the contents in the tubes was measured. The WAC or
224 FBC of lyophilized gelatins was calculated as follows:

225
$$\text{WAC or FBC (\%)} = [\text{weight of the tube contents after draining (g)}/\text{weight of}$$

226
$$\text{lyophilized gelatin (g)}] \times 100$$

227 The emulsion activity index (EAI) and emulsion stability index (ESI) of the gelatins
228 were measured according to the method of Zhang et al. (2020a) with minor modifications.
229 Briefly, gelatin solutions (w/v 1%) were mixed with soybean oil at a ratio of 4:1 and
230 homogenized (Phycostron, Niton Irika Kikai Seisakusho Co., Ltd., Tokyo, Japan) at 20 000
231 rpm for 3 min. Next, the gelatin-oil emulsion was diluted 100-fold with 0.1% sodium dodecyl
232 sulfate solution and vortexed for 10 s. Absorbance was measured at 500 nm, and EAI and ESI
233 were calculated using the following formulas:

$$234 \quad \text{EAI (m}^2\text{/g)} = (2 \times 2.303 \times A_0 \times \text{DF}) / (\emptyset \times C \times 10\,000)$$

$$235 \quad \text{ESI (min)} = (A_0 \times \Delta_t) / (A_0 - A_{30})$$

236 where A_0 is the absorbance of the sample at time zero, DF is the dilution factor, \emptyset is the oil
237 volumetric fraction, C is the protein concentration (g/mL) before emulsification, Δ_t is the
238 time interval (30 min), and A_{30} is the absorbance after 30 min.

239 Foaming expansion (FE) and foaming stability (FS) of the gelatin were measured
240 according to Renuka, Ravishankar, Zynudheen, Bindu, & Joseph (2019), with slight
241 modifications. Briefly, 2.5 mL gelatin solution (w/v 1%) was homogenized (Phycostron,
242 Niton Irika Kikai Seisakusho Co., Ltd.) in previously marked test tubes at 20 000 rpm for
243 2 min. The FE and FS were determined after incubation for 0 and 30 min at room temperature
244 using the following formulas:

$$245 \quad \text{FE (\%)} = (V_0 - V) / (V) \times 100$$

$$246 \quad \text{FS (\%)} = (V_{30} - V) / (V) \times 100$$

247 where V is the total volume before homogenization (mL), V_0 is the total volume after
248 homogenization (mL), and V_{30} is the total volume after 30 min (mL).

249

250 *2.9 Statistical analysis*

251

252 All experiments were replicated three times. Samples of the three replicates were
253 mixed for SDS-PAGE, Tricine-SDS-PAGE, amino acid, and Hyp content analyses. Where
254 applicable, data are presented as the mean \pm standard error (SE). Student's *t*-test or one-way
255 analysis of variance and Tukey-Kramer test were performed using Microsoft Excel add-on
256 statistical software (version 2.12, Social Survey Research Information Co., Ltd., Tokyo,
257 Japan). A *p* value of < 0.05 was considered to indicate statistical significance.

258

259 **3. Results and discussion**

260

261 *3.1 Yields of gelatins*

262

263 The yield of type B was significantly higher ($5.8 \pm 0.1\%$) than that of type A
264 ($3.6 \pm 0.1\%$) in sturgeon head. The yield of type A gelatin was similar to that of mackerel head
265 ($3.3\text{--}3.7\%$; Khiari, Rico, Martin-Diana, & Barry-Ryan, 2011) and higher than that of tiger
266 tooth croaker head (1.7% ; Elavarasan et al., 2017), but lower than the gelatin yields obtained
267 from mammalian and fish skin. For example, the gelatin yield from camel skin was 14.1%
268 (Abuibaid, AlSenaani, Hamed, Kittiphattanabawon, & Maqsood, 2020) and $9.4\text{--}12.5\%$ from
269 Amur sturgeon skin (Nikoo et al., 2013). The amount of gelatin obtained from approximately
270 500 g of sturgeon head, excluding the skull cartilage, was calculated to be approximately 18 g
271 for type A and 29 g for type B gelatin. The probable reason for the higher yield in type B
272 gelatin is discussed in Section 3.4.

273

274 *3.2 SDS-PAGE analysis*

275

276 Although the same amount of gelatin (10 μ g/lane) was loaded onto the gel for SDS-
277 PAGE, the thickness and intensities of the bands corresponding to the α - and β -chains of
278 gelatin were significantly greater in type A gelatin than in type B gelatin (Figs. 1A, B). The
279 intensity of the LMM bands (<100 kDa) was significantly higher for type B gelatin (Fig. 1B).
280 For example, the two dominant LMM bands (close to 37 kDa) in SDS-PAGE (Fig. 1A) and a
281 larger number of LMM bands (<46 kDa) in Tricine-SDS-PAGE (Fig. 1C) were observed for
282 type B gelatin. Peptide chains or LMM bands negatively affect gelatin properties such as gel-
283 forming ability (Nikoo et al., 2013), and gelatin hydrolysates less than 30 kDa are not
284 considered gelatin (Boran & Regenstein, 2009). These data strongly suggest that the type A
285 gelatin purity is higher than that of type B. The thickness of the gelatin bands obtained in this
286 experiment was comparable to those of fish head gelatins reported previously (Elavarasan et
287 al., 2017; Liu, Han, & Guo, 2009). The data of the yield and SDS-PAGE strongly suggest
288 that sturgeon heads can be used as a new material for industrial-scale gelatin manufacturing
289 in food and other industries.

