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

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## 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  $63.9\pm0.19\%$  and  $35.5\pm0.68\%$ . 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  $56.2\pm0.84\%$  for type I and  $31.8\pm1.13\%$  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.

39    **Keywords:**

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

42

## 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

biomedical use. After alkaline pretreatment of bovine hide type I collagen, however, Hattori et al. [11] have reported that collagen denaturation temperature was decreased, intramolecular cross-links involving proline and hydroxyproline were broken, and a portion of the collagen lost its ability to form fibrils. In addition, the proposed alkali-soluble collagen theory [12] suggests that some loss of collagen would occur during alkaline pretreatment. However, the effects of alkaline pretreatment on the yield and nature of fish collagen are still unclear. Moreover, specific effects on different types of collagen having different biochemical and physicochemical properties are unknown, both for mammalian and fish collagens.

Sturgeon is a highly valuable food fish, which is famous for its caviar. However, because of the long cultivation period required to obtain caviar, the production cost of sturgeon is higher than that of other fishes [13]. Except for caviar and meat, the lack of efficient utilization of sturgeon constrains the profitability of this aquaculture industry, and as a consequence, the expansion of sturgeon aquaculture is slow in some countries, including Japan. Therefore, the development of new commercial products from sturgeon by-products should support further growth of the sturgeon aquaculture industry. Our previous study [13] showed that sturgeon contains large amounts of type I collagen in the skin and swim bladder, and small but industrially extractable amounts of type II collagen in the notochord. Moreover, the type I collagen extracted from sturgeon was quick to reassemble into fibrils, suggesting its

suitability to fabricate biomedical materials. Type II collagen is highly valuable since its market availability is much less than that of type I collagen [13].

Because the extraction procedure employed in our previous study did not include alkaline pretreatment, the biochemical nature of alkali-pretreated collagen was not addressed. In fact, as far as we know, the biochemical nature of alkali-pretreated and non-pretreated fish collagen has not been compared, even though alkaline pretreatment is frequently employed for the extraction of fish collagen. Therefore, the present study clarified the effect of alkaline pretreatment on the extraction process, yield, biochemical nature and fibril-forming ability of types I and II collagen from bester sturgeon skin and notochord.

## **2 Materials and methods**

### *2.1 Isolation and purification of collagen*

A live, cultured bester sturgeon (1.24 m, 6.00 kg) was procured from the Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan. Skin and notochord were removed and washed with chilled tap water, lyophilized in a freeze dryer (FDU-830, Tokyo Rikakikai Co., Ltd., Tokyo, Japan), and stored at  $-30^{\circ}\text{C}$  until use.

Skin fat was removed over 24 h in 99.5% ethanol (two solution-changes) at  $4^{\circ}\text{C}$ , with a 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

in comparison with the dry weight of the initial tissues was calculated as the collagen yield.

Experiments were conducted for 4 times, and the data were expressed as means  $\pm$  standard errors (SE).

## *2.2 Amino acid analysis*

The amino acid composition was analyzed at the Instrumental Analysis Division, Equipment Management Center, Creative Research Institution, Hokkaido University. In brief, samples were hydrolyzed in 6 M HCl at 110°C for 24 h. The hydrolysates were evaporated, and the remaining materials were dissolved in a citric acid buffer, and then analyzed using an automated amino acid analyzer (JLC-500 V, JEOL Ltd., Tokyo, Japan). Samples were assayed three times, and the means were calculated to obtain amino acid compositions.

## *2.3 Soluble-collagen quantification*

The amount of solubilized collagen in each extraction step was measured using a soluble collagen quantification kit (Sircol Soluble Collagen Assay, Biocolor life science assays, Northern Ireland U.K.), following the manufacturer's protocol. Purified types I or II collagen from the skin and notochord of bester sturgeon was used for standards. The amount of 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%  $\beta$ -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

a quartz cell. CD spectra were measured at 190–250 nm wavelengths at 10°C under a scan speed of 50 nm/min with an interval of 0.1 nm. The rotatory angle at a fixed wavelength of 221 nm was then measured at 10–50°C with a rate of 1°C/min to determine the denaturation temperature of the collagen molecules.

