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Structural characterization of chondroitin sulfate from sturgeon bone

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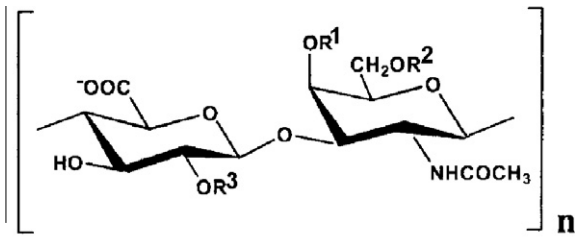
ABSTRACT

Chondroitin sulfate (CS) was purified for the first time from the bones of sturgeon and analyzed to evaluate its structure and properties. A single polysaccharide was extracted from sturgeon bone in a concentration of 0.28–0.34% for dry tissue and characterized as CS. By means of specific chondroitinases and HPLC separation of generated unsaturated repeating disaccharides, this polymer was found to be composed of ~55% of disaccharide monosulfated in position 6 of the GalNAc, ~38% of disaccharide monosulfated in position 4 of the GalNAc, and ~7% of nonsulfated disaccharide. The charge density was 0.93 and the ratio of 4:6 sulfated residues was equal to 0.69, a value confirmed by ^{13}C NMR experiments. Chondroitinase B confirmed that the purified sturgeon CS contained mainly GlcA (>99.5%) as uronic acid. PAGE analysis showed a CS having a high molecular mass with an average value of 39,880 according to HPSEC values producing a weight average molecular weight (Mw) of 37,500. On the basis of the data collected, it is reasonable to assume that CS isolated from sturgeon bone might be potentially useful for scientific and pharmacological applications, making this bony fish, which is generally discarded after ovary collection, a useful source of this polymer. Finally, this newly identified source of CS would enable the production of this macromolecule having a particular repeating disaccharide composition, structure, and biological properties.

1. Introduction

Chondroitin sulfate (CS) is a linear, complex, sulfated, polydisperse natural polysaccharide belonging to the class of macromolecules known as glycosaminoglycans (GAGs).[1,2] It is composed of alternate sequences of GlcA and differently sulfated residues of GalNAc linked by β-(1→3) bonds. Depending on the repeating disaccharide nature, CS with different structures is known to have different degrees of charge density and sulfate groups linked in various positions (see Fig. 1 for the structural nature of various CS disaccharides). Furthermore, it is a very heterogeneous polysaccharide in terms of relative molecular mass, chemical properties, biological and pharmacological activities.[1,3,4]

Recent evidence from glycobiology studies suggests that proteoglycans, and their complex polysaccharidic macromolecules (CS and other GAGs), are not only structural components but also participate in and regulate many cellular events and physiological processes.[5] As a consequence, GAGs are a class of macromolecules of great importance in the fields of biochemistry, pathology, and pharmacology. In fact, CS is currently recommended by EULAR[6]



$R_1 = R_2 = R_3 = H$: nonsulfated chondroitin
$R_1 = SO_3^-$ and $R_2 = R_3 = H$: chondroitin-4-sulfate, CSA
$R_2 = SO_3^-$ and $R_1 = R_3 = H$: chondroitin-6-sulfate, CSC
$R_2 = R_3 = SO_3^-$ and $R_1 = H$: chondroitin-2,6-disulfate, CSD
$R_1 = R_2 = SO_3^-$ and $R_3 = H$: chondroitin-4,6-disulfate, CSE
$R_1 = R_3 = SO_3^-$ and $R_2 = H$: chondroitin-2,4-disulfate, CSB
$R_1 = R_2 = R_3 = SO_3^-$: trisulfated chondroitin

Figure 1. Structures of repeating disaccharide units forming chondroitin sulfate. Minor disaccharides may be present, such as that characterized by a sulfate group in position 3 of glucuronic acid.

Abbreviations: CS, chondroitin sulfate; DS, dermatan sulfate; EULAR, The European League Against Rheumatism; GAG(s), glycosaminoglycan(s); IdoA, α-L-idopyranosyluronic acid; Mn, number-average molecular weight; Mw, weight-average molecular weight; Mz, *Z* average molecular weight; OA, osteoarthritis; SAX, strong anion exchange; SYSADOA, Symptomatic Slow Acting Drug for OA; ΔHexA, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid.