290

291 *3.3 Amino acid analysis and the relative gelatin content in the protein fraction*

292

293 Table 1 shows the amino acid compositions and relative gelatin contents of the
294 protein fractions of the samples. The glycine (Gly), proline (Pro), and Hyp contents of type A
295 gelatin were similar to those of purified collagen, and to reported values for Amur sturgeon
296 skin gelatin (Nikoo et al., 2011, 2013). Type B gelatin showed lower Gly, Pro, and Hyp
297 contents. Lower Hyp contents in type B resulted in lower relative gelatin content in the
298 protein fraction, suggesting that the differences in the ratio of gelatinous and non-gelatinous
299 proteins in both gelatins must be the reason for the distinctive amino acid compositions of
300 type A and B gelatins.

301

302 *3.4 Proximate composition of gelatins*

303

304 Type A gelatin showed higher relative gelatin content in the sample weight and lower
305 non-gelatinous protein content than type B gelatin (Table 2). Thus, the alkaline pretreatment
306 used to extract type B gelatin may lead to the extraction of more non-gelatinous proteins.
307 Alkaline pretreatment breaks down the amide groups of proteins (Johns & Courts, 1977)
308 without affecting the mother collagen molecules (Liu et al., 2015). Speculatively, the LMM
309 molecules of non-gelatinous proteins, which were produced by alkaline pretreatment, were
310 easily extracted. The LMM molecules likely originated from the dominating collagen-poor
311 muscle tissue in the sturgeon head. Our preliminary study revealed that collagen-poor muscle
312 occupies approximately 26.2% of the sturgeon head weight. The higher relative gelatin
313 content in the sample weight in type A was also supported by the banding pattern in SDS-
314 PAGE, wherein higher intensities of the α - and β -bands of gelatin were observed in type A
315 than in type B (Figs. 1A, B). Collectively, the two gelatin purity indicators—the relative
316 gelatin content in the sample weight and gelatin band intensities in SDS-PAGE—suggest
317 higher gelatin purity in type A than in type B.

318 The lipid contents of the sturgeon head gelatins were lower than those of tiger tooth
319 croaker head gelatin (3.2%) and similar to the head (0.7%–0.9%) or scale and fin gelatin
320 (0.8%–1.1%) of other fishes (Elavarasan et al., 2017; Khiari et al., 2011; Mirzapour-
321 Kouhdasht, Sabzipour, Taghizadeh, & Moosavi-Nasab, 2019). However, the gelatins in the
322 present study showed higher lipid contents than skin gelatin (0.2%–0.3%) from other fishes
323 (Renuka et al., 2019). Defatting was not performed in this study because hard clustering of
324 the mixed tissue occurred, rendering the samples unsuitable for defatting. This may have
325 resulted from preheating the head for tissue separation (Supplementary Fig. 1).

326 Ash contents were similar in the two gelatins but higher than in gelatins from the head
327 of mackerel and tiger tooth croaker (Elavarasan et al., 2017; Khiari et al., 2011). Large, thick
328 cranial bones covering and hardening the head may increase the ash content. Except for the
329 higher ash content, the proximate composition of the sturgeon head gelatins was close to that
330 of skin gelatins in other species (Tümerkan et al., 2019). The high ash content may categorize
331 the sturgeon head gelatins as low quality; thus, lowering the ash content is a future challenge
332 to increase the quality of these gelatins.

333 By multiplying the proximate composition and the yield (Section 3.1), fractional
334 yields could be estimated for gelatin, non-gelatinous proteins, and non-proteinaceous
335 substances (sum of lipid, ash, and others) in type A and type B samples. The respective
336 estimated fractional yields were 2.2%, 0.1%, and 1.3% (total 3.6%) for type A and 2.3%,
337 1.7%, and 1.8% (total 5.8%) for type B. Thus, a higher fraction of non-gelatinous protein is
338 the primary reason for the high overall yield in type B (Section 3.1 above).

339

340 *3.5 Transparency of gelatins*

341

342 The transparencies of BV, type A, and type B gelatin samples were $99.7\pm 0.1\%$,
343 $67.8\pm 0.4\%$, and $92.5\pm 0.3\%$, respectively (Table 2). Contamination of samples with inorganic,
344 proteinaceous, and mucous substances influences transparency (Elavarasan et al., 2017).
345 Therefore, the higher relative content of non-proteinaceous substances may significantly
346 lower the transparency of type A gelatin.

