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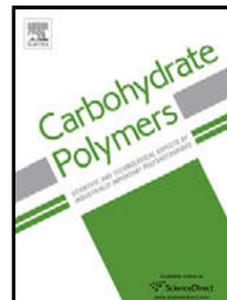
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1 **Isolation and structural characterization of chondroitin sulfate from bony fishes**

2 Francesca Maccari, Fabio Galeotti, Nicola Volpi

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4

5 **Highlights**

- 6 1. Chondroitin sulfate (CS) was purified from the bones of common fishes
- 7 2. The disulfated species $\Delta\text{Di}_{2,6\text{dis}}$ was present in all CS in a range of 1.3÷10.5%
- 8 3. These disaccharides may be a useful marker to check the CS marine origin
- 9 4. Spiny dogfish may be an alternative source of CS extracted from endangered
- 10 shark
- 11 5. CS from bony fishes may be useful for scientific and pharmacological
- 12 applications

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14

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4

5 **Running title**

6 CS from bony fishes

7

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18 **Abbreviations used**

19 CS, chondroitin sulfate. GlcA, β -D-glucopyranosyluronic acid. GAG(s),
20 glycosaminoglycan(s). GalNAc, *N*-acetyl-galactosamine. HPLC, high pressure liquid
21 chromatography. SAX, strong anion exchange.

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4

5 **Abstract**

6 Chondroitin sulfate (CS) was purified from the bones of common fishes, monkfish, cod,
7 spiny dogfish, salmon and tuna, and characterized in an effort to find alternative
8 sources and new peculiar structures of this complex biomacromolecule utilized in the
9 pharmaceutical and nutraceutical industry. Quantitative analyses yielded a CS content
10 ranging from 0.011% for cod up to 0.34% for monkfish. The disaccharide pattern
11 showed the presence of nonsulfated disaccharide, monosulfated species $\Delta\text{Di}6\text{s}$ and
12 $\Delta\text{Di}4\text{s}$, and disulfated disaccharides in different percentages. The disulfated species
13 $\Delta\text{Di}2,6\text{dis}$ was present in all CS extracts in a range of 1.3–10.5%. The presence of
14 these disulfated disaccharides may be a useful marker for the marine origin of CS. The
15 newly identified sources would certainly enable the production of CS with unique
16 disaccharide composition and properties.

17

18 **Keywords**

19 Glycosaminoglycans; Chondroitin sulfate; Fishes; Bones; Glycosaminoglycans

20

1 **1. Introduction**

2 Chondroitin sulfate (CS) is an anionic biomacromolecule belonging to the class of
3 glycosaminoglycans (GAGs) (Volpi, 2006; Lauder, 2009). It is a linear, sulfated,
4 polydisperse natural polysaccharide possessing structural microheterogeneity and a
5 wide variety of physiological functions (Volpi, 2006. Lauder, 2009). CS is composed of
6 alternate sequences of D-glucuronic acid (GlcA) and *N*-acetyl-D-galactosamine
7 (GalNAc) linked by $\beta(1\rightarrow3)$ bonds. Related to their specific origin and depending on the
8 type of source, CS with various carbohydrate backbones are known to possess
9 different degrees of charge density due to sulfate groups in varying amounts and linked
10 in different positions of GlcA and GalNAc. Moreover, CS is a considerably
11 heterogeneous polysaccharide in terms of relative molecular mass, chemical
12 properties, biological and pharmacological activities (Volpi, 2006; Sugahara, Mikami,
13 Uyama, Mizuguchi, Nomura, Kitagawa, 2003).

14 CS is a ubiquitous structural component of cells, tissues and organs and it is
15 implicated in the regulation of many cellular events and physiological processes
16 (Sugahara, Mikami, Uyama, Mizuguchi, Nomura, Kitagawa, 2003). Moreover, CS is
17 largely used as a biomacromolecule in the treatment of osteoarthritis via oral
18 administration (Sarzi-Puttini, Cimmino, Scarpa, Caporali, Parazzini, Zaninelli, Atzeni,
19 Canesi, 2005), and alone, or in combination with other active ingredients, it is utilized
20 as a dietary supplement based on studies confirming its safe and effective options for
21 the treatment of osteoarthritis symptoms (McAlindon, LaValley, Gulin, Felson, 2000).

22 CS is largely produced from animal sources by extraction and purification
23 processes (Volpi, 2006) generally utilising cartilages as raw material derived from
24 terrestrial animals such as bovine, porcine and avian (Volpi, 2007; Volpi, 2009), or from
25 marine organisms such as shark (Sugahara, Nandanaka, Takeda, Kojima, 1996), skate
26 (Lignot, Lahogue, Bourseau, 2003), squid (Cássaro & Dietrich, 1977) and sturgeon
27 (Maccari, Ferrarini, Volpi, 2010; Zhao, Zhou, Mao, Zou, Zhao, Bai, Yang, Wu, 2013).
28 However, its safety has been in question because of possible epidemics when it is
29 purified from land animal tissues, including mad-cow disease, foot-and-mouth disease,
30 and hog cholera (Volpi, 2006). Furthermore, there is a strong limitation of raw material
31 available for the production of unique CS from endangered animals such as sharks.
32 Moreover, due to its very complex heterogeneous structure, CS from different sources
33 may present disaccharides with different numbers, as well as sulfate group positions
34 located in different percentages inside the polysaccharide chains. These disaccharide
35 units are generally monosulfated but, depending on the origin, various disulfated

1 disaccharides (and possibly also a trisulfated one) may be present in the carbohydrate
2 backbone. In particular, highly sulfated disaccharides and possibly highly sulfated
3 oligosaccharide sequences inside the CS backbone may be responsible for closer and
4 more specific interactions with biological components (Sugahara, Nadanaka, Takeda,
5 Kojima, 1996; Sugahara, Mikami, Uyama, Mizuguchi, Nomura, Kitagawa, 2003).