## *2.6 Collagen fibril formation in vitro*

The fibril formation process of collagens was evaluated by the method of Zhang [13] with some modifications. Lyophilized collagens were dissolved in an HCl solution (pH 2.0) to 0.3% (w/v). The skin and notochord collagen solutions were mixed with a 45 mM Na-phosphate buffer (pH 7.4). The ratio of collagen solution: Na-phosphate buffer was 1:2 (v/v). The mixed solution was placed into a cell, and the subsequent fibril formation at 21°C was monitored by measuring increased turbidity via optical absorbance at 320 nm, using a spectral monitor (Hitachi High-technology Corporation, Tokyo, Japan). Fibril formation was monitored for 1 h and 10 h for the skin and the notochord collagens, respectively.

## *2.7 Morphology of fibrils*

The microstructure of skin and notochord collagen fibrils was observed using a scanning electron microscope (SEM; JSM6010LA, JEOL Ltd., Tokyo, Japan), as previously described

Zhang [13]. Collagen fibrils were formed using the same conditions as described above. Sample suspensions were centrifuged at 20,000×g for 20 min at 4°C to obtain precipitates of collagen fibrils. The fibrils were fixed with 2.5% (v/v) glutaraldehyde in a 0.1 M phosphate buffer (pH 7.4) for 3 h at room temperature, rinsed with the phosphate buffer to remove the fixative, dehydrated with a graded series of ethanol solutions, and then soaked in two 30-min changes of *t*-butyl alcohol solution. Finally, collagen fibrils were freeze-dried in a *t*-butyl alcohol solution with a freeze-drying device (JFD-320; JEOL Ltd.) and coated with gold-platinum using an auto fine coater (JFC-1600; JEOL Ltd.).

## *2.8 Degree of fibril formation*

The determination method of the fibril formation degree was followed by the method of Zhang [13]. Skin and notochord collagen fibrils were formed as shown in 2.6. After the centrifugation at 20,000×g for 20 min at 4°C, the protein concentration of the supernatant was measured following Lowry [16] using bovine serum albumin as a standard. The degree of collagen fibril formation was defined as the percentage of the decrease of collagen concentration in the solution after the experiment, which means the percent of collagen molecules that formed the fibrils.

## 2.9 Thermal stability of fibrils

The maximum transition temperature ( $T_m$ ) of re-assembled fibrils of skin and notochord collagens was measured by differential scanning calorimetry (EXSTAR DSC6100, SII Nano Technology Inc., Chiba, Japan). Collagen fibrils were prepared using the same conditions as described in 2.6. The sample solutions were centrifuged at  $20,000\times g$  for 20 min at  $4^\circ\text{C}$  to get precipitates of collagen fibrils. The precipitates were placed in an Al pan ( $70\ \mu\text{l}$ ), measured at  $20\text{-}60^\circ\text{C}$  at a rate of  $3^\circ\text{C}/\text{min}$  using Na-phosphate buffer pH 7.4 as a reference.

## 2.10 Statistical analysis

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

# 3 Results and discussion

## 3.1 Amino acid composition

The amino acid compositions of samples are shown in Table 1. The characteristic repeating unit of a collagen monomer is Glycine (Gly)-X-Y, where X and Y are often proline (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].



After alkaline pretreatment of the notochord, only two extractions were needed to obtain all the solubilized collagen. Combined with the results of the skin collagen extraction, we conclude that alkaline pretreatment not only removed non-collagenous proteins but also accelerated the solubilized collagen extraction process. Hattori et al [11] found that the extractability of bovine skin collagen, when treated with an alkaline solution, was much higher than that of non-treated tissue. In the present study, the same phenomenon was demonstrated for the first time for both types I and II fish collagens. Alkaline pretreatment might cause significant swelling of the collagen fibril structure [12]. After collagen fibrils are swollen, pepsin should more easily contact the collagen molecules to break their intermolecular cross-links.

