Comprehensive analysis of chondroitin sulfate and aggrecan in the head cartilage of bony fishes: Identification of proteoglycans in the head cartilage of sturgeon

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A R T I C L E   I N F O
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A B S T R A C T
Cartilage in the head of sturgeon or salmon has been gaining attention as a rich source of functional chondroitin sulfate (CS) or proteoglycans. Although the cartilage was found in the heads of other bony fishes, the structure of CS and its core protein, especially aggrecan, was not fully investigated. In this study, comprehensive analysis of CS and aggrecan in the head cartilage of 10 bony fishes including sturgeon and salmon was performed. The 4-O-sulfation to 6-O-sulfation ratio (4S/6S ratio; S: sulfate residue) of CS in Perciformes was ≥1.0, while the 4S/6S ratios of CS from sturgeons and salmon were less than 0.5. Dot blotting and proteomic analysis revealed that aggrecan was a major core protein in head cartilage of all bony fishes. These results suggest that the head cartilage of bony fishes is a promising source for the preparation of CS or proteoglycans as a health food ingredient.

1. Introduction
Glycosaminoglycans (GAGs) including chondroitin sulfate (CS), dermatan sulfate (DS), hyaluronan (HA), heparan sulfate (HS), heparin, and keratan sulfate (KS) are linear and acidic polysaccharides [1]. Sulfated GAGs are found as side chains of proteoglycans expressed on the cell surface and in the extracellular matrix, and participate in physiological processes through interactions with GAG-binding proteins [2]. HA, a non-sulfated GAG that exists in its free form, is also a major component in the extracellular matrix [3]. CS is composed of a repeating disaccharide unit, (-4)-β-D-GlcA-(1 → 3)-β-D-GalNAc-(1→3), where GlcA is glucuronic acid and GalNAc is N-acetylgalactosamine [1]. A disaccharide unit containing iduronic acid (IdoA) in the place of GlcA is found in DS, a stereoisoform of CS that differs in the C-5 configuration of the hexuronic acid moieties. The disaccharide units of CS (and DS) can be classified into six groups, based on the position and number of sulfate groups (S) in the disaccharide units including GlcA-GalNAc/IdoA-GalNAc (O-unit/iO-unit), GlcA-GalNAc(4S)/IdoA-GalNAc(4S) (A-unit/iA-unit), GlcA(2S)-GalNAc(4S)/IdoA-GalNAc(6S) (B-unit), GlcA-GalNAc(6S)/IdoA-GalNAc(6S) (C-unit/iC-unit), GlcA(2S)-GalNAc(6S)/IdoA(2S)-GalNAc(6S) (D-unit/iD-unit), and GlcA-GalNAc(4S,6S)/IdoA-GalNAc(4S,6S) (E-unit/iE-unit) [4]. Thus, the composition of disaccharides in CS (and DS) and the chain length are animal tissue- and cell type-specific [5–8]. For example, major disaccharides of CS in cartilage of terrestrial animals are A-units (50%–70%) and C-units (20%–40%), and the ratio of 4S/6S (A-/C-unit) is 4.50–7.00 in porcine, 1.50–2.00 in bovine, and 3.00–4.00 in chicken [9,10]. Although CS obtained from the cartilages of shark, salmon, and sturgeon are also constituted of A-units (13–29%) and C-units (31–71%), the 4S/6S ratios are 0.45–0.70 in shark, 0.36–0.69 in sturgeon, and 0.45 in salmon [10–13]. Shark CS, in particular, not only contains A-units and C-units but also D-units (approx. 20%) [10]. Furthermore, DS is found in porcine skin and intestinal mucosa, and CS containing E-units (more out of 30%) are found in cartilage.
than 20%) is prepared from squid cartilage [10,14].

CS is a popular ingredient in health foods as it is considered to benefit arthritis through cartilage repair. However, limitation to the availability of raw materials of CS is caused by overfishing of shark [15] and classical swine fever [16], leading to the high cost and low efficiency of CS extraction. For this reason, alternative resources are required for the effective preparation of CS.

