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*Note: The text in the table is in Japanese.*
Properties of collagen extracted from Amur sturgeon *Acipenser schrenckii* and assessment of collagen fibrils *in vitro*

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Abstract

The objective of this study was to assess the nature of the collagens from the Amur sturgeon to determine its possibility as a potential collagen source for biomedical applications. From a sturgeon (1.22 kg), 6.0 g (dry wt) of skin collagen (SC), 4.1 g of swim bladder collagen (SBC), and 0.4 g of notochord collagen (NC) were obtained. SC and SBC were characterized as type I, and NC as type II collagen. Denaturation temperatures of SC, SBC, and NC were calculated as 28.5, 30.5, and 33.5°C, respectively. Gene expression of the type I procollagen α2 chain of Amur sturgeon (ascollα2) was specifically higher than ascollα1 expression in the swim bladder, suggesting a unique composition of α chains in this organ. SC and SBC had better abilities of fibril formation with unique higher-order structures compared with porcine type I collagen. The maximum transition temperature (Tm) of reassembled fibrils formed in a buffer solution containing NaCl at 0 and 140 mM was 34.4°C and 38.9°C in SC, and 40.1°C and 40.7°C in SBC, respectively. These characteristic features suggested that sturgeon collagens could be used in the biomedical industries in future applications.

Keywords: Biochemical characteristics; Collagen fibril; Gene
1. Introduction

As one of the major proteins in animals, collagen accounts for approximately 30% of total protein [1]. At present, there are at least 29 different types of collagen named type I–XXIX, which play different roles in tissues, with each type having a distinctive amino acid sequence and molecular structure [2]. Among these, type I collagen is widely distributed in connective tissues, such as skin, tendon, and ligament, which is the most abundant collagen in vertebrate tissues. Collagen has been widely used in the food and cosmetics industries, medical research, and tissue engineering because it shows low antigenic activity, high cell adhesion properties, and biocompatibility [3]. However, concerns about the use of terrestrial animal collagens are growing because of the risk of contamination with diseases that are infectious to humans.

In recent years, fish collagen has received increasing attention. Its advantages include availability because of the high abundance of fish offal, as well as avoiding zoonosis and dietary/religious objections [4]. In addition, the components of collagen which possess immunogenicity, namely, telopeptides, can be removed by the pepsin during collagen extract [5]. Collagen without telopeptides is named atelocollagen, has an excellent safety profile for biomaterial use [5, 6]. Thus, fish atelocollagen is expected to have low antigenicity with less risk of pathogen infection, which would be an alternative to collagens of terrestrial animals for use in food, cosmetics, and biomedical materials [7].

Biochemical properties of collagens extracted from fish skin, scales, and bones have been
reported in many species [3, 4, 8-10]. In our previous study, collagens purified from Bester sturgeon showed high thermal stability and high ability of fibril formation [11]. Especially, type I collagen extracted from swim bladder (SBC) has excellent characteristics such as high solubility, low viscosity, and extremely fast kinetics to form large fiber bundles under certain conditions. On this basis, Mredha et al. [6] created stable, disk-shaped hydrogels with a concentric orientation of collagen fibers by controlled diffusion of neutral buffer through collagen solution at room temperature. The SBC hydrogels with thermal and mechanical stability suggest potentials as biomaterials for tissue engineering applications. However, as Bester sturgeon is a hybrid species, not cultured on a large-scale in the world, the industrial production of its collagens is limited.

In this study, we focused on another species, Amur sturgeon *Acipenser schrenckii*, which is widely cultured in the world because of its considerable economic and ecological value [12]. This study was designed to supply basic information of the collagens from the Amur sturgeon to determine its possibility as a potential collagen source for biomedical applications. Therefore, the present study provided information regarding the 1) biochemical nature, 2) fibril-forming ability of the Amur sturgeon type I atelocollagen, and 3) thermal stability of the atelocollagen molecules and the fibrils. In addition, 4) the primary structure of a proα chain and compositional differences of proα chains of type I collagen in the Amur sturgeon organs were also studied.
2. Materials and methods

2.1. Isolation and purification of collagen

Live aquacultured Amur sturgeon (0.67 m, 1.22 kg) were procured from the Nanae Fresh-Water Laboratory, Field Science Center for Northern Biosphere, Hokkaido University, Japan. There is no legal conservation or animal welfare restrictions for usage of live aquacultured sturgeon in Japan, Hokkaido, or Hokkaido University. Nevertheless, we humanely treated the live sturgeon, using deep anesthetic (2-phenoxyethanol solution) before dissection. Skin, swim bladder, and notochord were dissected out and stored at -30°C until use.

The collagen was extracted as previously described with slight modifications. Samples were washed with cold water (4°C) and cut into small pieces (0.5 × 0.5 cm). Fat of the skin was removed in 99.5% ethanol with a sample/solution ratio of 1:20 for 24 h with three changes of solution. The defatted skin was washed with cold water for 24 h. Then the samples of defatted skin, swim bladder, and notochord were continuously stirred in aqueous solution of HCl (pH 2.0) containing 0.5% (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 72 h at 4°C to extract collagens. The mixtures were centrifuged at 2,000 × g for 1 h to obtain the supernatants, and the residues of skin and notochord were re-extracted using the same conditions. Then the supernatants were sequentially filtered using 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 resultant precipitate was collected by centrifugation at 2,000 × g at 4°C for 90 min and dissolved in an aqueous HCl solution (pH 2.0). This process was repeated three times to purify the collagen. The extracted 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-drying (FDU-830; Tokyo Rikakikai Co., Ltd., Tokyo, Japan).