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as a SYSADOA (Symptomatic Slow Acting Drug for OA) drug in Europe in the treatment of knee osteoarthritis (OA) based on meta-analysis of numerous clinical studies.[7] Moreover, CS alone or in combination with glucosamine is utilized as a dietary supplement based on meta-analysis of studies confirming its safe and effective options for the treatment of symptoms of OA.[8]

CS, like other natural polysaccharides, is derived from animal sources by extraction and purification processes.[1] Commercial manufacture of CS relies at present on bovine,[9] porcine,[9] chicken,[10] or cartilaginous fish such as sharks[11] and skate[12,13] by-products, in particular, cartilage as raw material.

As previously illustrated, due to its very complex heterogeneous structure, CS from different sources may possess repeating disaccharides having various sulfate groups located, in different percentages, inside the polysaccharide chains (see Fig. 1). These repeating disaccharide units are generally monosulfated but, depending on the origin, various disulfated disaccharides (and possibly also a trisulfated one) may be present in the polysaccharide backbone. As a consequence, CS with different charge densities may be produced from various sources. Furthermore, as a result of the biosynthetic processes related to specific tissues and species, CSs with different grades of polymerization may be biosynthetized producing macromolecules having various molecular masses and polydispersity. Due to these structural variations, CS from different sources may have different properties and capacities.

The above-mentioned considerations have motivated us to look for alternative sources of this complex polysaccharide also considering the possibility of producing CS with a particular repeating disaccharide composition, structure, and activity. In this regard, the sturgeon belongs to one of the oldest families of bony fish in existence. It is a native of subtropical, temperate, and sub-Arctic rivers, lakes, and coastlines of Eurasia and North America but it is also found along the European Atlantic coast, including the Mediterranean Sea.[14] The common name is used for some 26 species of fish in the *Acipenseridae* family, including over 20 species commonly referred to as sturgeon and several closely related species that have distinct common names, notably sterlet, kaluga, and beluga. Collectively, the family is also known as the true sturgeons.[14] Where sturgeons are caught in large quantities, as in the rivers of southern Russia and in the great lakes of North America, their flesh is dried, smoked, or salted. The ovaries, which are large in size, are prepared for caviar, and the air bladder is used to produce one of the best kinds of gelatine,[15] while the rest of the animals is usually discarded. In this study, CS was extracted and purified from sturgeon bones and its structure characterized along with its important physico-chemical properties, thus demonstrating that another part of these animals might be exploited for commercial preparations.

2. Experimental

2.1. Materials and methods

Heparin from bovine intestinal mucosa, heparan sulfate from bovine kidney, CS from bovine trachea, DS from porcine intestinal mucosa, and HA from rooster comb were from Sigma–Aldrich (St. Louis, MO, USA). Papain from papaya latex (EC 3.4.22.2), specific activity of 16–40 units/mg protein, and deoxyribonuclease I, DNase I (EC 3.1.21.1) from bovine pancreas, specific activity of 10,000 units/mL, were from Sigma–Aldrich. Chondroitinase ABC from *Proteus vulgaris* (EC 4.2.2.4), specific activity of 0.5–2 units/mg, and chondroitinase B from *Flavobacterium heparinum* (EC 4.2.2.), specific activity of 100–300 units/mg, were from Sigma–Aldrich. Unsaturated chondro/dermato disaccharides [ΔDi0s (ΔUA-[1→3]-GalNAc), ΔDi4s (ΔUA-[1→3]-GalNAc-4s), ΔDi6s (ΔUA-[1→3]-GalNAc-6s), ΔDi2s (ΔUA-2s-[1→3]-GalNAc), ΔDi2,4dis (ΔDi-dis B, ΔUA-2s-[1→3]-GalNAc-4s), ΔDi2,6dis (ΔDi-dis D, ΔUA-2s-[1→3]-GalNAc-6s), Δdi4,6dis (ΔDi-dis E, ΔUA-[1→3]-GalNAc-4,6dis), and ΔDi2,4,6tris (ΔDi-tris, ΔUA-2s-[1→3]-GalNAc-4s,6s)] were from Seikagaku Corporation (Tokyo City, Japan). Stains-All (3,3′-dimethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine) was from Sigma. QAE Sephadex® A-25 anion-exchange resin was from Pharmacia Biotech (Uppsala, Sweden). Spectrapore dialysis tubing (Mr 1000 daltons cut off) was from Spectrum (Rancho Dominguez, CA, USA). All other reagents were of analytical grade.