347 3.6 Rheological properties

348

349 Fish gelatin is a widely used biopolymer that commonly has lower rheological
350 properties than mammalian gelatin (Huang et al., 2019). The present samples also showed a
351 lower gelling temperature than BV (Table 3). The gelling temperature of type A gelatin
352 (Table 3) was higher than that of type B gelatin and tiger tooth croaker head gelatin (11 °C;
353 Elavarasan et al., 2017), and similar to that of Amur sturgeon skin gelatin (13–14 °C; Nikko
354 et al., 2011, 2014). The gelling temperature correlated to the levels of the gelatin bands in
355 SDS-PAGE in the skin and bone gelatins of Nile perch (Muyonga, Cole, & Duodu, 2004);
356 thus, the higher intensities of α - and β -bands in type A gelatin (Fig. 1) might strongly
357 increase the gelling temperature of the present samples.

358 The melting temperatures of type A and type B gelatins were similar, but lower than
359 that of BV (Table 3). The melting temperature of the gelatins of the present study was higher
360 than those of gelatins from Amur sturgeon skin (20.3–22.6 °C; Nikoo et al., 2013, 2014) and
361 tiger tooth croaker head (20.3 °C; Elavarasan et al., 2017). These data suggest that the
362 melting temperature is not influenced by gelatin purity; thus, there are unknown factors
363 affecting the melting temperature of gelatins. The mechanism may be related to the effects of
364 pretreatment on the partial crosslinking of α -chains, which may stabilize the gelatin gel.

365 The breaking force-strain curve (Fig. 2) showed that the sturgeon head gelatins were
366 softer and more comfortable to deform with a smaller force than BV. The gel strength (N)
367 and breaking strain (%) values were obtained from the breaking force and strain at the
368 moment of gel fracture (Table 3). Type A gelatin showed higher gel strength and lower
369 breaking strain than type B gelatin. When the gel strengths of the head gelatin samples were
370 compared with previous data, measured at 6.67% gel concentration using a method similar to
371 this study, the strengths were higher than those of cod head (0.05–0.2 N) and croaker head

372 (0.4 N) gelatins (Arnesen & Gildberg, 2006; Elavarasan et al., 2017). Type A gelatin also
373 showed higher gel strength than Amur sturgeon skin gelatin (1.0–1.4 N) (Nikoo et al., 2014).
374 Cold-water fish show lower gelatin gel strength values (1.0 N or lower) than warm-water fish
375 (>2.0 N) (Gómez-Guillén et al., 2011). Based on these data, sturgeon head type A gelatin can
376 be categorized as medium quality with strength close to that of warm-water species, whereas
377 type B gelatin has low quality. Different methods, such as modification and crosslinking of
378 fish gelatins, are now present to overcome the limited industrial applications of fish gelatins
379 due to lower gel strength (Huang et al., 2019).

380 Various factors, such as the intensity of gelatin bands, amount of LMM fragments,
381 and amino acid composition (especially Hyp content), determine gel stability (Abuibaid et al.,
382 2020; Casanova et al., 2020; Tümerkan et al., 2019). The LMM fragments make fewer
383 intermolecular junctions, contributing to low gel strength (Intarasirisawat et al., 2007;
384 Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2010). Thus, the higher breaking
385 force of type A gelatin is reasonable as it showed higher purity—that is, higher relative
386 gelatin content in the sample weight (Table 2) and higher intensity of gelatin bands— with
387 lower LMM-fragments in SDS-PAGE (Fig. 1).

388 The breaking force-strain curve is commonly used in rheological analyses of gels in
389 food science because the factors impact food texture. For example, the breaking force-strain
390 relationships of the processed seafood-meat products *surimi* (Ueki, Matsuoka, Wan, &
391 Watabe, 2018) and *kamaboko* (Mao, Fukuoka, & Sakai, 2006) have been reported. Moreover,
392 the breaking force plays a key role in the field of biomedical materials for the pore
393 architectures and mechanical properties of gelatin-based cellular scaffolds (Fukushima, Ohji,
394 Hyuga, Matsunaga, & Yoshizawa, 2017). The present study is the first to report the breaking
395 force-strain curve of fish gelatins, providing useful information on gelatin quality for use in
396 the food, pharmaceutical, and biomedical industries.

397 *3.7 Structure of gelatins*

398

399 The structures of the gelatins are shown in Fig. 3. BV and type A gelatin did not show
400 any voids or pores at 6.67% gel concentration (Figs. 3A, B), whereas type B gelatin had small
401 homogeneous voids (Fig. 3C). The voids increased with decreasing sample concentrations,
402 resulting in thinner networks of the gel (Figs. 3D–H). BV showed only small voids at the
403 lowest concentration tested (1.50–3.00%) (Figs. 3D, G); however, type A gelatin showed
404 small voids at 3.00%, and the voids increased and became non-homogeneous in size at 1.50%
405 (Figs. 3E, H). Type B gelatin constituted a gel composed of fibrous networks at 3.00% (Fig.
406 3F) and did not form a gel at 1.50%. It is likely that rich non-collagenous proteins may inhibit
407 or nullify the intermolecular gelatin junctions in type B gels and result in large voids with
408 fine networks. Abuibaid et al. (2020) showed that camel skin gelatins with lower intensities
409 of gelatin bands produced gels with larger voids and a lower breaking force.

