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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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ABSTRACT

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, $[\rightarrow 4)$-β-$_D$-GlcA-$(1 \rightarrow 3)$-β-$_D$-GalNAc-$(1\rightarrow]_n$, 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), IdoA(2*S*)-GalNAc(4S) (B-unit), GlcA-GalNAc(6S)/IdoA-GalNAc (6S) (C-unit/iC-unit), GlcA(2*S*)-GalNAc(6S)/IdoA(2*S*)-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

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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 anti-aging, 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 sinensis* exhibited gastroprotective 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 (E.C. 4.2.2.4) from *Proteus vulgaris* was obtained from Sigma-Aldrich (St. Louis, MO, USA). Chondroitinase ACII (EC 4.2.2.5) from *Arthrobacter aurescens* 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-0S_{CS}, ΔDi-4S, ΔDi-6S_{CS}, ΔDiUA-2S, ΔDi-diS_E, ΔDi-diS_B, ΔDi-diS_D, ΔDi-TriS_{CS}), ΔDi-HA, and CS-A were obtained from Seikagaku Corp. (Tokyo, Japan). HA was obtained from Kikkoman Biochemifa Company (Tokyo, Japan). Protease inhibitor cocktail was obtained from Nacalai tesque (Tokyo, Japan). All the other analytical reagents used in this study were of analytical grade.

2.2. Extraction of crude proteoglycans or GAGs from head cartilage

Fish heads of Japanese amberjack (*Seriola quinqueradiata*), 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-PAGEL 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) was incubated in a reaction mixture (35 μL) containing 28.6 mM Tris-acetate (pH 8.0) and 50 mU of chondroitinase ABC. For HA analysis, 50 mU 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 spin columns were transferred to new microtubes and incubated in 16 μL of a reaction mixture (pH 7.0) containing 1 mU heparinase I, 1 mU heparinase II, 1 mU 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 (CTO-20A; Shimadzu Co., Tokyo, Japan), an autosampler (SIL-20 AC: Shimadzu Co., Tokyo, Japan), a double-plunger pump for the fluorogenic reagents (NP-FX (II)-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

(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-cyanoacetamide 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 Iduron (Manchester, UK) and were used as calibrants for the standard curve.

2.6. ^{1}H-nuclear magnetic resonance spectroscopy

The proton nuclear magnetic resonance (^{1}H 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_2O, 99% D) and lyophilized thrice to replace the exchangeable protons with deuterons. Then, the lyophilized samples were dissolved in 0.6 mL of D_2O 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 ^{1}H 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., Bartlesville, OK, USA) coupled with Easy-nLC 1200 (Thermo Fisher Scientific, Inc., Bartlesville, OK, USA). The peptides were separated using a nano-electrospray ionization column (NTCC analytical column, C18, φ75 μm × 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., Bartlesville, 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 *Acipenser ruthenus* genome (refxx) database (A_ruthenus_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 mmu; 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 notchord 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_D) was also detected in all of Perciforme 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,

