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Structural characterization of chondroitin sulfate from sturgeon bone

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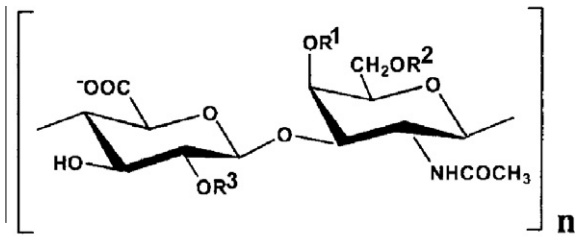
ABSTRACT

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