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# Biochemical characterisation and assessment of fibril-forming ability of collagens extracted from Bester sturgeon *Huso huso* × *Acipenser ruthenus*

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ABSTRACT

Collagens purified from Bester sturgeon organs were characterised biochemically, and their fibril-forming abilities and fibril morphologies formed *in vitro* clarified. Yields of collagens were 2.1%, 11.9%, 0.4%, 18.1%, 0.4%, 0.8% and 0.03% (collagen dry weight/tissue wet weight) from scales, skin, muscle, swim bladder, digestive tract, notochord and snout cartilage, respectively. Using SDS–PAGE and amino acid composition analyses, collagens from scales, skin, muscle, the swim bladder and digestive tract were characterised as type I, and collagens from the notochord and snout cartilage as type II. Denaturation temperatures of the collagens, measured using circular dichroism, were 29.6, 26.8, 29.0, 32.9, 31.6 and 36.3 °C in scales, skin, muscle, swim bladder, digestive tract, and notochord, respectively. For fibril formation, swim bladder and skin collagen showed a more rapid rate of increase in turbidity, a shorter time to attain the maximum turbidity, and formed thicker fibrils compared with porcine tendon type I collagen.

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## 1. Introduction

Collagen, in the form of elongated fibrils, contributes to the physiological functions and mechanical properties of skin, tendon, bone, cartilage and other tissues (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003). Collagen has been widely used in many industries such as the food, photographic film, cosmetic, and leather industries (Zhang, Liu, & Li, 2009). Furthermore, because of its low antigenic activity, high cell adhesion properties, biocompatibility, and biodegradability, collagen-based biomaterials have been applied in medical research, as well as tissue engineering (Parenteau-Bareil, Gauvin, & Berthod, 2010). The most common sources of collagen for biomaterials and tissue engineering are bovine skin and tendons, porcine skin, and rat tail (Parenteau-Bareil et al., 2010). However, concerns about the use of land animal collagens are growing because of the risk of common human diseases. Religious beliefs also restrict usage of porcine or bovine collagens in certain regions in the world. In recent years, as an alternative source to porcine or bovine collagen, fish collagen has received increasing attention, for its advantages of using highly abundant fish offal, and avoiding human diseases and religious objections (Jongjareonrak, Benjakul, Visessanguan, & Tanaka, 2005a). The biochemical nature of collagens extracted from fish skin, scales, and bones have been reported in many studies (Duan, Zhang, Du, Yao, & Konno, 2009; Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Nagai & Suzuki, 2000; Singh, Benjakul, Maqsood, & Kishimura, 2011; Zhang et al., 2009).

Sturgeon is highly valued as a food fish, and is especially famous for its caviar. Contemporary sturgeon culture was begun in the 1980s, and worldwide production had increased to ca. 25,000 t by 2008 (Zhang, Shimoda, Ura, Adachi, & Takagi, 2012). Thus, sturgeon culture now forms the basis of an important and promising industry. However, the culture cost of sturgeon is higher than other fishes, because caviar production requires long culture times. On the other hand, the lack of utilisation of other parts of sturgeon constrains the development of its culture. Therefore, we believe that if skin, offal and cartilage of sturgeon are used as a source of collagen, the value of the by-products would increase, and waste disposal problems could be alleviated.

Zeng et al. (2012) have reported the structure and characteristics of collagen extracted from cobia *Rachycentron canadum* skin for the utilisation in biomaterials, which should increase the value of cobia offal. However, the important collagen property, the fibril-forming ability, and morphology for biomaterial utilisation has not been reported. The collagen molecule is distinct from other proteins as its three polypeptide chains (α-chains) form a unique triple helical structure. The molecules self-assemble into fibrils *in vitro* when collagen solution is adjusted to an appropriate temperature, pH, and ionic strength (Kadler, Holmes, Trotter, & Chapman, 1996). After fibril formation, the low denaturation temperature of fish collagen can be improved to more than 40 °C, and

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therefore it is possible for use as a biomaterial for humans (Bae et al., 2009). However, there are few studies about the fibril-forming ability of fish collagens.

Bester, a hybrid sturgeon of *Huso huso* × *Acipenser ruthenus*, is suited to aquaculture because of its rapid growth, ability to grow in freshwater ponds, and the relative ease of inducing sexual maturation (Zhang et al., 2012). This fish is now widely cultured in China (Wei, Zou, Li, & Li, 2011) and Japan. The objective of this study was to extract and assess the biochemical nature of the collagens from the Bester sturgeon. In addition, the fibril-forming ability and morphology of fibrils formed *in vitro* were investigated.

## 2. Materials and methods

### 2.1. Extraction of collagens

A live cultured Bester sturgeon (length = 0.76 m, 2.00 kg) was procured from the Nanae Fresh-Water Laboratory, Field Science centre for Northern Biosphere, Hokkaido University, Japan. The fish was deeply anaesthetized in 2-phenoxyethanol solution and gutted. Scales, skin, muscle, swim bladder, digestive tract, notochord and snout cartilage were removed and washed with chilled tap water, then stored at −30 °C until use. Samples were washed with cold distilled water (4 °C) and cut into small pieces (0.5 × 0.5 cm). Skin fat was removed over 24 h in 99.5% ethanol (three solution changes) with a sample/solution ratio of 1:10. Scales and snout cartilage samples were removed, non-collagenous proteins extracted in 0.1 M NaOH with a sample/solution ratio of 1:10 for 6 h, and then samples were decalcified in 0.5 M EDTA with a sample/solution ratio of 1:10 for 24 h. After that, skin, scales and cartilage samples were washed with cold water for 24 h. To extract collagens, the samples were stirred continuously in a solution of HCl (pH 2.0) containing 0.1% (w/v) porcine pepsin (EC 3.4.23.1, 1:10,000, Wako Pure Chemical Industries Ltd., Osaka, Japan) with a sample/solvent ratio of 1:10 (w/v) for 48 h at 10 °C. The mixtures were centrifuged at 2000*g* for 1 h to get supernatants, and the residue was re-extracted in the same conditions. The supernatants were sequentially filtered with membrane filters with pore sizes of 3.0, 0.8, and 0.47 μm (Advantec, Tokyo, Japan). Collagen in the filtrate was precipitated by adding NaCl to a final concentration of 1 M. The resulting precipitate was collected by centrifugation at 2000*g* at 4 °C for 90 min, and dissolved in an HCl solution (pH 2.0). This process was repeated three times to purify the collagen. The purified collagen was dialyzed against 50 volumes of distilled water at 4 °C for 24 h with two changes of water. The dialysate was lyophilized by freeze dryer (FDU-830, Tokyo Rikakikai Co., Ltd., Tokyo, Japan). The percentage of dry weight of collagen extracted in comparison with the wet weight of the initial tissues was calculated as the collagen yield.