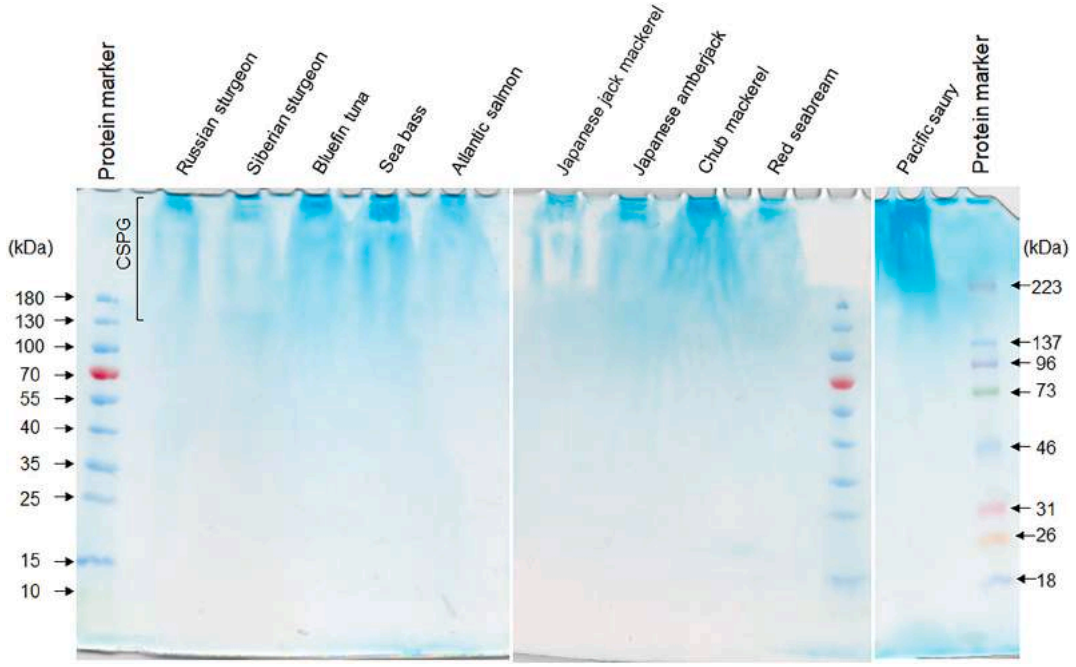

Fig. 1. High molecular weight proteoglycans containing chondroitin sulfate are present in the head cartilage of all bony fishes.
Ten μg of extracted proteins from head cartilage was separated by SDS-PAGE. The separated proteoglycans were stained with a 0.5% Alcian blue aqueous solution (pH 2.5).

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 aggrecan in the head cartilage of bony fishes

Several kinds of proteoglycans including aggrecan and epiphycan have been found in Chum salmon nasal cartilage [26,35]. Among them, three globular domains of aggrecan 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 aggrecan was present in the head cartilage of other bony fishes by dot blot analysis using an antibody against the G3 domain of aggrecan. Protein concentration-dependent immune reactivities were observed, suggesting that aggrecan may be conserved in all bony fishes (Fig. 3). Since genome sequence of the Sterlet sturgeon (*Acipenser ruthenus*) has recently been reported [24], the aggrecan gene was explored in the NCBI database (accession number VTUV00000000); six candidate genes were retrieved. Among them, the LOC117395495 gene corresponded to the aggrecan 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 aggrecans (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 aggrecan (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 aggrecan genes in Sterlet sturgeon.

3.4. Identification of proteoglycans in the skull of Russian sturgeon and Siberian sturgeon

Reduced-alkylated proteins were also digested using trypsin or endoproteinase Asp—N, and the resulting peptides were analyzed by nano LC-MS/MS. We detected 204 proteins in Russian sturgeon and 180 proteins in Siberian sturgeon (Supplementary Materials Tables S1, S2); 135 proteins were detected in both Russian and Siberian sturgeons. A few peptides of the brevican registered as aggrecan core protein (LOC117395495: XP_034774489.1) in the NCBI database were detected in Russian sturgeon only (Supplementary Materials Table S1). On the other hand, we found that three aggrecan core protein-like isoforms X1 and X2 (XP_034763398.1, XP_034763397.1, and XP_033902646.2) from LOC117428125 and LOC117964296 were abundant, indicating that LOC117428125 and LOC117964296 are aggrecan genes. The mass