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

SDS-PAGE was performed according to the method of Laemmli [13] and Zhang et al. [11]. The lyophilized collagens were dissolved in an aqueous HCl solution (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. Electrophoresis was performed on a 7.5% running gel. After electrophoresis, the gel was stained for 30 min using 0.1% Coomassie Brilliant Blue R250 solution. Type I collagen from the porcine tendon (Cellmatrix Type I-A; Nitta Gelatin Inc., Osaka, Japan), tilapia scale (Ti-03A; Taki Chemical Co., Ltd, Kakogawa, Japan), and type II collagen from chicken cartilage (Nippon Meat Packers, Inc., Osaka, Japan) were used as references. Precision Plus Protein Standards (Bio-Rad Laboratories, Inc., Hercules, CA,
USA) were used to estimate the molecular weight.

2.3. Amino acid analysis

The amino acid composition was analyzed at Instrumental Analysis Division, Equipment Management Center Creative Research Institution, Hokkaido University. In brief, samples were hydrolyzed 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 analyzed using an automated amino acid analyzer (JLC-500V; JEOL Ltd., Tokyo, Japan). Samples were assayed three times, and the averages were used to obtain amino acid compositions.

2.4. Circular dichroism (CD) measurement

CD spectra were measured using a JASCO model 725 spectrometer (JASCO, Tokyo, Japan), according to the method of Zhang et al. [11]. 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 denaturation temperature (Td) of the collagen molecule. Td was determined at the temperature with the fastest decreased speed of the CD [221] value.
2.5. Quantification of glycoside contents

Glycoside contents of collagens were performed by ProteNova Co. Ltd., Kagawa, Japan, according to the method of Yasuno et al. [14] with slight modifications. One mg lyophilized collagens were dissolved in 500 μL distilled water, then 50 μL dissolved collagen solution was hydrolyzed in 50 μL 8 M trifluoroacetic acid (TFA) at 100°C for 3 h. After acetylation by acetic anhydride, the solution was labeled by ABEE (p-aminobenzoic ethyl ester) reagent. ABEE-labeled monosaccharide solution was injected and analyzed by high-performance liquid chromatography (BioAssist eZ; Tosoh Corp., Tokyo, Japan). A PN-PAK C18 column (3.0 × 75 mm) (Protenova Co., Ltd., Takamatsu, Japan) was used at a flow rate of 0.5 mL/min with 0.2 M potassium borate buffer (pH 9.0)/acetonitrile solvent at 45°C, and detected using a fluorescence monitor (an excitation of 305 nm and emission of 360 nm).

2.6. Molecular cloning and gene expression analysis of the procollagen α2(I) gene in Amur sturgeon (ascollα2)

A live cultured Amur sturgeon (6.4 cm length, 0.97 g weight, 2 months old) was procured from the Nanae Fresh-Water Station, Field Science Center for Northern Biosphere, Hokkaido University, Japan. The fish was deeply anesthetized in 2-phenoxyethanol solution. Tissue samples were obtained and stored in -80°C until RNA extraction. Total RNA was extracted...
from the tissues followed by the methods in our previous study [15]. The cDNA from the skin was used to clone \textit{ascoli}la2. The PCR-based cloning strategy is shown in Fig. 1. The sequences and location of primers used for cloning are shown in Table 1. Molecular cloning of \textit{ascoli}la2 followed the method of \textit{ascoli}la1 used in our previous study [15].

To compare the gene expression levels of \textit{ascoli}la2 in the notochord, vertebral cartilage, snout cartilage, skin, scale, swim bladder, muscle, stomach, intestine, and fin, quantitative real-time PCR (qPCR) was used. Total RNA was extracted from each organ from three individuals of 3-month-old Amur sturgeons. Sample treatment and RNA extraction were conducted similarly to that for molecular cloning except for the tissue preservation methods, which used the RNAlater reagent (Life Technologies, Santa Cruz, CA, USA). Reverse transcription was performed using a Takara Prime Script RT reagent kit with gDNA Eraser Perfect Real Time (Takara Bio, Otsu, Japan). The PCR primers for a2qF (5$'$-AGG AGT CTG CAT GTC TAG TCT G-3$'$) and a2qR (5$'$-TGC AGG TCC AGG GGT ACC AGG TTC-3$'$) were used for \textit{ascoli}la2. To normalize the expression level, \(\beta\)-actin was used as a reference gene. Forward (5$'$-GAG GCT CAG AGC AAG AGA GGT ATC-3$'$) and reverse (5$'$-TTG AAG GTC TCA AAC ATG ATC TGG-3$'$) primers were used for measuring \(\beta\)-actin expression. Each 20-\(\mu\)L reaction contained cDNA corresponding to approximately 5 ng total RNA, 7 \(\mu\)L of FastStart Universal SYBR Green Master (Rox) (Roche, Basel, Switzerland) and 7.5 pmol of each primer. The specific amplification for each primer pair was confirmed by dissociation
curve analysis at the end of the PCR reaction. The qPCR was performed twice for each cDNA sample on a StepOne Plus Real-Time PCR instrument (Applied Biosystems, Foster City, CA, USA).