### 2.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) analysis

SDS–PAGE was performed according to the method of Laemmli (1970). The lyophilized collagens were dissolved in HCl (pH 3.0, 3 mg collagen/ml), and then mixed at a ratio of 1:2 (v/v) with the sample buffer (0.5 M Tris–HCl buffer, pH 6.8, with 4% SDS and 20% glycerol) containing 10% β-mercaptoethanol. The mixed solution was boiled for 5 min. Ten micrograms of protein were loaded in each lane. Electrophoresis was performed at 15 mA for the stacking gel and 20 mA for the 7.5% running gel. After electrophoresis, the gel was stained for 30 min using 0.1% Coomassie Brilliant Blue R250 solution and destained with a mixture of 20% ethanol, 5% acetic acid and 2.5% glycerin. Precision plus protein standards (Bio-Rad Laboratories, Inc., Hercules, CA, USA) were used to estimate the molecular weight. Quantitative analysis of band intensity was determined by AE-6932GXES Printgraph (ATTO Co., Tokyo, Japan) with CS Analyzer ver 3.0 (image analysis system).

### 2.3. Amino acid analysis

Samples were hydrolysed in 6 N HCl at 110 °C for 24 h. The hydrolysates were evaporated, and the remaining materials dissolved in citric acid buffer solution, and then analysed using an automated amino acid analyzer (JLC-500 V, JEOL Ltd., Tokyo, Japan). Samples were assayed three times and the averages were used to obtain amino acid compositions. In this study, the number of cysteine residues was obtained by first determining the number of cystine residues (a dimeric amino acid) then calculating cysteine from cystine. The number of tryptophan residues was not determined.

### 2.4. Circular dichroism (CD) measurement

CD spectra were measured using a JASCO model 725 spectrometer (JASCO, Tokyo, Japan). The measurement was performed according to the method of Ikoma et al. (2003). Lyophilized collagens were dissolved in an HCl solution (pH 3.0) 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. Then a rotatory angle at a fixed wavelength of 221 nm was measured at 10–50 °C with a rate of 1 °C/min to determine the collagen denaturation temperature (Td).

### 2.5. Collagen fibril formation in vitro

Collagen molecules self-assemble into fibrils *in vitro* (fibril formation) when collagen solution is adjusted to an appropriate temperature, pH, and ionic strength. Fibril formation of collagen from the skin and swim bladder was performed according to the method of Bae et al. (2009). Lyophilized collagens were dissolved in an HCl solution (pH 3.0) to 0.3% (w/v). The collagen solution was mixed with 0.1 M Na-phosphate buffer (pH 7.4) containing NaCl at 0, 70 and 140 mM. The ratio of the collagen solution/Na-phosphate buffer was 1:2 (v/v), and the final pH of the solution was 7.4. Porcine tendon collagen (Cellmatrix Type I-A, 0.3%, Nitta Gelatin Inc., Osaka, Japan) was used as a control. With fibril formation, the transparent solution would become white. The mixed solution was placed into a cell, and the resulting fibril formation at 21 ± 1 °C was monitored by the absorbance at 320 nm as the turbidity change using a spectral monitor GeneQuant *pro* (GE Healthcare Life Sciences, Tokyo, Japan). This is the measurement on the speed of collagen fibril formation in a short time.

### 2.6. Measurement of degree of collagen fibril formation

Skin and swim bladder collagen fibrils as well as porcine tendon collagen fibrils were formed for 24 h at 21 ± 1 °C, which was assumed to be the endpoint of fibril formation, using the same conditions as described above. Then the sample solution was centrifuged at 20,000*g* for 20 min to precipitate fibrils, and the protein content of the supernatant was measured based on the method of Lowry (Lowry, Rosebrough, Farr, & Randall, 1951) using bovine serum albumin as a standard. Degree of collagen fibril formation was defined as the percentage of the decrease of the collagen concentration in the solution after the experiment, which means the percent of collagen molecules that formed the fibrils.

### 2.7. Electron microscope observations

The microstructure of skin and swim bladder collagen fibrils was observed using a scanning electron microscope (SEM; JSM6010LA, JEOL Ltd., Tokyo, Japan). The collagen fibrils were formed using the same conditions as described above for 1 and 24 h at 21 ± 1 °C. The sample suspension of skin and swim bladder collagens was centrifuged at 20,000*g* for 20 min to get precipitates of collagen fibrils. The collagen fibrils were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.6) for 3 h at room temperature, and then rinsed with the phosphate buffer. The fibrils were sequentially soaked in 70%, 80%, 90%, 95% and 100% ethanol solutions for 30 min to dehydration, and then in two 30-min changes of t-butyl alcohol solution. Finally, collagen fibrils were freeze-dried in 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.). For comparison, porcine tendon type I collagen (Cellmatrix Type I-A, Nitta Gelatin Inc., Osaka, Japan) was treated similarly and observed.

