The effect of alkaline pretreatment on the biochemical characteristics and fibril-forming abilities of types I and II collagen extracted from bester sturgeon by-products

Dawei Meng[a]*, Hiroyuki Tanaka[b], Taishi Kobayashi[a], Hirosuke Hatayama[c], Xi Zhang[a, d], Kazuhiro Ura[b], Shunji Yunoki[c], Yasuaki Takagi[b]

[a]*Graduate School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan*

[b]*Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate, Hokkaido 041-8611, Japan*

[c]*Tokyo Metropolitan Industrial Technology Research Institute, Tokyo 135-0064, Japan*

[d]*College of Fisheries, Huazhong Agricultural University, Wuhan 430070, China*

E-mail-addresses

mengdawei@eis.hokudai.ac.jp (D. Meng), tanaka-h@fish.hokudai.ac.jp (H. Tanaka), zhangxi@mail.hzau.edu.cn (X. Zhang), hatayama.hirosuke@iri-tokyo.jp (H. Hatayama), kazu@fish.hokudai.ac.jp (K. Ura), yunoki.shunji@iri-tokyo.jp (S. Yunoki), takagi@fish.hokudai.ac.jp (Y. Takagi)

*Corresponding author Tel./fax: +81-138-40-5551, E-mail address: mengdawei@eis.hokudai.ac.jp

20

Abstract

Non-mammalian collagens have attracted increasing attention for industrial and biomedical use. We have therefore evaluated extraction conditions and the biochemical properties of collagens from aquacultured sturgeon. Pepsin-soluble type I and type II collagen were respectively extracted from the skin and notochord of bester sturgeon by-products, with yields of 63.9±0.19% and 35.5±0.68%. Collagen extraction efficiency was improved by an alkaline pretreatment of the skin and notochord (fewer extraction cycles were required), but the final yields decreased to 56.2±0.84% for type I and 31.8±1.13% for type II. Alkaline pretreatment did not affect the thermal stability or triple-helical structure of both types of collagen. Types I and II collagen formed re-assembled fibril structures *in vitro*, under different conditions. Alkaline pretreatment slowed down the formation of type I collagen fibrils and specifically inhibited the formation of thick fibril-bundle structures. In contrast, alkaline pretreatment did not change type II collagen fibril formation. In conclusion, alkaline pretreatment of sturgeon skin and notochord is an effective method to accelerate collagen extraction process of types I and II collagen without changing their biochemical properties. However, it decreases the yield of both collagens and specifically changes the fibril-forming ability of type I collagen.

Keywords:

Alkaline pretreatment; Type I collagen; Type II collagen; Collagen fibril; Thermal stability; By-products

1 Introduction

Collagen provides mechanical strength to tissues and forms a favorable matrix for cell attachment *in vivo*. It is widely used in the food, photographic film, cosmetic and pharmaceutical industries [1]. Because of its low antigenic activity, high cell adhesion properties, biocompatibility, and biodegradability, collagen is also used as a scaffolding material for tissue engineering. The most common collagen sources for biomedical use are bovine skin and tendons, porcine skin, and rat tail [2]. However, outbreaks of zoonoses such as bovine spongiform encephalopathy and foot-and-mouth disease have raised concerns about collagen products of mammalian origin. In addition, religious beliefs restrict the use of porcine collagens in some societies [3]. Therefore, alternative collagens extracted from fish by-products such as skin, scales, swim bladders and bones have attracted increasing attention [4, 5, 6, 7]. Also, the profitable utilization of fish by-products is a way to relieve fisheries waste disposal problems and to promote aquaculture development [8].

Alkaline pretreatment of tissues at low temperature is a common method to remove non-collagenous proteins and is widely used as a pretreatment for fish collagen extraction and purification [4, 9]. Alkaline pretreatment is also employed to reduce the endotoxin content of the collagen. Since endotoxins induce strong biological effects when they enter the human blood stream, even at very low concentrations [10], low-endotoxin collagen is desirable for

61 biomedical use. After alkaline pretreatment of bovine hide type I collagen, however, Hattori et
62 al. [11] have reported that collagen denaturation temperature was decreased, intramolecular
63 cross-links involving proline and hydroxyproline were broken, and a portion of the collagen
64 lost its ability to form fibrils. In addition, the proposed alkali-soluble collagen theory [12]
65 suggests that some loss of collagen would occur during alkaline pretreatment. However, the
66 effects of alkaline pretreatment on the yield and nature of fish collagen are still unclear.
67 Moreover, specific effects on different types of collagen having different biochemical and
68 physicochemical properties are unknown, both for mammalian and fish collagens.
69 Sturgeon is a highly valuable food fish, which is famous for its caviar. However, because
70 of the long cultivation period required to obtain caviar, the production cost of sturgeon is
71 higher than that of other fishes [13]. Except for caviar and meat, the lack of efficient
72 utilization of sturgeon constrains the profitability of this aquaculture industry, and as a
73 consequence, the expansion of sturgeon aquaculture is slow in some countries, including
74 Japan. Therefore, the development of new commercial products from sturgeon by-products
75 should support further growth of the sturgeon aquaculture industry. Our previous study [13]
76 showed that sturgeon contains large amounts of type I collagen in the skin and swim bladder,
77 and small but industrially extractable amounts of type II collagen in the notochord. Moreover,
78 the type I collagen extracted from sturgeon was quick to reassemble into fibrils, suggesting its

79 suitability to fabricate biomedical materials. Type II collagen is highly valuable since its
80 market availability is much less than that of type I collagen [13].
81 Because the extraction procedure employed in our previous study did not include alkaline
82 pretreatment, the biochemical nature of alkali-pretreated collagen was not addressed. In fact,
83 as far as we know, the biochemical nature of alkali-pretreated and non-pretreated fish collagen
84 has not been compared, even though alkaline pretreatment is frequently employed for the
85 extraction of fish collagen. Therefore, the present study clarified the effect of alkaline
86 pretreatment on the extraction process, yield, biochemical nature and fibril-forming ability of
87 types I and II collagen from bester sturgeon skin and notochord.
88
89 **2 Materials and methods**
90 *2.1 Isolation and purification of collagen*
91 A live, cultured bester sturgeon (1.24 m, 6.00 kg) was procured from the Nanae Fresh-
92 Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan.
93 Skin and notochord were removed and washed with chilled tap water, lyophilized in a freeze
94 dryer (FDU-830, Tokyo Rikakikai Co., Ltd., Tokyo, Japan), and stored at −30°C until use.
95 Skin fat was removed over 24 h in 99.5% ethanol (two solution-changes) at 4°C, with a
96 sample:solution ratio of 1:10 (w/v). Defatted skin and notochord were cut into small pieces

97 (approximately 0.5×0.5cm) for collagen extraction. The tissues were then divided into alkali-
98 pretreated and non-pretreated groups. In the alkali-pretreated group, the tissues were
99 continuously stirred in a solution of 0.1 M NaOH for 12 h at 4°C, with a sample:solution ratio
100 of 1:50 (dry w/v; two solution-changes). After alkaline pretreatment, the tissues were washed
101 with cold distilled water until the pH value was neutralized, and were then lyophilized.
102 The following extraction and purification procedures were conducted at 4°C. To extract
103 collagen, the alkali-pretreated and non-pretreated tissues were stirred continuously in a
104 solution of HCl (pH 2.0) containing 0.1% (dry w/v) porcine pepsin (EC 3.4.23.1, 1:10,000,
105 Wako Pure Chemical Industries Ltd., Osaka, Japan) for 48 h, with a sample:solvent ratio of
106 1:100 (dry w/v). The mixtures were then centrifuged at 2,000×*g* for 90 min (Model 6800,
107 KUBOTA Manufacturing Corporation, Tokyo, Japan) to obtain supernatants. The supernatants
108 were kept at 4°C until use, whereas the precipitates were re-extracted several times under the
109 same conditions until no residue remained in the solutions obtained. Next, the supernatants
110 were combined and salted-out by adding NaCl to a final concentration of 1 M. The precipitate
111 was collected by centrifugation at 10,000×*g* for 30 min, and re-dissolved in HCl solution (pH
112 2.0). The solution was dialyzed for 24 h with a 100-kDa dialysis membrane, against 50
113 volumes of distilled water, with two changes of the distilled water. Finally, the dialysate was
114 lyophilized and stored at −30°C until use. The percentage of dry weight of collagen extracted