410

411 *3.8 Functional properties*

412

413 The WAC of type A gelatin samples was higher than that of type B, and of other fish
414 gelatins reported previously (Renuka et al., 2019; Zhang et al., 2020a; Table 4). However, the
415 WAC of the sturgeon head gelatins was much lower than that of bovine bone gelatin. WAC
416 may relate to several factors such as amino acid composition, especially Hyp content and
417 hydrophilic groups of amino acids (Ninan, Jose, & Aliyamveetil, 2014; Ranasinghe et al.,
418 2020; Shyni et al. 2014). However, the hydrophilic amino acid contents in type A gelatin
419 (375/1000 residues) were lower than those in type B (438/1000 residues). Thus, differences
420 in Hyp content—that is, the relative gelatin content (Tables 1, 2)—may be a reason for the
421 higher WAC in type A than in type B.

422 A high fat-binding capacity (FBC) is a characteristic feature of type A and B gelatins
423 (Table 4). These values were much higher than those of BV and fish gelatins reported
424 previously (Renuka et al., 2019; Zhang et al., 2020a), except for that of tuna skin gelatin
425 (Tümerkan et al., 2019). Type A and B gelatins would, therefore, be good fat-binding agents
426 for foods or other purposes. The hydrophobic amino acid content (Ala, Gly, Ile, Leu, Met,
427 Phe, Pro, and Val) of protein molecules positively affects the FBC (Renuka et al., 2019).
428 However, the present results suggest that non-gelatinous inclusions such as ash and sugar
429 (Table 2) or other unknown factors may positively affect the FBC, as type A gelatin contains
430 more hydrophobic amino acids (625/1000 residues) compared to type B (562/1000 residues,
431 Table 1).

432 Gelatin has surface-active and amphiphilic properties and is useful as an oil-in-water
433 emulsifier (Zhang et al., 2020b). The emulsifying factors EAI and ESI were higher in type B
434 than in type A and BV (Table 4), indicating a greater migration ability from water to the
435 interface between oil and water. Several factors—such as molecular mass distribution, amino
436 acid composition, interfacial tension, isoelectric point, and protein aggregation—influence
437 the emulsion activity (Bkhairia, Mhamdi, Jridi, & Nasri, 2016; Ding et al., 2021; Zhang et al.,
438 2020b). The higher emulsion activity of type B gelatin might be due to more LMM fragments
439 (Fig. 1C) or non-collagenous proteins (Tables 1, 2), since the gelatin with more peptide
440 fragments showed higher solubility, surface activity, and creaming stability, and resulted in
441 higher emulsion activity (Ranasinghe et al., 2020; Zhang et al., 2020b). Furthermore, amino
442 acids such as aspartic acid, glutamic acid, leucine, isoleucine, and Trp are potential
443 emulsifiers for oil (Yiase, 2015); these amino acids, except Trp, were higher in type B gelatin
444 (Table 1). In addition, the higher emulsification activity of proteins correlates with higher
445 surface hydrophobicity (Tan, Karim, Uthumporn, & Ghazali, 2020). Therefore, the higher
446 emulsifying activity of type B gelatin suggests that alkali-pretreatment might increase the

447 surface activity and hydrophobicity of gelatin more than acid pretreatment. The EAI of type
448 B gelatin was higher than that of previously reported fish gelatins (Alfaro, Balbinot, Weber,
449 Tonial, & Machado-Lunkes, 2015) or camel gelatins (Abuibaid et al., 2020), and close to that
450 of frog skin gelatin, but lower than that of tuna and chicken skin gelatins (Tümerkan et al.,
451 2019). The ESI of type B gelatin was even higher than that of frog, tuna, chicken, and camel
452 skin gelatins (Abuibaid et al., 2020; Tümerkan et al., 2019).

453 The foaming activities, FE and FS, of type B gelatin were similar to those of BV and
454 higher than those of type A (Table 4). These data suggest that the migration of type B gelatin
455 led to the adsorption of more air at the newly made gas-liquid surface—rapidly reorganized
456 the protein molecules—and also led to a stronger interaction with hydrophobic residues
457 (Alfaro et al., 2015; Gómez-Guillén et al., 2011). The foaming properties of the sturgeon
458 head gelatins were higher than those of tiger tooth croaker and pink perch skin and bone
459 gelatins, but lower than those of giant catfish skin (Jongjareonraka et al., 2010; Koli et al.,
460 2012) and camel gelatin (Abuibaid et al., 2020).