6 The above considerations have motivated us to look for alternative sources of
7 this complex polysaccharide also considering the possibility of producing CS with
8 peculiar disaccharide composition and structure responsible for specific activities. In
9 this regard, bony fishes could serve as an excellent new source for commercial CS
10 thanks to their abundance and possible extensive culture programs, as well as the
11 reutilization of their bones discarded as side-products of fish meat processing. In fact,
12 to date, no detailed CS composition and content from the bones of the most common
13 and caught fishes have been determined. In this study, CS macromolecules were
14 extracted and purified from the bones of monkfish, cod, spiny dogfish, salmon and
15 tuna, and their structure characterized along with their important structural properties.

17 2. Material and methods

18 2.1. Material

19 GAGs standard, heparan sulfate from bovine kidney, CS from bovine trachea,
20 dermatan sulfate from porcine intestinal mucosa, and hyaluronic acid (hyaluronan)
21 from rooster comb, were from Sigma-Aldrich (St. Louis, MO, USA). Papain from
22 papaya latex (EC 3.4.22.2), specific activity of 16-40 units/mg protein, and
23 deoxyribonuclease I, DNase I (EC 3.1.21.1) from bovine pancreas, specific activity of
24 10,000 units/ml, were procured from Sigma-Aldrich. Chondroitinase ABC, chondroitin
25 ABC lyase, from *Proteus vulgaris* (EC 4.2.2.4), specific activity of 0.5-2 units/mg, and
26 chondroitinase B, chondroitin B lyase, from *Flavobacterium heparinum* (EC 4.2.2.),
27 specific activity of 100-300 units/mg, were from Sigma-Aldrich. Unsaturated
28 chondro/dermato disaccharides [Δ Di0s (Δ UA-[1 \rightarrow 3]-GalNAc), Δ Di4s (Δ UA-[1 \rightarrow 3]-
29 GalNAc-4s), Δ Di6s (Δ UA-[1 \rightarrow 3]-GalNAc-6s), Δ Di2s (Δ UA-2s-[1 \rightarrow 3]-GalNAc),
30 Δ Di2,4dis (Δ Di-dis B, Δ UA-2s-[1 \rightarrow 3]-GalNAc-4s), Δ Di2,6dis (Δ Di-dis D, Δ UA-2s-
31 [1 \rightarrow 3]-GalNAc-6s), Δ di4,6dis (Δ Di-dis E, Δ UA-[1 \rightarrow 3]-GalNAc-4,6dis), and Δ Di2,4,6tris
32 (Δ Di-tris, Δ UA-2s-[1 \rightarrow 3]-GalNAc-4s,6s)] were from Seikagaku Corporation (Tokyo
33 City, Japan) and Sigma-Aldrich. Stains-All (3,3' - dimethyl - 9 - methyl - 4,5,4',5' -
34 dibenzothiacarbocyanine) was from Sigma-Aldrich. QAE Sephadex® A-25 anion-
35 exchange resin was from Pharmacia Biotech (Uppsala, Sweden). Spectrapore dialysis

1 tubing (Mr 1,000 daltons cut off) was from Spectrum (Rancho Dominguez, CA, USA).
2 All other reagents were of analytical grade.

3 Monkfish, codfish, spiny dogfish, salmon and tuna were obtained from a local
4 market in Modena, Italy, and identified as to the species and area in which they were
5 caught.