115 in comparison with the dry weight of the initial tissues was calculated as the collagen yield.
116 Experiments were conducted for 4 times, and the data were expressed as means ± standard
117 errors (SE).
118
119 *2.2 Amino acid analysis*
120 The amino acid composition was analyzed at the Instrumental Analysis Division,
121 Equipment Management Center, Creative Research Institution, Hokkaido University. In brief,
122 samples were hydrolyzed in 6 M HCl at 110°C for 24 h. The hydrolysates were evaporated,
123 and the remaining materials were dissolved in a citric acid buffer, and then analyzed using an
124 automated amino acid analyzer (JLC-500 V, JEOL Ltd., Tokyo, Japan). Samples were assayed
125 three times, and the means were calculated to obtain amino acid compositions.
126
127 *2.3 Soluble-collagen quantification*
128 The amount of solubilized collagen in each extraction step was measured using a soluble
129 collagen quantification kit (Sircol Soluble Collagen Assay, Biocolor life science assays,
130 Northern Ireland U.K.), following the manufacturer's protocol. Purified types I or II collagen
131 from the skin and notochord of bester sturgeon was used for standards. The amount of
132 solubilized collagen in each extract was expressed as the percentage to the sum of the

133 solubilized collagen of all extracts.

134

135 *2.4 Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)*

136 SDS-PAGE was performed according to the method of Laemmli [14]. The lyophilized
137 collagens were dissolved in HCl (pH 2.0, 1 mg collagen/ml) and then mixed at a ratio of 1:1
138 (v/v) with sample buffer (0.5 M Tris–HCl buffer, pH 6.8, with 4% SDS and 20% glycerol)
139 containing 10% β-mercaptoethanol. The mixed solution was boiled for 5 min. Ten
140 micrograms of the mixture were loaded onto each lane. Electrophoresis was performed at 15
141 mA for the stacking gel and 20 mA for the 7.5% running gel. After electrophoresis, the gel
142 was stained for 30 min with a 0.1% Coomassie Brilliant Blue R250 solution and destained
143 with a mixture of 20% ethanol, 5% acetic acid, and 2.5% glycerin. Precision plus protein
144 standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used to estimate the
145 molecular weight.

146

147 *2.5 Circular dichroism (CD) measurement*

148 CD spectra were measured using a JASCO model 725 spectrometer (JASCO, Tokyo,
149 Japan). The measurement was performed following the method of Ikoma et al [15].
150 Lyophilized collagens were dissolved in an HCl solution (pH 2.0) to 1 mg/ml and placed into

151 a quartz cell. CD spectra were measured at 190–250 nm wavelengths at 10°C under a scan
152 speed of 50 nm/min with an interval of 0.1 nm. The rotatory angle at a fixed wavelength of
153 221 nm was then measured at 10–50°C with a rate of 1°C/min to determine the denaturation
154 temperature of the collagen molecules.
155
156 *2.6 Collagen fibril formation in vitro*
157 The fibril formation process of collagens was evaluated by the method of Zhang [13] with
158 some modifications. Lyophilized collagens were dissolved in an HCl solution (pH 2.0) to
159 0.3% (w/v). The skin and notochord collagen solutions were mixed with a 45 mM Na-
160 phosphate buffer (pH 7.4). The ratio of collagen solution: Na-phosphate buffer was 1:2 (v/v).
161 The mixed solution was placed into a cell, and the subsequent fibril formation at 21°C was
162 monitored by measuring increased turbidity via optical absorbance at 320 nm, using a spectral
163 monitor (Hitachi High-technology Corporation, Tokyo, Japan). Fibril formation was
164 monitored for 1 h and 10 h for the skin and the notochord collagens, respectively.
165
166 *2.7 Morphology of fibrils*
167 The microstructure of skin and notochord collagen fibrils was observed using a scanning
168 electron microscope (SEM; JSM6010LA, JEOL Ltd., Tokyo, Japan), as previously described

169 Zhang [13]. Collagen fibrils were formed using the same conditions as described above.
170 Sample suspensions were centrifuged at 20,000×*g* for 20 min at 4°C to obtain precipitates of
171 collagen fibrils. The fibrils were fixed with 2.5% (v/v) glutaraldehyde in a 0.1 M phosphate
172 buffer (pH 7.4) for 3 h at room temperature, rinsed with the phosphate buffer to remove the
173 fixative, dehydrated with a graded series of ethanol solutions, and then soaked in two 30-min
174 changes of t-butyl alcohol solution. Finally, collagen fibrils were freeze-dried in a *t*-butyl
175 alcohol solution with a freeze-drying device (JFD-320; JEOL Ltd.) and coated with gold-
176 platinum using an auto fine coater (JFC-1600; JEOL Ltd.).
177
178 *2.8 Degree of fibril formation*
179 The determination method of the fibril formation degree was followed by the method of
180 Zhang [13]. Skin and notochord collagen fibrils were formed as shown in 2.6. After the
181 centrifugation at 20,000×*g* for 20 min at 4°C, the protein concentration of the supernatant was
182 measured following Lowry [16] using bovine serum albumin as a standard. The degree of
183 collagen fibril formation was defined as the percentage of the decrease of collagen
184 concentration in the solution after the experiment, which means the percent of collagen
185 molecules that formed the fibrils.
186

187 *2.9 Thermal stability of fibrils*

188 The maximum transition temperature (Tm) of re-assembled fibrils of skin and notochord
189 collagens was measured by differential scanning calorimetry (EXSTAR DSC6100, SII Nano
190 Technology Inc., Chiba, Japan). Collagen fibrils were prepared using the same conditions as
191 described in 2.6. The sample solutions were centrifuged at 20,000×*g* for 20 min at 4°C to get
192 precipitates of collagen fibrils. The precipitates were placed in an Al pan (70 µl), measured at
193 20-60°C at a rate of 3°C/min using Na-phosphate buffer pH 7.4 as a reference.

194

195 *2.10 Statistical analysis*

196 Data are expressed as means ± standard errors. Significance was set at $p < 0.05$.
197 Statistical analyses were performed using student's *t*-test, in Microsoft Excel add-in statistical
198 software (SSRI, Tokyo, Japan).

199

200 **3 Results and discussion**

201 *3.1 Amino acid composition*

202 The amino acid compositions of samples are shown in Table 1. The characteristic
203 repeating unit of a collagen monomer is Glycine (Gly)-X-Y, where X and Y are often proline
204 (Pro) and hydroxyproline (Hyp), respectively [17, 18]. Furthermore, collagen has abundant

alanine (Ala) (approximately 10% of total amino acids), but low amounts of cysteine (Cys), methionine (Met) and threonine (Thr) [19, 20]. Results of the amino acid analysis show that both tissues contained Gly as the major amino acid (skin 32.2%, notochord 30.4%) and were rich in Pro, Hyp and Ala. These data support the relatively high collagen content of the sturgeon skin and notochord. Hyp is an imino acid, which is unique to collagen and often used to calculate collagen content [21]. From the Hyp content, the collagen contents of the tissues were calculated as 85.7% in the skin and 75.5% in the notochord.