Recently, aggrecan, a major component of proteoglycan containing CS in cartilaginous tissues, derived from salmon nasal cartilage has become increasingly popular as an ingredient in health food. In fact, salmon proteoglycan demonstrates biological properties such as antiaging, inhibition of angiogenesis, attenuation of inflammatory responses [17–21]. In the case of sturgeons, the backbone and skull remains after food processing are gaining attention as an untapped natural source of CS. CS derived from certain species of sturgeon namely hybrid type: Huso dauricus × Acipenser schrenckii, Acipenser schrenckii, and Acipenser stenosis exhibited gastroteprotective activity [15], reduced pain caused by osteoarthritis [22], and protected chondrocytes [23], respectively. Therefore, head cartilage of other bony fishes, usually leftover after food processing, may be a promising source of CS proteoglycans; however, there is little information on the composition of core proteins and the structure of CS of the head cartilage.

In this study, we performed a comprehensive analysis of CS structure in proteoglycans derived from the head cartilage of 10 edible bony fishes including Siberian sturgeon (Acipenser baerii), Russian sturgeon (Acipenser gueldenstaedtii), and Atlantic salmon (Salmo salar). Interestingly, the 4S/6S ratio of CS in Perciformes was more than 1.0, while it was less than 0.5 in CS from sturgeon and salmon. Aggrecan protein was detected in the head cartilage of all bony fishes tested in this study. These results suggest that the head cartilage of Perciformes may be a promising source for the preparation of CS and proteoglycans as a health food ingredient. We further analyzed the composition of proteoglycans in the skulls of Russian and Siberian sturgeons using nano LC-MS/MS, and the aggrecan protein from LOC117428125 and LOC117964296 genes registered in the National Center for Biotechnology Information (NCBI) database [24] were found to be abundant in the skull of sturgeons. Considering that the structure of CS from the sturgeon’s skull is similar to that from the nasal cartilage, the sturgeon skull is a promising source for the formulations with proteoglycans as a health food ingredient.

2. Materials and methods

2.1. Materials

Chondroitinase ABC (EC 4.2.2.4) from Proteus vulgaris was obtained from Sigma-Aldrich (St. Louis, MO, USA). Chondroitinase ACII (EC 4.2.2.5) from Arthrobrauer aureus was prepared as described previously [25]. Heparinase II was purchased from Iduron (Manchester, UK). Heparinase I (EC 4.2.2.7), heparinase III (EC 4.2.2.8), CS unsaturated disaccharides (ΔDi-6SΔS, ΔDi-4S, ΔDi-6SΔG, ΔDiΔUA2S, ΔDiΔdiSΔ, ΔDiΔdiSΔ, ΔDiΔtriSΔ, ΔDi-ΔHA), and CS-A were obtained from Seikagaku Corp. (Tokyo, Japan). HA was obtained from Kikkoman Research Institute (Miyazaki, Japan). Crude proteoglycans were prepared from squid cartilage [10,14] and Atlantic salmon (Salmo salar). CS in cartilaginous tissues, derived from salmon nasal cartilage has been found to be abundant in the skull of sturgeons. Considering that the backbone and skull remains after food processing are gaining attention as a natural source of CS, CS derived from certain species of sturgeon namely hybrid type: H. dauricus × A. schrenckii, A. schrenckii, and A. stenosis exhibited a gastroteprotective activity [15], reduced pain caused by osteoarthritis [22], and protected chondrocytes [23], respectively. Therefore, head cartilage of other bony fishes, usually leftover after food processing, may be a promising source of CS proteoglycans; however, there is little information on the composition of core proteins and the structure of CS of the head cartilage.

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2.2. Extraction of crude proteoglycans or GAGs from head cartilage

Fish heads of Japanese amberjack (Seriola quinquemaculata), Bluefin tuna (Thunnus orientalis), Sea bass (Lateolabrax japonicus), Japanese jack mackerel (Trachurus japonicus), Chub mackerel (Scomber japonicus), Pacific saury (Cololabis saira), Red seabream (Pagrus major) and Atlantic salmon (Salmo salar) were purchased from the food market in Funabashi, Chiba, Japan. Nasal cartilage of Chum salmon (Oncorhynchus keta) was also obtained from Nihon Pharmaceutical Co. Ltd. (Tokyo, Japan). Siberian sturgeon (Acipenser baerii: 10-month old, 54.0 cm of body length, 504.1 g of body weight) and Russian sturgeon (Acipenser gueldenstaedtii: 10-month old, 52.4 cm of body length, 559.6 g of body weight) were kindly provided by Miyazaki Prefectural Fisheries Research Institute (Miyazaki, Japan). Crude proteoglycans were extracted according to the method of Kakizaki et al. with minor modifications [26]. The dried head cartilage (20 to 50 mg) was cut into small pieces and suspended in 1 mL of acetone to remove lipids. The precipitates were collected by centrifugation and dried. The residual dry cartilage was suspended in 3.2 mL of buffer A (4 M guanidine hydrochloride, 50 mM sodium acetate, pH 6.0 and 1 × protease inhibitor cocktail), and the resulting supernatant was collected using an Amicon Ultra centrifugal Filter 30 K device (EMD Millipore, Burlington, MA, USA). After buffer exchange with PBS including 1 × protease inhibitor cocktail, the protein concentration was determined using the Bradford method [27].