2.2. Purification of sturgeon CS

Sturgeon bones (~50 g) were defatted by grinding with 100 mL of acetone, followed by filtration and drying at 60 °C for 24 h. The pellet was solubilized (1 g/10 mL) in 100 mM Na–acetate buffer pH 5.5 containing 5 mM EDTA and 5 mM cysteine. 50 mg of papain were added per g of tissue and the solution incubated for 24 h at 60 °C in a stirrer. After boiling for 10 min, the mixture was centrifuged at 5000*g* for 15 min, and three volumes of ethanol saturated with sodium acetate were added to the supernatant and left at +4 °C for 24 h. The precipitate was recovered by centrifugation at 5000*g* for 15 min and dried at 60 °C for 6 h. The dried precipitate was dissolved in 50 mL of 50 mM NaCl. After centrifugation at 10,000*g* for 10 min, the supernatant was applied to a column (2 cm × 40 cm) packed with QAE Sephadex® A-25 anion-exchange resin equilibrated with the same NaCl solution. GAGs were eluted with a linear gradient of NaCl from 50 mM to 1.2 M in 150 min using low-pressure liquid chromatography (Biological LP chromatography system from BioRad) at a flow of 1 mL/min. Two volumes of ethanol were added to the collected fractions corresponding to fractionated species of polysaccharides evaluated by uronic acid assay[16] and agarose gel electrophoresis.[17,18] After precipitation at 4 °C and centrifugation at 10,000*g* for 10 min, the pellet was dried at 60 °C and solubilized in 20 mM Tris-Cl buffer pH 8.0 containing 2 mM $MgCl_2$ and treated with DNAse I (750 mg) at 37 °C for 12 h. After boiling for 5 min, NaCl concentration was brought to 16% and the GAGs were precipitated by adding 80% methanol. The recovered precipitate (~0.1 g) was solubilized in 20 mL doubly distilled water, dialyzed overnight at 4 °C, and freeze-dried for further characterization.

2.3. Agarose gel electrophoresis

Agarose gel electrophoresis in barium acetate–1,2-diaminopropane was performed as reported elsewhere[17,18] with minor modifications. A Pharmacia Multiphor II (from Pharmacia LKB Biotechnology, Uppsala, Sweden) electrophoretic cell instrument was used. Agarose gel was prepared at a concentration of 0.5% in 0.04 M barium acetate buffer pH 5.8. The run was in 0.05 M 1,2-diaminopropane (buffered at pH 9.0 with acetic acid) for 150 min at 50 mA. After migration, the plate was soaked in cetyltrimethylammonium bromide 0.1% solution for at least 6 h, dried and stained with toluidine blue.[18] Extracted CS was also evaluated by agarose gel electrophoresis after treatment with various lyases.

2.4. Enzymatic treatments and disaccharide evaluation

After treatment of purified CS with chondroitinase ABC or B, the generated unsaturated disaccharides were separated and quantified by strong anion-exchange (SAX)-HPLC using an HPLC equipment from Jasco equipped with a 150 × 4.6-mm stainless-steel column spherisorb 5-SAX (5 μm, trimethylammoniopropyl groups $Si–CH_2–CH_2–CH_2–N^+(CH_3)_3$ in Cl^- form, from Phase Separations Limited, Deeside Industrial Park, Deeside Clwyd, U.K.) and detection at 232 nm. Isocratic separation was performed using 50 mM NaCl pH 4.00 for 5 min followed by a linear gradient from 5 to

60 min of 50 mM NaCl to 1.2 M NaCl pH 4.00, at a flow rate of 1.2 mL/min. Authentic unsaturated standard disaccharides were used for qualitative and quantitative purposes.

2.5. CS molecular mass determination

The molecular mass of sturgeon CS was determined by PAGE according to Edens et al.[19] 20 μg of the purified CS were layered on the gel and the calibration curve was constructed by using oligosaccharide standards of known molecular mass prepared from CS. The gel was stained with toluidine blue (0.1% in acetic acid 1%) for 30 min. After decoloration with acetic acid 1%, molecular mass evaluation was performed after densitometric acquisition.