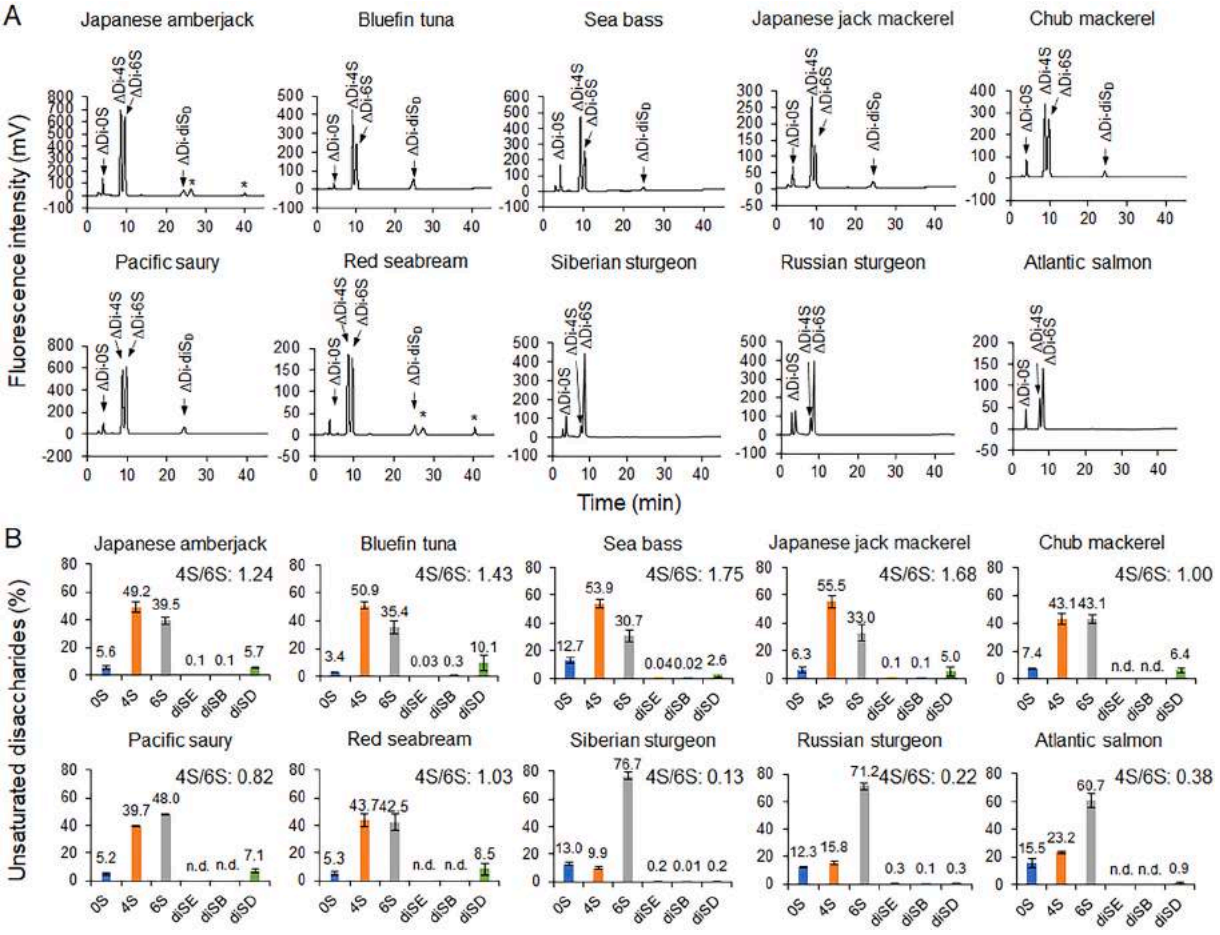

Fig. 2. Disaccharide composition analysis of chondroitin sulfate from the head cartilage of bony fishes.
Chromatograms of unsaturated disaccharides of CS (A) and disaccharide composition (B). Two μg of extracted proteoglycans from the head cartilage were treated with chondroitinase ABC, and the resulting unsaturated disaccharides were analyzed by HPLC with fluorescence detection. *: unidentified peak. Data are expressed as the mean ± SD. ($n = 3$) of three independent experiments.