461

462 **4. Conclusion**

463

464 The present study clearly showed the high potential of sturgeon head as a new source
465 of fish gelatin. Using the two indicators of gelatin purity—the relative gelatin content based
466 on the Hyp content of the sample weight and the intensities of gelatin bands in SDS-PAGE—
467 we found that gelatin purity was positively related to rheological properties, but negatively
468 related to functional properties, except for WAC. Type A gelatin had a lower yield, higher
469 purity, higher gel strength, and higher gelling temperature; whereas type B gelatin had a
470 higher yield along with higher emulsifying factors and foam-forming activities. This is the
471 first study to evaluate the relationships between gelatin purity and the properties and

472 functionalities of fish gelatin. Therefore, the parameters used in this study would be a useful
473 basis for future gelatin studies. This study suggests that type A gelatin is suitable for use in
474 biomaterials and pharmaceuticals because of its higher purity and rheological properties,
475 whereas type B gelatin is suitable for use in foods or cosmetics based on its yield and
476 functional properties.

477

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479

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483

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701 **Figure captions**

702

703 **Fig. 1.**

704 7.5% SDS-PAGE banding patterns (A), the intensity of α -, β -, and γ -bands and low
705 molecular mass bands (<100 kDa) in SDS-PAGE (B), and 15.5% Tricine SDS-PAGE
706 banding patterns (C) of bovine bone gelatin (BV) and type A and type B gelatins extracted
707 from the head of kalamtra sturgeon. M, molecular marker. All gelatins were loaded as 10
708 $\mu\text{g/lane}$. Positions of α -, β -, and γ -bands were shown by arrows. Different letters in each band
709 show significant difference ($p < 0.05$), in B.

710

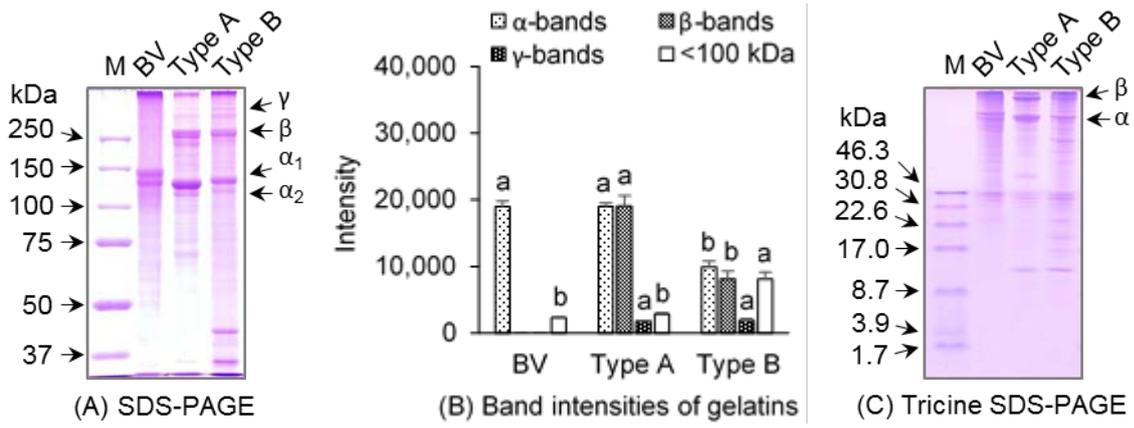
711 **Fig. 2.**

712 Typical breaking force-breaking strain curves of bovine bone gelatin (BV), and type A and
713 type B gelatins extracted from the head of kalamtra sturgeon. The arrows indicate the
714 breaking points of the gels where the breaking forces and breaking strains of gelatins were
715 obtained.

716

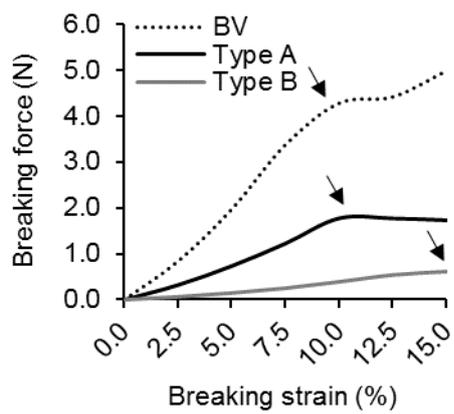
717 **Fig. 3.**

718 Scanning electron microscopy of bovine bone gelatin (BV; A, D, G), and type A (B, E, H)
719 and type B (C, F) gelatins extracted from the head of kalamtra sturgeon. A–C, 6.67% gelatin
720 (w/v); D–F, 3.00% gelatin; G and H, 1.50% gelatin. Scale bars, 10 μm .