The molecular mass and polydispersity of CS were also determined by HPSEC using CS fractions of known molecular mass.[20] 50 μg of the extracted CS were injected into the column. The mobile phase was composed of 125 mM Na_2SO_4 and 2 mM NaH_2PO_4 adjusted to pH 6.0 with 0.1 N NaOH. Flow rate was 0.9 mL/min. Standards were solubilized in the mobile phase at a concentration of 10 mg/mL and 10 μL (100 μg) were injected into HPLC. Columns were Protein Pak 125 (Waters, cod. 84601, 7.8 mm × 30 cm) and Protein Pak 300 (Waters, cod. T72711, 7.5 mm × 30 cm) assembled in series. The retention times were plotted against the logarithm of molecular mass for standard heparins. The curve that fits the experimental data is a third grade polynomial with the formula $y(fx) = -ax^3 + bx^2 - cx + d$ performed by the Jasco Borwin program. The number average molecular weight (Mn), the weight average molecular weight (Mw), the *Z* average molecular weight (Mz), and the polydispersity index (Mw/Mn) were calculated by the Jasco Borwin GPC software ver 4.1.

2.6. NMR analysis

The ^{13}C NMR spectrum of purified CS was recorded by a Bruker AMX400 WB spectrometer operating at 100.61 MHz. The sample was prepared by dissolving 50 mg in 2.0 mL of D_2O at a high level of deuteration (99.997%) to avoid the presence of a relatively high percentage of water. The spectra were recorded at a temperature of 33 °C and pH 6.5, unless specified. ^{13}C chemical shifts (δ, ppm) are quoted with respect to external sodium 4,4-dimethyl-4-silapentane-1-sulfonate (0.0 ppm).

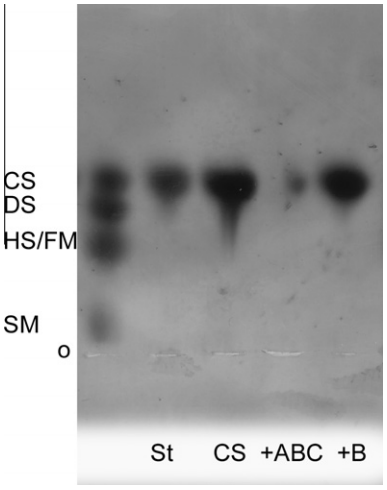

Figure 2. Agarose gel electrophoresis stained with toluidine blue of the chondroitin sulfate purified from sturgeon bones (CS) before and after treatment with chondroitinase ABC (+ABC) or B (+B). CS, chondroitin sulfate standard (also marked St). DS, dermatan sulfate. HS, heparan sulfate. FM, fast-moving heparin. SM, slow-moving heparin. o = origin.