## 3. Results and discussion

### 3.1. Yields of collagens

Yields of collagens are summarised in Table 1. The yield of swim bladder collagen (18.1% on a wet weight basis, 37.7% on a dry weight basis) was the highest on a percent basis, and snout cartilage (0.03% and 0.2%, on a wet and dry weight basis, respectively) was the lowest. The amount of collagen obtained from one fish (length = 0.76 m, 2.00 kg) was highest from the skin (6010 mg) and the swim bladder (4400 mg). The yield of collagen from sturgeon skin (11.9%, wet weight basis) was therefore higher than that from bigeye snapper skin (7.5%; Jongjareonrak et al., 2005a), and similar to skin yields from brownstripe red snapper (13.7%; Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005b) and striped catfish (12.8%; Singh et al., 2011). The yield of collagen from sturgeon skin (34.1%, dry weight basis) was higher than that from black drum skin (18.1%), but lower than that from largefin longbarbel catfish skin (44.8%) and ocellate puffer fish skin (55.4%) (Nagai, Araki, & Suzuki, 2002; Zhang et al., 2009). The comparable high yield of skin and swim bladder collagen suggests the possibility for industrial production.

In the collagen extraction process, the solubility of swim bladder collagen was much higher than that of others. The collagen molecule has a unique triple helical structure, and the two terminal ends are non-helical parts known as the telopeptide region. Covalent bonds through the condensation of aldehyde groups at the telopeptide region, as well as the triple helical region, form an inter-molecular cross-linked structure, leading to a decrease in the solubility of collagen (Duan et al., 2009; Singh et al., 2011). Pepsin has been reported to cleave peptides in the telopeptide region without damaging the integrity of the triple helix structure (Jongjareonrak et al., 2005b). Thus pepsin-digestion extracts collagen molecules without cross-links at the triple helical region. Therefore, the more rapid extraction of collagen from the swim bladder under pepsin digestion suggests that inter-molecular cross-links at the triple helical region of the collagen molecules in this tissue are likely to be fewer than those in other organs.

**Table 1**
Yields (%, based on the wet and dry weight of initial samples) and dry weight (mg) of collagens purified from Bester sturgeon (0.76 m, 2.00 kg).

| Samples | Yields/wet (%) | Yields/dry (%) | Yields (mg) |
|---|---|---|---|
| Scale | 2.1 | 3.0 | 80 |
| Skin | 11.9 | 34.1 | 6010 |
| Muscle | 0.4 | 2.0 | 692 |
| Swim bladder | 18.1 | 37.7 | 4400 |
| Digestive tract | 0.4 | 2.8 | 105 |
| Notochord | 0.8 | 5.1 | 460 |
| Snout cartilage | 0.03 | 0.2 | 2 |

### 3.2. SDS–PAGE

The SDS–PAGE patterns of collagens are shown in Fig. 1. Collagen from scales, skin, muscle, swim bladder and digestive tract consisted of two α-chains (ca. 120 and 100 kDa) as the major constituents, and the density of the 120-kDa band (α1-chain) was higher than that of the 100-kDa band (α2-chain), at a ratio of approximately 2:1 (scale 2.1:1, skin 2.1:1, muscle 2.0:1, swim bladder 2.0:1, digestive tract 2.1:1). The molecular weight of these bands, as well as the band pattern, suggest that these collagens are most likely to be classified as type I collagen. On the other hand, the ratio which is little different from 2:1 might show the possibility of the presence of other type collagen in the extracted collagens, such as the type III collagen which is present in skin and intestines and the type V collagen which is present in tissues where type I collagen is expressed (Parenteau-Bareil et al., 2010; Wang et al., 2014). In addition, the α3-chain has been reported to exist in skin type I collagen of white sturgeon, Bester sturgeon, and many other teleost fish (Kimura, 1992). However, it could not be determined whether the α3-chain existed in the collagens in this study, because the migration similarity of the α3 and α1 chains prevents separation of the former from the latter using SDS–PAGE (Kimura, 1992). To determine whether these collagens contain the α3-chain, CM-cellulose chromatography should be used (Kimura & Ohno, 1987). Notochord collagen and snout cartilage collagen of the Bester sturgeon consisted of only one α-chain as the major constituent (Fig. 1), which may be classified as type II collagen, as type II collagen comprises three identical α chains (Foegeding, Lanier, & Hultin, 1996; Kittiphattanabawon, Benjakul, Visessanguan, & Shahidi, 2010). Miller and Mathews (1974) also reported the collagen from notochord and cartilage of sturgeon is comprised of a single type of α-chain, which was similar to α1 (II) chain of higher vertebrates.

### 3.3. Amino acid composition

Amino acid compositions of Bester collagens are shown in Table 2. All collagens had glycine as the major amino acid, and were also rich in alanine, proline and hydroxyproline. Generally, glycine in collagen represents approximately one third of the total residues and occurs as every third residue in collagen molecules, except for the telopeptide regions (first 14 amino acid residues from the N-terminus and the last 10 of the C-terminus) (Singh et al., 2011; Zhang et al., 2009).

For type II collagen of the notochord and snout cartilage, the glutamic acid, leucine, hydroxylysine, and imino acid (proline+hydroxyproline) contents were higher than those of type I collagens, and yet the contents of serine, alanine, and lysine were lower (Table 2). In addition, the degree of hydroxylation of lysine was calculated to be 62% in notochord collagen and 56% in snout cartilage (Table 3), which was much higher than those in type I collagens such as scale (25%) and skin (29%) collagens. Seyer and Vinson (1974) reported that the high degree of lysine hydroxylation was one of the criteria to identify type II collagen from type I collagen. Therefore, notochord collagen and snout cartilage collagen are thought to be type II collagen, in accordance with the result of SDS–PAGE.

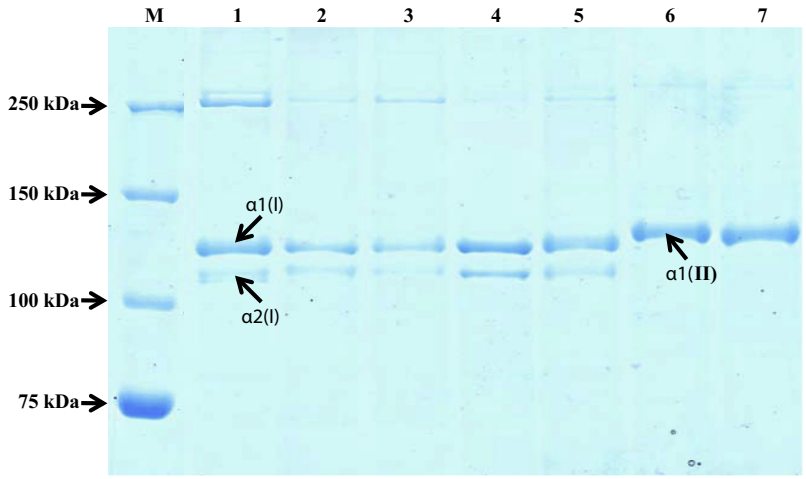


**Fig. 1.** SDS–PAGE of collagens from Bester sturgeon. M, high molecular weight marker; lane 1: scale collagen; lane 2: skin collagen; lane 3: muscle collagen; lane 4: swim bladder collagen; lane 5: digestive tract collagen; lane 6: notochord collagen; lane 7: snout cartilage collagen.