### *3.3 Yields of collagens*

The yields of purified collagen are summarized in Table 2. The yields of skin and notochord collagen were  $63.9 \pm 0.19\%$  and  $35.5 \pm 0.68\%$  (dry weight basis), respectively. These yields were much higher than our previous study (skin, 31.4%; notochord, 5.1%) [13]. Incomplete extraction might be the main factor that limited the yield in our previous study. In 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  $56.2\pm0.84\%$  and  $31.8\pm1.13\%$   
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 ( $63.9\pm0.19\%$ ) 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 ( $35.5\pm0.68\%$ ) 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  $35.5 \pm 0.68\%$ . 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  $\alpha$ -chains as the major constituents (approximately 120 kDa and 100 kDa), whereas the non-pretreated notochord collagen had

only one  $\alpha$ -chain (approximately 130 kDa), indicating that the skin and notochord collagen were type I and type II, respectively. For both tissues, the alkaline pretreatment did not affect the band pattern.

High molecular weight bands, which might be cross-linked  $\alpha$ -chains (that is,  $\beta$ - and  $\gamma$ -chains), showed lower intensities in the notochord collagens compared with the skin collagens, suggesting that the number of cross-links was much less in the notochord collagen. Cross-links are one of the factors that make collagen extraction difficult. As already stated in section 3.2, the notochord collagen was more difficult to extract compared with the skin collagen. However, the SDS-PAGE revealed that number of cross-links could not explain the difficulty of collagen extraction in notochord. Also, the  $\beta$ - and  $\gamma$ -chain band intensities from bester sturgeon skin presented in the present study are much lower compared with those from carp and bigeye snapper skin [4, 5]. Therefore, our results suggest that inter- or intra-molecular cross-links in sturgeon skin collagen are less than those of other fishes.

### *3.5 Thermal stability of collagen molecule*

CD spectra of skin and notochord collagens are shown in Fig. 3A. All collagens had a rotatory maximum at 221 nm and a crossover point (zero rotation) at approximately 212 nm, which are typical characteristics of the collagen triple-helical conformation [13].

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

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

327

### 3.6 Collagen fibril formation *in vitro*

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

Calf and human type II collagen have been reported to have a longer lag time for assembly and lower fibril formation rate than type I collagen [34, 35]. Notbohm [36] investigated human type II collagen fibril formation in 30 mM phosphate buffer (pH 7.4) for 600 min. In the present study, a lower buffer concentration and a longer observation time were employed compared with our previous study [13], and fibril formation by type II collagen from a fish species was achieved for the first time (Fig. 4B). In contrast to the skin type I collagen, alkaline pretreatment showed little effect on fibril formation by the notochord type II collagen. The different influences of alkaline pretreatment may be explained by different characteristics and mechanisms of fibril formation between type I and type II collagen. This is the first study to demonstrate that alkaline pretreatment has different influences on *in vitro* fibril formation by types I and II fish collagen.

Collagen fibril formation is affected by many factors, such as ionic strength, ionic species, pH value, temperature and others [37]. The present study included only a preliminary examination of the effects of alkaline pretreatment. For the industrial use of sturgeon skin type I and notochord type II collagen, it will be necessary to further optimize conditions for fibril formation.

### 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

buffer concentration and a longer reaction time were better for fibril formation by type II collagen compared with type I collagen.

In addition, to investigate whether these collagen fibrils are similar to the fibrils formed in the body, the D-period of fibrils was determined by transmission electron microscopy (data not shown). Non-pretreated type I collagen showed  $68\pm 12$  nm D-period. However, the D-periods of alkali-pretreated type I collagen and two type II collagen preparations were not confirmed because of insufficient resolution. Meanwhile, we found the diameter of type II collagen was much smaller than type I collagen, confirming our SEM study. Collagen fibril formation condition and observation condition need further improvement.

### *3.8 Degree of fibril formation*

The degree of skin and notochord collagen fibril formation was assessed after 1 h and 10 h of fibrillogenesis, respectively, and the results are shown in Fig. 7. Alkali-pretreatment showed no significant influences on the degree of type I and type II collagen fibril formation: all samples showed values higher than 90%. These values were higher than those of barramundi and tilapia skin type I collagens, in which fibrils were formed for 24 h [40].