6 **2.2. Purification of bony fish CS**

7 Fish bones (~10 g) were defatted by grinding with 100 mL of acetone, filtration
8 and drying at 60°C for 24 h. The pellet was solubilized (1 g/10 mL) in 100 mM Na-
9 acetate buffer pH 5.5 containing 5 mM EDTA and 5 mM cysteine. 60 mg of papain
10 were added per g of tissue and the solution incubated for 24 h at 60°C in a stirrer. After
11 boiling for 10 min, the mixture was centrifuged at 5,000 g for 15 min, and three volumes
12 of ethanol saturated with sodium acetate were added to the supernatant and stored at
13 +4°C for 24 h. The precipitate was recovered by centrifugation at 5,000 g for 15 min
14 and dried at 60°C for 6 h. The dried precipitate was dissolved in 50 mL of 50 mM NaCl.
15 After centrifugation at 10,000 g for 10 min, the supernatant was applied to a column (2
16 cm x 40 cm) packed with QAE Sephadex® A-25 anion-exchange resin equilibrated with
17 the same NaCl solution. GAGs were eluted with a linear gradient of NaCl from 50 mM
18 to 1.2 M from 0 to 150 min using low-pressure liquid chromatography (Biologic LP
19 chromatography system from BioRad) at a flow of 1 mL/min. Two volumes of ethanol
20 were added to the collected fractions corresponding to single species of GAGs
21 evaluated by uronic acid assay (Cesaretti, Luppi, Maccari, Volpi, 2003) and agarose-
22 gel electrophoresis (Volpi, 1993; Volpi & Maccari, 2002), and polysaccharide(s)
23 precipitated at 4°C. After centrifugation at 10,000 g for 10 min, the pellet was dried at
24 60°C and solubilized in 20 mM Tris-Cl buffer pH 8.0 containing 2 mM MgCl₂ and
25 treated with DNase I (750 mg) at 37°C for 12 h. After boiling for 5 min, NaCl
26 concentration was brought to 16% and the GAGs were precipitated by adding 80%
27 methanol. The recovered precipitate was solubilized in 20 mL bidistilled water, dialyzed
28 overnight at 4°C and freeze-dried for further structural characterization.

29 **2.3. Agarose-gel electrophoresis**

30 Purified bony fish CS samples were evaluated for the presence of other complex
31 GAGs by agarose-gel electrophoresis performed in barium acetate/1,2-diaminopropane
32 (Volpi, 1993; Volpi & Maccari, 2002). A Pharmacia Multiphor II (from Pharmacia LKB
33 Biotechnology, Uppsala, Sweden) electrophoretic cell instrument was used. Agarose-
34 gel was prepared at a concentration of 0.5% in 0.04 M barium acetate buffer pH 5.8.
35 The run was in 0.05 M 1,2-diaminopropane (buffered at pH 9.0 with acetic acid) for 240

1 min at 50 mA. After migration, the plate was soaked in cetyltrimethylammonium
2 bromide 0.1% solution for at least 6 hours, dried and stained with toluidine blue.

3 **2.4. Enzymatic treatment and constitutive disaccharide determination**

4 After treatment of the purified CS samples with chondroitinase ABC (or
5 chondroitinase B), the generated unsaturated disaccharides were separated and
6 quantified by strong anion-exchange (SAX) by means of HPLC equipment from Jasco
7 equipped with a 150 x 4.6-mm stainless-steel column spherisorb 5-SAX (5 μ m,
8 trimethylammoniopropyl groups Si-CH₂-CH₂-CH₂-N⁺(CH₃)₃ in Cl⁻ form, from Phase
9 Separations Limited, Deeside Industrial Park, Deeside Clwyd, U.K.) and detection at
10 232 nm. Isocratic separation was performed using 50 mM NaCl pH 4.00 for 5 min
11 followed by a linear gradient from 5 to 60 min of 50 mM NaCl to 1.2 M NaCl pH 4.00,
12 at a flow rate of 1.2 mL/min. Authentic unsaturated standard disaccharides were used
13 for qualitative and quantitative purposes.

14 **2.5. CS molecular mass determination**

15 The molecular mass of bony fish CS was determined by PAGE according to
16 Edens *et al.* (Edens, al-Hakim, Weiler, Rethwisch, Fareed, Linhardt, 1982). 25 μ g of
17 the purified CS determined by uronic acid assay (Cesaretti, Luppi, Maccari, Volpi,
18 2003) were layered on the gel. The related calibration curve was constructed by using
19 oligosaccharide standards of known molecular mass prepared from CS (Buzzega,
20 Maccari, Volpi, 2010). After a run of 30 min at 50 mA, the gel was stained with toluidine
21 blue (0.1% in acetic acid 1%) for 30 min followed by destaining in 1% acetic acid.
22 Molecular mass evaluation was performed by densitometric acquisition of bands and
23 comparison of their migration times on the calibration curve constructed by plotting
24 retention times of standards against their logarithm of molecular mass values.

26 **3. Results**

27 The fish bones were defatted with organic solvents, subjected to proteolytic
28 treatment, and GAGs extracted after degradation of DNA by DNase and after
29 fractionation on an anion-exchange resin. Agarose-gel electrophoresis (Volpi, 1993;
30 Volpi & Maccari, 2002) specific for sulfated GAGs confirmed the presence of CS as a
31 unique polysaccharide (Figure 1). This was also established by specific treatment with
32 chondroitinase B specific for dermatan sulfate showing no presence of this
33 polysaccharide in CS preparations (see below). The quantitative analyses performed
34 by means of the carbazole test for uronic acids (Cesaretti, Luppi, Maccari, Volpi, 2003)
35 yielded a CS content ranging from 0.011% for cod up to 0.34% for monkfish (Table 1).

