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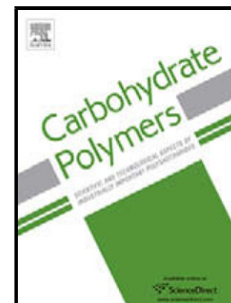
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**Isolation and structural characterization of chondroitin sulfate from bony fishes**

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**Highlights**

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

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**Running title**

CS from bony fishes

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**Abbreviations used**

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

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

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**Abstract**

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

**Keywords**

Glycosaminoglycans; Chondroitin sulfate; Fishes; Bones; Glycosaminoglycans

## 1. Introduction

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

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

CS is largely produced from animal sources by extraction and purification processes (Volpi, 2006) generally utilising cartilages as raw material derived from terrestrial animals such as bovine, porcine and avian (Volpi, 2007; Volpi, 2009), or from marine organisms such as shark (Sugahara, Nandanaka, Takeda, Kojima, 1996), skate (Lignot, Lahogue, Bourseau, 2003), squid (Cássaro & Dietrich, 1977) and sturgeon (Maccari, Ferrarini, Volpi, 2010; Zhao, Zhou, Mao, Zou, Zhao, Bai, Yang, Wu, 2013). However, its safety has been in question because of possible epidemics when it is purified from land animal tissues, including mad-cow disease, foot-and-mouth disease, and hog cholera (Volpi, 2006). Furthermore, there is a strong limitation of raw material available for the production of unique CS from endangered animals such as sharks. Moreover, due to its very complex heterogeneous structure, CS from different sources may present disaccharides with different numbers, as well as sulfate group positions located in different percentages inside the polysaccharide chains. These 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. In particular, highly sulfated disaccharides and possibly highly sulfated oligosaccharide sequences inside the CS backbone may be responsible for closer and more specific interactions with biological components (Sugahara, Nadanaka, Takeda, Kojima, 1996; Sugahara, Mikami, Uyama, Mizuguchi, Nomura, Kitagawa, 2003).

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

## 2. Material and methods

### 2.1. Material

GAGs standard, heparan sulfate from bovine kidney, CS from bovine trachea, dermatan sulfate from porcine intestinal mucosa, and hyaluronic acid (hyaluronan) 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 procured from Sigma-Aldrich. Chondroitinase ABC, chondroitin ABC lyase, from *Proteus vulgaris* (EC 4.2.2.4), specific activity of 0.5-2 units/mg, and chondroitinase B, chondroitin B lyase, from *Flavobacterium heparinum* (EC 4.2.2.), specific activity of 100-300 units/mg, were from Sigma-Aldrich. Unsaturated chondro/dermato disaccharides [ $\Delta$ Di0s ( $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc),  $\Delta$ Di4s ( $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc-4s),  $\Delta$ Di6s ( $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc-6s),  $\Delta$ Di2s ( $\Delta$ UA-2s-[1 $\rightarrow$ 3]-GalNAc),  $\Delta$ Di2,4dis ( $\Delta$ Di-dis B,  $\Delta$ UA-2s-[1 $\rightarrow$ 3]-GalNAc-4s),  $\Delta$ Di2,6dis ( $\Delta$ Di-dis D,  $\Delta$ UA-2s-[1 $\rightarrow$ 3]-GalNAc-6s),  $\Delta$ di4,6dis ( $\Delta$ Di-dis E,  $\Delta$ UA-[1 $\rightarrow$ 3]-GalNAc-4,6dis), and  $\Delta$ Di2,4,6tris ( $\Delta$ Di-tris,  $\Delta$ UA-2s-[1 $\rightarrow$ 3]-GalNAc-4s,6s)] were from Seikagaku Corporation (Tokyo City, Japan) and Sigma-Aldrich. Stains-All (3,3' - dimethyl - 9 - methyl - 4,5,4',5' - dibenzothiacarbocyanine) was from Sigma-Aldrich. QAE Sephadex® A-25 anion-exchange resin was from Pharmacia Biotech (Uppsala, Sweden). Spectrapore dialysis

tubing (Mr 1,000 daltons cut off) was from Spectrum (Rancho Dominguez, CA, USA).