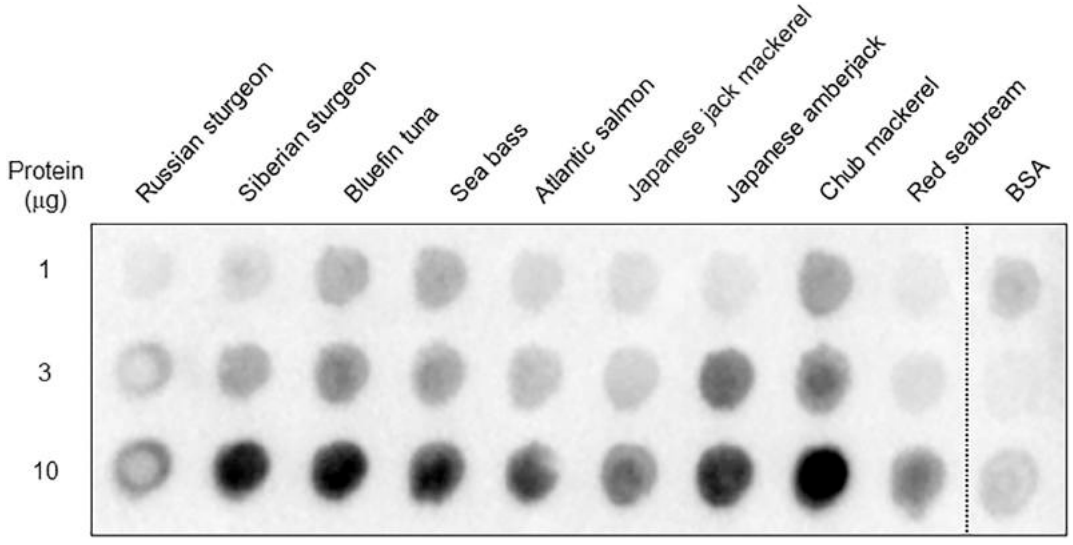

Fig. 3. Dot blot analysis of aggrecan in the head cartilage of bony fishes.

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. 4. Deduced amino acid sequences of the LOC117428125 and LOC117964296 genes in Sterlet sturgeon (*Acipenser ruthenus*).
Multiple sequence alignments were generated by CLUSTALW (https://www.genome.jp/tools-bin/clustalw). Amino acid sequences of aggrecan from Russian sturgeon (*Acipenser gueldenstaedtii*) and Siberian sturgeon (*Acipenser baerii*) were determined using LC-MS/MS. The identified amino acid sequences are shown in red. Ser-Gly dipeptides are underlined. Identical amino acids are shown with asterisks.

LOC117428125) at the GAG attachment site were successfully detected when the samples were digested using Asp-N (proteinase) (Fig. 4).

Other proteoglycans (epiphycan, decorin, mimecan-like, biglycan b, 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.

Position	Observed	Mr(expt)	Mr(calc)	ppm	Score	Expect	Peptide obtained by trypsinization
78–93	553.9768	1658.9087	1658.909	−0.23	50	0.00074	R.ISKEGESVILVATEGK·V
81–93	666.3563	1330.698	1330.698	−0.0075	65	3.10E-05	K.EGESVILVATEGK·V
96–103	500.7239	999.4332	999.4331	0.11	36	0.0053	R.VTSEYMDR.V
96–124	1105.8885	3314.6436	3314.6414	0.65	37	0.026	R.VTSEYMDRVTMVSYPLVPTDATLEISELR.T
125–132	449.7167	897.4188	897.4192	−0.44	49	0.00046	R.TSDSGIYR.C
133–151	735.3338	2202.9795	2202.9773	0.97	67	9.90E-06	R.CEVMHGIEDSEDTVDIQVK.G + Carbamidomethyl (C)
164–170	451.237	900.4594	900.4593	0.15	34	0.025	R.YTLTFEK.A
173–185	696.849	1391.6835	1391.6827	0.59	72	4.70E-06	K.SACIQNSATIATR.E + Carbamidomethyl (C)
186–210	970.7635	2909.2688	2909.2675	0.43	50	0.00038	R.EQLQAAYDDGFHQCDAGWLSDQTVR.Y + Carbamidomethyl (C)
211–217	435.235	868.4554	868.4555	−0.15	30	0.047	R.YPIHSPR.E
218–231	543.5718	1627.6935	1627.6937	−0.1	29	0.033	R.EGCYGDKDEFPGVR.T + Carbamidomethyl (C)
255–263	500.261	998.5074	998.5073	0.13	49	0.00074	K.VFYSTSPAK·F
264–275	737.8297	1473.6449	1473.6446	0.22	70	2.90E-06	K.FSFSEAEEQCIK.L + Carbamidomethyl (C)
630–640	406.5355	1216.5846	1216.5836	0.79	33	0.035	R.GEVFHASSVER.F
676–685	537.2902	1072.5658	1072.5665	−0.7	49	0.0015	R.AGWLLDGSVR.Y
686–699	506.2698	1515.7877	1515.7868	0.6	35	0.03	R.YPITTPRPLCGGGK.T + Carbamidomethyl (C)
700–707	454.765	907.5155	907.5127	3.02	55	9.70E-05	K.TGVITVYR.F
721–727	497.7078	993.4011	993.4014	−0.3	28	0.021	K.YDAYCFR.A + Carbamidomethyl (C)
1504–1514	699.2895	1396.5645	1396.5639	0.41	34	0.0049	K.DLETCEEGWMK·F + Carbamidomethyl (C)
1515–1521	472.7055	943.3964	943.397	−0.68	35	0.0053	K.FQGNCYR.H + Carbamidomethyl (C)
1527–1534	503.2352	1004.4557	1004.4563	−0.56	48	0.00077	R.ETWVDAER.R
1538–1570	1293.6032	3877.7877	3877.7853	0.61	117	1.60E-10	R.EISSHLVSIMTPEEQDYVNNNAQDYQWIGLNDR.T
1630–1645	568.6119	1702.8138	1702.8131	0.46	55	0.00027	K.KGTMSCGSPPLVENAR.M + Carbamidomethyl (C)
1631–1645	525.9132	1574.7179	1574.7181	−0.12	43	0.0024	K.GTMSCGSPPLVENAR.M + Carbamidomethyl (C)
1652–1661	422.2264	1263.6574	1263.6571	0.25	54	0.00039	R.DRYEVNSIIR.Y
1654–1661	497.2717	992.5288	992.5291	−0.28	34	0.033	R.YEVNSIIR.Y
1662–1671	437.5285	1309.5637	1309.5622	1.12	33	0.018	R.YQCNHGFTQR.H + Carbamidomethyl (C)
1672–1677	367.7373	733.4601	733.4599	0.3	33	0.0051	R.HLPVIR.C
1680–1688	544.244	1086.4734	1086.473	0.39	34	0.0099	K.ADGEWEQPR.V
1689–1699	678.8276	1355.6407	1355.6391	1.19	48	0.00097	R.VECIETTVYSR.R + Carbamidomethyl (C)