1 Purified bony fish CS samples were subjected to treatment with chondroitin ABC
2 lyase and the unsaturated disaccharides produced were analysed by SAX-HPLC
3 (Figure 2). Apart from the monosulfated disaccharide $\Delta\text{Di}2\text{s}$ and the trisulfated species
4 $\Delta\text{Di}2,4,6\text{tris}$ determined in trace amounts ($<0.1\%$), chondroitinase ABC produced
5 various unsaturated disaccharides in different percentages from bony fish CS samples
6 (Table 1). In particular, the nonsulfated disaccharide $\Delta\text{Di}0\text{s}$ was found ranging from 1.6
7 up to 12.9 in monkfish, while monosulfated disaccharides $\Delta\text{Di}6\text{s}$ and $\Delta\text{Di}4\text{s}$ were
8 evaluated to be 27.3 ± 55.7 and 25.3 ± 63.2 , respectively, for a 4s/6s ratio ranging from
9 0.45 to 2.23 (Table 1). Interestingly, disulfated disaccharides were observed in CS
10 samples extracted from all bony fishes even if in different percentages. The disulfated
11 species $\Delta\text{Di}2,6\text{dis}$ was present in all CS extracts in a range of $1.3\pm 10.5\%$ (Table 1)
12 while the disulfated disaccharide $\Delta\text{Di}4,6\text{dis}$ was found in elevated concentration only in
13 monkfish CS (6.3%). The presence of disulfated disaccharides produced a great
14 overall charge density of bony fishes CS ranging from 0.95 up to 1.06.

15 Due to the incapacity of chondroitinase ABC to distinguish between GlcA and
16 iduronic acid (Maccari, Ferrarini, Volpi, 2010), chondroitinase B specific for iduronic
17 acid was used. Chondroitinase B produced $<0.5\%$ of the expected disaccharides, in
18 particular $\Delta\text{di}4\text{s}$ (not shown), confirming that CS is the main polysaccharide purified
19 from bony fishes, as also observed by agarose-gel electrophoresis (see above).

20 Figure 3 shows the PAGE analysis of bony fish CS extracts calculated on a
21 calibration curve of CS fractions of known molecular masses. CS from monkfish shows
22 a larger polydispersity and a higher molecular mass value of 48680 than other
23 samples. CS extracts from the other bony fishes have values ranging from 13460 to
24 32940 (Table 1).

25

26 4. Discussion

27 CS was extracted from the bones of five different species of very common fishes,
28 monkfish, codfish, spiny dogfish, salmon and tuna, as part of a search for new sources
29 of this biomacromolecule, also considering the possibility of using the huge amount of
30 bones discarded as side-products of fish meat processing. Moreover, it is useful to
31 investigate other marine organisms as alternative and peculiar sources of abundant CS
32 for the manufacture of CS-containing pharmaceuticals and nutraceuticals. The most
33 relevant structural characteristic common to CS purified from the different bony fishes
34 was the presence of disulfated disaccharides in various percentages and types. In
35 particular, the disaccharide disulfated in position 2 of GlcA and 6 of GlcNAc, named

1 disaccharide D, was determined in all species in variable amounts from 1.3 up to
2 10.5% of total disaccharides. This disaccharide is a typical constituent of CS purified
3 from cartilaginous fishes such as shark, dogfish and skate where it is present in
4 percentages even higher than 10% (Volpi, 2007; Gargiulo, Lanzetta, Parrilli, De Castro,
5 2009). Moreover, monkfish CS shows the presence of a high content of another
6 disulfated disaccharide, named disaccharide E disulfated in position 4 and 6 of GlcNAc,
7 which is typical from squid (Habuchi, Moroi, Ohtake, 2002). It is worth mentioning that
8 CS purified from terrestrial sources, i.e. avian, porcine and bovine cartilages, shows
9 either no presence or trace of disulfated disaccharides (Volpi, 2007; Volpi, 2009). Thus,
10 these disulfated disaccharides from bony fishes may be a useful marker to check the
11 origin of purified CS, from marine rather than terrestrial sources.

12 Commercial CS is predominantly purified from bovine trachea, porcine nasal
13 septa, chicken keel and shark cartilage (Volpi, 2007; Volpi, 2009). However, frequent
14 occurrences of animal epidemics, including mad-cow disease, foot-and-mouth disease,
15 and hog cholera, concern the continued use of CS from terrestrial sources. So far,
16 shark and skate cartilages have been important commercial sources. Moreover, the
17 extract from shark cartilage having a very peculiar pattern of major disaccharide units
18 rich in sulfate groups located at the position 6 of GlcNAc displays many biological
19 activities (Mizumoto, Murakoshi, Kalayanamitra, Deepa, Fukui, Kongtawelert, Yamada,
20 Sugahara, 2013; Sugahara, Nadanaka, Takeda, Kojima, 1996). However, still obtaining
21 CS from shark cartilage might become problematic in the future, as the price of this raw
22 material has been increasing and the shark has been declared to be an endangered
23 animal. In the light of these concerns, spiny dogfish stand out as alternative source of
24 pure CS. In fact, spiny dogfish CS has ~56% of 6-sulfated disaccharide with a 4s/6s
25 ratio of 0.45 while that shark was around ~50% with a 4s/6s ratio of 0.45 ± 0.70 (Volpi,
26 2007). Comparatively, shark CS possesses $5 \pm 20\%$ of the disulfated disaccharide D
27 while that from spiny dogfish was ~10%.