All other reagents were of analytical grade.

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

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

Fish bones (~10 g) were defatted by grinding with 100 mL of acetone, 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. 60 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 5,000 g for 15 min, and three volumes of ethanol saturated with sodium acetate were added to the supernatant and stored at +4°C for 24 h. The precipitate was recovered by centrifugation at 5,000 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 x 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 from 0 to 150 min using low-pressure liquid chromatography (Biologic LP chromatography system from BioRad) at a flow of 1 mL/min. Two volumes of ethanol were added to the collected fractions corresponding to single species of GAGs evaluated by uronic acid assay (Cesaretti, Luppi, Maccari, Volpi, 2003) and agarose-gel electrophoresis (Volpi, 1993; Volpi & Maccari, 2002), and polysaccharide(s) precipitated at 4°C. After 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<sub>2</sub> 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 was solubilized in 20 mL bidistilled water, dialyzed overnight at 4°C and freeze-dried for further structural characterization.

## **2.3. Agarose-gel electrophoresis**

Purified bony fish CS samples were evaluated for the presence of other complex GAGs by agarose-gel electrophoresis performed in barium acetate/1,2-diaminopropane (Volpi, 1993; Volpi & Maccari, 2002). 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 240



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

#### 2.4. Enzymatic treatment and constitutive disaccharide determination

After treatment of the purified CS samples with chondroitinase ABC (or chondroitinase B), the generated unsaturated disaccharides were separated and quantified by strong anion-exchange (SAX) by means of HPLC equipment from Jasco equipped with a 150 x 4.6-mm stainless-steel column spherisorb 5-SAX (5 µm, trimethylammoniopropyl groups Si-CH<sub>2</sub>-CH<sub>2</sub>-CH<sub>2</sub>-N<sup>+</sup>(CH<sub>3</sub>)<sub>3</sub> in Cl<sup>-</sup> 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 bony fish CS was determined by PAGE according to Edens *et al.* (Edens, al-Hakim, Weiler, Rethwisch, Fareed, Linhardt, 1982). 25 µg of the purified CS determined by uronic acid assay (Cesaretti, Luppi, Maccari, Volpi, 2003) were layered on the gel. The related calibration curve was constructed by using oligosaccharide standards of known molecular mass prepared from CS (Buzzega, Maccari, Volpi, 2010). After a run of 30 min at 50 mA, the gel was stained with toluidine blue (0.1% in acetic acid 1%) for 30 min followed by destaining in 1% acetic acid. Molecular mass evaluation was performed by densitometric acquisition of bands and comparison of their migration times on the calibration curve constructed by plotting retention times of standards against their logarithm of molecular mass values.

### 3. Results

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

Purified bony fish CS samples were subjected to treatment with chondroitinase ABC lyase and the unsaturated disaccharides produced were analysed by SAX-HPLC (Figure 2). Apart from the monosulfated disaccharide  $\Delta\text{Di}2\text{s}$  and the trisulfated species  $\Delta\text{Di}2,4,6\text{tris}$  determined in trace amounts ( $<0.1\%$ ), chondroitinase ABC produced various unsaturated disaccharides in different percentages from bony fish CS samples (Table 1). In particular, the nonsulfated disaccharide  $\Delta\text{Di}0\text{s}$  was found ranging from 1.6 up to 12.9 in monkfish, while monosulfated disaccharides  $\Delta\text{Di}6\text{s}$  and  $\Delta\text{Di}4\text{s}$  were evaluated to be  $27.3\pm55.7$  and  $25.3\pm63.2$ , respectively, for a 4s/6s ratio ranging from 0.45 to 2.23 (Table 1). Interestingly, disulfated disaccharides were observed in CS samples extracted from all bony fishes even if in different percentages. The disulfated species  $\Delta\text{Di}2,6\text{dis}$  was present in all CS extracts in a range of  $1.3\pm10.5\%$  (Table 1) while the disulfated disaccharide  $\Delta\text{Di}4,6\text{dis}$  was found in elevated concentration only in monkfish CS (6.3%). The presence of disulfated disaccharides produced a great overall charge density of bony fishes CS ranging from 0.95 up to 1.06.