**Table 2**
Amino acid composition of collagens from Bester sturgeon (expressed as residues/1000 total amino acid residues).

| | Scale | Skin | Muscle | Swim bladder | Digestive tract | Notochord | Snout cartilage |
|---|---|---|---|---|---|---|---|
| Asp | 48 | 47 | 48 | 45 | 48 | 48 | 48 |
| Thr | 23 | 24 | 25 | 26 | 23 | 21 | 20 |
| Ser | 42 | 43 | 42 | 42 | 43 | 31 | 34 |
| Glx | 76 | 69 | 78 | 77 | 76 | 96 | 86 |
| Gly | 340 | 344 | 330 | 335 | 340 | 322 | 333 |
| Ala | 116 | 121 | 118 | 121 | 110 | 95 | 102 |
| Cys/2 | 2 | 2 | 2 | 1 | 1 | 1 | 1 |
| Val | 17 | 17 | 19 | 17 | 17 | 16 | 16 |
| Met | 9 | 9 | 9 | 10 | 10 | 8 | 8 |
| Ile | 11 | 11 | 13 | 12 | 11 | 11 | 10 |
| Leu | 17 | 17 | 19 | 16 | 19 | 29 | 26 |
| Tyr | 2 | 1 | 2 | 2 | 2 | 2 | 2 |
| Phe | 11 | 12 | 13 | 12 | 12 | 13 | 12 |
| HyLys | 9 | 10 | 10 | 13 | 13 | 24 | 22 |
| Lys | 27 | 25 | 26 | 23 | 22 | 15 | 17 |
| His | 4 | 4 | 4 | 4 | 4 | 5 | 5 |
| Arg | 53 | 52 | 51 | 54 | 52 | 51 | 52 |
| Hypro | 76 | 78 | 80 | 88 | 82 | 81 | 87 |
| Pro | 117 | 114 | 111 | 102 | 115 | 131 | 119 |
| Imino acid | 193 | 192 | 191 | 190 | 197 | 212 | 206 |

**Table 3**
Hydroxylation degree of lysine residues of collagens from the Bester sturgeon (expressed as residues/1000 total amino acid residues).

| | Scale | Skin | Muscle | Swim bladder | Digestive tract | Notochord | Snout cartilage |
|---|---|---|---|---|---|---|---|
| Lys | 27 | 25 | 26 | 23 | 22 | 15 | 17 |
| HyLys | 9 | 10 | 10 | 13 | 13 | 24 | 22 |
| Hydroxylation | 25% | 29% | 28% | 36% | 37% | 62% | 56% |

### 3.4. Thermal stability

CD spectra of the collagens from the scales, skin, muscle, swim bladder, digestive tract and notochord of Bester sturgeon are shown in Fig. 2a. All collagens showed a rotatory maximum at 221 nm and a crossover point (zero rotation) at about 212 nm, which are typical characteristics of the collagen triple helical conformation (Ikoma et al., 2003). Mensuration of collagen denaturation temperature is shown in Fig. 2b. When the temperature rose, the CD [221] values decreased due to the decomposition of the collagen triple helical structure. Denaturation temperature (Td) was determined as the temperature with the fastest decreased speed of the CD [221] value. Denaturation temperatures of Bester sturgeon were as follows: scale collagen 29.6 °C, skin collagen 26.8 °C, muscle collagen 29.0 °C, swim bladder collagen 32.9 °C, digestive tract collagen 31.6 °C, and notochord collagen 36.3 °C. This suggests that the inside tissue possesses a higher Td. In the same species, type II collagen seems to have the higher Td than type I collagen (Amudeswari, Liang, & Chakrabarti, 1987). Thus, the highest Td in the notochord collagen may be attributed to its collagen type (type II). However, for type I collagen, the reason why inside tissue has a higher Td is not known. We could not measure Td of snout cartilage because the amount of purified collagen from this organ was too small. The Tds of type I collagens in fish species are positively correlated with their preferred environmental water temperature (Singh et al., 2011).

The Td of notochord collagen was higher than some tropical fish type I collagens, as described above, and similar to those of

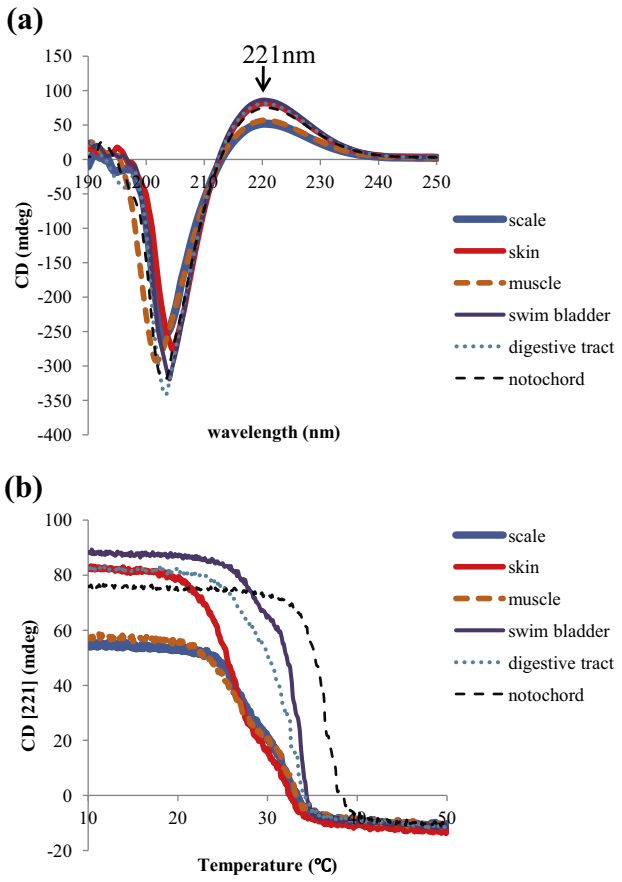


**Fig. 2.** CD spectra (a) and temperature effect on the CD spectra at 221 nm, (b) of the collagens from scale, skin, muscle, swim bladder, digestive tract and notochord of Bester sturgeon.