Crude GAGs were also extracted from dried head cartilage by actinase E digestion and recovered by ethanol precipitation as described previously [5]. In this case, 1.1–1.3 g of the dried skull of sturgeon and 0.29–3.01 g of nasal cartilage of salmon were used.

2.3. Detection of proteoglycans by Alcian blue staining and immunostaining

The proteins extracted from the head cartilage were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (using e-PAGE 5–20%, ATTO Corporation, Tokyo, Japan). The gels were stained and visualized using a 0.5% (w/v) Alcian blue aqueous solution (pH 2.5).

To detect the aggrecan protein using a mouse monoclonal antibody, (12/21/1-C-6; reduced and alkylated G3 domain at the C-terminus), 20 μg of crude proteins in 0.1 mL of PBS (pH 8.0) containing 20 mM dithiothreitol and 0.1% SDS were incubated at 95 °C for 10 min. After incubation, the reduced proteins were alkylated using 40 mM iodoacetamide for 30 min. The resulting reduced-alkylated proteins were applied to dot blotter with a PVDF membrane at the specified amount. Immunostaining was performed based on the method developed by Nielsen et al. [28] using Chemi-Lumi One Ultra reagent (Nacalai tesque, Tokyo, Japan). Proteins were detected using a ChemiDoc XRS Plus device (Bio-Rad Laboratories, Inc., CA, USA).

2.4. Disaccharide analysis of GAGs by high performance liquid chromatography and cellulose acetate membrane electrophoresis of GAGs

To analyze the unsaturated disaccharides of CS, crude proteoglycans (2 μg) or crude GAGs (2 μg) were incubated in a reaction mixture (35 μL) containing 28.6 mM Tris-acetate (pH 8.0) and 50 μM of chondroitinase ABC. For HA analysis, 50 μM of chondroitinase ACII was used instead of chondroitinase ABC. After incubation for 16 h at 37 °C, the depolymerized GAG samples were boiled and evaporated, and the unsaturated disaccharides from CS and HA were collected using the Amicon Ultra centrifugal Filter 30 K device. The remaining HS samples in the filters of a reaction mixture (pH 7.0) containing 1 μM heparinase I, 1 μM heparinase II, 1 μM heparinase III, 31.3 mM sodium acetate, and 3.13 mM calcium acetate for 16 h at 37 °C. The post-column HPLC system was constructed with two HPLC pumps (LC-20AD: Shimadzu Co., Tokyo, Japan), a column oven (CTC-20A; Shimadzu Co., Tokyo, Japan), an autosampler (SIL-20 AC: Shimadzu Co., Tokyo, Japan), a double-plunger pump for the fluoroergic reagents (NP-FX (B)-1 U; Nihon Seimitsu Kagaku Co., Ltd., Tokyo, Japan), a dry reaction bath (DB-5; Shimamura Instrument), and Fluorescence Detector (FP-4025: Jasco, Tokyo, Japan). A DOCOSIL column (150 mm × 4.6 mm i.d.; Senshu Kagaku, Tokyo, Japan) was used at 60 °C. The HPLC conditions were as follows: eluent A, 1.2 mM tetrabutylammonium hydrogen sulfate in 13% methanol; eluent B, 1.2 mM tetrabutylammonium hydrogen sulfate and 0.2 M NaCl in 13% methanol; gradient for CS, 0–10 min (1% eluent B), 10–11 min
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(1–10% eluent B), 11–30 min (10% eluent B), 30–35 min (10–60% eluent B), 35–40 min (60% eluent B), 40–41 min (60–1% eluent B), 41–50 min (1% eluent B), gradient for HS, 0–10 min (1–4% eluent B), 10–11 min (4–15% eluent B), 11–20 min (15–25% eluent B), 20–22 min (25–53% eluent B), 22–29 min (53% eluent B), 29–30 min (53–1% eluent B), 30–40 min (1% eluent B); flow rate, 1.0 mL/min. The reaction reagents, aqueous 0.5% (w/v) 2-cyanoacetoamide and 0.25 M NaOH, were delivered at the same flow rate of 0.25 mL/min by a double-plunger pump. The mixture reaction was performed at 110 °C in dry reaction bath and detected fluorometrically (Ex. 346 nm, Em. 410 nm). Unsaturated disaccharide of HA was determined according to the method of Toyoda et al. [29].