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

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

#### 4. Discussion

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

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

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

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

## 12 13 **References**

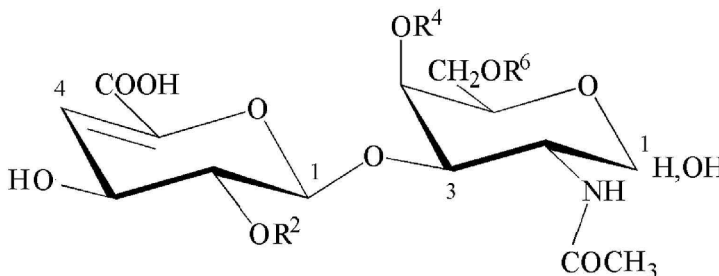
- 14 Buzzega, D., Maccari, F., Volpi, N. (2010). Determination of molecular mass values of  
15 chondroitin sulfates by fluorophore-assisted carbohydrate electrophoresis  
16 (FACE). *J Pharm Biomed Anal*, 51, 969-972.
- 17 Cesaretti, M., Luppi, E., Maccari, F., Volpi, N. (2003). A 96-well assay for uronic acid  
18 carbazole reaction. *Carbohydr Polym*, 54, 59-61.
- 19 Edens, R.E., al-Hakim, A., Weiler, J.M., Rethwisch, D.G., Fareed, J., Linhardt, R.J.  
20 (1982). Gradient polyacrylamide gel electrophoresis for determination of  
21 molecular weights of heparin preparations and low-molecular-weight heparin  
22 derivatives. *J Pharm Sci*, 81, 823-827.
- 23 Galeotti, F., Maccari, F., Volpi, N. (2014). Selective removal of keratan sulfate in  
24 chondroitin sulfate samples by sequential precipitation with ethanol. *Anal  
25 Biochem*, 448, 113-115.
- 26 Gargiulo, V., Lanzetta, R., Parrilli, M., De Castro, C. (2009). Structural analysis of  
27 chondroitin sulfate from *Scyliorhinus canicula*: a useful source of this  
28 polysaccharide. *Glycobiology*, 19, 1485-1491.
- 29 Habuchi, O., Moroi, R., Ohtake, S. (2002). Enzymatic synthesis of chondroitin sulfate E  
30 by N-acetylgalactosamine 4-sulfate 6-O-sulfotransferase purified from squid  
31 cartilage. *Anal Biochem*, 310, 129-136.
- 32 Lauder, R.M. (2009). Chondroitin sulphate: a complex molecule with potential impacts  
33 on a wide range of biological systems. *Complement Ther Med*, 17, 56-62.