2.7. Collagen fibril formation in vitro

Collagen molecules can self-assemble into fibrils in appropriate solution [16]. In this study, the fibril formation of type I collagen from the skin and swim bladder was performed according to the method of Zhang et al. [11]. In brief, lyophilized collagens were dissolved in an aqueous HCl solution (pH 3.0) to 0.3% (w/v). The collagen solution was mixed with 0.1 M Na$_2$HPO$_4$ buffer (pH 7.4) containing NaCl at 0, 70, and 140 mM, at the ratio of the collagen solution/buffer of 1:2 (v/v). The resulting fibril formation at $21 \pm 1^\circ$C was monitored by absorbance at 320 nm as the turbidity change using a spectral monitor, GeneQuant pro (GE Healthcare Life Sciences, Tokyo, Japan). This measured the speed of collagen fibril formation in a short time.

2.8. Morphology of fibrils formed in vitro

The morphology of collagen fibrils was observed using a scanning electron microscope (SEM; JSM6010LA, JEOL Ltd.), as previously described by Zhang et al. [11] with slight modifications. The collagen fibrils were formed using the same conditions as described above.
for 1 h and 24 h at 21 ± 1°C. The sample solutions of skin and swim bladder collagens were
centrifuged at 20,000 × g for 20 min, and that of notochord was centrifuged for 1 h, to get
precipitates of collagen fibrils. Then, collagen fibrils were fixed with 2.5% (v/v)
glutaraldehyde in 0.1 M Na₂HPO₄ buffer (pH 7.6) for 3 h at room temperature, and then
rinsed with the same buffer. The fibrils were sequentially dehydrated in 70, 80, 90, 95, and
100% ethanol solutions for 30 min, and then in two 30-min changes of t-butyl alcohol.
Finally, collagen fibrils were freeze-dried in t-butyl alcohol 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, type II collagen from chicken cartilage (Nippon Meat Packers, Inc.)
was treated similarly and observed.

2.9. Morphology of collagen fibrils in vivo

Tissues of skin and swim bladder of Amur sturgeon were also treated to observe the
collagen fibrils in vivo. Skin and swim bladder were dissected out from another Amur
sturgeon (0.67 m, 1.1 kg) and cut into small pieces. After washing in distilled water, the
pieces were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M Na₂HPO₄ buffer (pH 7.6) and the
same process was used as for fibrils formed in vitro. After freeze-drying in t-butyl alcohol
with a freeze-drying device, the tissue surface was peeled off by forceps to expose the inner
part (dermal dense layer of skin), then coated with gold-platinum.
2.10. Thermal stability of reassembled fibrils

The maximum transition temperature (Tm) of reassembled fibrils of skin and swim bladder collagens was measured by differential scanning calorimetry (EXSTAR DSC6100; SII NanoTechnology Inc., Chiba, Japan). Collagen fibrils were prepared using the same conditions as described above for collagen fibril formation in vitro. The sample solutions were centrifuged at 20,000 × g for 30 min to get precipitates of collagen fibrils. The precipitates (about 15–20 mg wet weight) were placed in an Al pan (70 μL), and measured at 25–60°C at a rate of 3°C/min using Na₂HPO₄ buffer (pH 7.4) as a reference.

3. Results and discussion

3.1. Yields of collagens

From an Amur sturgeon (0.67 m, 1.22 kg), 6.0, 4.1, and 0.4 g (dry wt) of purified collagens were obtained from the skin, swim bladder and notochord, respectively. Yields (% based on the wet weight of initial samples) were calculated as 13.4, 16.5, and 1.7%, respectively. The yield of swim bladder collagen was the highest on a percent basis, but the greatest amount of collagen was obtained from the skin because of the largest amount of the
tissue. Yields of collagens from Amur sturgeon skin, swim bladder, and notochord were similar to those from the Bester sturgeon [11].

3.2. SDS-PAGE

The SDS-PAGE patterns of collagens from the skin and swim bladder of Amur sturgeon were similar with the pattern of type I collagen from porcine skin as well the tilapia scale which is widely used in biomedical application (Fig. 2). Both SC and SBC consisted of two major bands (ca. 120 and 100 kDa), and the density of the 120-kDa band was higher than that of the 100-kDa band. From the molecular weight of these bands, as well as the band pattern, the 120-kDa band was judged as an α1-chain, and the 100-kDa band as an α2-chain. Additionally, the patterns were almost the same as the type I collagen from the skin of Amur sturgeon [17]. High molecular weight components, which seemed to be crosslinked α-chains, were also observed in SC and SBC. However, SBC had much lower band intensities of high molecular weight components than those of SC, tilapia scale, and porcine skin (Fig. 2), which suggested that the intramolecular crosslinks of SBC were specifically poorer. Such specific characteristics of Amur sturgeon SBC was also similar to those of Bester SBC. Whether the α3-chain existed in the collagens of SC and SBC in this study could not be determined because the migration similarity of the α3 and α1 chains prevented separation of the former from the latter using SDS-PAGE [18]. The α3-chain has been reported to exist in the skin of
the white sturgeon, Bester sturgeon, and many other teleost fish species, using CM-cellulose chromatography [18, 19].

NC of Amur sturgeon consisted of only one α-chain as the major constituent, which was similar to that of chicken cartilage type II collagen (Fig. 2). Therefore, NC is likely to be classified as type II collagen, which comprises three identical α chains [11, 20]. In addition, the NC pattern was similar to that of type II collagen extracted from Amur sturgeon cartilage, and the slight, higher molecular weight band above the major α-chain was speculated to be other collagen types, such as type IX and XI collagens as minor components [21].