28 Keratan sulfate has been detected in many batches of CS extracted in particular
29 from shark cartilage, averaging approximately 16% of the total GAGs, while its content
30 was found to be very low (<1%) in those produced from bovine cartilage (Pomin,
31 Piquet, Pereira, Mourão, 2012). The removal of KS from CS requires further steps of
32 purification (Galeotti, Maccari, Volpi, 2014). CS purified from bony fishes was
33 determined according to previous published methodologies (Galeotti, Maccari, Volpi,
34 2014) and KS was found in the order of <1% (not shown), comparable to levels
35 measured in bovine cartilage. As a result, CS extracted from bony fishes requires less

1 complicated purification procedure in obtaining a highly pure product compatible with
2 its use in pharmaceutical and nutraceutical applications.

3 In conclusion, various CS polysaccharides have been purified for the first time
4 from several bony fishes and their structural disaccharide composition and properties
5 evaluated. On the basis of the data collected, it is reasonable to assume that CS
6 isolated from various bony fishes might be potentially useful for scientific and
7 pharmacological applications, making the bones of these fishes, generally discarded
8 after collecting meat, a useful source of this polymer. Finally, these newly identified
9 sources of CS would enable the production of this macromolecule having a unique
10 disaccharide composition and properties, as in the case of spiny dogfish, making it a
11 useful alternative source of CS extracted from endangered shark.

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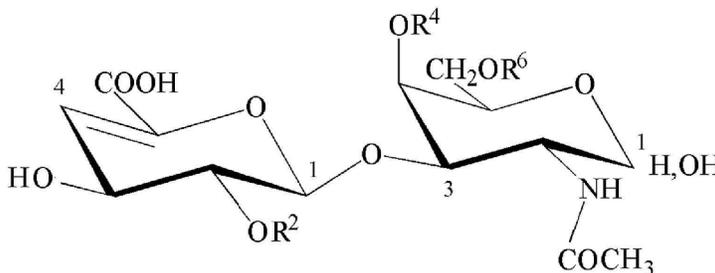
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- 7
8

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1 Table 1. Amount, disaccharide composition and charge density, and molecular mass
2 values of CS purified from various bony fishes.

3



| | Monkfish | Codfish | Spiny Dogfish | Salmon | Tuna |
|--|----------|---------|---------------|--------|-------|
| Total CS% (w/w) | 0.340 | 0.011 | 0.280 | 0.100 | 0.023 |
| Δ Di-0s (Δ UA-GalNAc) (%) | 12.9 | 3.4 | 7.4 | 7.7 | 1.6 |
| Δ Di-6s (Δ UA-GalNAc,6s) (%) | 28.2 | 27.3 | 55.7 | 37.3 | 28.3 |
| Δ Di-4s (Δ UA-GalNAc,4s) (%) | 51.0 | 59.8 | 25.3 | 51.2 | 63.2 |
| Δ Di2s (Δ UA2s-GalNAc) (%) | Trace | Trace | Trace | Trace | Trace |
| Δ Di2,6dis, (Δ UA2s-GalNAc6s) (%) | 1.3 | 9.5 | 10.5 | 3.5 | 4.6 |
| Δ Di4,6dis (Δ UA-GalNAc4,6dis) (%) | 6.3 | Trace | 0.8 | 0.2 | 1.1 |
| Δ Di2,4dis (Δ UA2s-GalNAc4s) (%) | 0.4 | Trace | 0.3 | 0.1 | 1.2 |
| Δ Di2,4,6tris (Δ UA2s-GalNAc4,6dis) | Trace | Trace | Trace | Trace | Trace |
| 4s/6s | 1.81 | 2.19 | 0.45 | 1.37 | 2.23 |
| Charge density | 0.95 | 1.06 | 1.04 | 0.96 | 1.05 |
| Molecular Mass | 48680 | 18120 | 13460 | 20070 | 32940 |

4

5 The scheme illustrates the CS unsaturated disaccharides produced by the action of
6 chondroitinase lyases. R_2 , R_4 and R_6 may be sulfate groups or H. Minor disaccharides
7 may be present such as that characterized by a sulfate group in position 3 of glucuronic
8 acid.

9 CS, chondroitin sulfate. Δ UA, 4,5-unsaturated uronic acid. GalNAc, *N*-acetyl-
10 galactosamine. s, sulfate group. The percentage of each identified disaccharide was
11 determined by purified standards (Seikagaku Co./Sigma-Aldrich) and reported as
12 weight percent. Trace indicates values lower than 0.1%. Charge density was calculated
13 by considering the number of sulfated groups per disaccharide unit. Quantitative data
14 are means of three repetitions.

15

1 **Legends to Figures**

2 Figure 1. Agarose-gel electrophoresis stained with toluidine blue of the chondroitin
3 sulfate samples purified from various bony fishes. CS St., chondroitin sulfate standard
4 of European Pharmacopea. DS, dermatan sulfate. HS, heparan sulfate. O, origin.