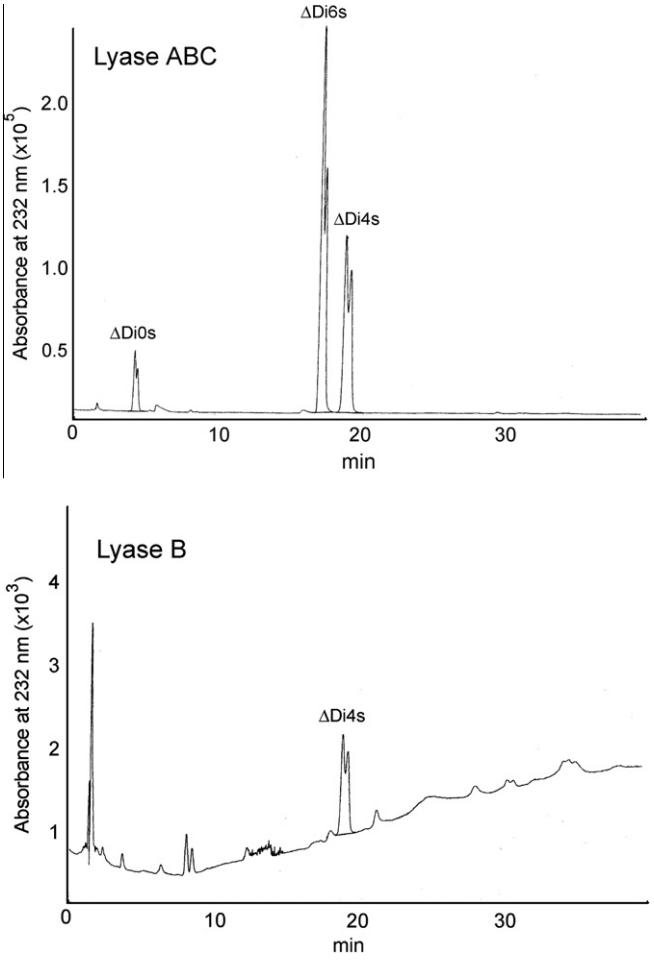

Figure 3. SAX-HPLC of the unsaturated repeating disaccharides produced by treating CS isolated from sturgeon bone with chondroitinase ABC (Lyase ABC) or chondroitinase B (Lyase B). ΔDi0s, ΔUA-[1→3]-GalNAc. ΔDi6s, ΔUA-[1→3]-GalNAc-6s. ΔDi4s, ΔUA-[1→3]-GalNAc-4s.

3. Results and discussion

After defatting with organic solvents, extraction by proteolytic treatment, and degradation of DNA by using DNAse, GAGs from the sturgeon bones were fractionated on an anion-exchange resin. The carbazole test for uronic acids[16] and agarose gel electrophoresis[17,18] confirmed the presence of a single polysaccharide, that is, CS (Fig. 2), in the recovered fractions. Quantitative analyses yielded ~0.34% CS for dry tissue by carbazole test[16] and ~0.28% by agarose gel electrophoresis.

To characterize the structure of sturgeon bone CS, the purified CS was subjected to treatment with two lyases, chondroitinase ABC and B, and the unsaturated disaccharides produced were analyzed by SAX-HPLC (Fig. 3) for a full characterization of the constituent repeating disaccharides. The chondroitinase ABC was able to produce three unsaturated disaccharides, the nonsulfated Δdi0s [ΔUA1→3GalNAc], the monosulfated Δdi6s [ΔUA1→3GalNAc6(SO_4)], and the Δdi4s [ΔUA1→3GalNAc4(SO_4)]. No disulfated or trisulfated disaccharides were generated, thus confirming the absence of these species inside the CS carbohydrate backbone. This polymer was found to be composed of ~55% of disaccharide monosulfated in position 6 of the GalNAc, ~38% of disaccharide monosulfated in position 4 of the GalNAc, and ~7% on nonsulfated disaccharide, with a charge density of ~0.93 and a 4/6-sulfated ratio of 0.69. Due to the incapacity of chondroitinase ABC to distinguish between GlcA and iduronic acid (IdoA),[1] chondroitinase B