3.3. Amino acid composition

The amino acid compositions of Amur SC, SBC, and NC are shown in Table 2. Generally, glycine in collagen represents approximately one third of the total residues, supporting its occurrence as every third residue in collagen molecules [3, 20]. Amur SC and SBC were also rich in alanine, proline, and hydroxyproline but comparatively low amounts of cysteine (~0.2%), methionine (~1.24–1.33%) and threonine were present, showing typical characteristics of type I collagen [22, 23]. In NC, glutamic acid, leucine, hydroxylysine, and imino acid (proline + hydroxyproline) contents were higher than in SC and SBC, but those of serine, alanine, and lysine were lower. Moreover, the degree of hydroxylation of lysine was calculated to be 60% in NC, which was much higher than those in SC (22.6%) and SBC.
A high degree of lysine hydroxylation was reported to be one of the criteria used to identify type II collagen [24]. In addition, our previous study also revealed that the notochord of Amur sturgeon highly expressed mRNA of the proα1 chain of type II collagen [15]. However, NC had a different amino acid composition compared with Amur cartilage type II collagen [21], and this could be related to the different mixture with other types of collagen.

Imino acid contents were reported to have a major influence on the thermal stability of collagen; the higher the imino acid content, the more stable the helical structure [1, 25]. This occurs because proline + hydroxyproline-rich zones of collagen molecules are most likely to be involved in the formation of junction zones that are stabilized by hydrogen bonding, and the pyrrolidine rings of proline and hydroxyproline impose restrictions on the conformation of the polypeptide chain, both of which help to strengthen the triple helix [26]. In addition, the degree of hydroxylation of proline residues also influences the stability of the collagen helix structure owing to interchain hydrogen bonding via a bridging water molecule as well as direct hydrogen bonding to a carbonyl group [27]. For SC, SBC, and NC, the imino acid contents were 217, 221, and 235, and the hydroxylation degrees of proline were calculated to be 47.5%, 57.5%, and 48.9% (Table 3). The results suggested NC and SBC might have higher thermal stability than SC. On the other hand, the lysine and hydroxylysine have biological roles in self-assembly and stabilization of the molecule through pyridinium cross-links.
formation [28, 29]. The total content of lysine and hydroxylysine in Amur SC, SBC, and NC are almost the same, yet the degree of lysyl hydroxylation was highest in NC, followed by SBC and SC (Table 3). The high lysyl hydroxylation degree is frequently found in highly insoluble collagens with a high crosslinking degree [29]. Actually, the loss of NC extract was much more than SC and SBC as its insoluble property.

3.4. Thermal stability

CD spectra of the SC, SBC, and NC of Amur sturgeon are shown in Fig. 3A. All collagens have a rotatory maximum at 221 nm and a crossover point (zero rotation) at ca. 212 nm, which are typical characteristics of the collagen triple helical conformation [11]. Collagen denaturation is shown in Fig. 3B. Tds of SC, SBC, and NC were calculated as 28.5°C, 30.5°C, and 33.5°C, respectively. This result substantiates the assumption of thermal stability based on collagen imino acid compositions and hydroxylation degrees of different tissues in the same fish, as described above. However, Td of SC in this study was lower than that of the skin collagen of Amur sturgeon (32.52°C) [17], which may be explained, in part, by individual differences and the method of purification of the collagen.

Tds of SC, SBC, and NC in Amur sturgeon were lower than those of Bester sturgeon [11]. The difference may correlate with the Bester sturgeon preference of higher environmental temperatures than Amur sturgeon. The Bester sturgeon genetic ancestor, Beruga, is originally
distributed around the Caspian Sea and the Black Sea. Such a habitat difference may affect the
difference in collagen thermal stability in these two species.

3.5 Molecular cloning and gene expression analysis of ascolla2

As ascolla1 data have been reported in our last study [15], this study focused on the
ascolla2 analysis and the comparison between ascolla2 and ascolla1, the same tissue
samples with the last study [15] were used. The open reading frame (ORF) region was 4,071
bases, encoding 1,357 amino acids in ascolla2 (Fig. 4). One nucleotide sequence was
identified in ascolla2 and deposited in the DNA Data Base of Japan (DDBJ) with accession
number LC055022. The deduced amino acid sequence of Ascolla2 was produced (Fig. 4).
The amino acid sequence of Ascolla2 showed 76% homology to goldfish, rainbow trout, and
zebrafish Colla2, and 71% to cattle and human Colla2. On the other hand, Ascolla2 showed
only 54% homology to Ascolla1.

As shown in Fig. 4, the deduced amino acid sequence of Ascolla2 included a signal
peptide, N- and C-propeptides, telopeptides, and a triple-helical domain, which was similar to
that of rainbow trout [30]. The sequences of Lys-Gly-His-Arg (KGHR) and Lys-Gly-Leu-Arg
(KGLR) (underlined in Fig. 4) appeared close to the N- and C-termini in the triple-helical
domain. These were likely substrates for cross-linking of fibrils [31] and were well conserved
in other type 1 collagens in all species shown in the above phylogenetic analysis. The residues
assumed important for collagen function, three Arg-Gly-Asp (RGD boxed by dotted lines in Fig. 4), could be possible cell-binding sites in Ascol1a2; they were also conserved in most of the species examined in the present study. Additionally, the positions of Cys residues and Asn-X-Thr (glycosylation site) in the C-terminal propeptide were conserved. A sequence of Lys-Gly-His-Arg (cross-linking site of fibrils) [30] was also conserved. These observations suggested that the cloned sequence was the proα chain of Col1, and these collagens in Amur sturgeons were expected to share basic functions of fibrillar collagen common in many vertebrate species.