From the results obtained in sections 3.6-3.8, the effects of alkaline pretreatment on type I 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.  $T_m$  was obtained as the endothermic peak of the DSC curve. For type I collagen, non-pretreated collagen showed higher  $T_m$  ( $40.14 \pm 0.31^\circ\text{C}$ ) than alkali-pretreated collagen ( $37.26 \pm 0.08^\circ\text{C}$ ), which may be attributed to the thick fibril-bundle structure in the non-pretreated preparation. In addition, comparing with  $T_m$  of type I collagen molecules ( $27.0^\circ\text{C}$ ), the  $T_m$  of type I collagen fibrils was higher by  $10\text{--}12^\circ\text{C}$ . 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  $34\text{--}35^\circ\text{C}$  on both preparations. These temperatures were close to the denaturation temperatures of non-pretreated and alkali-pretreated type II collagen molecules ( $31\text{--}32^\circ\text{C}$ ). These data suggested

that although type II collagen fibrils were assembled, the thermally stable fibril structures were not formed in the present condition, and more suitable fibril formation condition need to be discussed in the future.

#### **4 Conclusions**

This study demonstrated that a large amount of type I and type II collagen could be extracted from the skin and notochord of better sturgeon, and suggested that both tissues are promising sources of fish collagen for industrial use. Alkaline pretreatment accelerated the collagen extraction process of both types of collagen, although it also reduced the yields. Although alkaline pretreatment did not affect the thermal stability of either type I or type II collagen molecules, it lowered the fibril-forming speed and  $T_m$  and changed the fibril morphology of the type I collagen. Since there has been no previous study on the effects of alkaline pretreatment on types I and II fish collagen, this study provides basic data describing the nature of these collagens as well as their suitability for industrial use.

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558

**Figure captions**

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

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

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

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

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

577 B) non-pretreated skin collagen. (C & D) alkali-pretreated skin collagen. Scale bars: 10  $\mu\text{m}$  in  
578 A & C, 5  $\mu\text{m}$  in B & D.

579

580 **Fig. 6** Scanning electron micrographs of notochord collagen fibrils formed at 21°C after 10 h.

581 (A & B) non-pretreated notochord collagen. (C & D) alkali-pretreated notochord collagen.

582 Scale bars: 10  $\mu\text{m}$  in A & C, 5  $\mu\text{m}$  in B & D.

583

584 **Fig. 7** The degree of fibril formation of skin collagen and notochord collagen of sturgeon.

585 White, non-pretreated skin collagen; spots, alkali-pretreated skin collagen; stripes, non-

586 pretreated notochord collagen; black, alkali-pretreated notochord collagen. Columns and bars

587 show mean and standard error of the means of three experiments.

588

589 **Fig. 8** DSC curves of re-assembled fibrils of skin and notochord collagens. The black and

590 white arrowheads indicated the maximum transition temperatures of non-pretreated and

591 alkali-pretreated skin collagen, respectively.

1 **Table 1**

2 Amino acid composition of non-pretreated tissues, alkali-pretreated tissues, and purified

3 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
Ile	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