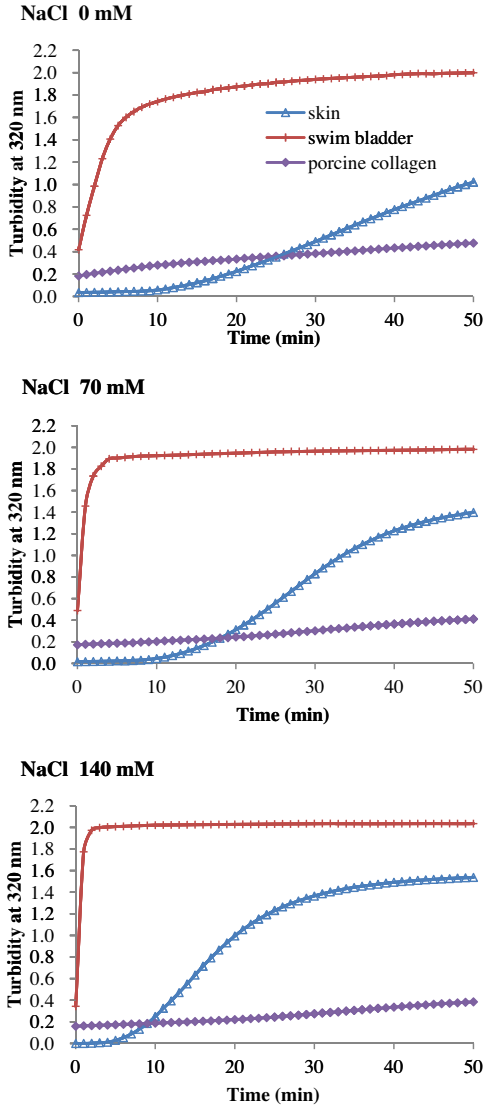


**Fig. 3.** Effects of NaCl concentrations on the *in vitro* progress of fibril formation of skin and swim bladder collagen of Bester sturgeon and porcine collagen.

collagens in brownbanded bamboo shark and blacktip shark cartilages (34.56–36.73 °C), which were suggested to be a mixture of type I and II collagens (Kittiphattanabawon et al., 2010). However, the Td of notochord collagen was lower than calf skin type I collagen (40.8 °C) (Duan et al., 2009) and chick sternal cartilage type II collagen (43.8 °C) (Cao & Xu, 2008). Differences in Tds amongst collagens of different species were reported to be correlated to the different imino acid contents (proline and hydroxyproline) (Kittiphattanabawon et al., 2005). The mechanism is assumed as proline and hydroxyproline rich zones of collagen molecules that are most likely involved in the formation of junction zones that help to strengthen the triple helix structure (Singh et al., 2011).

### 3.5. Process of collagen fibril formation in vitro

Collagen molecules have the ability to self-assemble from solution, and it is now generally accepted that all of the information needed for fibril formation is contained in the collagen molecules themselves (Helseth & Veis, 1981). In this study, the fibril-forming ability of skin and swim bladder type I collagen was studied, as the yields of these two collagens were much higher than others. Therefore, the production of collagen from these tissues on an industrial scale may be possible. Fibril formation was monitored by the increase in turbidity at 320 nm (Fig. 3). For type I collagen, swim bladder collagen showed a more rapid rate of increase in turbidity, and a shorter time to attain the maximum turbidity than skin collagen at the same NaCl concentration. There was no lag time of fibril formation in swim bladder collagen, which suggests a high speed of nucleation when compared with skin collagen. Additionally, swim bladder and skin collagen both showed a rapid rise in turbidity compared with porcine tendon type I collagen. Thus, it can be concluded that sturgeon type I collagen had a higher ability of fibrillogenesis than porcine collagen in the present experimental conditions.

Compared with the fibril formation rate of porcine tendon collagen, those of swim bladder and skin collagen increased with increasing ionic strength by the addition of NaCl (Fig. 3), which suggests that NaCl concentration had a more pronounced effect on the process of collagen fibrillogenesis in sturgeon. In contrast, the rates of fibril formation of red stingray skin type I collagen and salmon skin type I collagen were suppressed by high NaCl concentrations (Bae et al., 2009; Yunoki, Nagai, Suzuki, & Munekata, 2004). Different characteristics of collagen fibril formation may be explained in part by species differences. In addition, they may also be affected by the method of purification of the collagen, the solvent system employed in the fibrillogenesis experiment, and the condition under which fibril formation is initiated (Williams, Gelman, Poppke, & Piez, 1978). Fibrillogenesis appears to be an

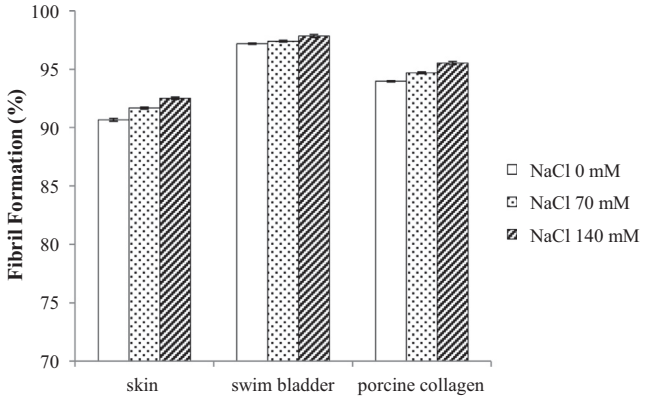


**Fig. 4.** Effects of NaCl concentrations on degree of fibril formation of skin collagen and swim bladder collagen of Bester sturgeon and porcine collagen. Error bars show standard deviation.

entropy-driven self-assembly process, which is driven by the loss of solvent molecules from the surface of protein molecules and result in assemblies with a circular cross-section, which minimizes the surface area/volume ratio of the final assembly (Kadler et al., 1996). As different NaCl concentration resulted in the different ionic strength of solution which may affect the assembly process of collagen molecules, leading to different speed of collagen formation. On the other hand, in the lag phase, during which no measurable change occurs in turbidimetric analysis, was interpreted as collagen self-assembly involved in a nucleation mechanism analogous to the formation of crystals from a supersaturated inorganic salt solution (Helseth & Veis, 1981). The general principles of collagen fibril formation are accepted, yet molecular mechanism of the assembly process is less known.