The comparison of the amino acid composition of procollagen and the triple helical domain of Ascol1a2 and Ascol1a1 (data of Ascol1a1 from Zhang et al., [15]) is shown in Table 4. In both the Ascol1a2 and Ascol1a1, Gly was most abundant, and Pro and Ala followed. The triple helical domain of Ascol1a1 contained no Tyr, but it included three Tyr in Ascol1a2. In the triple helical domain, Ala, Glu, and Pro were more abundant in Ascol1a1 than Ascol1a2. In contrast, Leu, Asn, and Val were more abundant in Ascol1a2 than Ascol1a1.

Table 5 shows the number of amino acid residues of Gly-Pro-Pro and Gly-Gly in the triple-helical domain, which probably influences collagen stability. The numbers of Gly-Pro-Pro and Gly-Gly were almost the same between Ascol1a2 and Ascol1a1 and were similar to that of zebrafish Col1a1, however, much lower than those of mammalian collagen. In comparison with Col1a1s among several vertebrates [32], there were less Gly-Pro-Pro and
more Gly-Gly units in fish than in other species. The Gly-Pro-Pro sequence, whose content is reflected in the content of Gly-Pro-Hyp, stabilizes the tripeptide unit, and Gly-Gly, which likely contributes to the partial skew in the triple helix, decreases thermal stability [31]. Thus, the results are in accordance with the low environmental temperature of these fish with the lower thermal stability of collagen.

Gene expressions of \textit{ascoll1a2} and \textit{ascoll1a1} (data of \textit{ascoll1a1} from Zhang et al. [15]), standardized by the expression of \(\beta\)-actin, are shown in Fig. 5 (A). The type I collagen gene \textit{ascoll1a2} appeared relatively ubiquitously, suggesting the similar trend with \textit{ascoll1a1}, and its expression in notochord was extremely low (below the detection limit). This was in accordance with the data of SDS-PAGE and amino acid composition of Amur sturgeon, which showed type II collagen was the major collagen in the notochord. Type I collagen expression levels varied among the organs, with swim bladder, fin, snout cartilage, scale, skin, stomach, muscle, and intestine, being from more to less.

Different gene expression ratios of \textit{ascoll1a1} and \textit{ascoll1a2} were found in different organs as shown in Fig. 5 (B). Type I collagens of many bony fish species are comprised of three slightly different chains, named \(\alpha_1(\text{I})\), \(\alpha_2(\text{I})\), and \(\alpha_3(\text{I})\), and two types of collagen molecules are believed to exist: \([\alpha_1(\text{I})_2\alpha_2(\text{I})]\) and \([\alpha_1(\text{I})\alpha_2(\text{I})\alpha_3(\text{I})]\) [19]. Thus, if the ratio of gene expression of \(\alpha_1/\alpha_2\) is near 2, this suggests \([\alpha_1(\text{I})_2\alpha_2(\text{I})]\) is the main type, and the ratio of gene expression of \(\alpha_1/\alpha_2\) near 1 suggests \([\alpha_1(\text{I})\alpha_2(\text{I})\alpha_3(\text{I})]\) is the main type. Therefore, different
ratios of collagen gene expressions among different organs suggested that the organs had two
type I collagen molecules in different ratios. In the present study, for example, [α1(I)2α2(I)]
was the main type in scale and fin, but in muscle, [α1(I)α2(I)α3(I)] was the main type. The
compositional differences in collagen molecules may result in the different amino acid
compositions of type I collagens, since the amino acid composition of Ascol1a2 and Ascol1a1
are different as already described. This is in consistent with the result of small different amino
acid compositions in type I collagens from different organs by biochemical analysis obtained
in skin and swim bladder. Also, the compositional differences in Ascol1a1 and Ascol1a2 in
the collagen fibrils from different organs would be expected to produce distinct collagen
characteristics in distinct organs. In the studies of Alaska pollack and rainbow trout, organ-
specific differences in subunit structures (different compositions of α chains) of skin, muscle,
and swim bladder type I collagens were also reported, which could affect the properties of
collagen [19, 30]. Additionally, recombinant human type I collagen consisting of an α1(I)3
heterotrimer was reported to have higher thermal stability than the [α1(I)2α2(I)] heterotrimer
[33, 34].

Interestingly, in swim bladder, the gene expression ratios of α1/α2 were lower compared
with other organs (below 0.5); therefore, in addition to [α1(I)2α2(I)] and [α1(I)α2(I)α3(I)]
collagens, another type I collagen molecule must be present. We speculate the collagen is
[α3(I)2α2(I)] and/or α3(I)3. In addition, the unique collagen molecular composition may relate
to the biochemical and physicochemical characteristics of swim bladder collagen, such as the fibril-forming ability. For sturgeon collagen, α3 chains have been reported to exist in the skin type I collagen of white sturgeon and Bester sturgeon [18, 19]. These data strongly suggested that the existence of α3 was organ specific, and thus, different compositions of α chains may have had significant effects on collagen properties.