Crude GAGs (10 μg) were subjected to cellulose acetate membrane electrophoresis and visualized using Alcian blue staining as described previously [5].

2.5. Estimation of the average molecular weight of CS

The molecular weight of the CS was determined by gel filtration chromatography. Chromatography was performed using an Asahipak 510 HQ column (7.6 mm, i.d. × 300 mm; Showa Denko K.K., Tokyo, Japan) that was eluted with 10 mM ammonium bicarbonate at a flow rate of 0.3 mL/min. The effluent was monitored at 204 nm. The chromatograms were recorded and analyzed using the Chromato-PRO-GPC data processing software (Run Time Instruments, Kanagawa, Japan). CS of molecular weights dp6, dp10, and dp20 were purchased from Lduron (Manchester, UK) and were used as calibrants for the standard curve.

2.6. H$^1$ nuclear magnetic resonance spectroscopy

The proton nuclear magnetic resonance (H$^1$ NMR) spectra of the purified polysaccharides were recorded on a Bruker AVANCE600 (600 MHz) spectrometer in the FT mode. Each polysaccharide (approximately 3 mg) for NMR analysis was dissolved in deuterium oxide (D$_2$O, 99% D) and lyophilized thrice to replace the exchangeable protons with deuterium. Then, the lyophilized samples were dissolved in 0.6 mL of D$_2$O and transferred to the NMR tube. The one-dimensional (1D) NMR experiments were recorded with a relaxation time of 2.0 s at a probe temperature of 60 °C. 1D H$^1$ NMR spectra were recorded with an acquisition time of 3.4 s and a number of 64 scans.

2.7. Identification of sturgeon proteoglycans by nano LC-MS/MS

Ten μg of crude protein was reduced with dithiothreitol, and alkylated with iodoacetamide. Aliquots of samples were subjected to SDS-PAGE and the electrophoresis was stopped as soon as the protein entered the running gel. The protein bands were visualized with Coomassie Brilliant Blue (CBB), and excised. The protein bands were digested with TPCK-treated trypsin (Worthington Biochemical Co, Lakewood, NJ, USA) or endoproteinase Asp-N (Roche) at 37 °C overnight. Each digestion mixture was analyzed using a Q Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Inc., Bartslelives, OK, USA) coupled with Easy-nLC 1200 (Thermo Fisher Scientific, Inc., Bartslelives, OK, USA). The peptides were separated using a nanoelectrospray ionization column (NTCC analytical column, C18, 0.75 mm × 150 mm, 3 μm; Nikkyo Technos, Japan) with a linear gradient of 0–99% buffer (100% acetonitrile and 0.1% formic acid) at a flow rate of 300 nL/min over 60 min. MS and MS/MS data were acquired using a data-dependent TOP10 method. The MS and MS/MS data were quantified using the Proteome Discoverer 2.4 (Thermo Fisher Scientific, Inc., Bartslelives, OK, USA). A q-value threshold of 0.05 was employed and the MASCOT search engine Version 2.7 (Matrix Science, London, UK) was used against the Aciipients rhabenus genome (refnx) database (A_rhabenus_20210317, 66,973 sequences; 44,603,069 residues) [24]. The following parameters were employed: type of search, MS/MS ion search; enzyme, trypsin or Asp-N-ambic; fixed modification, none; variable modifications, acetyl (Protein N-term), oxidation (M), Gln > pyro-Glu (N-term Q), carbamidomethyl (C); mass values, monoisotopic; peptide mass tolerance, 15 ppm; fragment mass tolerance, ±30 mnu; maximum missed cleavages, 3 (trypsin) or 4 (Asp-N-ambic); instrument type, ESI-TRAP.