### 3.6. Degree of collagen fibril formation

The degrees of collagen fibril formation of Bester sturgeon were assessed after 24 h of fibrillogenesis, and the results are shown in Fig. 4. The degree of skin collagen fibril formation ranged from 91% to 93%, and was slightly increased by increasing NaCl concentration. The degree of swim bladder collagen fibril formation ranged from 97% to 98%, was higher than that of skin collagen and porcine collagen, and also slightly increased by increasing NaCl concentration. In a study of red stingray collagen, the degree of fibril formation was also slightly increased with increasing NaCl concentration (Bae et al., 2009). In contrast to these collagens, the degree of salmon skin collagen fibril formation was decreased by increasing NaCl concentration (Yunoki et al., 2004). Taken together, the results from sturgeon collagen and the reported results from stingray and salmon skin reveal that the degree of collagen fibril formation varies greatly depending on the source of collagen.

### 3.7. SEM observation of collagen fibrils

SEM images of skin, swim bladder, and porcine tendon collagen fibrils after 1 h of incubation are illustrated in Fig. 5. The unordered structural appearance of fibrils in sturgeon collagen indicates that fibrillogenesis occurs in all collagens and solutions. Sturgeon collagen showed a similar fibril character, and the fibrils seemed to become thicker with increasing NaCl concentration. For porcine collagen, only a few fibrils were observed in the images at 140 mM NaCl (e.g. Fig. 5i), suggesting a much lower fibril-forming ability of porcine collagen than sturgeon collagen in a short time.

SEM images of skin collagen, swim bladder collagen and porcine tendon collagen fibrils after 24 h incubation are illustrated in Fig. 6. Fibrils of skin collagen showed an unordered structure and were

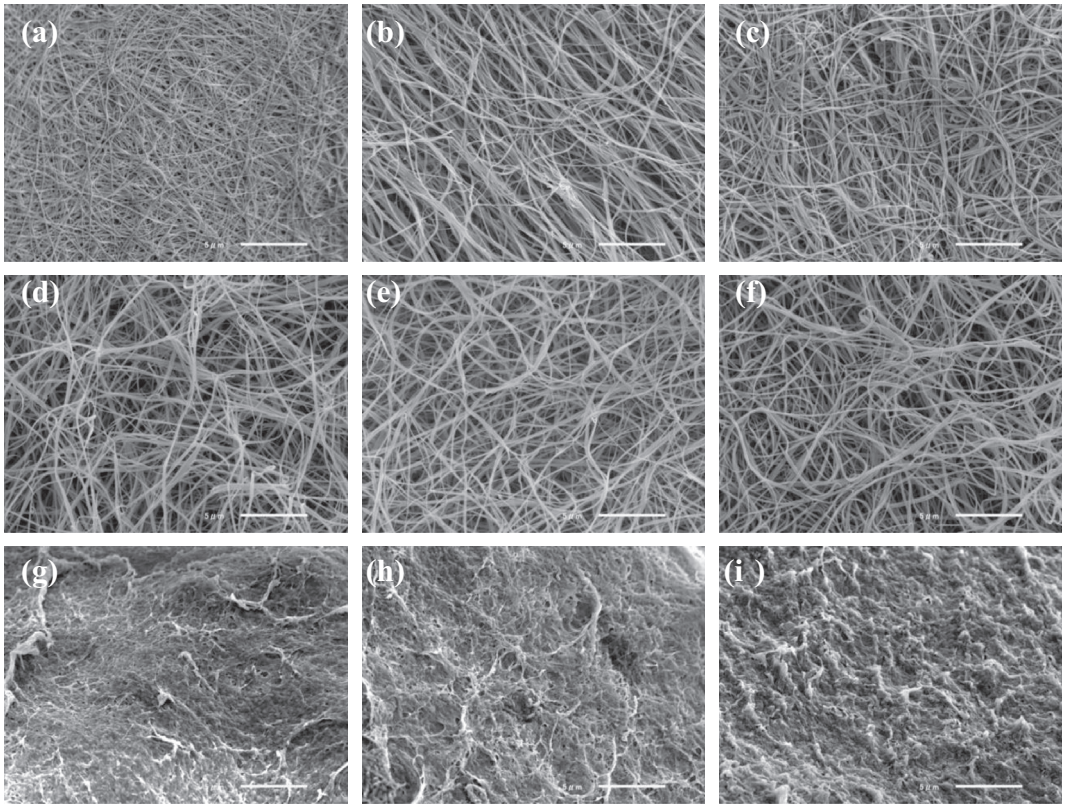


**Fig. 5.** Scanning electron micrographs of fibrils formed at 21 ± 1 °C for 1 h. (a–c) sturgeon skin with NaCl concentration of 0, 70 and 140 mM; (d–f) sturgeon swim bladder with NaCl concentration of 0, 70 and 140 mM; (g–i) porcine collagen with NaCl concentration of 0, 70 and 140 mM. Scale bars, 5 μm.