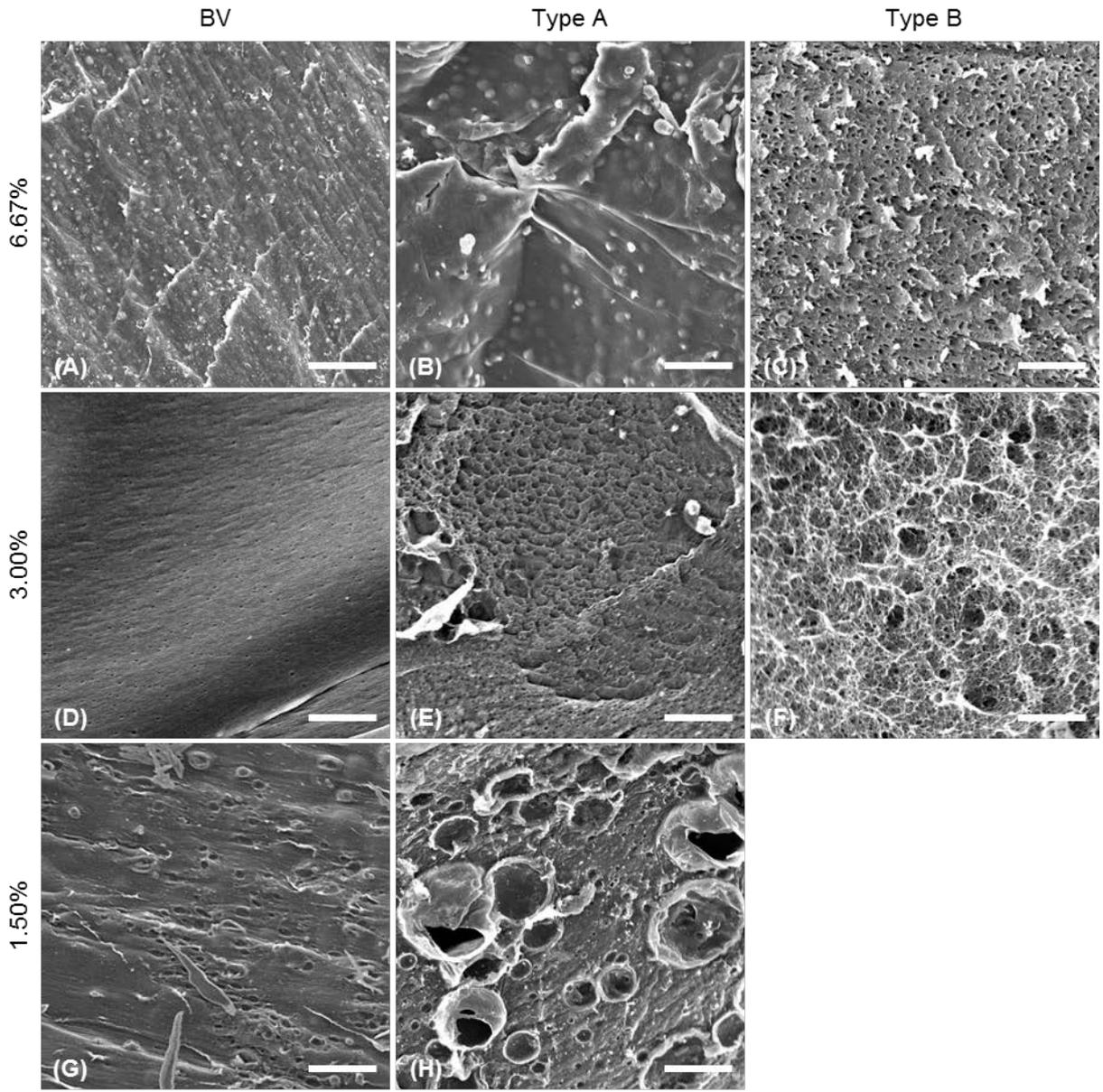
722

Fig. 1.



723

724 **Fig. 2.**



725

726 **Fig. 3.**

673 **Table 1**

674 Amino acid composition (residues/1000 residues) and gelatin content (% in the protein
 675 fraction) of purified collagen extracted from the sturgeon skin and type A and type B gelatins
 676 extracted from the head of kalamtra sturgeon.

Amino acids	<u>Amino acid composition (residues/1000 residues)</u>		
	Purified collagen	Type A	Type B
Ala	115	114	101
Arg	50	49	47
Asp	50	53	75
Cys	0	1	2
Glu	71	78	101
Gly	346	322	227
His	5	10	14
Hyls	9	9	6
Hyp	65	61	37
Ile	13	14	29
Leu	19	24	48
Lys	24	32	52
Met	10	11	19
Phe	13	17	25
Pro	116	104	79
Ser	51	51	53
Thr	24	26	36
Tyr	2	5	15
Val	16	19	34
Gelatin content*	100.0	93.9	56.9

677

678 *Relative gelatin content (%) in the protein fraction of sample. Values of each sample were
 679 obtained from the mixed samples of three replicates.