6 **Table 2**

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

10

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

11

Fig.1

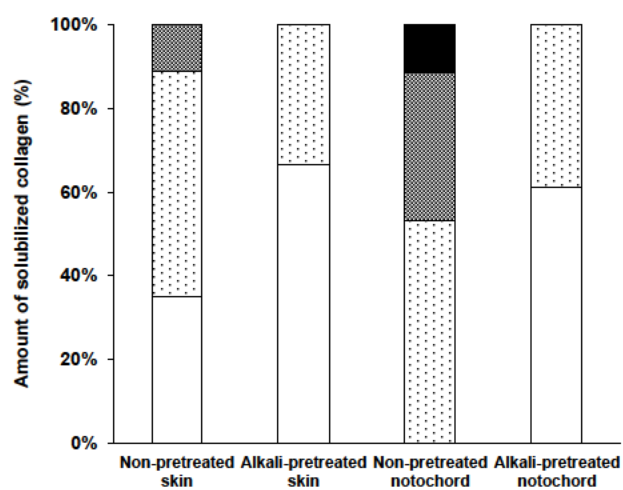


Fig.2

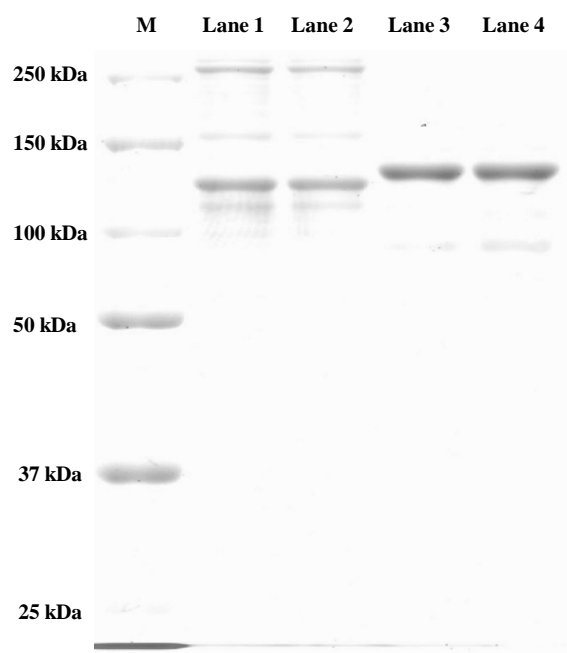




Fig.3

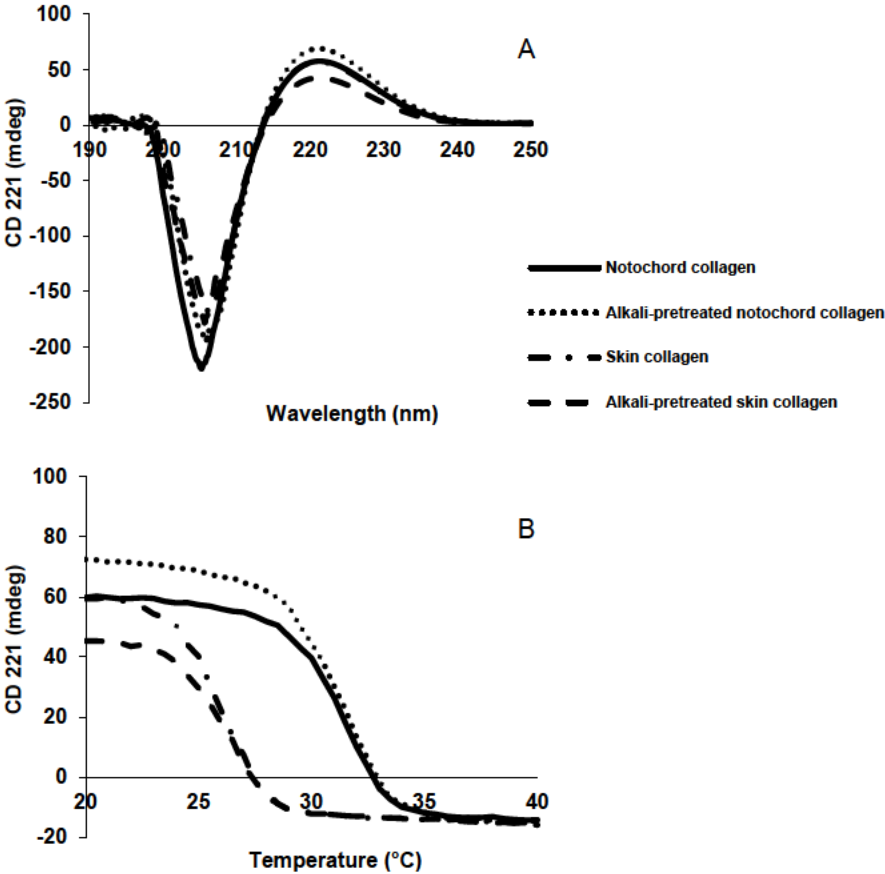


Fig.4

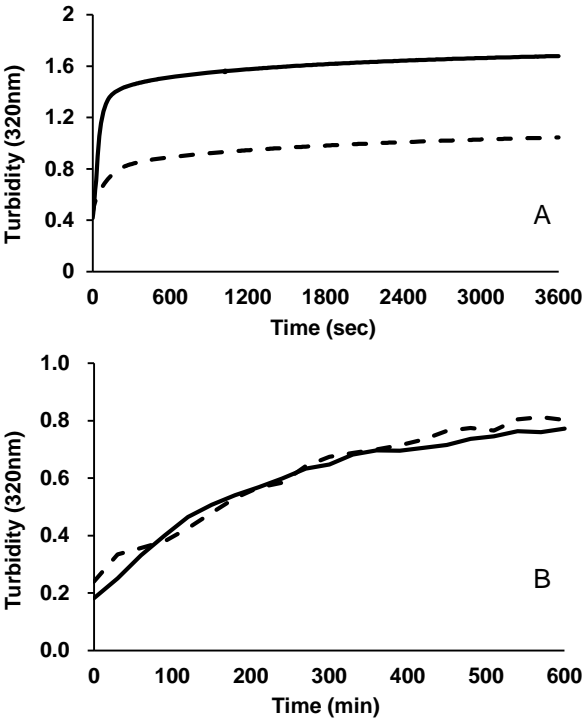


Fig.5