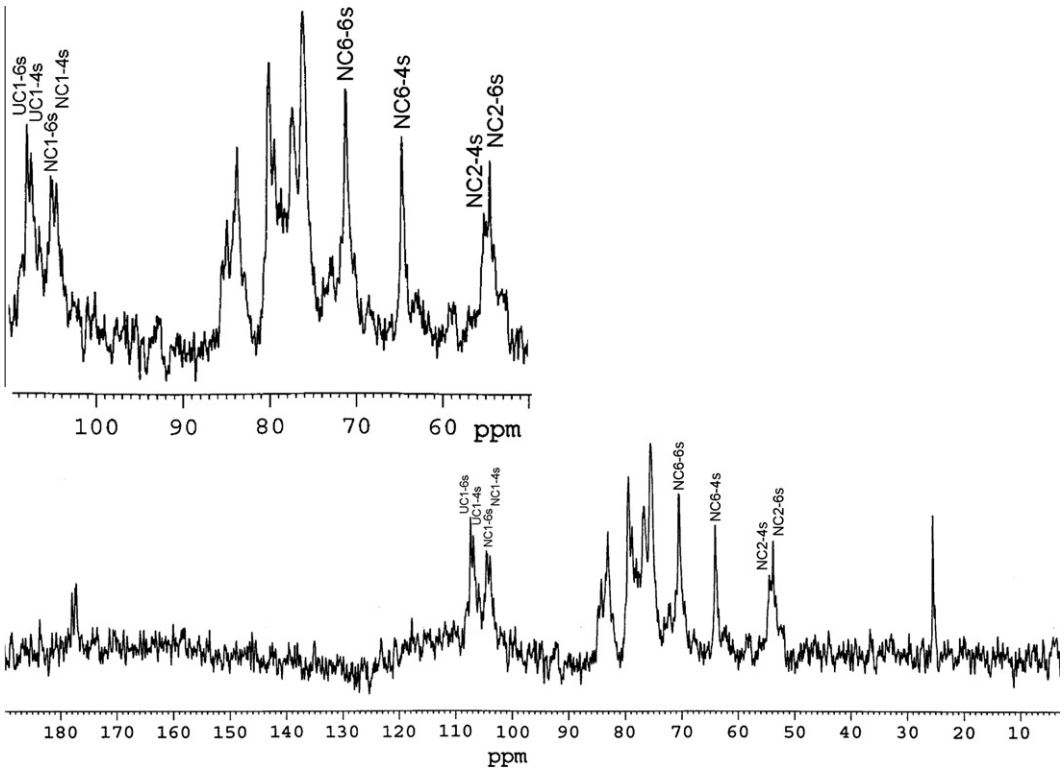

Figure 4. ^{13}C NMR spectrum of the purified sturgeon CS. Chemical shift is reported as ppm. Inset illustrates the expanded spectrum in the region 50–110 ppm. The spectra were recorded by a Bruker AMX400 WB spectrometer operating at 100.61 MHz at a temperature of 33 °C and pH 6.5.

(Fig. 3), specific for IdoA, was used. This last lyase produced <0.5% of the expected repeating disaccharides, in particular Δdi4s (Fig. 3), confirming that the purified sturgeon CS mainly contained GlcA (>99.5%) as uronic acid. These data were also in agreement with the results of agarose gel electrophoresis of the sample treated with both chondroitinases (Fig. 2).

The ^{13}C NMR spectrum of the sturgeon CS is shown in Figure 4. All signals were found in the region 50-110 ppm[21] except for those of carbonyl (around 177–178 ppm) and acetamido methyl carbons (all at 25.6 ppm). Inspection of the 50–70 and 100–110 ppm regions (Fig. 4 inset) revealed a relatively high amount of CS sulfated in position 4 or 6 of the GalNAc confirming the 4/6-sulfated ratio obtained by HPLC of repeating disaccharides. The signals at 107.3 and 104.6 were assigned to the C1 of the GlcA and to the C1 of GalNAc-6SO_4, respectively, and signals at 106.3 and 103.6 were assigned to the C1 of the GlcA and to the C1 of GalNAc-4SO_4 (Fig. 4).[21] Finally, the signal at 70.5 was related to C6 of GalNAc-6SO_4 and the signal at 64.1 was assigned to C6 of GalNAc-4SO_4.

Figure 5A reports the PAGE analysis (densitometric acquisition is illustrated in Fig. 5B) of sturgeon CS showing an average molecular mass of 39,880 calculated on a calibration curve of CS fractions of known molecular masses. Figure 5C illustrates the HPSEC profile of the same extract obtained by UV detection at 214 nm and the third grade polynomial calibration curve used to determine the size-exclusion chromatography parameters. The number average molecular weight (Mn) was 28,030, the weight average molecular weight (Mw) was 37,500, in agreement with the PAGE result, the *Z* average molecular weight (Mz) was 48,250, and the dispersity index (Mw/Mn) was 1.3378.

It is well known that CS from different sources may have variable structures and properties. In particular, the disaccharide repeating units may differ for the number and position of substitution of sulfate groups, as well as for their amount. These repeating disaccharide units are generally monosulfated but, depending on the origin, various disulfated disaccharides (and possibly also a trisulfated one) may be present in the carbohydrate backbone[9,21–24] producing CS having different charge densities. Furthermore, CS samples may also possess various molecular masses and polydispersities depending on the source. To date, considering these structural aspects, CS from sturgeon shows distinctive properties and characteristics. In fact, CS purified from 'terrestrial' sources, that is, avian, porcine, and bovine cartilages, generally shows Mw values between 13,000 and 26,000,[22,23] lower than those observed for sturgeon CS, ~37,000–40,000. On the contrary, CS from cartilaginous fishes, shark and raja, has greater MW values, ~50,000–70,000.[22,23] As a consequence, sturgeon bone CS possesses a molecular mass value intermediate between cartilaginous fishes and pure terrestrial cartilages. Furthermore, we should bear in mind that, for the first time, we have been able to give a full characterization of CS from bones as opposed to previous common CS cartilage sources.