680 **Table 2**

681 Proximate composition (%) of type A and type B gelatins extracted from the head of kalamtra
 682 sturgeon.

Components	<u>Proximate composition (%)</u>	
	Type A	Type B
Gelatin [†]	60.3	39.7
Non-gelatinous proteins [†]	4.0	30.0
Lipid	2.4±0.1	1.1±0.2*
Ash	19.6±1.2	17.3±1.2
Others	13.6±1.1	12.0±1.2
Total	100.0	100.0

683

684 All values are expressed as percent in the sample weight. [†]Values were obtained from the
 685 mixed samples of three replicates. Other values are the mean ± standard error of three
 686 replicates. *Significantly different from the value in type A gelatin (p < 0.05).

687 **Table 3**

688 Rheological properties of bovine bone gelatin (BV) and type A and type B gelatins extracted
689 from the head of kalamtra sturgeon.

Rheological properties	<u>Gelatins</u>		
	BV	Type A	Type B
Gelling temperature (°C)	22.4±0.1 ^a	13.1±0.1 ^b	8.2±0.2 ^c
Melting temperature (°C)	29.3±0.1 ^a	23.4±0.1 ^b	23.1±0.1 ^b
Gel strength (N)	4.4±0.1 ^a	2.0±0.0 ^b	0.7±0.0 ^c
Breaking strain (%)	11.1±0.8 ^b	12.1±0.4 ^b	15.8±1.2 ^a

690

691 All values are the mean ± standard error of three replicates. Different letters in the superscript
692 at the row show significant difference ($p < 0.05$).

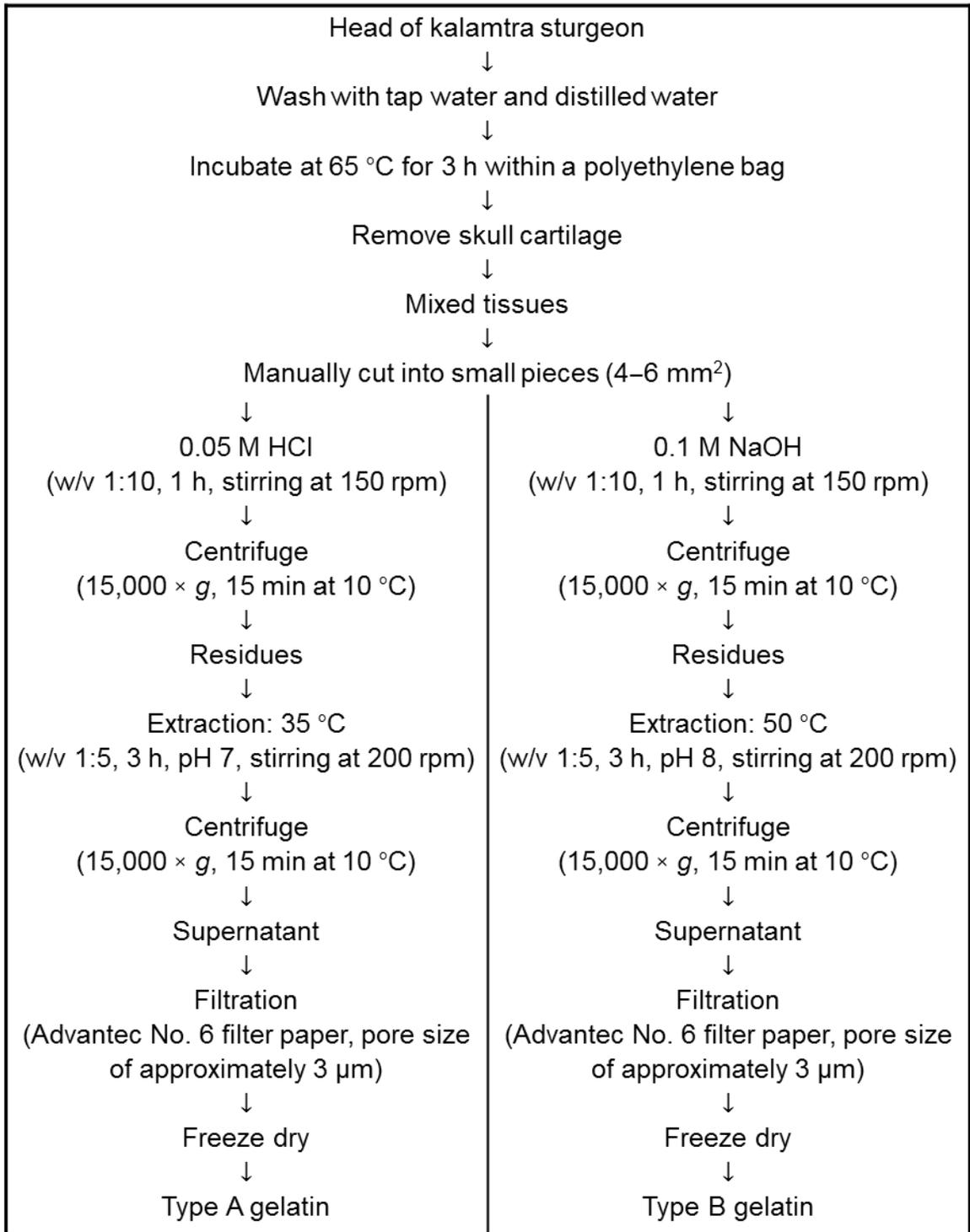
693 **Table 4**

694 Functional properties of bovine bone gelatin (BV) and type A and type B gelatins extracted
 695 from the head of kalamtra sturgeon.