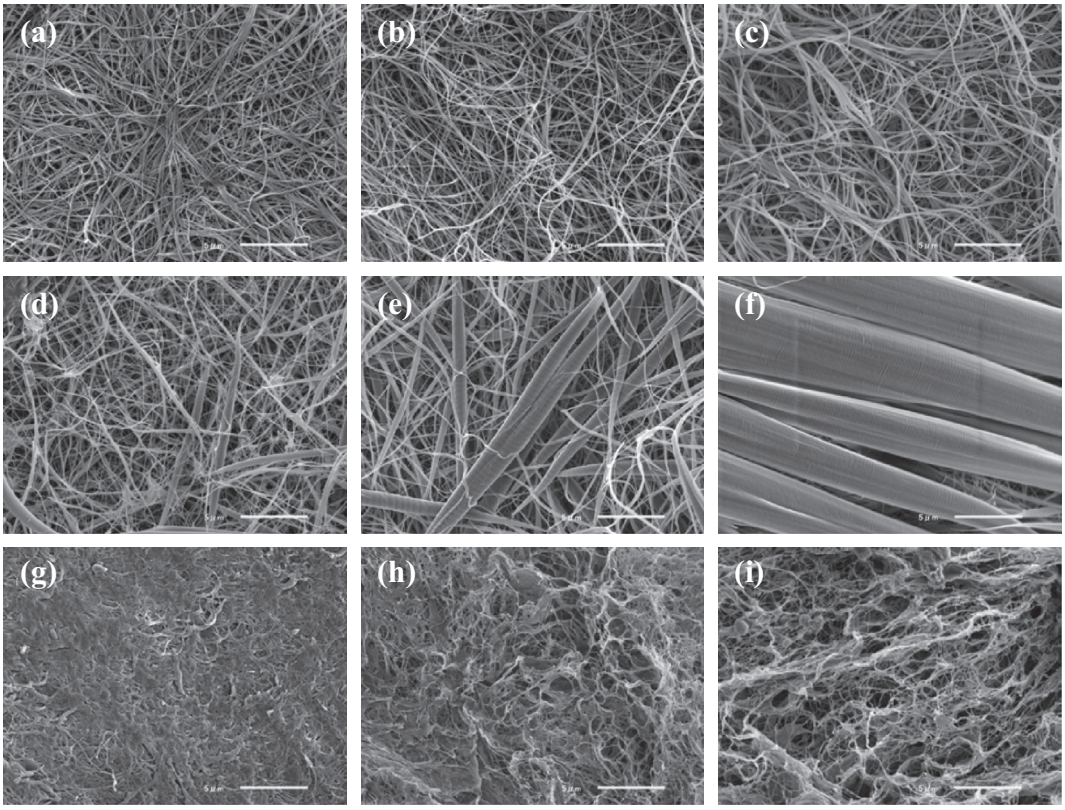


**Fig. 6.** Scanning electron micrographs of fibrils formed at 21 ± 1 °C for 24 h. (a–c) sturgeon skin with NaCl concentration of 0, 70 and 140 mM; (d–f) sturgeon swim bladder with NaCl concentration of 0, 70 and 140 mM; (g–i) porcine collagen with NaCl concentration of 0, 70 and 140 mM. Scale bars, 5 μm.

thicker than the fibrils from 1 h incubation (Fig. 6a–c). For swim bladder collagen, some fusiform structures characterised by a transverse periodic banding pattern (one unit width ≈0.45 μm) were observed (Fig. 6e). When NaCl concentration was increased, more fibrils formed the bigger fusiform structures. At 140 mM NaCl, almost all the fibrils formed bigger fusiform structures, and the average width of a unit's banding pattern became narrower (one unit width ≈0.22 μm) (Fig. 6f). In porcine collagen, a few fibrils only were observed at 0 mM NaCl (Fig. 6g). At 70 and 140 mM NaCl (Fig. 6h and i), the fibrils were obvious. However, the fibrils were more slender than those from skin and swim bladder collagens. Thus, sturgeon type I collagens may have a higher fibril-forming ability than type I porcine collagen.

In sturgeon skin and swim bladder collagen, the effect of increasing NaCl concentration on fibril formation is an increase in the thickness of the fibrils. In swim bladder collagen, increasing NaCl concentration promotes a characteristic fusiform structure after 24 h incubation. The formation process of the fusiform structure was not clarified in the present experiments. However, some SEM images appeared to show the fusion of several fibrils into a fusiform fibril. Therefore, our working hypothesis is as follows: first, collagen is assembled to form fibrils, then several fibrils are fused into one fusiform structure. The reason for the construction of the banding pattern is unknown. The fact that a fusiform structure was observed only after 24 h incubation suggests that fusion of collagen fibrils takes longer than fibril formation itself. Although the reason why only swim bladder collagen has the ability to form a fusiform structure is not clear, it may relate to the ability of rapid fibril formation in this collagen.

There are some studies in terrestrial vertebrates on the morphology of collagen fibrils formed *in vitro*, such as type I collagens from calf skin (Holmes, Capaldi, & Chapman, 1986), rat tail tendon (Williams et al., 1978), and chick embryo tendon (Miyahara, Njieha, & Prockop, 1982). However, there are no data on the effects of NaCl concentrations on the morphology of fibrils. Similarly, for fish fibrils, there are a few morphological studies only, such as on type I collagen from salmon skin (Yunoki et al., 2004), shark skin (Nomura, Toki, Ishii, & Shirai, 2000) and red stingray skin (Bae et al., 2009), and no data on the effects of NaCl concentrations on the morphology of formed fibrils. Therefore, this study is possibly the first to clarify the effects of NaCl concentrations on the detailed morphology of fibrils of fish type I collagen formed *in vitro*. Moreover, to the best of our knowledge, this is the first report about the fusiform structure of collagen fibrils. The effect of NaCl and/or ionic strength on the morphology of collagen fibrils formed *in vitro* is worthy of study when it is intended to apply collagens for medical purposes such as a cell culture system and tissue engineering, because collagen morphology of the scaffold will affect cellular responses. Further assessments on the biomechanical strength of collagen gels, as well as cellular responses against the gels, are needed to industrialise biomaterials made of sturgeon collagens.