3. Results and discussion

3.1. Isolation of CS proteoglycans derived from the head cartilage of bony fishes

First, a tissue assumed to be nasal cartilage was removed from bony fishes, and was confirmed to be cartilage by Alcian blue staining (data not shown). The wet weights of partial nasal cartilages (n = 1) were as follows: Japanese amberjack: 1.66 g, Bluefin tuna: 12.6 g, sea bass: 1.51 g, Japanese jack mackerel: 0.52 g, Chub mackerel: 2.31 g, Pacific saury: 0.96 g, Red seabream: 0.83 g and Atlantic salmon: 4.07 g. Although sturgeon is bony fish, almost the entire forehead bone (skull) is cartilaginous [30]. Therefore, the skull of each sturgeon was removed and weighed; the weights were as follows: Russian sturgeon: 4.82 g, Siberian sturgeon: 3.28 g. Because structural analysis data of CS from the backbone and cartilage have been reported [12,13,31], the backbone and notochord were also removed and weighed. The wet weights of the backbone were as follows: Russian sturgeon: 3.56 g, Siberian sturgeon: 2.64 g and those of the notochord were as follows: Russian sturgeon: 2.13 g, Siberian sturgeon: 1.83 g. Next, proteoglycans in the head cartilage were extracted using guanidine hydrochloride as described in the “Materials and methods” section, and 2.7–7.5 mg/mL of proteoglycan solutions were obtained. Thus, proteoglycan (10 μg of protein) samples were subjected to SDS-PAGE and visualized by Alcian blue staining. Because the chain length of CS is not uniform [32,33], proteoglycans having CS were detected as smeared bands whose molecular weight was higher than 180 kDa in all of the bony fishes tested in this study (Fig. 1). When proteoglycan samples were treated with chondroitinase ABC, the smeared bands disappeared (data not shown). This result suggests that the smear bands were proteoglycans containing CS, and proteoglycans are abundantly present in the head cartilage of all bony fishes.

3.2. Unsaturated disaccharide analysis of the chondroitin sulfate moieties of proteoglycans

Unsaturated disaccharide analysis of GAGs attached to proteoglycans from head cartilage was performed by high performance liquid chromatography (HPLC) with post-column derivatization and fluorescence detection. As shown in Fig. 2, we found that the ratios of 4-O-sulfation to 6-O-sulfation (4S/6S ratio) of CS in the head cartilage of Perciformes (except for the Pacific saury) were greater than 1.0, suggesting the similarity of the 4S/6S ratio with the commercially available CS from terrestrial animals [9,10]. For example, the 4S/6S ratios of CS from Bluefin tuna, Sea bass, and Japanese jack mackerel were greater than 1.4, while the 4S/6S ratios of Japanese amberjack, Chub mackerel and Red seabream were around 1.0. In addition, a small amount of D-unit (ΔDi-diS$_i$) was also detected in all of Protocorme samples. On the other hand, the disaccharide compositions of CS from Russian and Siberian sturgeons were similar to that of CS from Atlantic salmon. Particularly, the 4S/6S ratio of CS in sturgeon was 0.13–0.22, and trace amount of D-unit was detected, suggesting the similarity of CS from Atlantic salmon (Fig. 2). The contents of CS in the head cartilage were expressed as the total amount of unsaturated disaccharides, and it was found that the CS levels in Perciformes were similar to those in salmon and sturgeons (Supplementary Materials Fig. S1).

To evaluate our extraction method of proteoglycans from head cartilage, the CS of proteoglycans from the backbone and notochord of the sturgeon’s were also analyzed, because the disaccharide composition of CS is tissue dependent. As shown in Supplementary Materials Fig. S2,
the major disaccharide of CS in the sturgeon backbone was C-unit, and the 4S/6S ratio of CS was 0.56 in the Russian sturgeon and 0.64 in the Siberian sturgeon. On the other hand, the major disaccharide of CS in the sturgeon notochord was A-unit, and the 4S/6S ratio of CS was 14.4 in the Russian sturgeon and 11.7 in the Siberian sturgeon. Therefore, the disaccharide compositions of CS of the backbone and notochord obtained in this study are similar to those of CS from sturgeon as previously reported [12,34]. This result suggests that the proteoglycans from the head cartilage of bony fishes were favorably extracted using guanidine hydrochloride. Overall, these results suggest that the structure of CS in Perciformes (except for Pacific saury) was similar to that of commercially available CS from terrestrial animals [9,10]. Furthermore, the amount of CS in the head cartilage was similar among the 10 bony fishes.