After the alkaline pretreatment, the Hyp content in skin and notochord increased, and the collagen content of the alkali-pretreated tissues was calculated as 98.8% for the skin and 90.7% for the notochord. Meanwhile, the contents of amino acids that were rich in collagen (Gly, Ala, and Pro) in skin and notochord increased, whereas those poor in collagen (Cys, Met, and Thr) decreased obviously with alkaline pretreatment. These results suggested that alkaline pretreatment efficiently removed non-collagenous proteins, especially from the skin. There were no significant differences in amino acid composition between the purified non-pretreated and alkali-pretreated collagens from either tissue. Thus, alkaline pretreatment did not affect collagen amino acid composition.

3.2 Soluble-collagen analysis

223 We conducted the analysis three times and obtained almost same results. The typical
224 results were shown in Fig. 1.
225 Three extractions were needed for the non-pretreated skin to fully solubilize the tissue.
226 The second extract contained 53.8% of total solubilized collagen, which was more than the
227 first (35.2%) and the third (11.0%) extracts. For the alkali-pretreated skin, 66.6% of total
228 solubilized collagen was obtained in the first extraction, and all solubilized collagen was
229 extracted after the second. Thus, the alkaline pretreatment accelerated the collagen extraction
230 process in the skin.
231 For the non-pretreated notochord, only 0.1% of the total solubilized collagen was obtained
232 in the first extraction. The second extract contained 53.2%. After that, the solubilized collagen
233 content decreased with repeated extractions, and approximately 36.2% of the solubilized
234 collagen was obtained in the third time extraction. Thus, the notochord collagen was more
235 difficult to extract compared with the skin collagen. The precise reason is as yet unknown, but
236 it is possible that notochord contains more tightly-packed collagen tissue than skin, reflecting
237 their different physiological roles in the body. Sturgeon insufficiently develops cartilaginous
238 vertebrae and the notochord functions as the major axis of the body. Therefore, the notochord
239 has a strong but flexible collagenous sheath that resists high pressure [22, 23].

240 After alkaline pretreatment of the notochord, only two extractions were needed to obtain
241 all the solubilized collagen. Combined with the results of the skin collagen extraction, we
242 conclude that alkaline pretreatment not only removed non-collagenous proteins but also
243 accelerated the solubilized collagen extraction process. Hattori et al [11] found that the
244 extractability of bovine skin collagen, when treated with an alkaline solution, was much
245 higher than that of non-treated tissue. In the present study, the same phenomenon was
246 demonstrated for the first time for both types I and II fish collagens. Alkaline pretreatment
247 might cause significant swelling of the collagen fibril structure [12]. After collagen fibrils are
248 swollen, pepsin should more easily contact the collagen molecules to break their
249 intermolecular cross-links.
250
251 *3.3 Yields of collagens*
252 The yields of purified collagen are summarized in Table 2. The yields of skin and
253 notochord collagen were 63.9±0.19% and 35.5±0.68% (dry weight basis), respectively. These
254 yields were much higher than our previous study (skin, 31.4%; notochord, 5.1%) [13].
255 Incomplete extraction might be the main factor that limited the yield in our previous study. In
256 the present study, the increased number of extraction cycles increased collagen yields and

257 avoided waste. Also, a filtration process was avoided in the present study. This may be
258 another reason for the reduced loss of solubilized collagen.
259 The collagen yields of alkali-pretreated tissues decreased to 56.2±0.84% and 31.8±1.13%
260 for skin and notochord, respectively. This result suggests that collagens were lost during the
261 alkaline pretreatment, and the loss of skin collagen was greater than that of notochord
262 collagen. The existence of alkali-soluble collagen was previously reported for mammals [11].
263 The present results suggest that sturgeon skin contains more alkali-soluble collagen than
264 notochord. Combined with the amino acid composition and the soluble-collagen analyses, it
265 can be concluded that the alkaline pretreatment improved collagen extraction efficiency, but at
266 the expense of greater collagen losses.
267 The yield of collagen from sturgeon skin (63.9±0.19%) in the present study was
268 significantly higher than that from catfish skin (44.8%), ocellate puffer fish skin (55.4%), and
269 black drum skin (18.1%) [1, 24, 25]. It is difficult to compare the notochord collagen yield
270 with other fishes or mammals because there have been few other studies on notochord
271 collagen utilization. Even so, the yield of notochord collagen (35.5±0.68%) was same levels
272 or higher than that from cuttlefish skin (35.4%), black drum skin (18.1%), and rhizostomous
273 jellyfish (35.2%) [25, 26, 27]. From these results, we conclude that sturgeon skin and

notochord are suitable for large-scale industrial production of type I and type II collagen, respectively.

On the other hand, the collagen content of notochord was estimated as 75.5% of the tissue weight, but the yield of type II collagen was only 35.5±0.68%. We suppose that the loss was mainly generated during the salting-out process; the 1 M NaCl might not completely precipitate type II collagen from the extraction solution. In another study, a higher concentration of NaCl (4 M) was used to precipitate type II collagen from Amur sturgeon cartilage [28]. We tried salting-out using 2 M NaCl to obtain a greater yield, but the resulting collagen could not form a fibril structure (data not shown). Instead of the salting-out method, Cao [29] used a chromatographic separation method to purify collagen. Considering the cost of the method (especially on an industrial scale), we did not employ chromatographic separation in the present study. Studies on more efficient salting-out conditions should be pursued in the future.

3.4 SDS-PAGE

The SDS-PAGE patterns of collagens are shown in Fig. 2. As reported in our previous study [13], the non-pretreated skin collagen had two α-chains as the major constituents (approximately 120 kDa and 100 kDa), whereas the non-pretreated notochord collagen had

292 only one α-chain (approximately 130 kDa), indicating that the skin and notochord collagen
293 were type I and type II, respectively. For both tissues, the alkaline pretreatment did not affect
294 the band pattern.
295 High molecular weight bands, which might be cross-linked α-chains (that is, β- and γ-
296 chains), showed lower intensities in the notochord collagens compared with the skin
297 collagens, suggesting that the number of cross-links was much less in the notochord collagen.
298 Cross-links are one of the factors that make collagen extraction difficult. As already stated in
299 section 3.2, the notochord collagen was more difficult to extract compared with the skin
300 collagen. However, the SDS-PAGE revealed that number of cross-links could not explain the
301 difficulty of collagen extraction in notochord. Also, the β- and γ-chain band intensities from
302 bester sturgeon skin presented in the present study are much lower compared with those from
303 carp and bigeye snapper skin [4, 5]. Therefore, our results suggest that inter- or intra-
304 molecular cross-links in sturgeon skin collagen are less than those of other fishes.
305
306 *3.5 Thermal stability of collagen molecule*
307 CD spectra of skin and notochord collagens are shown in Fig. 3A. All collagens had a
308 rotatory maximum at 221 nm and a crossover point (zero rotation) at approximately 212 nm,
309 which are typical characteristics of the collagen triple-helical conformation [13].

Measurement of the collagen denaturation temperature is shown in Fig. 3B. As the temperature increased, CD values at 221 nm decreased following decomposition of the collagen triple-helical structure. The denaturation temperature of each collagen preparation was determined as the temperature with the fastest decrease in CD value. As previously shown by Zhang et al. [13], the denaturation temperature of non-pretreated notochord collagen (31.8°C) and alkali-pretreated notochord collagen (32.0°C) were higher than those of non-pretreated skin collagen (27.0°C) and alkali-pretreated skin collagen (26.8°C).

No obvious differences were observed between the alkali-pretreated and non-pretreated collagens, suggesting that alkaline pretreatment did not affect the triple-helical structure. Bowes [30] found that some peptide links involving proline and hydroxyproline were broken when bovine skin was treated with NaOH for 14 days at 20°C. Hattori [11] reported that the denaturation temperature of bovine hide collagen decreased by 6°C after treatment with 0.75 M NaOH for several days at 20°C. Thus, the denaturation of collagen molecules may be induced by lengthy alkaline pretreatment at high temperature. In the present study, non-collagenous proteins were removed by pretreatment with 0.1 M NaOH for only 12 h at 4°C. Similar alkaline pretreatment conditions have been widely used in many other collagen and gelatin extraction studies [4, 9, 31].