- 1 Lignot, B., Lahogue, V., Bourseau, P. (2003). Enzymatic extraction of chondroitin  
2 sulfate from skate cartilage and concentration-desalting by ultrafiltration. *J*  
3 *Biotechnol*, 103, 281-284.
- 4 Maccari, F., Ferrarini, F., Volpi, N. (2010). Structural characterization of chondroitin  
5 sulfate from sturgeon bone. *Carbohydr Res*, 345, 1575-1580.
- 6 McAlindon, T.E., LaValley, M.P., Gulin, J.P., Felson, D.T. (2000). Glucosamine and  
7 chondroitin for treatment of osteoarthritis: a systematic quality assessment and  
8 meta-analysis. *JAMA*, 283, 1469-1475.
- 9 Mizumoto, S., Murakoshi, S., Kalayanamitra, K., Deepa, S.S., Fukui, S., Kongtawelert,  
10 P., Yamada, S., Sugahara, K. (2013). Highly sulfated hexasaccharide sequences  
11 isolated from chondroitin sulfate of shark fin cartilage: insights into the sugar  
12 sequences with bioactivities. *Glycobiology*, 23, 155-168.
- 13 Pomin, V.H., Piquet, A.A., Pereira, M.S., Mourão, P.A. (2012). Residual keratan sulfate  
14 in chondroitin sulfate formulations for oral administration. *Carbohydr Polym*, 90,  
15 839-846.
- 16 Sarzi-Puttini, P., Cimmino, M.A., Scarpa, R., Caporali, R., Parazzini, F., Zaninelli, A.,  
17 Atzeni, F., Canesi, B. (2005). Osteoarthritis: an overview of the disease and its  
18 treatment strategies. *Semin Arthritis Rheum*, 35(1 Suppl 1), 1-10.
- 19 Sugahara, K., Nadanaka, S., Takeda, K., Kojima, T. (1996). Structural analysis of  
20 unsaturated hexasaccharides isolated from shark cartilage chondroitin sulfate D  
21 that are substrates for the exolytic action of chondroitin ABC lyase. *Eur J*  
22 *Biochem*, 239, 871-880.
- 23 Sugahara, K., Mikami, T., Uyama, T., Mizuguchi, S., Nomura, K., Kitagawa, H. (2003).  
24 Recent advances in the structural biology of chondroitin sulfate and dermatan  
25 sulfate. *Curr Opin Struct Biol*, 13, 612-620.
- 26 Volpi, N. (1993). "Fast moving" and "slow moving" heparins, dermatan sulfate, and  
27 chondroitin sulfate: qualitative and quantitative analysis by agarose-gel  
28 electrophoresis. *Carbohydr Res*, 247, 263-278.
- 29 Volpi, N., Ed. (2006). *Chondroitin sulfate: structure, role and pharmacological activity*.  
30 Academic Press, Amsterdam, Boston, Heidelberg, London, New York, Oxford,  
31 Paris, San Diego, San Francisco, Singapore, Sydney, Tokyo.
- 32 Volpi, N. (2007). Analytical aspects of pharmaceutical grade chondroitin sulfates. *J*  
33 *Pharm Sci*, 96, 3168-3180.
- 34 Volpi, N. (2009). Quality of different chondroitin sulfate preparations in relation to their  
35 therapeutic activity. *J Pharm Pharmacol*, 61, 1271-1280.

- 1 Volpi, N. & Maccari, F. (2002). Detection of submicrogram quantities of  
2 glycosaminoglycans on agarose-gels by sequential staining with toluidine blue  
3 and Stains-All. *Electrophoresis*, 23, 4060-4066.
- 4 Zhao, T., Zhou, Y., Mao, G., Zou, Y., Zhao, J., Bai, S., Yang, L., Wu, X. (2013).  
5 Extraction, purification and characterisation of chondroitin sulfate in Chinese  
6 sturgeon cartilage. *J Sci Food Agric*, 93, 1633-1640.
- 7
- 8

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
$\Delta$ Di-0s ( $\Delta$ UA-GalNAc) (%)	12.9	3.4	7.4	7.7	1.6
$\Delta$ Di-6s ( $\Delta$ UA-GalNAc,6s) (%)	28.2	27.3	55.7	37.3	28.3
$\Delta$ Di-4s ( $\Delta$ UA-GalNAc,4s) (%)	51.0	59.8	25.3	51.2	63.2
$\Delta$ Di2s ( $\Delta$ UA2s-GalNAc) (%)	Trace	Trace	Trace	Trace	Trace
$\Delta$ Di2,6dis, ( $\Delta$ UA2s-GalNAc6s) (%)	1.3	9.5	10.5	3.5	4.6
$\Delta$ Di4,6dis ( $\Delta$ UA-GalNAc4,6dis) (%)	6.3	Trace	0.8	0.2	1.1
$\Delta$ Di2,4dis ( $\Delta$ UA2s-GalNAc4s) (%)	0.4	Trace	0.3	0.1	1.2
$\Delta$ Di2,4,6tris ( $\Delta$ 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.  $\Delta$ 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