3.6. Collagen fibril formation in vitro

Fibril-forming collagens are most commonly used in the production of collagen-based biomaterials [35]. Thus, the process of collagen fibril formation in vitro was clarified in the present study (Fig. 6). SBC (type I collagen) showed a more rapid rate of increase in turbidity, and a shorter time to attain the maximum turbidity, than SC (type I collagen) at the same NaCl concentration. The lag time of fibril formation in SBC was not observed in all the conditions examined, indicating the high speed of SBC nucleation. These processes of fibril formation in type I collagens were similar to those of Bester sturgeon collagens, which were reported in our previous study, and further suggested their higher ability for fibrillogenesis than porcine tendon type I collagen [11]. The rapid fibril formation is a desirable character for biomaterial applications, as reported in Mredha et al., 2015 and 2017, Moroi et al., 2019 [6, 36, 37]. However, NC (type II collagen) showed no obvious absorbance change in the conditions used in this study. After 24 h, NC produced white precipitation (data not shown), suggesting that
fibrillogenesis proceeded to a certain extent. Type II collagen was reported to have a longer
lag time for assembly and lower fibril formation rate than type I collagen in calf and human
[38,39]. Additionally, glycosylation of collagen has been reported to result in a longer lag
phase and a slower growth rate of collagen fibril formation, probably owing to a decrease of
hydrophobic interactions in the early stage of fibril formation [40]. Amudeswari et al. also
reported that the rate of fibril formation decreased after glycosylation, and increased after
deglycosylation [41]. In this study, the glycoside content in Amur sturgeon collagens was
quantified (Table 6). The major glycosides were galactose and glucose, the same as those
reported in mammalian collagens [42]. The glycoside content of NC was higher than SC and
SBC. For type I collagen, the glycoside content of SBC was lower than that of SC. Thus, the
higher fibril formation speed of SBC could, in part, be explained by its lower glycoside
content.

When the ionic strength was increased by the addition of NaCl, the fibril formation rate of
SBC and SC was increased (Fig. 6) as in the case of the Bester collagens [11], suggesting that
NaCl concentration influenced the process of collagen fibrillogenesis in sturgeons. In the case
of fibril formation of SC in Amur, under higher NaCl concentration, the lag time was longer,
yet the speed was faster, and higher final turbidity was attained. This is different from the case
in Bester SC. Different characteristics of collagen fibril formation may, therefore, be
explained by species differences [11].
3.7. SEM observation of the morphology of fibrils formed in vitro and in vivo

Fibril morphology is an important factor of collagenous cell scaffolds because the topography of extracellular matrix including collagen is recognized as an important cellular regulator [43]. Thus, in the present study, SEM images of SC and SBC revealed the collagen fibrils of Amur sturgeon. After 1 h of incubation, unordered fibril structures in SC and SBC were observed (Fig. 7), indicating that fibrillogenesis occurred in both collagens in all conditions. The fibril structures of SC and SBC of Amur sturgeon were similar to those of Bester sturgeon [11]. Additionally, the SC fibrils seemed to become thicker with increasing NaCl concentration.

After 24 h incubation, fibrils of SC and SBC (Fig. 8) were generally thicker than those after 1 h. The fibrils of SC showed an unordered structure at 0 mM NaCl (Fig. 8A), with a diameter of 0.1–0.4 μm, thicker than skin collagen fibrils of Amur sturgeon in vitro (diameter of 40–60 nm), reported in another study [17]. The reasons for such differences may be the different conditions of the fibrillogenesis experiments and the methods of purification of the collagen. When NaCl concentration was increased, fibrils became thicker and had a more ordered alignment. Moreover, several fibrils were coiled to make rope-like, higher-order structures as NaCl concentration increased (Fig. 8B and C). When the NaCl concentration was 140 mM, the diameter of the structure was from 0.4 to 1.6 μm, which may, therefore, be
called a fiber because of its size. For SBC, the fibrils showed an entangled structure at 0 mM NaCl (Fig. 8D). At 70 mM NaCl, some large and fusiform higher-order structures that were characterized by transverse periodic banding patterns were observed (Fig. 8E). The average width of a unit of the banding pattern was about 0.625 μm. When NaCl concentration was increased to 140 mM, more fibrils became fusiform (Fig. 8F). The maximum width of the fusiform structure reached 3.9 μm, which may also be called a fiber.

Skin and swim bladder tissues of Amur sturgeon were observed by SEM (Fig. 9). SC fibrils formed in vivo (Fig. 9A) were much thinner compared with those formed in vitro. Furthermore, in vivo fibrils were aligned closely in the same direction, forming a plate-like structure. Thus, a rope-like structure is a characteristic feature of SC fibrils formed in vitro and suggested the possibility of showing characteristic bioactivity compared with SC fibrils in vivo. In the swim bladder tissue (Fig. 9B), collagen fibrils with a transverse periodic banding pattern with a unit of 0.71–0.89 μm were observed. Thus, SBC fibrils formed in vitro suggested the possibility of bioactivity like collagen fibrils in vivo, because their morphology is similar.