3.3. Identification of aggregcan in the head cartilage of bony fishes

Several kinds of proteoglycans including aggregcan and epiphycan have been found in Chum salmon nasal cartilage [26,35]. Among them, three globular domains of aggregcan termed as G1, G2 and G3 are conserved from fish to humans, though the amino acid sequence of the CS domain (GAG attachment site) has low similarity among species [26]. Thus, we examined whether aggregcan was present in the head cartilage of other bony fishes by dot blot analysis using an antibody against the G3 domain of aggregcan. Protein concentration-dependent immune reactivities were observed, suggesting that aggregcan may be conserved in all bony fishes (Fig. 3). Since genome sequence of the Sterlet sturgeon (Acipenser ruthenus) has recently been reported [24], loci of aggregcan were explored in the NCBI database (accession number VTUV00000000); six candidate genes were retrieved. Among them, the LOC117395495 gene corresponded to the aggregcan core protein, though it appeared under the name "brevican". For this reason, we searched for other candidates, and found that the deduced amino acid sequences of the LOC117428125 (chromosome 19) and LOC117964296 (chromosome 27) genes were highly similar (Fig. 4), and their G1, G2, and G3 domains were similar to those of Chum salmon, zebrafish, and human aggregcans (Supplementary Materials Fig. S3A). The complete cDNA sequence of XM_034046755.2 (LOC117428125) corresponded to 7514 nucleotides and encoded 1721 amino acids with a predicted molecular mass of 182,872 Da (Fig. 4).

On the other hand, the complete cDNA sequence of XM_034907506.1 (LOC117964296) corresponded to 7579 nucleotides and encoded 1719 amino acids with a predicted molecular mass of 182,691 Da (Fig. 4). The Sterlet sturgeon has consensus sequences at N-glycosylation sites (Asn-X-Thr or Asn-X-Ser) within the G1 and G2 domains at Asn-239, Asn-333, Asn-654, and Asn-710, which are conserved among species examined in this study (Supplementary Materials Fig. S3A). The interglobular domain (IGD) region of Sterlet sturgeon as well as salmon and zebrafish showed low similarity to that in human [36]. The number of Ser-Gly dipeptides in the CS attachment site of LOC117428125 and LOC117964296 was 93 and 92 (Fig. 4), respectively, which was nearly identical to the number in zebrafish (85 Ser-Gly dipeptides) and higher than that in Chum salmon aggregcan (43 Ser-Gly dipeptides) [26]. Furthermore, the amino acid sequences of a carbohydrate recognition domain (CRD, lectin-like domain) and a complement binding protein-like (CBP) module of Sterlet sturgeon were highly conserved among fishes examined in this study (Supplementary Materials Fig. S3B). Based on this observation, we deduced that LOC117428125 and LOC117964296 were aggregcan genes in Sterlet sturgeon.
spectra of the aggrecan peptides from sturgeon are shown in Table 1 (Russian sturgeon) and Supplementary Materials Table S3 (Siberian sturgeon); the identified sequence coverage was 19% (Fig. 4). It is assumed that the inhibition of protease digestion at the GAG attachment site due masking by CS may have caused the low sequence coverage [26]. However, two peptides (positions: 1229–1246, 1300–1320 in Fig. 2.)
Other proteoglycans (epiphycan, decorin, mimecan-like, biglycan, biglycan-like, basement membrane-specific heparan sulfate proteoglycan core protein, lumican, and brevican), collagens and extracellular matrix (ECM) proteins (matrilin-4, thrombospondin-1, and hyaluronan and proteoglycan link protein 1) were also identified in the skull of Russian and Siberian sturgeons (Supplementary Materials Tables S1 and S2). Considering that proteoglycans (aggrecan, epiphycan, decorin, biglycan, epiphycan, lumican, and basement membrane-specific heparan sulfate proteoglycan), collagens, and ECM proteins (matrilin-4, thrombospondin-1, and hyaluronan and proteoglycan link protein 1) were also found in the nasal cartilage of Chum salmon [35], their compositions in the sturgeon skull may be similar to that in the nasal cartilage of salmon.