The repeating disaccharide pattern determination gives us more information on the various CS samples and related origins[22,23] as it is possible to determine the charge density values (as sulfate group number per disaccharide unit) and the 4-sulfated/6-sulfated ratio (4s/6s ratio as the ratio between the sulfated groups located in position 4 and 6 on GalNAc). In fact, CS produced from various species shows different qualitative and quantitative repeating disaccharide patterns and values. In particular, 'terrestrial' CS samples from bovine, porcine, and avian cartilages shows the same charge density values, from 0.90 to 0.96 (0.93 for sturgeon CS), due to the absence of disulfated (and trisulfated) disaccharides and to the presence of 6–8% of the nonsulfated disaccharide, but different 4s/6s ratios. In fact, the 4-sulfated disaccharide content in bovine CS is almost double that of the monosulfated disaccharide in position 6 producing a 4s/6s ratio in the range of 1.5–2.5. The percentage of the 4-sulfated disaccharide increases in comparison with the 6-sulfated one in avian CS causing a 4s/6s ratio in the range of 3.0–4.0, and

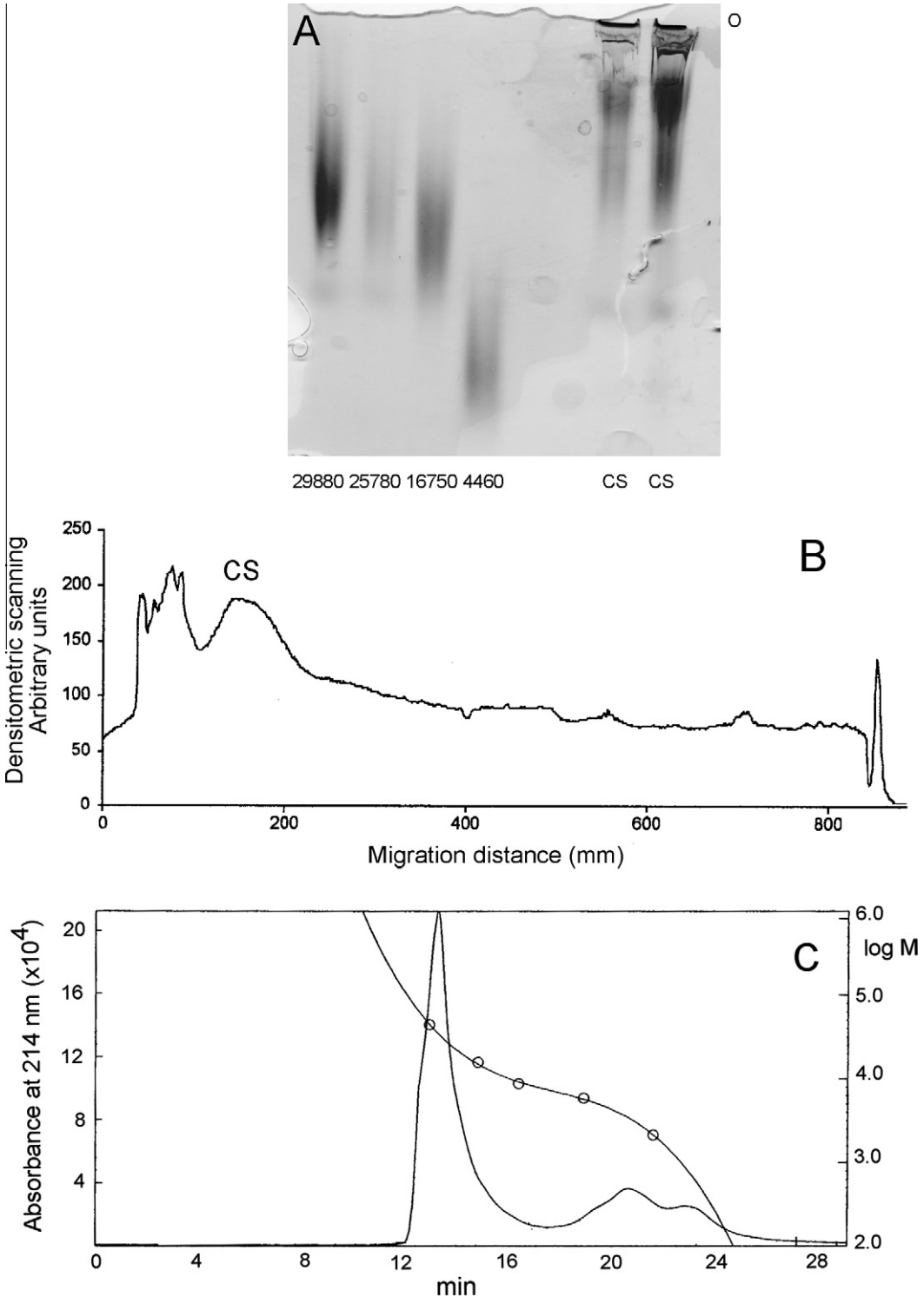

Figure 5. (A) PAGE analysis of sturgeon CS at two different concentrations with the related densitometric scanning (B). The calibration curve was constructed using saccharide standards of known molecular mass prepared from CS and having masses of 29,880, 25,780, 16,750, and 4460. Gels were stained with 0.1% toluidine blue in 1% acetic acid for 30 min. After decoloration with 1% acetic acid, molecular mass evaluation was performed through densitometric acquisition (B). (C) HPSEC analysis of sturgeon CS. The calibration curve was constructed using saccharide standards having mass values of 29,880, 8700, 4460, 3700, and 2130 (see the third grade polynomial curve inside the HPSEC profile).

porcine CS has the lowest amount of the 6-sulfated disaccharide with a high 4s/6s ratio in the range of 4.5–7.0. On the contrary, CS samples from cartilaginous fishes, shark,[22,23] dogfish,[25] and skate[22,23] have particular charge density values greater than approx. 1.0 due to the presence of disulfated disaccharides, and a 4s/6s ratio lower than 0.7 in the case of shark CS due to the presence of a higher percentage of 4-sulfated groups with respect to 6-sulfated ones, and between 1.0 and 1.4 in the case of dogfish and skate polysaccharides due to a similar percentage of the two monosulfated disaccharides. Also in this context, sturgeon CS shows intermediate characteristics between cartilaginous fishes and pure terrestrial cartilages. In fact, this CS from the bony fish