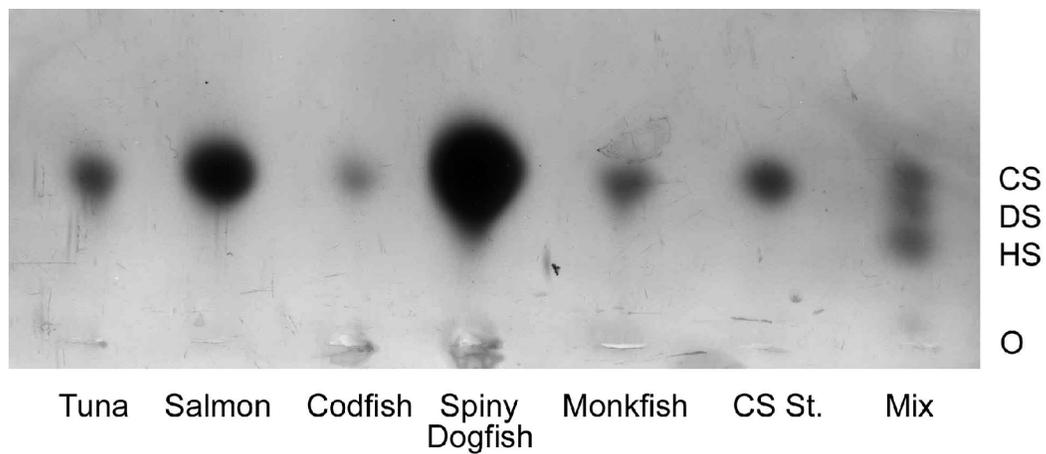
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6 Figure 2. SAX-HPLC separation of the unsaturated disaccharides produced by CS
7 isolated from various bony fishes and treated with chondroitin ABC lyase. Δ Di0s, Δ UA-
8 GalNAc. Δ Di6s, Δ UA-GalNAc6s. Δ Di4s, Δ UA-GalNAc4s. Δ Di2,6dis, Δ UA2s-GalNAc6s.
9 Δ Di4,6dis, Δ UA-GalNAc4,6dis. Δ Di2,4dis, Δ UA2s-GalNAc4s. The identity of
10 disaccharide species was assured by co-elution with purified standards (Seikagaku
11 Co./Sigma-Aldrich)

12

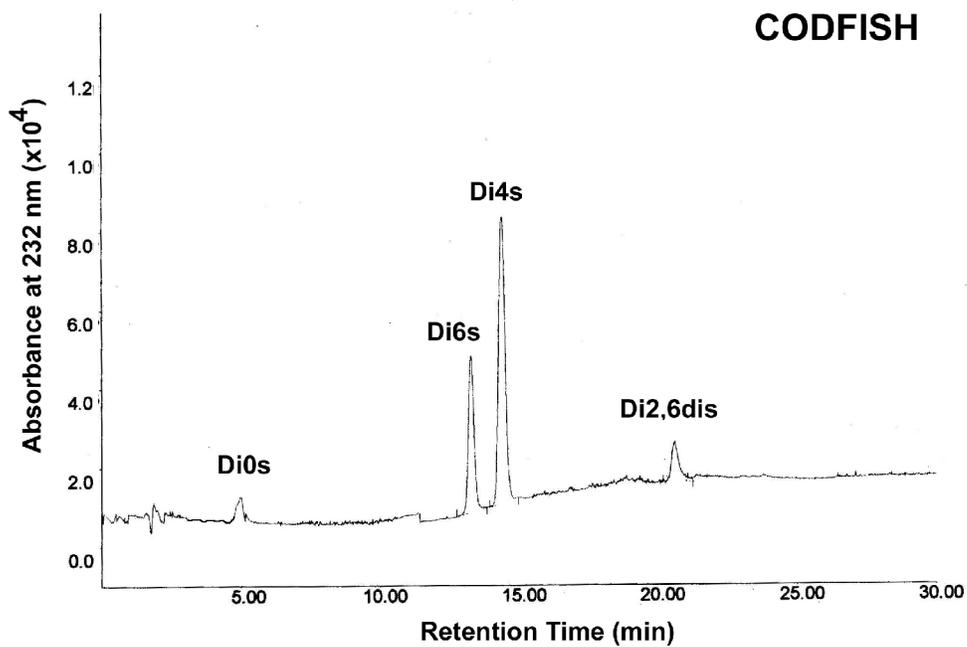
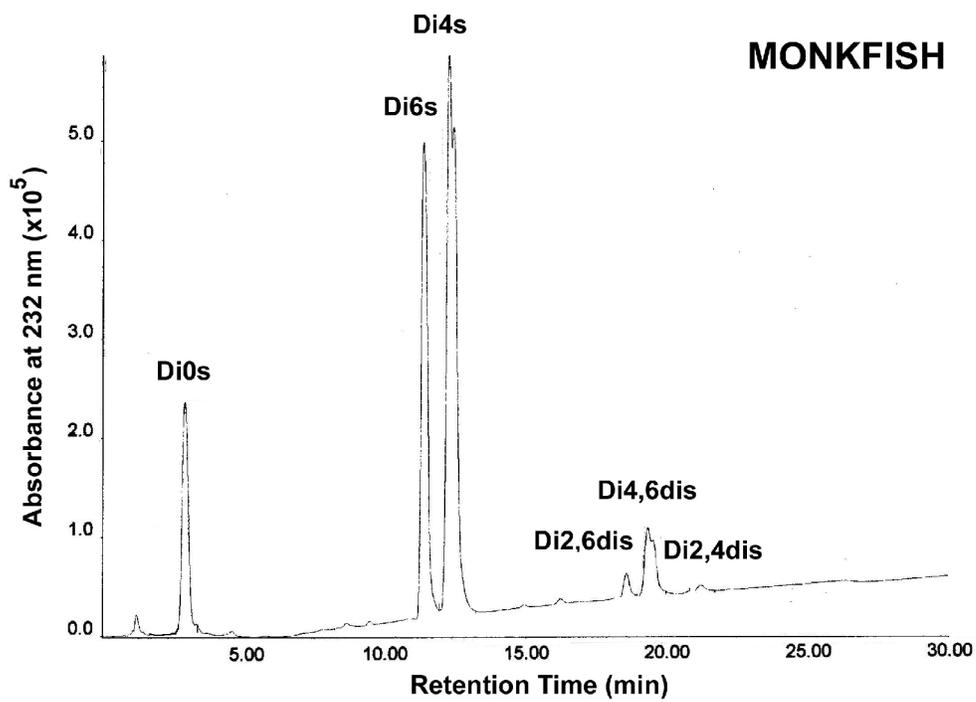
13 Figure 3. PAGE analysis of various bony fishes CS. The calibration curve was
14 constructed by using CS standards of known molecular mass prepared from CS and
15 having masses of 27300, 16770 and 7330. Gel was stained with toluidine blue (0.1% in
16 acetic acid 1%) for 30 min. After decoloration with acetic acid 1%, molecular mass
17 evaluation was performed after densitometric acquisition.