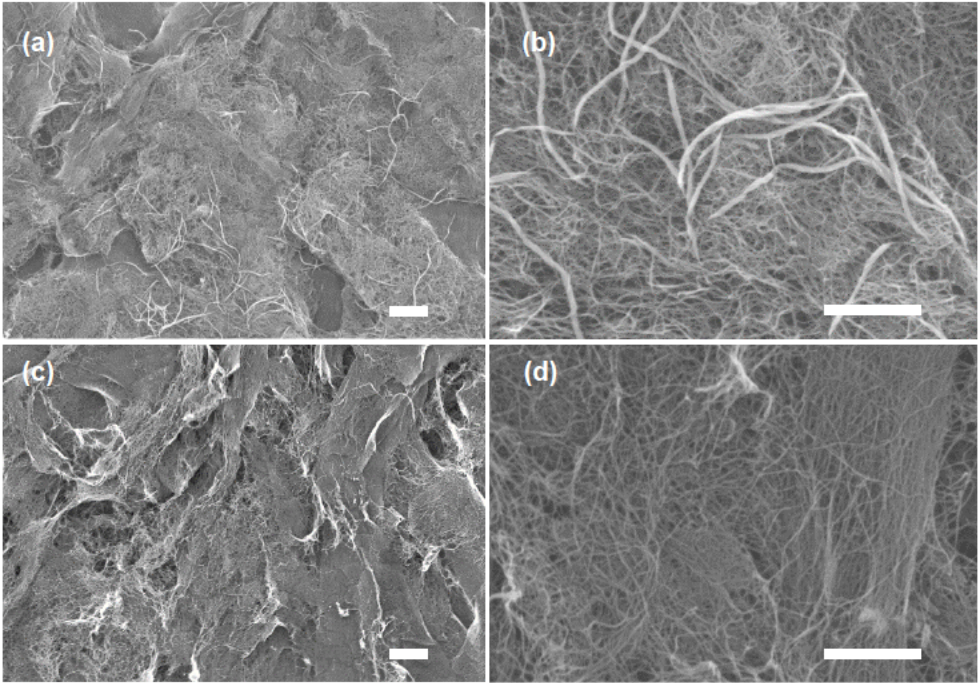


Fig.6

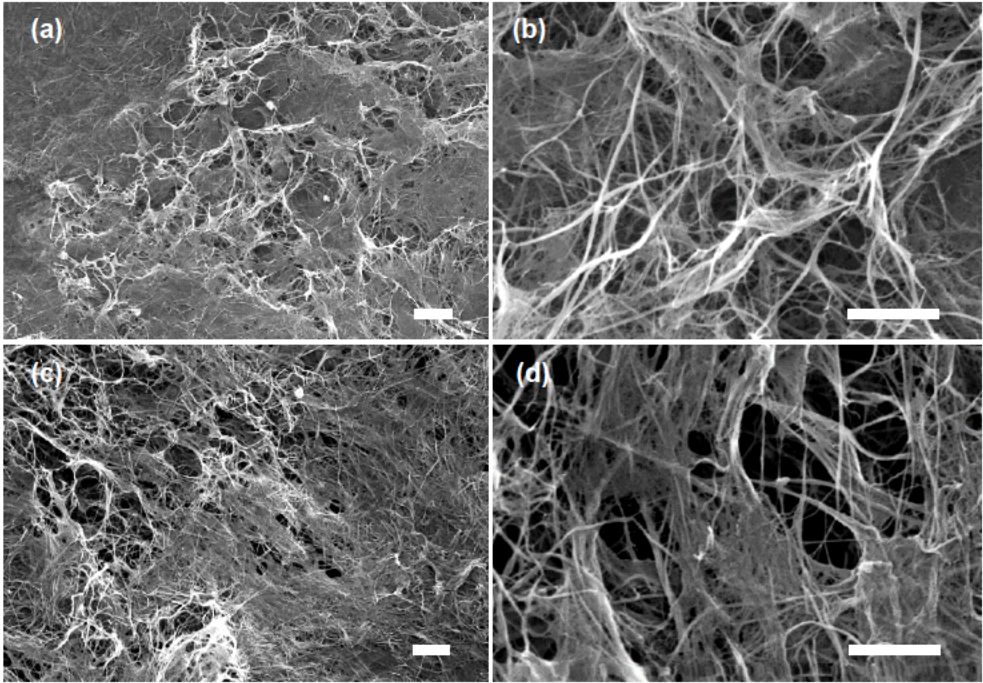


Fig.7

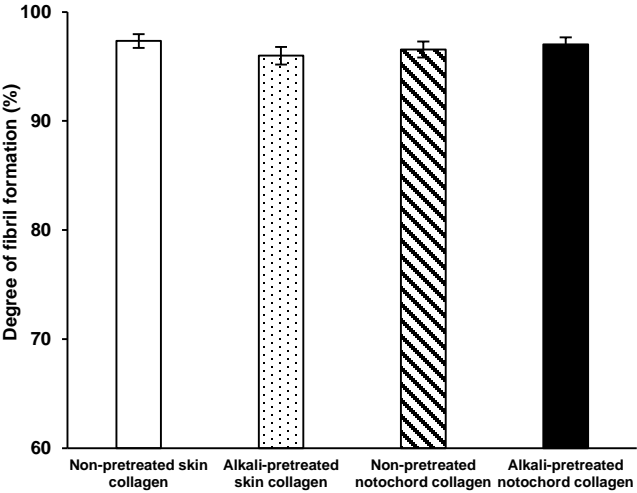
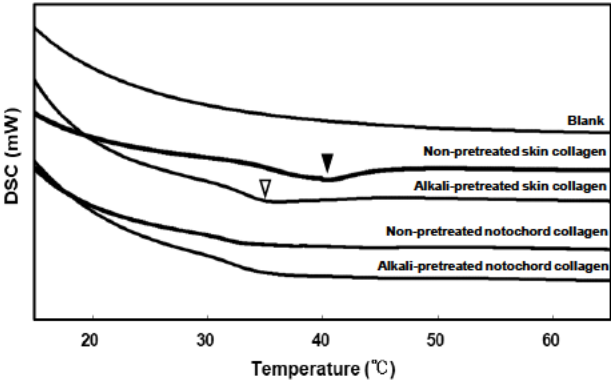