In addition, the higher-order, coiled structure may be a characteristic feature of Amur SC, because no similar structures were observed in Bester SC [11]. For Amur SBC, the fusiform fibrils with a transverse periodic banding pattern were similar to those of Bester SBC, although the average width of the banding pattern was narrower in Bester SBC. Thus, the
fibril-forming nature of sturgeon collagens may be species specific. In SC and SBC of both sturgeons, increasing NaCl concentration promoted unique higher-ordered structures. Some SEM images that looked like fusions of several fibrils to make a fusiform fibril were also observed in Amur sturgeon, which agreed with the working hypothesis reported in Bester sturgeon: SBC collagen molecules first assembled to form fibrils, then several fibrils fused into one fusiform structure [11]. Similarly, in SC, collagen molecules first assembled to form fibrils, then several fibrils coiled to form a rope-like structure. This was in accordance with the process of collagen fiber formation [35]; first tropocollagen self-assembled into fibrils ranging from 10 to 300 nm in diameter, then aggregated fibrils formed a collagen fiber with a diameter ranging from 0.5 to 3 μm. Thus, the structures of SC and SBC formed in the 140 mM NaCl after 24 h incubation could be called fibers. The ability of Amur sturgeon collagens to form higher-ordered fibers in vitro is a unique characteristic for their biomaterial application. Also, formation of such higher-ordered fibers is characteristic feature of sturgeon collagens, which have never observed in porcine tendon collagen [11]. NC (type II collagen) assembled into fibrils arranged in network-like structures after 24 h incubation, as reported in our previous study in Bester NC [10], and the fibrils formed from NC were thinner and shorter (Fig. 10). This result was in agreement with the result of bovine type II collagen and human type II collagen [38, 39]. By contrast to the cases observed in SC and SBC, NaCl concentration of the solution seemed to have no obvious effect on the fibril
configuration formed from NC. Fibrils of chicken type II collagen were also observed in this study, and network-like structures were visible at 70 mM and 140 mM NaCl.

3.8. Thermal stability of reassembled fibrils

The thermal stability of reassembled fibrils is important for their application to tissue engineering. Differential scanning calorimetry (DSC) curves of reassembled fibrils of SC and SBC are shown in Fig. 11. Tm was obtained as the endothermic peak of the DSC curve. For SC fibrils formed in buffer solution for 1 h containing NaCl at 0 and 140 mM, the Tms were 34.4°C and 38.9°C, respectively. For SBC fibrils formed in buffer solution for 1 h containing NaCl at 0 and 140 mM, the Tms were 40.1°C and 40.7°C, respectively. Thus, the addition of NaCl increased the Tm in both SC and SBC fibrils. Compared with the Td of collagen molecules, the thermal stability of collagen fibrils was higher by 6–10°C. Especially for SBC fibrils, the Tm was higher than 37°C, suggesting its possibility as a biomaterial for humans.

The incubation time for fibril formation had almost no effect on the Tm. Therefore, the thermal stability of reassembled fibrils was hypothesized to be determined at the initial stage of fibril formation. In the later stage, several fibrils will fuse to form the higher-order structure, but the thermal stability would not be changed.

4. Conclusions
This study demonstrated that a significant quantity of type I collagen could be extracted from skin, swim bladder, and type II collagen from notochord of Amur sturgeon, which may be a promising resource for industrial use. Because there is little type II collagen on the market, sturgeon notochord is a promising resource for industrialization. In addition, the successful cloning and deduced amino acid sequences provided information on the primary structure of pro\(\alpha_2\) chains in Amur sturgeon. This was the first comparative analysis of the characteristics of \(ascol1a1/Ascol1a1\) and \(ascol1a2/Ascol1a2\) in a sturgeon species, at the molecular level. The \(ascol1a1\) and \(ascol1a2\) qPCRs revealed that type I collagen mRNAs were expressed in considerable amount in cartilage but were at nondetectable levels in notochord, suggesting that notochord is a good source of type II collagen with very limited amount of type I collagen contamination. Moreover, a higher fibril-forming ability with unique higher-order structures and better thermal stability of type I collagen fibrils were found. These results suggested the possibility that Amur sturgeon collagens could be used in biomedical industries. As most studies on fish collagen have dealt with the biochemical nature for utilization as food, and there have been only a few studies proposed as biomaterials, so this study provided basic data, which may help to open a new field of industrial use for fish collagen.

Acknowledgments
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References


https://www.nature.com/articles/227680a0.


https://doi.org/10.1271/bbb.61.1944.


http://www.jbc.org/content/256/14/7118.short.


https://doi.org/10.1016/0006-291X(74)90923-1.


https://doi.org/10.1007/BF01114964.


https://doi.org/10.1016/S0174-173X(87)80011-0.


Table 1

Primer sequences used for cloning of *ascolla2* in Amur sturgeon.

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<tr>
<td>R1</td>
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</tr>
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Amino acid composition of collagens from the skin (SC), swimming bladder (SBC), and notochord (NC) of Amur sturgeon (expressed as residues/1000 total amino acid residues).

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Table 3
Comparison of numbers of imino acid, proline (Pro), hydroxyproline (Hypro), lysine (Lys), and hydroxylysine (Hylys) residues, and hydroxylation degree of proline and lysine residues of collagens from the skin (SC), swimming bladder (SBC), and notochord (NC) of Amur sturgeon (expressed as residues/1000 total amino acid residues).

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Table 4. Amino-acid composition of the α1 chain and α2 chain of type I procollagen in Amur sturgeon based on deduced amino acid sequences.

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*data from Zhang et al., 2016 [15].
Table 5. Number of Gly-Gly and Gly-Pro-Pro repeats in the triple helical domain of α chains. For each organism, the temperature of life is in parentheses (for the poikilotherms; the indicated life temperature is a breeding temperature). The nucleotide sequences of collagen α chains used for sequence analyses were taken from the EMBL.

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<td>(Present study)</td>
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<td>a2</td>
<td>31</td>
<td>4</td>
</tr>
<tr>
<td>Human (37°C)</td>
<td>K01228</td>
<td>a1</td>
<td>42</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>J03464</td>
<td>a2</td>
<td>26</td>
<td>4</td>
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</tbody>
</table>
Table 6. Glycosides in collagen of the skin (SC), swimming bladder (SBC), and notochord (NC) of Amur sturgeon (expressed as glycosides/mg lyophilized collagen).