Position	Observed	Mr(expt)	Mr(calc)	ppm	Score	Expect	Peptide obtained by Asp-N treatment
91–98	438.2512	874.4878	874.4872	0.66	32	0.032	T.EGKVRVTS.E
119–126	467.7459	933.4773	933.4767	0.67	36	0.019	L.EISELRTS·D
127–140	841.3677	1680.7209	1680.7236	−1.62	44	0.0034	S.DSGIYRCEVMHGIE.D + Carbamidomethyl (C); Oxidation (M)
169–185	462.9956	1847.9531	1847.9523	0.43	41	0.0091	F.EKAKSACIQNSATIATR.E + Carbamidomethyl (C)
193–199	439.6586	877.3027	877.3025	0.29	31	0.015	Y.DDGFHQC·D + Carbamidomethyl (C)
554–569	834.9271	1667.8396	1667.8267	7.76	49	0.0011	L.ENGAVIASPQHLQAAY.E
1229–1239	601.8346	1201.6547	1201.6554	−0.62	44	0.0026	V.DSSLVKVTEKP·D
1229–1246	987.0083	1972.0021	1972.0001	1.03	58	0.0002	V.DSSLVKVTEKPDVEQELG.E
1311–1320	1055.5736	1054.5663	1054.5659	0.42	34	0.013	I.EVAPKPTVSQ.E
1538–1552	858.4128	1714.8111	1714.8084	1.6	59	0.00014	R.EISSHLVSIMTPEEQ.D + Oxidation (M)
1595–1602	902.3538	901.3465	901.3454	1.24	30	0.031	P.DNYFSAGE.D
1603–1610	538.2382	1074.4618	1074.4627	−0.86	36	0.019	E.DCVVMIWH.E + Carbamidomethyl (C); Oxidation (M)

Crude GAGs were analyzed by cellulose acetate membrane electrophoresis and visualized using Alcian blue in the presence or absence of chondroitinase ABC (Fig. 5). As a result, the migration of crude GAGs was nearly equal to that of CS standard, whereas the crude GAGs treated with chondroitinase ABC were disappeared. This result indicates that most of the GAGs present was CS. The structure of sturgeon CS was investigated by 600 MHz ^{1}H NMR spectroscopy (Fig. 6). The anomeric H-1 (4.50 ppm), H-2 (3.38 ppm), and H-3 (3.58 ppm) signals of GlcA and anomeric H-1 (4.58 ppm), H-2 (4.02 ppm), H-4 (4.18 ppm), and H-6 (4.23 ppm) signals of GalNAc observed were similar to the signals seen in CS from the Atlantic salmon or Chum 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