## Legends to Figures

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

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

Figure 3. PAGE analysis of various bony fishes CS. The calibration curve was constructed by using CS standards of known molecular mass prepared from CS and having masses of 27300, 16770 and 7330. 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.



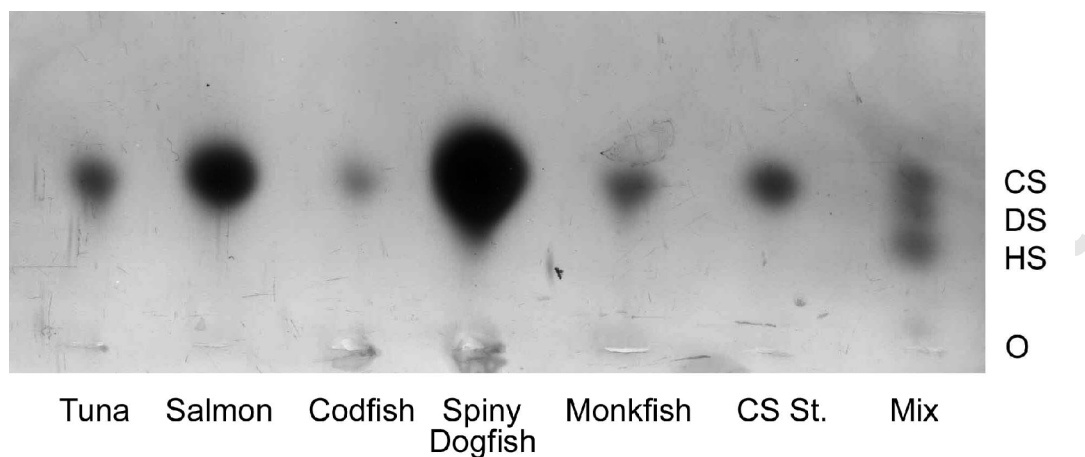
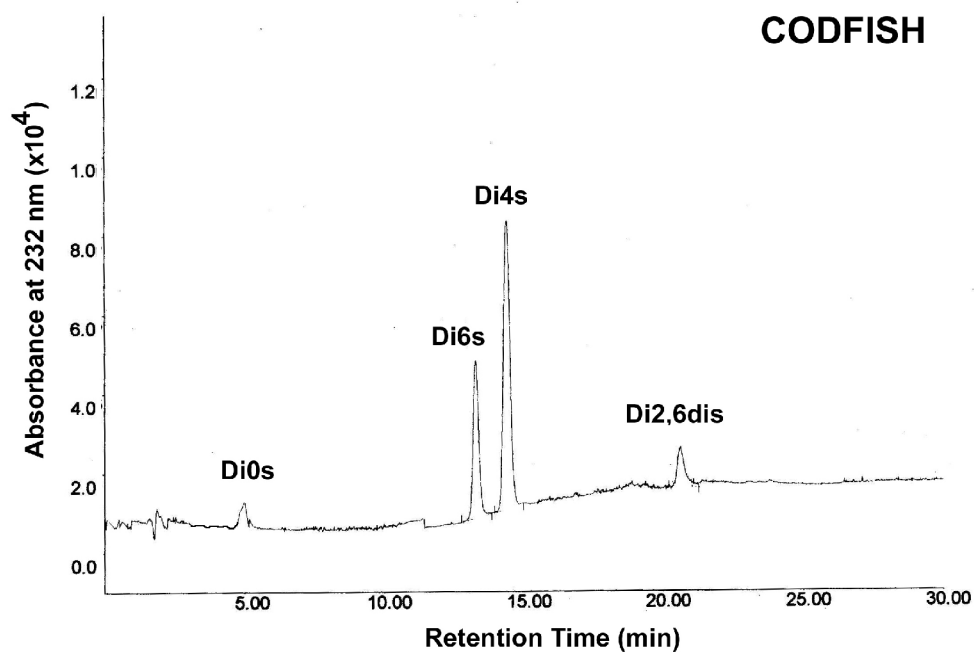
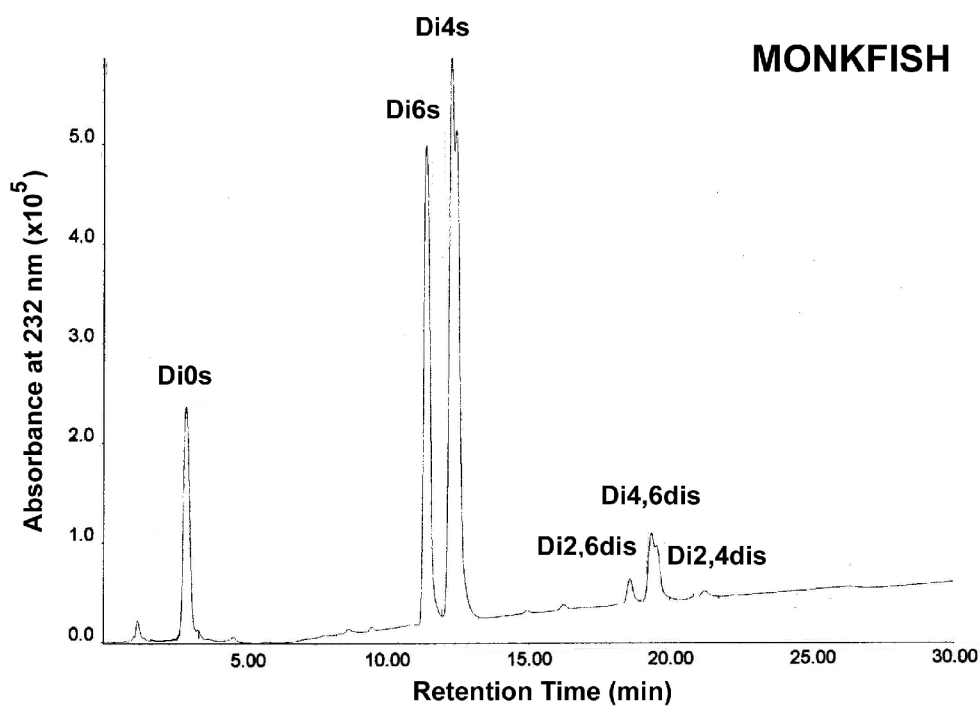
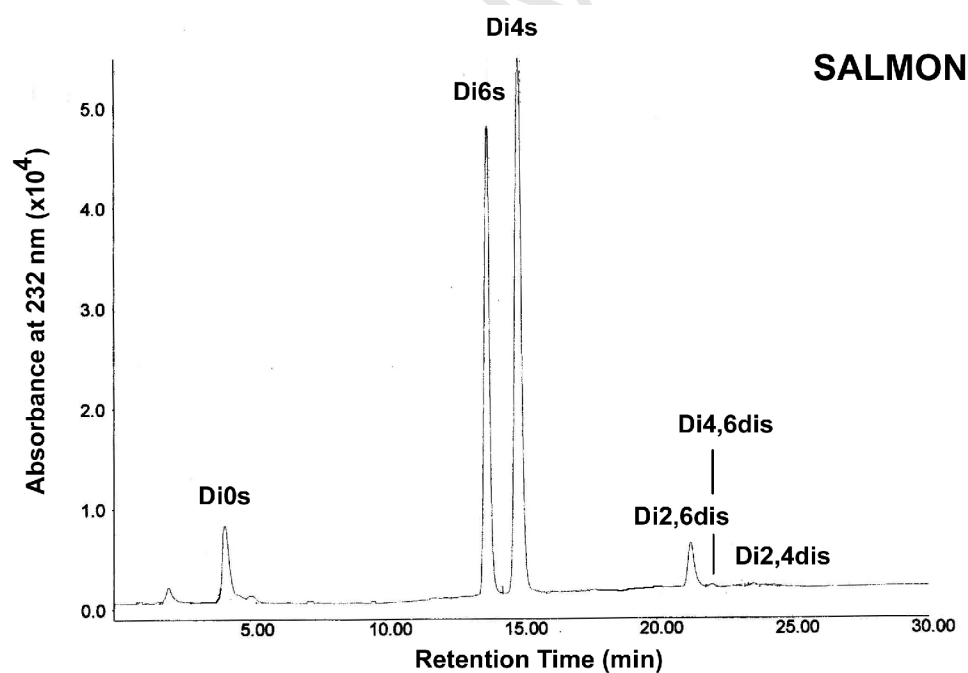
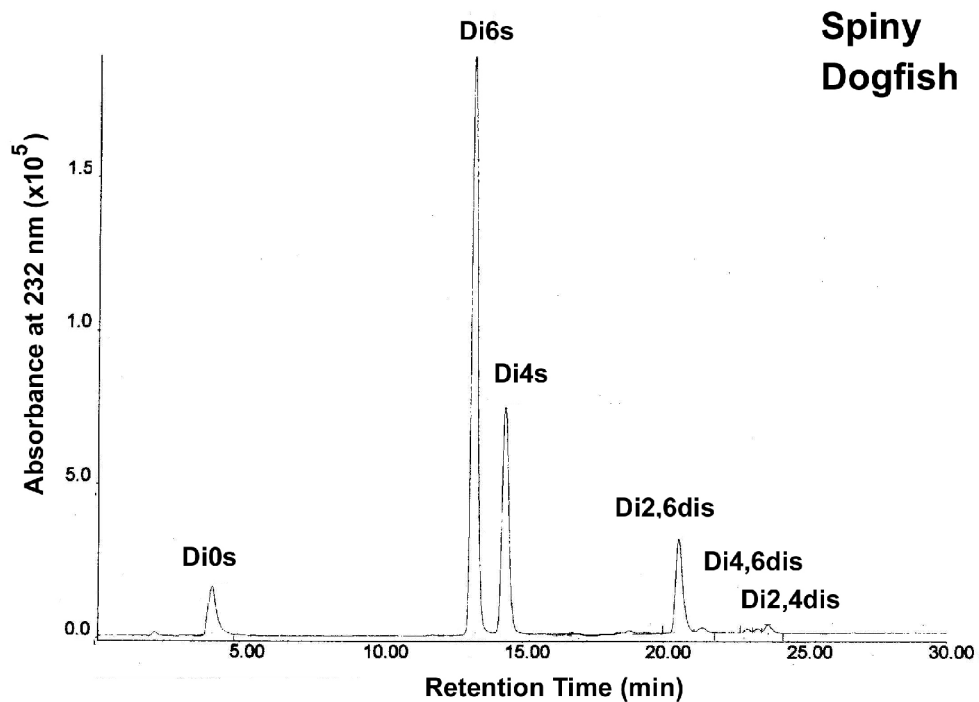