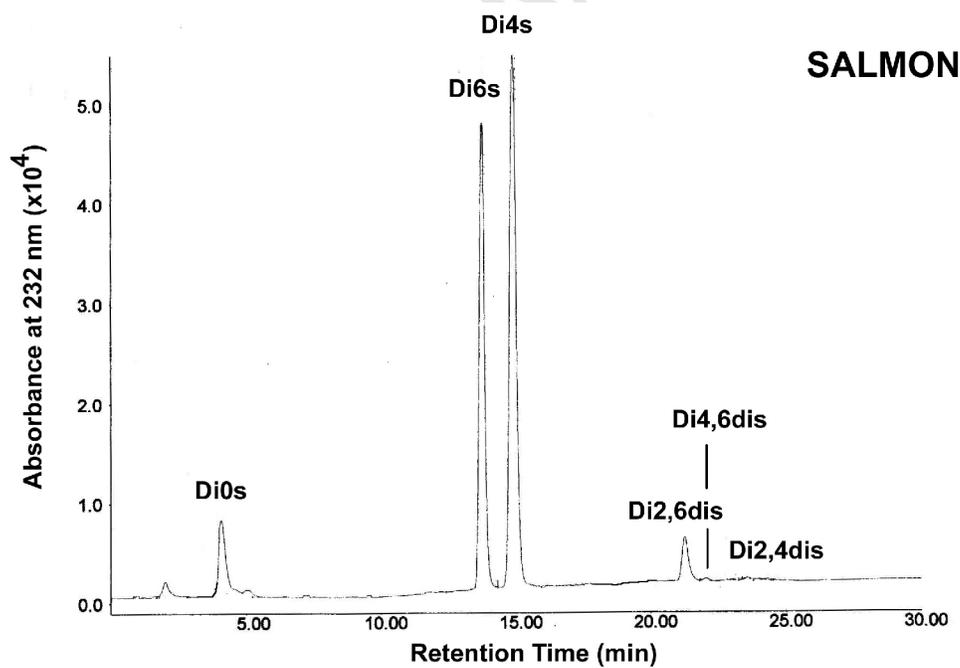
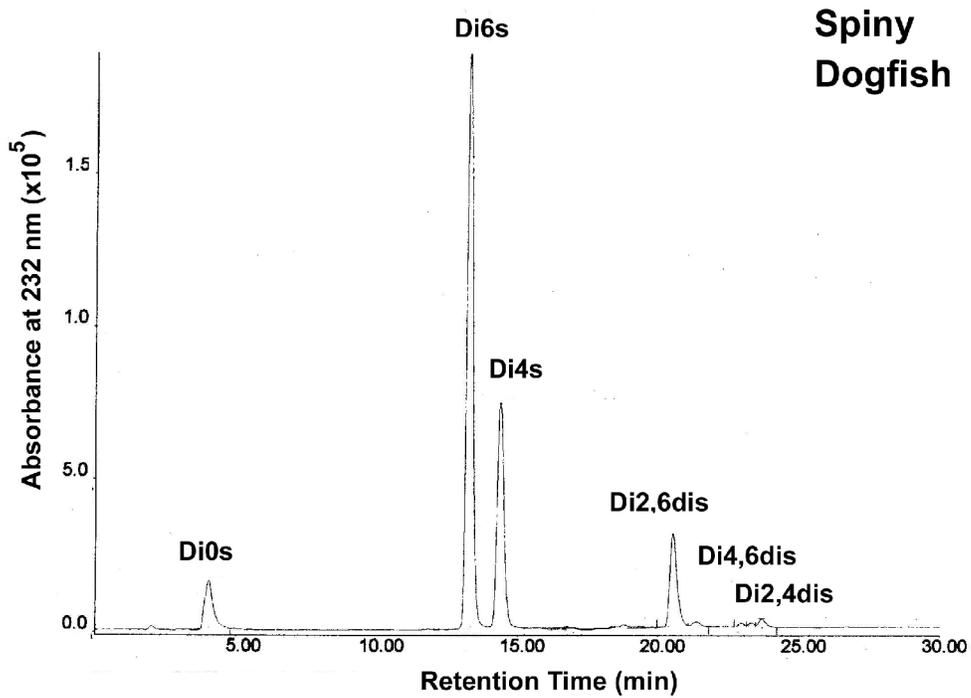
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Figure 1