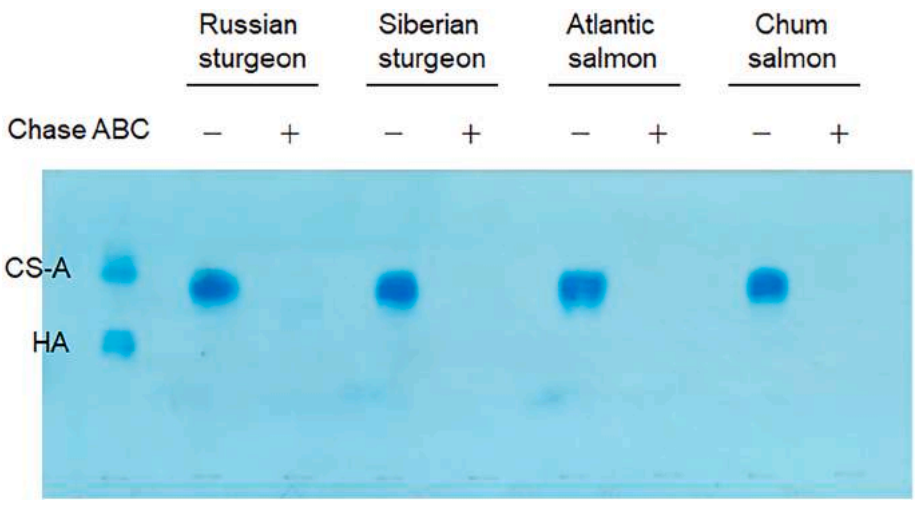

Fig. 5. Cellulose acetate membrane electrophoresis of crude GAGs extracted after actinase E digestion and ethanol precipitation.
Crude GAGs (10 μg) treated with or without of chondroitinase ABC were subjected to cellulose acetate membrane electrophoresis. GAGs were visualized by Alcian blue staining.

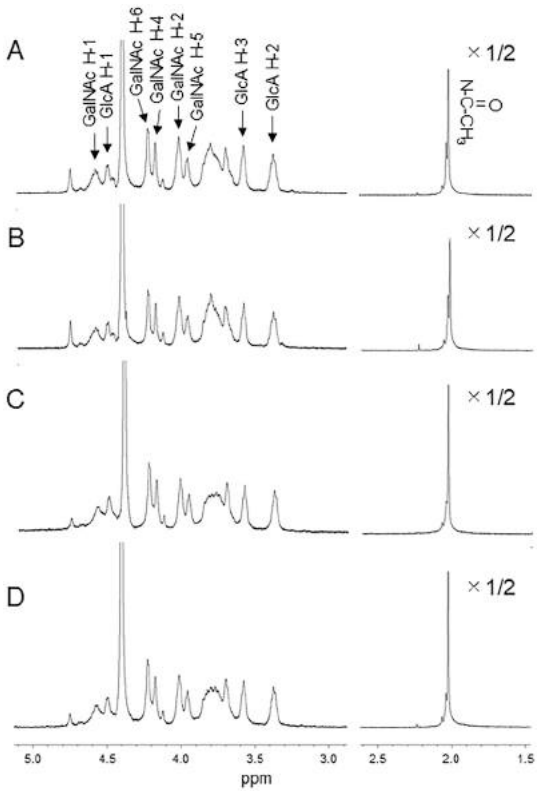

Fig. 6. ^{1}H NMR of CS derived from head cartilage in Chum salmon (A), Atlantic salmon (B), Russian sturgeon (C), and Siberian sturgeon (D).

which was prepared using guanidine hydrochloride (Fig. 7A, 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 salmons (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.

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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.