328 *3.6 Collagen fibril formation in vitro*

329 Fibril formation during incubation at 21°C was monitored by optical absorbance change at
330 320 nm. The progression of fibril formation *in vitro* by skin and notochord collagen is shown
331 in Fig. 4. For skin collagen, the non-pretreated preparation showed a higher rate of turbidity
332 increase than the alkali-pretreated preparation (Fig. 4A), indicating that the fibril-forming
333 speed was slowed down by the alkaline pretreatment. Also, the final turbidity of the non-
334 pretreated preparation was approximately two times higher than that of the alkali-pretreated
335 preparation. Possible factors that affect the final turbidity are thickness and number (amount)
336 of fibrils formed in the reaction fluid. Thus, we further examined the morphology of fibrils
337 and the degree of fibril formation in section 3.7 and 3.8. A similar inhibition of fibril
338 formation speed by alkaline pretreatment was observed for bovine skin type I collagen [11].
339 Suzuki [32] reported that alkaline pretreatment acted as a competitive inhibitor of fibril
340 formation in steer hide type I collagen. These researches used the turbidity method to show
341 the inhibition of alkaline pretreatment on mammalian collagen fibril formation speed. The
342 mechanisms by which alkaline pretreatment inhibits fibril formation have not been identified.
343 One possible mechanism may be the modification of collagen amino acid side-chains, which
344 might destroy intra- and inter-molecular cross-links [33].

345 Calf and human type II collagen have been reported to have a longer lag time for assembly
346 and lower fibril formation rate than type I collagen [34, 35]. Notbohm [36] investigated
347 human type II collagen fibril formation in 30 mM phosphate buffer (pH 7.4) for 600 min. In
348 the present study, a lower buffer concentration and a longer observation time were employed
349 compared with our previous study [13], and fibril formation by type II collagen from a fish
350 species was achieved for the first time (Fig. 4B). In contrast to the skin type I collagen,
351 alkaline pretreatment showed little effect on fibril formation by the notochord type II
352 collagen. The different influences of alkaline pretreatment may be explained by different
353 characteristics and mechanisms of fibril formation between type I and type II collagen. This is
354 the first study to demonstrate that alkaline pretreatment has different influences on *in vitro*
355 fibril formation by types I and II fish collagen.
356 Collagen fibril formation is affected by many factors, such as ionic strength, ionic species,
357 pH value, temperature and others [37]. The present study included only a preliminary
358 examination of the effects of alkaline pretreatment. For the industrial use of sturgeon skin
359 type I and notochord type II collagen, it will be necessary to further optimize conditions for
360 fibril formation.
361
362 *3.7 Morphology of fibrils formed in vitro*

SEM images of skin type I collagen fibrils after 1 h of incubation are shown in Fig. 5. The unordered, net-like appearance of fibrils in both non-pretreated and alkali-pretreated samples indicates that fibrillogenesis occurred under these experimental conditions. The fibril morphology was notably different between non-pretreated and alkali-pretreated collagens: thick fibril-bundle structures were formed only by the non-pretreated type I collagen. These bundles are likely to have been formed by the assembly of thin fibrils via spiral winding. Based on the proposed process of collagen fiber formation [2], we hypothesize that collagen molecules first assembled to form fibrils, then several fibrils coiled to form a fibril-bundle structure. In light of the results of the turbidity measurement experiment, the formation of these bundles might increase the turbidity of the non-pretreated collagen. This result also indicates that alkaline pretreatment specifically inhibited bundle formation. The morphological factors of fibrils, including diameter, shape, and orientation, exert substantial effects on cell behaviors such as adhesion, alignment, proliferation, and differentiation [38]. Therefore, fibril morphology has significant impacts on the functionality of medical materials fabricated with collagen fibrils. Based on this observation, alkaline pretreatment is possibly a method that could be used to control type I collagen fibril morphology. The effect of alkaline pretreatment on the speed and morphology of fibril formation by mammalian type I collagen

is already known. However, the present study is the first to report similar effects for piscine type I collagen.

The type II collagen formed many thin fibrils with a non-uniform network structure, for both non-pretreated and alkali-pretreated collagens (Fig. 6). There were no morphological differences between the two preparations. This finding is also consistent with the results of the turbidity measurement experiment: non-pretreated and alkali-pretreated type II collagens showed similar turbidity increases, indicating that alkaline pretreatment did not affect the fibril-forming ability of type II collagen. This is the first study to show the effects of alkaline pretreatment on the fibril-forming ability of type II collagen.

Bae et al. [39] showed that increasing NaCl concentration promoted fibril formation by red stingray type I collagen. In our previous study, similar phenomena were demonstrated using bester sturgeon skin and swim bladder type I collagens [13]. We also examined the effects on fibril formation by notochord type II collagen of increasing NaCl concentrations in the 0.1 M Na-phosphate buffer (pH 7.4), but no collagen fibrils were formed when NaCl was added. The morphology of the type II collagen after 1 h was not a fibril structure (data not shown). In contrast to the type I collagen, high NaCl concentration seems to inhibit type II collagen fibril formation. Fibrils were visible when the buffer concentration was lowered and the fibril-forming time was prolonged compared with our previous study [13]. Thus, a lower

398 buffer concentration and a longer reaction time were better for fibril formation by type II
399 collagen compared with type I collagen.
400 In addition, to investigate whether these collagen fibrils are similar to the fibrils formed in
401 the body, the D-period of fibrils was determined by transmission electron microscopy (data
402 not shown). Non-pretreated type I collagen showed 68±12 nm D-period. However, the D-
403 periods of alkali-pretreated type I collagen and two type II collagen preparations were not
404 confirmed because of insufficient resolution. Meanwhile, we found the diameter of type II
405 collagen was much smaller than type I collagen, confirming our SEM study. Collagen fibril
406 formation condition and observation condition need further improvement.
407
408 *3.8 Degree of fibril formation*
409 The degree of skin and notochord collagen fibril formation was assessed after 1 h and 10 h
410 of fibrillogenesis, respectively, and the results are shown in Fig. 7. Alkali-pretreatment
411 showed no significant influences on the degree of type I and type II collagen fibril formation:
412 all samples showed values higher than 90%. These values were higher than those of
413 barramundi and tilapia skin type I collagens, in which fibrils were formed for 24 h [40].
414 From the results obtained in sections 3.6-3.8, the effects of alkaline pretreatment on type I
415 collagen fibril formation could be summarized as the decrease in the formation speed and the

inhibition of the thick bundle-structure fibril formation with no influence on the degree of fibril formation. In contrast, alkaline pretreatment did not influence type II collagen fibril formation.

3.9 Thermal stability of collagen fibrils

DSC curves of re-assembled fibrils of types I and II collagen were obtained three times, and the typical curves are shown in Fig. 8. Tm was obtained as the endothermic peak of the DSC curve. For type I collagen, non-pretreated collagen showed higher Tm (40.14±0.31°C) than alkali-pretreated collagen (37.26±0.08°C), which may be attributed to the thick fibril-bundle structure in the non-pretreated preparation. In addition, comparing with Tm of type I collagen molecules (27.0°C), the Tm of type I collagen fibrils was higher by 10–12°C. This result is consistent with the report that the thermal stability of collagen molecules is enhanced by molecular interaction [41]. This result might be of interest for its use as biomaterials for medical usages.