<table>
<thead>
<tr>
<th>Glycosides (μg)</th>
<th>SC</th>
<th>SBC</th>
<th>NC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Galactose</td>
<td>2.08</td>
<td>0.49</td>
<td>31.79</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.64</td>
<td>0.65</td>
<td>33.67</td>
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</table>
**Figure captions**

**Fig. 1.** Name and position of PCR primers and the lengths of amplicons for cDNA cloning of collagen pro-α2(I) chain in the Amur sturgeon. After PCR, each amplicon was subcloned and sequenced. Primers in black letters were designed from a sequence comparison among certain species other than Amur sturgeon, and those in red letters were designed from sequences newly obtained in the present study. As forward and reverse primers for 5′ RACE and 3′ RACE, respectively, universal primers supplied by the manufacturer were used.

**Fig. 2.** SDS–PAGE of collagens from pig, tilapia, chick, and Amur sturgeon. M, high molecular weight marker; 1: porcine skin type I collagen; 2: tilapia scale type I collagen; 3: chicken cartilage type II collagen; 4: skin collagen of Amur; 5: swim bladder collagen of Amur; 6: notochord collagen of Amur.

**Fig. 3.** CD spectra (A) and temperature effect on the CD spectra at 221 nm (B) of the collagens from the skin (SC), swim bladder (SBC), and notochord (NC) of Amur sturgeon.

**Fig. 4.** Deduced amino acid sequences of collagen pro-α2(I) chain of the Amur sturgeon, Ascoll1a2. The vertical line indicates the predicted cleavage site for the signal peptide, and arrows indicate cleavage sites for the N- and C-telopeptides. The triple-helical domains are
bracketed. The potential cell-binding and integrin-binding sites are boxed by solid and dotted lines, respectively. The putative cross-linking site is underlined. The positions of Cys residues in the N- and C-propeptide and Asn-X-Thr (glycosylation site) in the C-terminal propeptide are indicated by closed and open circles, respectively.

Fig. 5. Relative expression of ascolla1 and ascolla2 (A) and expression ratio of ascolla1/ascolla2 (B) in various tissues in 3-month-old Amur sturgeons. Relative mRNA represents the mean ± S.E of samples from three different fish. In the notochord, expressions of ascolla1 and ascolla2 were below the detection limit (ND). Note that a logarithmic scale is used for the relative expression level.

Fig. 6. Effects of NaCl concentrations on the in vitro progress of fibril formation of skin collagen (SC), swim bladder collagen (SBC), and notochord collagen (NC) of Amur sturgeon.

Fig. 7. Scanning electron micrographs of collagen fibrils formed at 21±1°C for 1 h. (A–C): Amur sturgeon skin collagen with NaCl concentration of 0, 70 and 140 mM; (D–F): Amur sturgeon swim bladder collagen with NaCl concentration of 0, 70 and 140 mM. Scale bars, 5 μm.
Fig. 8. Scanning electron micrographs of collagen fibrils formed at 21±1°C for 24 h. (A–C): Amur sturgeon skin collagen with NaCl concentration of 0, 70 and 140 mM; (D–F): Amur sturgeon swim bladder collagen with NaCl concentration of 0, 70 and 140 mM. Scale bars, 5 μm.

Fig. 9. Scanning electron micrographs of skin (A) and swim bladder (B) of Amur sturgeon in vivo. Scale bars, 5 μm.

Fig. 10. Scanning electron micrographs of collagen fibrils formed at 21±1°C for 24 h. (A–C): Amur sturgeon notochord collagen with NaCl concentration of 0, 70 and 140 mM; (D–F): chicken cartilage type II collagen with NaCl concentration of 0, 70 and 140 mM. Scale bars, 5 μm.

Fig. 11. DSC curves of collagen fibrils of skin (A) and swim bladder (B) from Amur sturgeon. 1, fibrils formed at 21±1°C for 1 h with NaCl concentration of 0 mM; 2, fibrils formed at 21±1°C for 24 h with NaCl concentration of 0 mM; 3, fibrils formed at 21±1°C for 1 h with NaCl concentration of 140 mM; 4, fibrils formed at 21±1°C for 24 h with NaCl concentration of 140 mM.
Figure 1
Figure 2

- 250 kDa
- 150 kDa
- 100 kDa
- 75 kDa
- 50 kDa

M 1 2 3 4 5 6 M

α1(I) α2(I) α1(II)
Figure 3

(A) Wavelength (nm)

(B) Temperature (ºC)
Figure 5

(A) Relative expression to β-actin

(B) Expression ratio of ascollα1/ascollα2
Figure 6

**NaCl 0 mM**

**NaCl 70 mM**

**NaCl 140 mM**
Figure 8
Figure 9

(A) [Image]

(B) [Image]
Figure 11

(A) Heat Flow (mW) vs. Temperature (°C)

(B) Heat Flow (mW) vs. Temperature (°C)
A significant quantity of type I and type II collagen could be extracted from Amur sturgeon.

Biochemical properties of both type I and type II collagens were studied, and organ collagen specificity was found.

Skin collagen and swim bladder collagen had better abilities of fibril formation compared with porcine type I collagen.

Analysis of the characteristics of the primary structure of type I collagen at the molecular level was clarified.