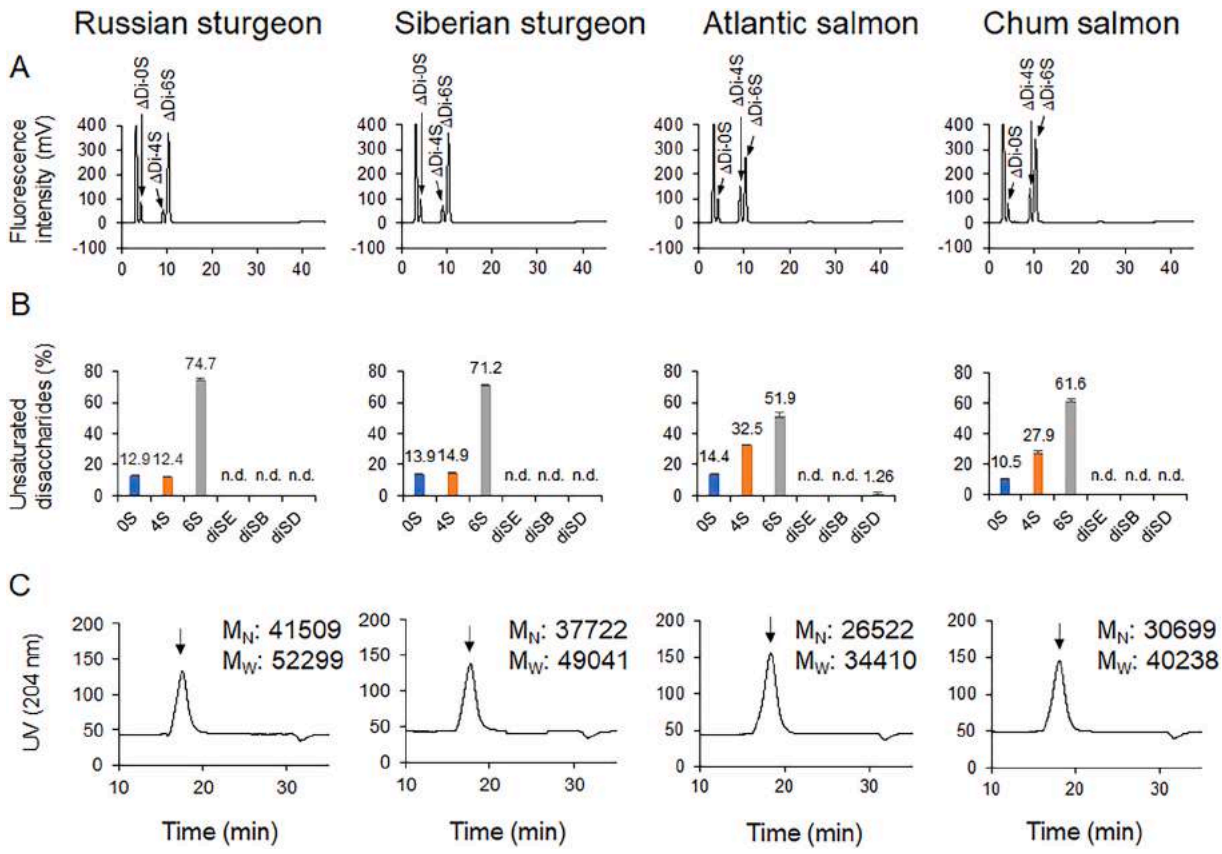

Fig. 7. Disaccharide composition and molecular weight of CS derived from head cartilage of sturgeons and salmons.
(A) Chromatogram of unsaturated disaccharides of CS derived from sturgeons and salmons. 2 μg of CS was treated with chondroitinase ABC, and the resulting unsaturated disaccharides were analyzed by HPLC with fluorescence detection. (B) Disaccharide composition of CS in the head cartilage of sturgeon and salmon. Data are expressed as the mean ± SD. ($n = 3$) of three independent experiments. (C) Determination of the molecular weight of CS in head cartilage of sturgeon and salmon. CS was subjected to gel filtration chromatography to obtain the number average (M_N) and weight average (M_W) of molecular weight. The retention time of CS consisted of dp6, dp10, and dp20 were 23.64, 22.53, and 21.21 min, respectively. Results were reproducible in duplicate determinations.

Declaration of competing interest

The authors declare no conflict of interest.

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