Functionalities	Gelatins		
	BV	Type A	Type B
WAC (%)	1123.6±82.1 ^a	560.0±24.9 ^b	2.1±0.2 ^c
FB <u>C</u> (%)	153.4±6.3 ^b	2283.4±69.6 ^a	2199.7±45.2 ^a
EAI (m ² /g)	5.4±0.1 ^b	6.3±0.5 ^b	45.02±0.3 ^a
ESI (min)	53.2±2.6 ^b	59.8±1.4 ^b	423.4±44.2 ^a
FE (%)	34.5±0.3 ^a	27.8±1.1 ^b	34.8±0.4 ^a
FS (%)	27.5±1.0 ^a	9.0±0.7 ^b	29.8±0.4 ^a

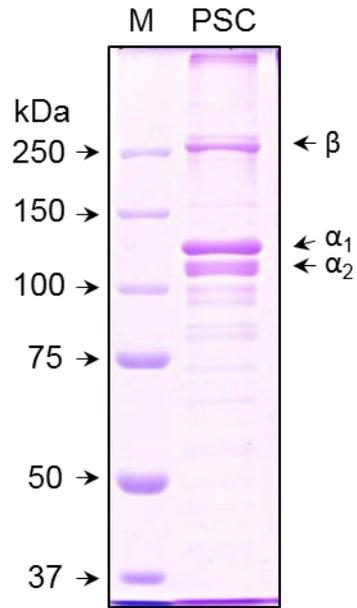
696

697 WAC, water absorbing capacity; FBC, fat binding capacity; EAI, emulsion activity index;
 698 ESI, emulsion stability index; FE, foam expansion; and FS, foam stability. All values are the
 699 mean ± standard error of three replicates. Different letters in the superscript at the row show
 700 significant difference (p < 0.05).



Supplementary Fig. 1.

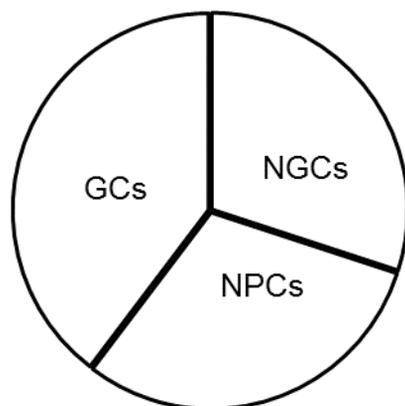
Protocols used for the extraction of type A and type B gelatins from the mixed tissue of the kalamtra sturgeon head.



Supplementary Fig. 2.

SDS-PAGE (7.5% gel) patterns of purified skin collagen (5 μ g/lane) using a mixture of kalamtra and bester sturgeon skin. M, molecular maker; PSC, purified skin collagen. Positions of type I collagen α_1 -, α_2 -, and β -bands were shown by arrows.

Total sample



Here,
Sample is composed of gelatin, non-gelatinous proteins and non-proteinaceous substances;
thus,
 $GCs + NGCs + NPCs = 100 (\%)$

Protein fraction in the sample is GCs + NGCs.

We can write as,
 $\rightarrow GCp (\%) = GCs / (GCs + NGCs) \times 100$
 $\rightarrow NGCs(\%) = 100 GCs/GCp - GCs$

Where,
GCp, Relative gelatin content in the protein fraction of the sample (%)
GCs, Relative gelatin content in the sample (%)
NGCs, Relative non-gelatinous protein content in the sample (%)
NPCs, Non-proteinaceous content [lipid, ash, carbohydrate, etc.] in the sample (%)

In this study, GCp and GCs was measured from the Hyp content using the following equations;

$GCp (\%) = [(Hyp \text{ of the sample}) / (Hyp \text{ of the purified collagen})] \times 100$,
where Hyp is the number of hydroxyproline expressed as Hyp residues/1,000 amino acid residues. Since amino acids come from protein fraction (gelatin and non-gelatinous protein) of the sample, and Hyp is the gelatin- and collagen-specific amino acid, the percentage of Hyp number in the sample to that in the purified collagen shows the relative gelatin content in the protein fraction of the sample.

$GCs (\%) = [(Hyp \text{ of the sample}) / (Hyp \text{ of the purified collagen})] \times 100$,
where Hyp is the concentration (nmol Hyp/ μ g dry sample) of the gelatin sample or the purified collagen. In this case, Hyp concentration is amount of Hyp in the total sample (or collagen) weight. Thus, the percentage of Hyp in the sample to that in the purified collagen shows the relative gelatin content in the sample.

Supplementary Fig. 3.

Equation to calculate the non-gelatinous protein contents based on both relative gelatin content in the total protein fraction and in the total sample weight.