sturgeon has no disulfates (or trisulfated) disaccharides typical of cartilaginous fishes but it shows a greater percentage of 6-sulfated disaccharide than 4-sulfated disaccharide, with an overall 4s/6s ratio of ~0.7 very close to that of shark cartilages.

GAGs constitute a considerable fraction of the glycoconjugates located on cellular membranes and in the extracellular matrix of virtually all mammalian tissues and also other vertebrates and many invertebrates. CS is particularly interesting for further studies and applications because it is expressed in many tissues and organs. In fact, growing evidence suggests that this GAG is an important cofactor in a variety of cell behaviors, in the development of the CNS, and it also acts as a receptor for various pathogens.[1,5] Biological activities of CS chains possibly involve various growth factors and chemokines, and these functions are closely associated with the sulfation patterns and characteristics of the polysaccharide chains.[5] As a consequence, CS functions and possible therapeutic applications are closely related to its specific structure and properties. With this aim, new bioactive sources of CS, such as from sturgeon bone, may represent potential drugs for future research and development and would enable the production of this polysaccharide having a distinctive repeating disaccharide composition, structure, and possible biological properties.

In conclusion, CS was purified for the first time from a bony fish and analyzed to evaluate its structure and properties also in relationship to similar polysaccharides extracted from cartilaginous fish and terrestrial avian and mammalian species. Furthermore, on the basis of the data collected, it is reasonable to assume that CS isolated from sturgeon might be potentially useful for scientific and pharmacological applications, making this bony fish, which is generally discarded after ovary collection, a useful source of this polymer.

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