3.5. Structural analysis of chondroitin sulfate derived from Russian and Siberian sturgeons

To compare the precise structure of CS from the Russian and Siberian sturgeons to CS from salmon, crude GAGs were prepared from head cartilage by actinase E digestion and recovered by ethanol precipitation as described previously [5]. The resulting crude GAGs delivered 62.5 mg/g dry powder from the Russian sturgeon, 74.3 mg/g dry powder from Siberian sturgeon, 50.6 mg/g dry powder from the Atlantic salmon, and 45.2 mg/g dry powder from the Chum salmon.
Table 1

Identified peptide sequences of the aggrecan core protein-like from Russian sturgeon.

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Crude GAGs were analyzed by cellulose acetate membrane electrophoresis and visualized using Alcian blue in the presence or absence of chondroitinase ABC. The resulting disaccharide composition was similar to that of sturgeon CS in the nasal cartilage of salmon. Most of the CS found in the skull of sturgeon is similar to that found in the nasal cartilage of salmon. This result suggests that almost all of the CS found in the skull of sturgeon is similar to that found in the nasal cartilage of salmon. Disaccharide composition of CS in crude GAGs extracted by actinase E digestion and ethanol precipitation was analyzed by HPLC. The resulting disaccharide composition was similar to that of sturgeon CS.
which was prepared using guanidine hydrochloride (Fig. 7 A, B). The contents of CS in crude GAGs were expressed as total amount of unsaturated disaccharides, and it was found that the CS amount in crude GAGs of sturgeons was nearly equal to that of salmon (Supplementary Materials Fig. S4). Furthermore, HA levels were also determined by HPLC following treatment with chondroitinase ACII [25,29] , and HA levels in crude GAGs were noted to be one-hundredth of the corresponding CS level (Supplementary Materials Fig. S5). These results strengthen the suggestion that most of the GAG present was CS. The molecular weight of CS was measured by gel filtration chromatography and it was suggested that molecular weight of CS from sturgeons was slightly higher than that of CS from salmon (Fig. 7C). Overall, these results suggest that the structure of CS from sturgeons was similar to that of CS from salmon, which has been recognized as a health food ingredient.

4. Conclusions

In this study, we found that the 4S/6S ratio of CS in Perciformes (except that of Pacific saury) was more than 1.0, suggesting the similarity of the 4S/6S ratio to CS in terrestrial animals. Aggrecan was observed in the head cartilage of all bony fishes, and the CS levels were similar among 10 bony fishes. These results suggest that the nasal cartilage of Perciformes could be a promising alternative source for the preparation of CS and proteoglycans. In the case of sturgeons, the CS structure was similar to that in salmon thus far recognized as functional ingredient of health foods. In addition, aggrecan encoded by the LOC117428125 and LOC117964296 genes registered in the NCBI database was a major core protein in the skulls of the Russian and Siberian sturgeons. These results suggest that the skull of sturgeons is a promising source of proteoglycans as well as CS as a health food ingredient. Although commercially available CS has been prepared from the cartilages of porcine, chicken, shark, ray, salmon, and squid [9] , proteoglycans can only be sourced from salmon nasal cartilage. In addition, proteoglycans in micronized salmon nasal cartilage can be easily extracted by incubation with 4% acetic acid or 4 M MgCl$_2$ instead of 4 M guanidine hydrochloride [37] . Further studies are being undertaken to evaluate the biological activity of sturgeon proteoglycans to facilitate its application.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2022.03.125.

CRediT authorship contribution statement

KS, Conceptualization, Investigation, Writing-original draft; TS, Investigation, Writing-review & editing; MT, Investigation; KS, Investigation; SO, Investigation; ND, Investigation, Writing-review & editing; KN, Supervision; TW, Supervision; RJL, Investigation, Supervision, Writing-review & editing; TT, Conceptualization, Supervision, Writing-review & editing; KH, Conceptualization, Project administration, Writing-review & editing.
Declaration of competing interest

The authors declare no conflict of interest.

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[20] K. Higashi, Y. Okamoto, T. Mano, T. Wada, T. Toida, A simple HPLC method for determination of the molecular weight of CS derived from head cartilage of sturgeons and salmon. Data were expressed as the mean ± SD. (n = 3) of three independent experiments.


Y. Tatara, et al., Epiphycan from salmon nasal cartilage is a novel type of large leucine-rich proteoglycan, Glycobiology 23 (2013) 993–1003.