Fig.8



## **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



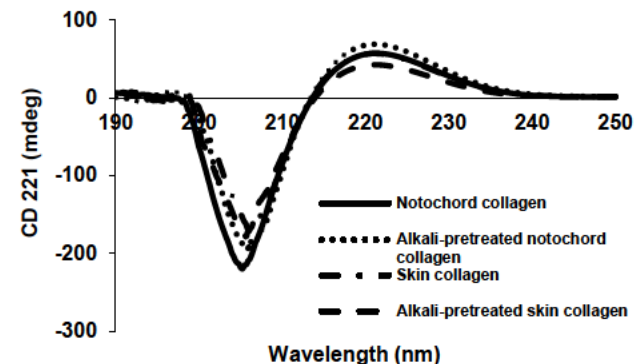
**Notochord**

Type II collagen

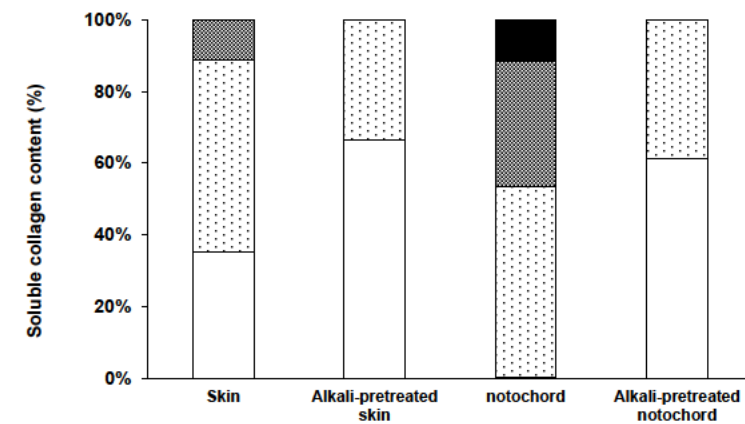
## ***Efficient utilization of Bester sturgeon by-products***

**Alkaline pretreatment  
Non pretreatment**

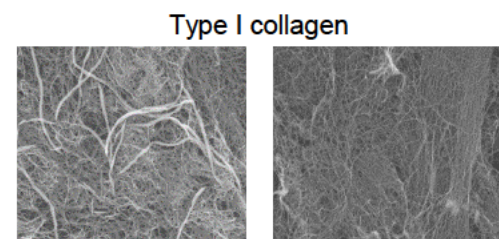
**Collagen  
extraction**



**Triple helical structure**

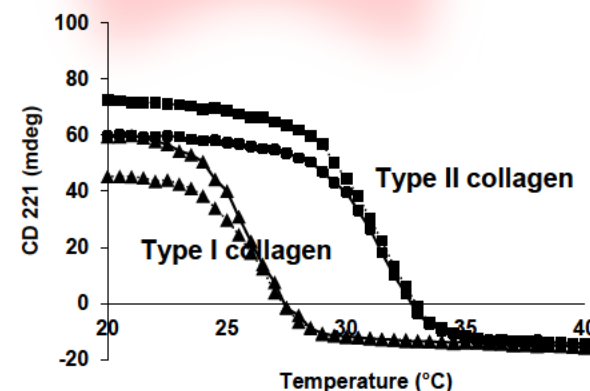


**Extraction speed**

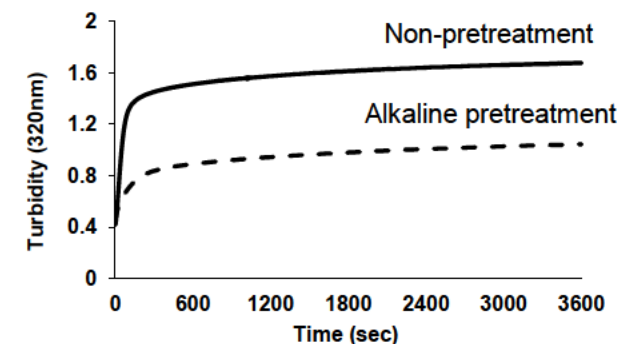


Non pretreatment Alkaline pretreatment

**Fibril morphology**



**Thermal stability**



**Fibril formation**

**Collagen  
biochemical  
characteristics**