For type II collagen, no typical endothermic peaks were found on both non-pretreated and alkali-pretreated collagens. There were only slight changes in the curves at about 34–35°C on both preparations. These temperatures were close to the denaturation temperatures of non-pretreated and alkali-pretreated type II collagen molecules (31–32°C). These data suggested

434 that although type II collagen fibrils were assembled, the thermally stable fibril structures
435 were not formed in the present condition, and more suitable fibril formation condition need to
436 be discussed in the future.
437
438 **4 Conclusions**
439 This study demonstrated that a large amount of type I and type II collagen could be
440 extracted from the skin and notochord of bester sturgeon, and suggested that both tissues are
441 promising sources of fish collagen for industrial use. Alkaline pretreatment accelerated the
442 collagen extraction process of both types of collagen, although it also reduced the yields.
443 Although alkaline pretreatment did not affect the thermal stability of either type I or type II
444 collagen molecules, it lowered the fibril-forming speed and Tm and changed the fibril
445 morphology of the type I collagen. Since there has been no previous study on the effects of
446 alkaline pretreatment on types I and II fish collagen, this study provides basic data describing
447 the nature of these collagens as well as their suitability for industrial use.
448
449 **Acknowledgments**
450 This work was partially supported by a Grant-in-Aid for Scientific Research (B)
451 18H02273 from the Japan Society for the Promotion of Science. We thank Nicholas Rufaut,

Ph.D., from Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

References

[1] M. Zhang, W. Liu, G. Li, Isolation and characterisation of collagens from the skin of largefin longbarbel catfish (*Mystus macropterus*), Food Chem. 115 (2009) 826–831.

[2] R. Parenteau-Bareil, R, Gauvin, F, Berthod, Collagen-based biomaterials for tissue engineering applications, Materials. 3 (2010) 1863–1887.

[3] A. Jongjareonrak, S. Benjakul, W. Visessanguan, T. Nagai, M. Tanaka, Isolation and characterisation of acid and pepsin-solubilised collagens from the skin of brownstripe red snapper (Lutjanus vitta), Food Chem. 93 (2005) 475–484.

[4] R. Duan, J. Zhang, X. Du, X. Yao, K. Konno, Properties of collagen from skin, scale and bone of carp (*Cyprinus carpio*), Food Chem. 112 (2009) 702–706.

[5] P. Kittiphattanabawon, S. Benjakul, W. Visessanguan, T. Nagai, M. Tanaka, Characterisation of acid-soluble collagen from skin and bone of bigeye snapper (*Priacanthus tayenus*), Food Chem. 89 (2005) 363–372.

[6] T. Nagai, N. Suzuki, Isolation of collagen from fish waste material-skin, bone and fins, Food Chem. 68 (2000) 277–281.

[7] J. Zhang, R. Duan, C. Ye, K. Konno, Isolation and characterization of collagens from scale

of silver carp (*Hypophthalmichthys molitrix*), J. Food Biochem. 34 (2010) 1343–1354.

[8] J.Y. Je, Z.J. Qian, H.G. Byun, S.K. Kim, Purification and characterization of an antioxidant peptide obtained from tuna backbone protein by enzymatic hydrolysis, Process Biochem. 42 (2007) 840–846.

[9] E. Zelechowska, M. Sadowska, M. Turk, Isolation and some properties of collagen from the backbone of Baltic cod *(Gadus morhua),* Food Hydrocoll. 24 (2010) 325–329.

[10] F.B. Anspach, Endotoxin removal by affinity sorbents. J. Biochem. Biophys. Methods 49 (2001) 665-681.

[11] S. Hattori, E. Adachi, T. Ebihara, T. Shirai, I. Someki, S. Irie, Alkali-treated collagen retained the triple helical conformation and the ligand activity for the cell adhesion viaα2β1 integrin, J. Biochem. 125 (1999) 676–684.

[12] D.S. Liu, G. Wei, T. Li, J. Hu, N. Lu, J. Regenstein, P. Zhou, Effects of alkaline pretreatments and acid extraction conditions on the acid-soluble collagen from grass carp *(Ctenopharyngodon idella)* skin, Food Chem. 172 (2015) 836–843.

[13] X. Zhang, M. Ookawa, Y. Tan, K. Ura, S. Adachi, Y. Takagi, Biochemical characterization and assessment of fibril-forming ability of collagens extracted from Bester sturgeon *Huso huso* × *Acipenser ruthenus*, Food Chem. 160 (2014) 305–312.

[14] U. K. Laemmli, Cleavage of structural proteins during assembly of head of bacteriophage

T4, Nature 277 (1970) 680–685.

[15] T. Ikoma, H. Kobayashi, J. Tanaka, D. Walsh, S. Mann, Physical properties of type I collagen extracted from fish scales of *Pagrus major* and *Oreochromis niloticus*, Int. J. Biol. Macromol. 32 (2003) 199–204.

[16] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[17] K. Kadler, D. Holmes, J. Trotter, J. Chapman, Collagen fibril formation, Biochem. J. 316 (1996) 1–11.

[18] E.A. Foegeding, T.C. Lanier, H.O. Hultin, Characteristics of edible muscle tissues. In O. R. Fennema (Ed.), Food chemistry. Marcel Dekker, Inc., New York, (1996) pp. 879–942.

[19] P. Singh, S. Benjakul, S. Maqsood, H. Kishimura, Isolation and characterisation of collagen extracted from the skin of striped catfish (*Pangasianodon hypophthalmus*), Food Chem. 124 (2011) 97–105.

[20] R.K. Owusu-Apenten, Protein digestibility-corrected amino acid scored, In Food Protein Analysis: Quantitative Effects On Processing. Marcel Dekker, New York, (2002) pp381.

[21] K. Hofman, B. Hall, H. Cleaver, S. Marshall, High-throughput quantification of hydroxyproline for determination of collagen, Anal. Biochem. 417 (2011) 289–291.

[22] J.H. Long, Morphology, mechanics, and locomotion: the relation between the notochord

and swimming motions in sturgeon, Environ. Biol. Fish. 44 (1995) 199–211.

[23] D.L. Stemple, Structure and function of the notochord: an essential organ for chordate development, Development 132 (2005) 2503–2512.

[24] T. Nagai, Y. Araki, N. Suzuki, Collagen of the skin of ocellate puffer fish *(Takifugu rubripes)*, Food Chem. 78 (2002) 173–177.

[25] M. Ogawa, M. Moody, R. Portier, J. Bell, M. Schexnayder, J. Losso, Biochemical properties of black drum and sheepshead seabream skin collagen, J. Agric. Food. Chem. 51 (2003) 8088–8092.

[26] T. Nagai, E. Yamashita, K. Taniguchi, N. Kanamori, N. Suzuki, Isolation and characterization of collagen from the outer skin waste material of cuttlefish *(Sepia lycidas)*, Food Chem. 72 (2001) 425–429.

[27] T. Nagai, W. Worawattanamateekul, N. Suzuki, T. Nakamura, T. Ito, K. Fujiki, M. Nakao, T. Yano, Isolation and characterization of collagen from rhizostomous jellyfish *(Rhopilema asamushi)*, Food Chem. 70 (2000) 205–208.

[28] Q. Liang, L. Wang, W. Sun, Z. Wang, J. Xu, H. Ma, Isolation and characterization of collagen from the cartilage of Amur sturgeon (*Acipenser schrenckii*), Process Biochem. 49 (2014) 318–323.