Figure 1





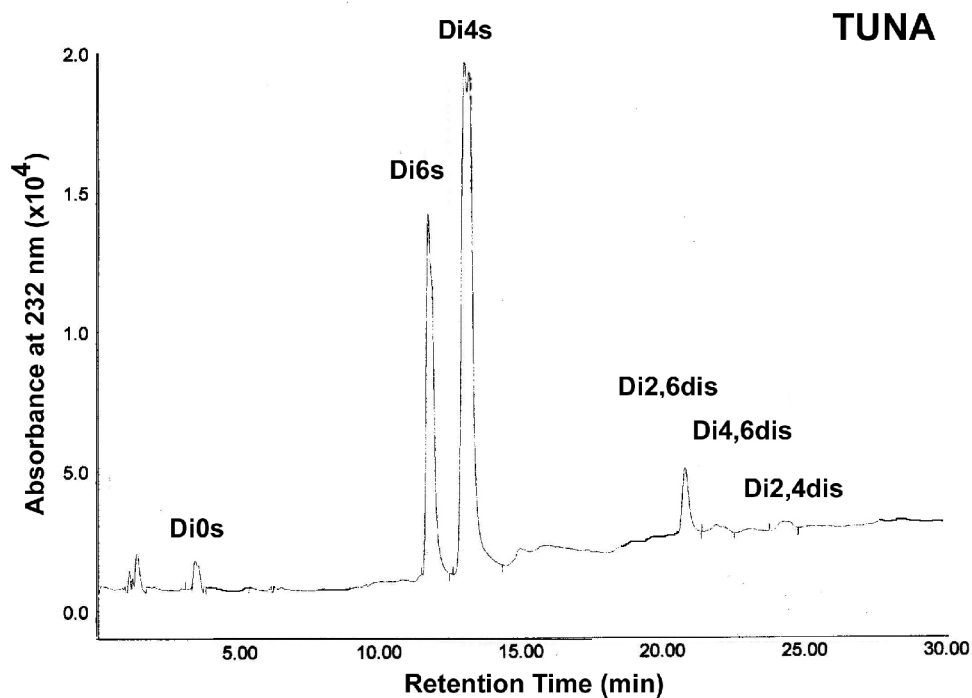


Figure 2

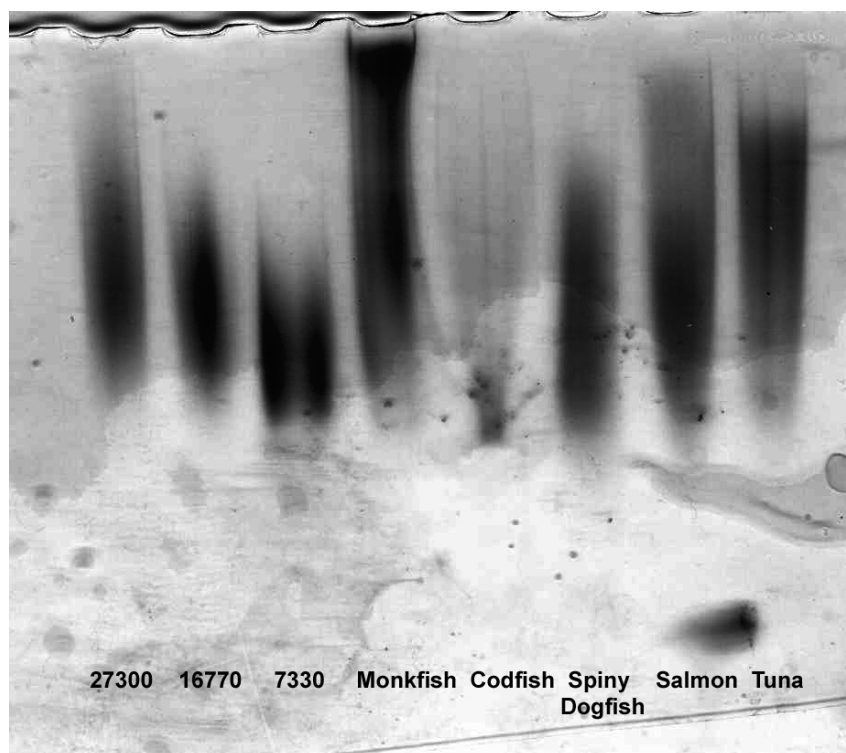


Figure 3