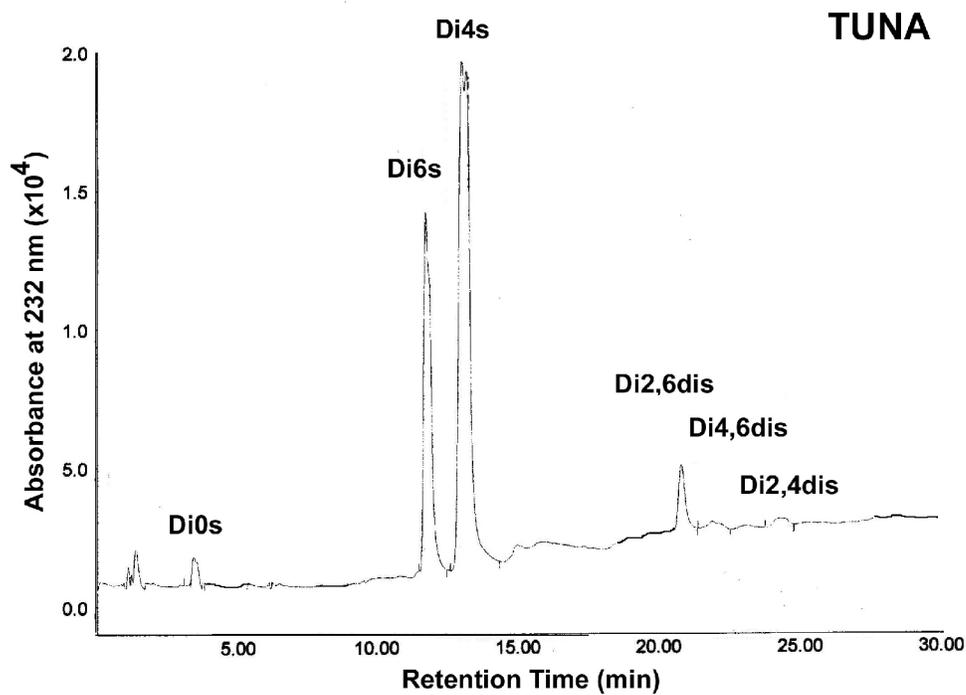


Figure 2

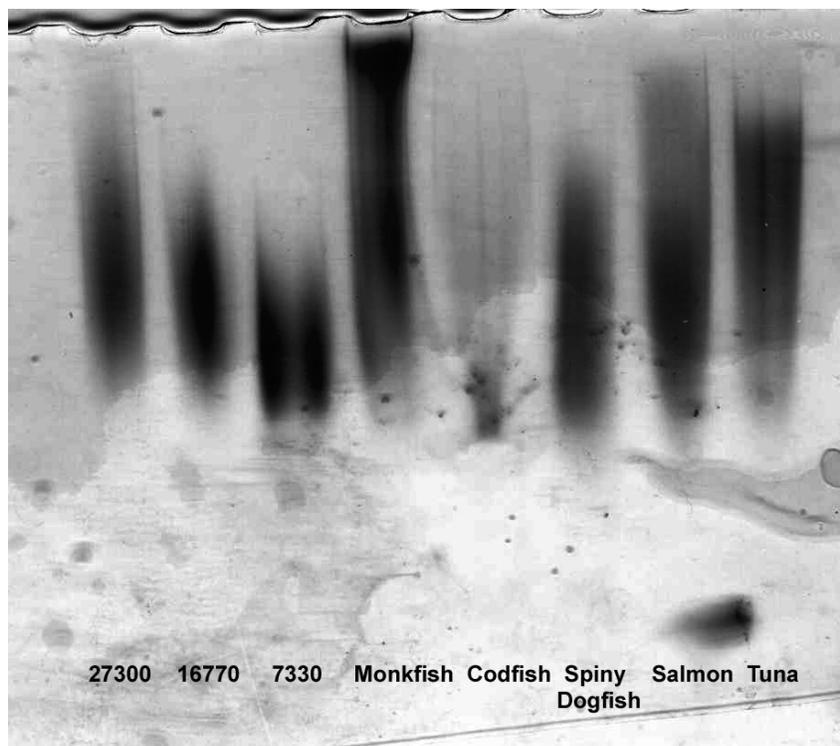


Figure 3