[29] H. Cao, S. Y. Xu, Purification and characterization of type II collagen form chick sternal

524 cartilage, Food Chem. 108 (2008) 439–445.
525 [30] J.H. Bowes, R.H. Kenten, The effect of alkalis on collagen, Biochem. J. 43 (1948) 365–
526 372.
527 [31] M. Ahmad, S. Benjakul, Extraction and characterization of pepsin-solubilized collagen
528 from the skin of unicorn leatherjacket *(Aluterus monocerous)*, Food Chem. 120 (2010)
529 817–824.
530 [32] Y. Suzuki, I. Someki, E. Adachi, S. Hattori, Interaction of collagen molecules from the
531 aspect of fibril formation acid-soluble, alkali-treated, and MMP1-digested fragments of
532 Type I collagen, J. Biochem. 126 (1999) 54–67.
533 [33] G. Boran, J.M. Regenstein, Fish Gelatin, Advance in Food and Nutrition Research Vol.
534 60 (2010) 119–143.
535 [34] D.E. Birk, F.H. Silver, Collagen fibrillogenesis *in vitro*: comparison of types I, II, and III.
536 Arch. Biochem. Biophys. 235 (1984) 178–185.
537 [35] B. Williams, R.Gelman, D. Poppke, K.A. Piez, Collagen fibril formation. Optimal in
538 vitro conditions and preliminary kinetic results, J. Biol. Chem. 253 (1978) 6578–6585.
539 [36] H. Notbohm, M. Nokelainen, J. Myllyharju, P.P. Fietzek, P.K. Muller, K.I. Kivirikko,
540 Recombinant human type II collagens with low and high levels of hydroxylysine and its
541 glycosylated forms show marked differences in fibrillogenesis *in vitro*, J. Biol. Chem. 274

542 (1999) 8988–8992.
543 [37] A. Fertala, A.L. Sieron, Y. Hojima, D.J. Prockop, Self-assembly into fibrils of collagen II
544 by enzymic cleavage of recombinant procollagen II, J. Biol. Chem. 269 (1994) 11584–
545 11589.
546 [38] K. Wolf, S. Alexander, V. Schacht, L.M. Coussens, U.H. von Andrian, J. Rheenen, E.
547 Deryugina, P. Friedl, Collagen-based cell migration models *in vitro* and *in vivo*, Semin.
548 Cell Dev. Biol. 20 (2009) 931-941.
549 [39] I. Bae, K. Osatomi, A. Yoshida, A. Yamaguchi, K. Tachibana, T. Oda, K. Hara,
550 Characteristics of a self-assembled fibrillar gel prepared from red stingray collagen, Fish.
551 Sci. 75 (2009) 765–770.
552 [40] L. Wei, G.H. Xia, Y.C. Li, X.R. Shen, C. Li, Comparison of characteristics and fibril-
553 forming ability of skin collagen form barramundi *(Lates calcarifer)* and tilapia
554 *(Oreochromisniloticus)*, Int. J. Biol. Macromol. 107 (2018) 549–559.
555 [41] M. L. Zou, H. Yang, H.B. Wang, H.Y. Wang, J.T. Zhang, B.M. Wei, H.J. Zhang, D. Xie,
556 Detection of type I collagen fibrils formation and dissociation by a fluorescence method
557 based on thioflavin T, Int. J. Biol. Macromol. 92 (2016) 1175–1182.
558

559 **Figure captions**

560 **Fig. 1** Solubilized collagen content after each extraction step, expressed as a percentage of
561 total solubilized collagen obtained. White, first extraction; spots, second extraction; stripes,
562 third extraction; black, fourth extraction.

563

564 **Fig. 2** SDS-PAGE of collagens from skin and notochord. M, molecular weight marker; lane 1:
565 non-pretreated skin collagen; lane 2: alkali-pretreated skin collagen; lane 3: non-pretreated
566 notochord collagen; lane 4: alkali-pretreated notochord collagen.

567

568 **Fig. 3** (A) CD spectra of the collagens from non-pretreated and alkali-pretreated skin and
569 notochord. (B) Temperature effect on the CD spectra at 221 nm of collagens from non-
570 pretreated and alkali-pretreated skin and notochord.

571

572 **Fig. 4** Fibril formation *in vitro* by (A) skin type I collagen and (B) notochord type II collagen,
573 measured by optical absorbance at 320 nm. Solid line, non-pretreated preparations; dotted
574 line, alkali-pretreated preparations.

575

576 **Fig. 5** Scanning electron micrographs of skin collagen fibrils formed at 21°C after 1 h. (A &

B) non-pretreated skin collagen. (C & D) alkali-pretreated skin collagen. Scale bars: 10 μm in A & C, 5 μm in B & D.

Fig. 6 Scanning electron micrographs of notochord collagen fibrils formed at 21°C after 10 h. (A & B) non-pretreated notochord collagen. (C & D) alkali-pretreated notochord collagen. Scale bars: 10 μm in A & C, 5 μm in B & D.

Fig. 7 The degree of fibril formation of skin collagen and notochord collagen of sturgeon. White, non-pretreated skin collagen; spots, alkali-pretreated skin collagen; stripes, non-pretreated notochord collagen; black, alkali-pretreated notochord collagen. Columns and bars show mean and standard error of the means of three experiments.

Fig. 8 DSC curves of re-assembled fibrils of skin and notochord collagens. The black and white arrowheads indicated the maximum transition temperatures of non-pretreated and alkali-pretreated skin collagen, respectively.

Table 1

Amino acid composition of non-pretreated tissues, alkali-pretreated tissues, and purified collagens (expressed as residues / 1000 total amino acid residues).

Amino acids	Skin	Alkali-pretreated skin	Skin collagen	Alkali-pretreated skin collagen	Notochord	Alkali-pretreated notochord	Notochord collagen	Alkali-pretreated notochord collagen
Asx	54	51	49	49	56	53	51	49
Thr	27	26	25	25	26	23	22	21
Ser	46	46	47	47	37	35	35	34
Glu	76	73	71	70	99	97	92	9
Gly	322	337	341	343	304	320	324	329
Ala	110	113	114	115	82	83	91	93
Cys	3	2	0	0	2	1	1	0
Val	22	20	17	17	25	22	18	17
Met	12	11	10	10	12	12	8	8
Iie	15	13	12	12	18	16	13	12
Leu	25	20	18	18	39	36	31	30
Tyr	7	5	2	2	8	5	4	2
Phe	16	15	13	13	15	14	14	13
Hylys	8	9	8	8	25	27	23	24
Lys	30	25	25	24	19	17	16	16
His	6	5	4	4	9	8	6	6
Arg	53	53	53	53	50	49	51	51
Hypro	58	64	70	71	64	69	77	79
Pro	110	113	120	120	111	113	123	124
Imino acid	168	177	190	191	175	182	210	213

Table 2

Extracted collagen yields (%, dry weight basis). All data are expressed as mean ± standard errors of four experiments. *Significantly different ($P < 0.05$) compared with non-pretreated skin (student`s *t*-test).

Samples	Non-pretreated skin	Alkali-pretreated skin	Non-pretreated notochord	Alkali-pretreated notochord
Yields (%)	63.9±0.19	56.2±0.84*	35.5±0.68	31.8±1.13

Fig.1

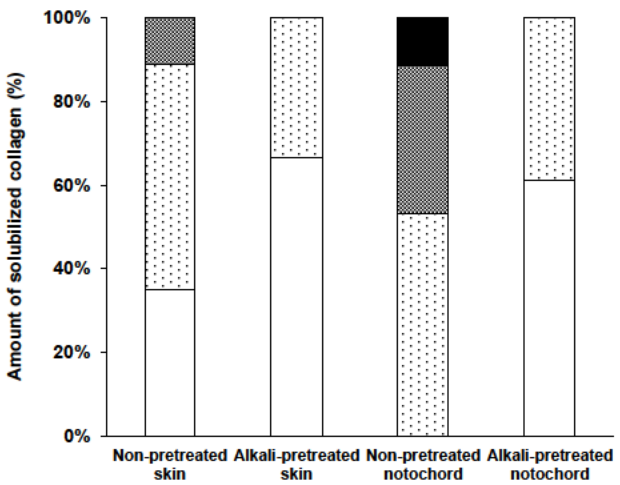

Amount of solubilized collagen (%)
100%
80%
60%
40%
20%
0%
Non-pretreated skin
Alkali-pretreated skin
Non-pretreated notochord
Alkali-pretreated notochord