## 4. Conclusion

This study assessed the biochemical nature of collagens purified from the Bester sturgeon *H. huso* × *A. ruthenus*. In addition, details of the fibril-forming ability and morphology of fibrils formed *in vitro* were clarified. A quantity of collagens could be extracted from skin, swim bladder and the notochord of Bester sturgeon. Collagens from scales, skin, muscle, the swim bladder and digestive tract were characterised as type I collagen, and collagens from the notochord and snout cartilage characterised as type II collagen. Sturgeon collagens have two characteristic features: relatively higher thermal stabilities compared with other fish species, and a better fibril-forming ability in skin and swim bladder type I

collagen compared with mammalian type I collagen. The big fusiform fibril structures of swim bladder collagen suggest its special utility for enhancing mechanical strength of collagen-based biomaterials. Such high thermal stability and high ability of fibril formation will open a new field of industrial utilisation for sturgeon collagens as biomaterials, which may increase the economic value of sturgeon and accelerate aquaculture development of this fish.

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## References

Amudeswari, S., Liang, J. N., & Chakrabarti, B. (1987). Polar-apolar characteristics and fibrillogenesis of glycosylated collagen. *Collagen and Related Research, 7*, 215–223.

Bae, I., Osatomi, K., Yoshida, A., Yamaguchi, A., Tachibana, K., Oda, T., et al. (2009). Characteristics of a self-assembled fibrillar gel prepared from red stingray collagen. *Journal of Fisheries Science, 75*, 765–770.

Cao, H., & Xu, S. Y. (2008). Purification and characterization of type II collagen from chick sternal cartilage. *Food Chemistry, 108*, 439–445.

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

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

Helseth, D. L., & Veis, A. (1981). Collagen self-assembly *in vitro*: Differentiating specific telopeptide-dependent interactions using selective enzyme modifications and the addition of free amino telopeptide. *Journal of Biological Chemistry, 256*, 7118–7128.

Holmes, D. F., Capaldi, M. J., & Chapman, J. A. (1986). Reconstitution of collagen fibrils *in vitro*; the assembly process depends on the initiating procedure. *International Journal of Biological Macromolecules, 8*, 161–166.

Ikoma, T., Kobayashi, H., Tanaka, J., Walsh, D., & Mann, S. (2003). Physical properties of type I collagen extracted from fish scales of *Pagrus major* and *Oreochromis niloticus*. *International Journal of Biological Macromolecules, 32*, 199–204.

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

Jongjareonrak, A., Benjakul, S., Visessanguan, W., & Tanaka, M. (2005a). Isolation and characterisation of collagen from bigeye snapper (*Priacanthus marcracanthus*) skin. *Journal of the Science of Food and Agriculture, 85*, 1203–1210.

Kadler, K. E., Holmes, D. F., Trotter, J. A., & Chapman, J. A. (1996). Collagen fibril formation. *Journal of Biochemisty, 316*, 1–11.

Kimura, S. (1992). Wide distribution of the skin type I collagen a3 chain in bony fish. *Comparative Biochemistry and Physiology, 102B*(2), 255–260.

Kimura, S., & Ohno, Y. (1987). Fish type I collagen: Tissue-specific existence of two molecular forms, (a1)2a2 and a1a2a3 in Alaska Pollack. *Comparative Biochemistry and Physiology, 88B*(2), 409–413.

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

Kittiphattanabawon, P., Benjakul, S., Visessanguan, W., & Shahidi, F. (2010). Isolation and characterization of collagen from the cartilages of brownbanded bamboo shark (*Chiloscyllium punctatum*) and blacktip shark (*Carcharhinus limbatus*). *Food Science and Technology, 43*, 792–800.

Laemmli, U. K. (1970). Cleavage of structural proteins during assembly of head of bacteriophage T4. *Nature, 277*, 680–685.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Miller, E. J., & Mathews, M. B. (1974). Characterization of notochord collagen as a cartilage-type collagen. *Biochemical and Biophysical Research Communications, 60*, 424–430.

Miyahara, M., Njieha, F. K., & Prockop, D. J. (1982). Formation of collagen fibrils *in vitro* by cleavage of procollagen with procollagen proteinases. *Journal of Biological Chemistry, 257*, 8442–8448.

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

Nagai, T., & Suzuki, N. (2000). Isolation of collagen from fish waste material-skin, bone and fins. *Food Chemistry, 68*, 277–281.

Nomura, Y., Toki, S., Ishii, Y., & Shirai, K. (2000). Improvement of the material property of shark type I collagen by composing with pig type I collagen. *Journal of Agricultural and Food Chemistry, 48*, 6332–6336.

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

Seyer, J. M., & Vinson, W. C. (1974). Synthesis of type I and type II collagen by embryonic chick cartilage. *Biochemical and Biophysical research communications, 58*(1), 272–279.

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

Wang, L., Liang, Q., Wang, Z., Xu, J., Liu, Y., & Ma, H. (2014). Preparation and characterisation of type I and V collagens from the skin of Amur sturgeon (*Acipenser schrenckii*). *Food Chemistry, 148*, 410–414.

Wei, Q. W., Zou, Y., Li, P., & Li, L. (2011). Sturgeon aquaculture in China: Progress, strategies and prospects assessed on the basis of nation-wide surveys (2007–2009). *Journal of Applied Ichthyology, 27*, 162–168.

Williams, B. R., Gelman, R. A., Poppke, D. C., & Piez, K. A. (1978). Collagen fibril formation. *Journal of Biological Chemistry, 253*, 6578–6585.

Yunoki, S., Nagai, N., Suzuki, T., & Munekata, M. (2004). Novel biomaterial from reinforced salmon collagen gel prepared by fibril formation and cross-linking. *Journal of Bioscience and Bioengineering, 98*, 40–47.

Zeng, S., Yin, J., Yang, S., Zhang, C., Yang, P., & Wu, W. (2012). Structure and characteristics of acid and pepsin-solubilized collagens from the skin of cobia (*Rachycentron canadum*). *Food Chemistry, 135*, 1975–1984.

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

Zhang, X., Shimoda, K., Ura, K., Adachi, S., & Takagi, Y. (2012). Developmental structure of the vertebral column, fins, scutes and scales in bester sturgeon, a hybrid of beluga *Huso huso* and *sterlet Acipenser ruthenus*. *Journal of Fish Biology, 81*, 1985–2004.