Fig.2

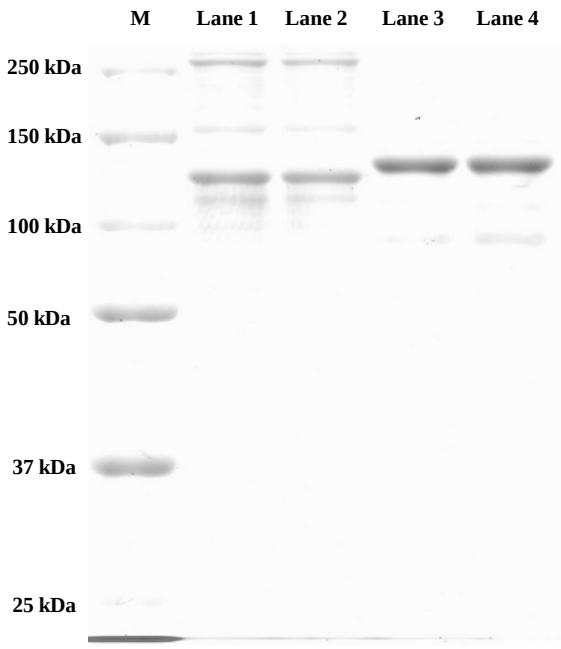

M
Lane 1
Lane 2
Lane 3
Lane 4
250 kDa
150 kDa
100 kDa
50 kDa
37 kDa
25 kDa

Fig.3

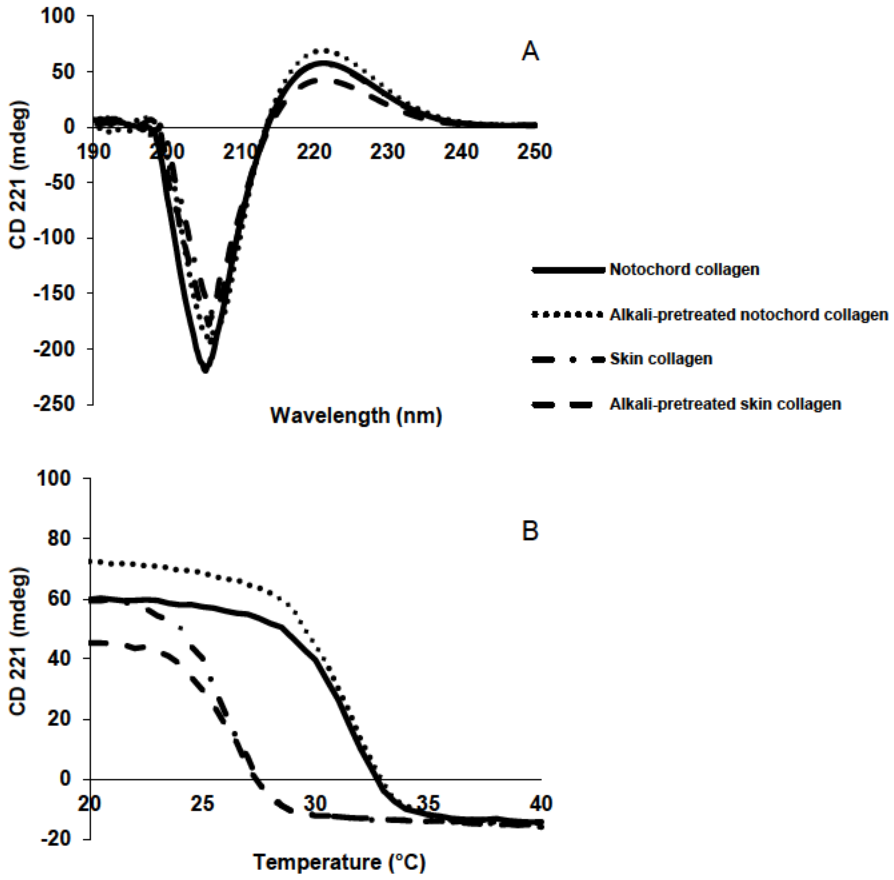

A
100
50
0
-50
-100
-150
-200
-250
190
200
210
220
230
240
250
CD 221 (mdeg)
Wavelength (nm)
Notochord collagen
Alkali-pretreated notochord collagen
Skin collagen
Alkali-pretreated skin collagen
B
100
80
60
40
20
0
-20
20
25
30
35
40
CD 221 (mdeg)
Temperature (°C)

Fig.4

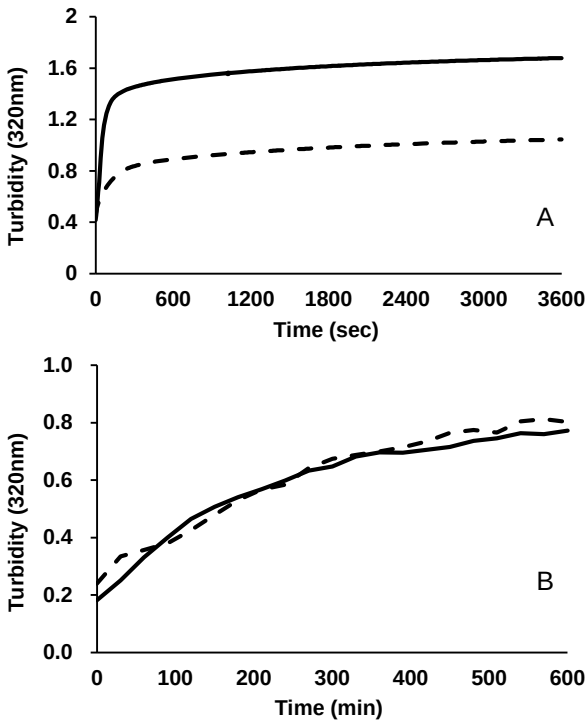

Turbidity (320nm)
2
1.6
1.2
0.8
0.4
0
0
600
1200
1800
2400
3000
3600
Time (sec)
A
Turbidity (320nm)
1.0
0.8
0.6
0.4
0.2
0.0
0
100
200
300
400
500
600
Time (min)
B

Fig.5

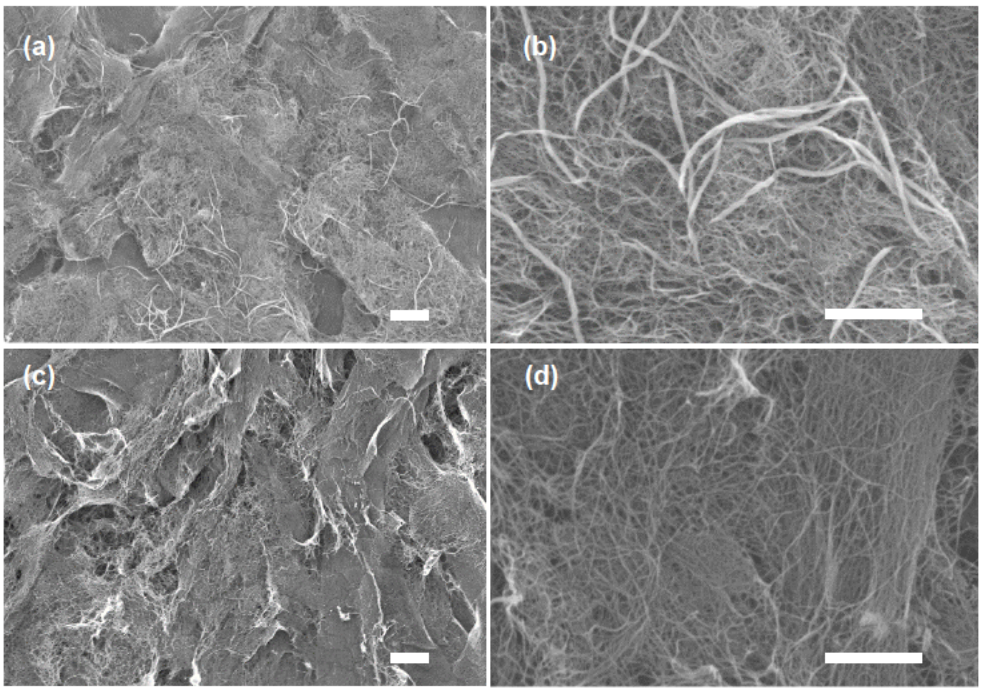


Fig.6

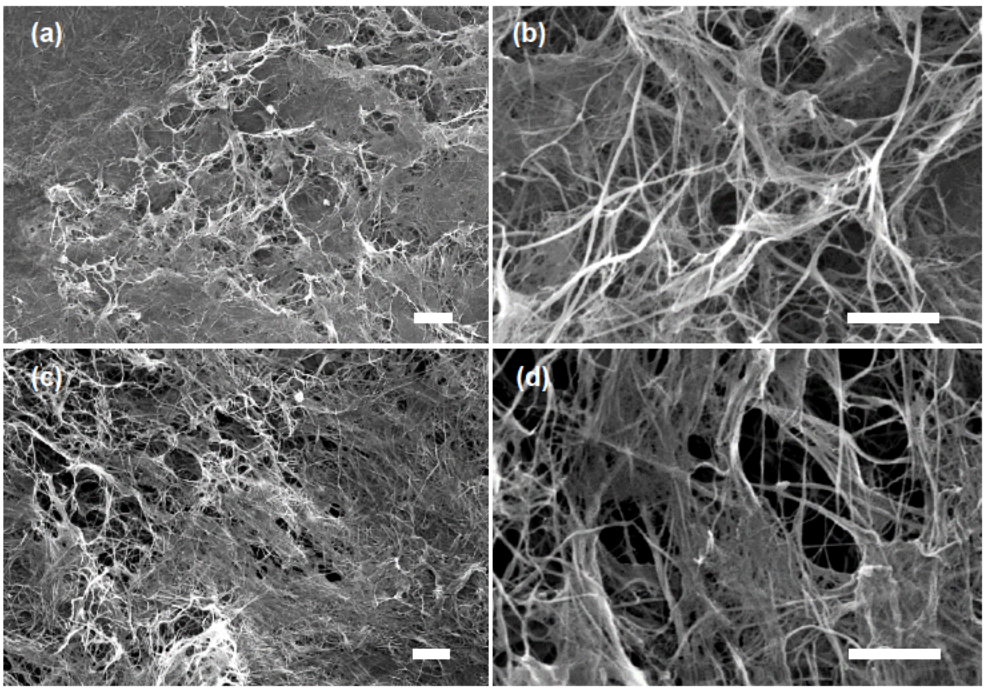


Fig.7

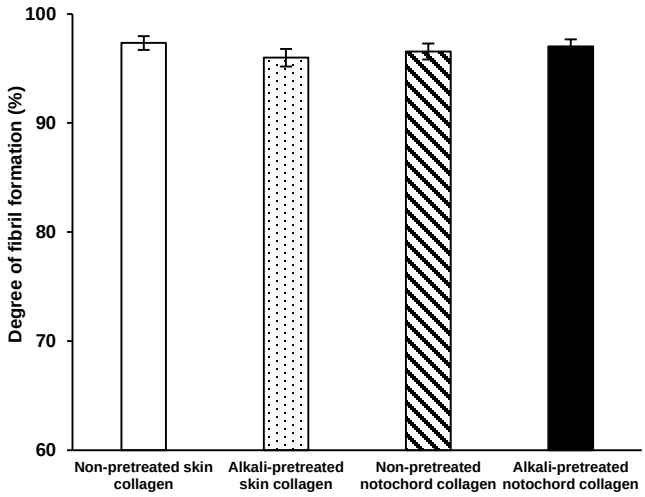
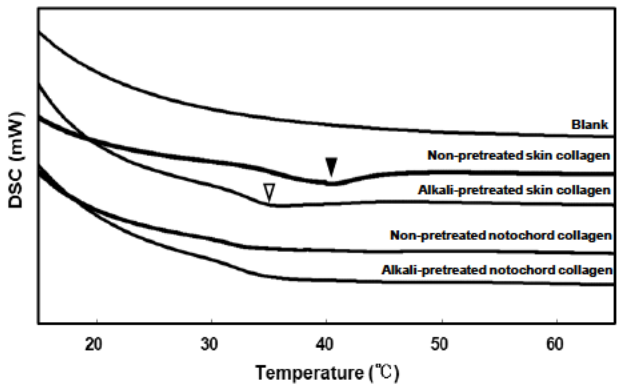

100
90
80
70
60
Degree of fibril formation (%)
Non-pretreated skin collagen
Alkali-pretreated skin collagen
Non-pretreated notochord collagen
Alkali-pretreated notochord collagen

Fig.8


DSC (mW)
Blank
Non-pretreated skin collagen
Alkali-pretreated skin collagen
Non-pretreated notochord collagen
Alkali-pretreated notochord collagen
20
30
40
50
60
Temperature (℃)

Highlights

• We have evaluated extraction conditions and the biochemical properties of collagens from by-products of aquacultured sturgeon.
• Alkaline pretreatment accelerated collagen solubilization (fewer extraction cycles were required) but evoked decrease of collagen yields in both types I and II collagen.
• Alkaline pretreatment had no effects on the thermal stability and triple helical structure of either type I or type II collagen.
• Alkaline pretreatment slowed down the formation of type I collagen fibrils and specifically inhibited the formation of thick fibril-bundle structures.

Bester Sturgeon

By-products

Skin

Type I collagen

Notochord

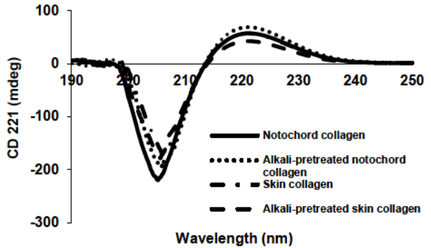
Type II collagen

Efficient utilization of Bester sturgeon by-products

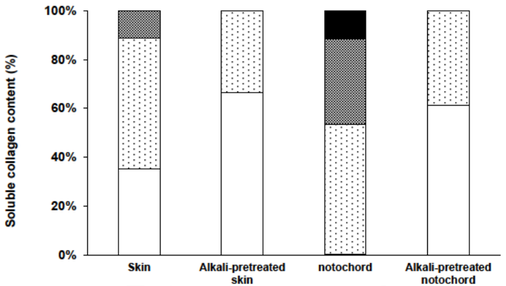
Alkaline pretreatment
Non pretreatment

Collagen extraction

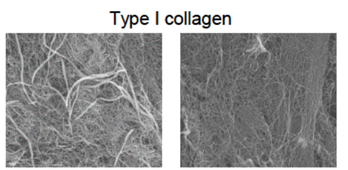
Collagen biochemical characteristics



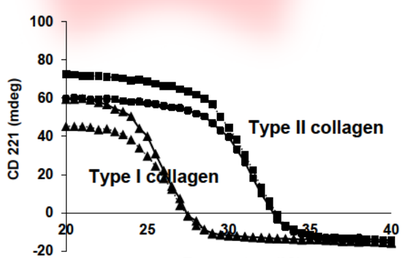
Triple helical structure



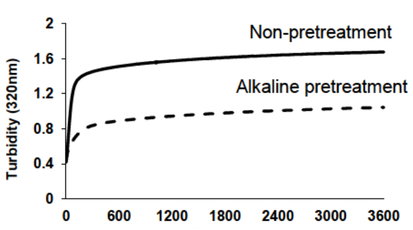
Extraction speed



Fibril morphology



Thermal stability



Fibril formation