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Structural analysis of chondroitin sulfate from *Scyliorhinus canicula:* A useful source of this polysaccharide

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Chondroitin sulfate (CS), a constituent of proteoglycans, is a key component of the connective tissues and it is widely used as a precautionary drug for joint diseases; for this reason, the increased demand of this polysaccharide has posed the problem to identify new and secure sources of this product. In this context, CS from the cartilage of the lesser spotted dogfish (*Scyliorhinus canicula,* a cartilaginous fish) was isolated and investigated through chemical and spectroscopical techniques. The structural elucidation was performed on the entire polysaccharide and confirmed analyzing the products obtained via ABC lyase treatment. As a result, its compositional analysis disclosed the occurrence of CS-A, CS-C, CS-D, and CS-0S motifs in the ratio of 41, 32, 19.8, and 8.2%, respectively. Additionally, two different glycopeptides were isolated and characterized via NMR, providing information on the linkage oligosaccharide region joining the glycosaminoglycan chain to the core protein. Therefore, chondroitin sulfate from *Scyliorhinus canicula* appears very similar to that isolated from shark, a cartilaginous and taxonomically related fish, with the main difference residing in the major percentage of the CS-A motif. In the light of the results obtained, *Scyliorhinus canicula* chondroitin sulfate possesses a chemical structure compatible for the formulation of commercial and pharmaceutical products.

Keywords: 2D-NMR/chondroitin sulfate/lesser spotted dogfish/linkage region/RPIP-HPLC

Introduction

Chondroitin sulfate (CS) is a linear anionic polysaccharide, it is characterized by a repeating disaccharide unit composed of glucuronic acid (GlcA) and *N*-acetylated galactosamine (GalNAc), arranged in the sequence [4)-β-GlcA-(1→3)-β-GalNAc-(1→]; this regular structure is masked during the biosynthesis by the introduction of sulfate groups at different positions of sugar backbone. Accordingly, chondroitin sulfate type A (CS-A) is mainly sulfated at *O*-4 of GalNAc, CS-C at *O*-6 of GalNAc, CS-D at positions 6 of GalNAc and 2 of GlcA, and CS-E is sulfated at both 4 and 6 positions of GalNAc (Malavaki et al. 2008).

The chondroitin sulfate, such as the other glycosaminoglycans, is linked to the core protein through a tetrasaccharide junction, composed of xylose, galactose, and glucuronic acid. The whole assemblage, generically referred as proteoglycan, is widely spread in connective tissues both on the cellular membrane (Yanagishita and Hascall 1992) and in the extracellular matrix (Iozzo 1998).

In this last case, aggrecan is the principal CS proteoglycan in the cartilage, where it is found associated with hyaluronic acid, collagen, and other proteins; this multimeric aggregate confers to the tissue its hydrate gel-like and elastic characteristics. The role of aggrecan is to draw water into the extracellular matrix which swells and expands acquiring its compressive resilience.

Aggrecan molecular modifications are regulated by many cellular and extracellular events; however, individual age appears to play an important role on the cartilage composition, influencing the sulfation pattern of chondroitin and proteolytic cleavage of aggrecan yielding to an extracellular matrix with a reduced amount of poorly sulfated chondroitin. These events play a central role in joint degenerative diseases such as osteoarthritis and rheumatoid arthritis (Hardingham and Bayliss 1990). Additionally, cartilage breakdown products with antigenic properties are observed and their release into synovial fluid induce synovial inflammation (Volpi 2006).

Pharmacological management of the osteoarthritis is based on the use of analgesics, steroidal and nonsteroidal antiinflammatory drugs, in combination with chondroitin sulfate polysaccharide, resulting in the increasing demand of this material, and in the search of new sources able to provide this polysaccharide with a sulfation pattern compatible with human physiology.

In this regard, a pertinent example is given from oversulfated chondroitin (Guerrini et al. 2008), which was used as an additive in heparin formulation in order to increase, fraudulently, the anticoagulant titer of the preparation. This component, when administered intravenously, was found to induce severe anaphylactic reactions that led patients to death (Guerrini et al. 2008); whereas its effects, when taken orally, are not reported yet.

On the basis of the above consideration, *Scyliorhinus canicula*, even known as lesser spotted dogfish, has been considered as an alternative source of this polysaccharide; it is a cartilaginous fish belonging to the *Scryliohinidae* family of the *Carcharhino*formers order (Delabre et al. 1998). This species is quite common all around the coast of the Mediterranean Sea, where it has no marketability because it is not used in the population diet; despite this, every year a large number of specimens die because they are erroneously captured during fishing expeditions. The dead animals are usually thrown back to the sea, discarding the possibility of isolating chondroitin sulfate from their tissues and

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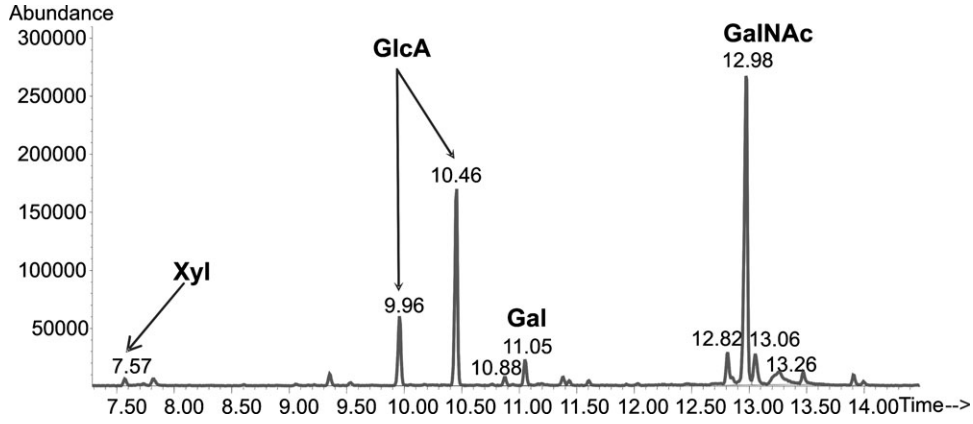

Fig. 1. GC-MS chromatogram and attribution of acetylated methylglycosides from chondroitin sulfate isolated from *Scyliorhinus canicula.* Composition is comparable with that considered compatible for human use since unusual substituents as fucose (RT 7.65) or glucose (RT 11.50) were not detected.

to use it in commercial preparations. This problem descends from the lack of information on its chemical structure, an issue addressed in the present investigation.

Results and discussion

Chondroitin sulfate purification and chemical characterization

Chondroitin sulfate was purified denaturing the cartilage tissue at 65°C: in this condition, the complex formed from proteoglycan–hyaluronic acid and other components disassembles and all the proteic components are accessible to papain digestion. CS obtained after repeated ethanol precipitations was further purified via ion-exchange gel chromatography, which eluted this highly charged polymer at high ionic strength (700 mM NaCl) with a final calculated yield of 1.5% w/w with respect to the fresh tissue.

Monosaccharide composition was compatible with that expected for chondroitin polysaccharide and its linkage oligosaccharide (glucuronic acid, galactosamine, xylose, and galactose, Figure 1). Iduronic acid (IdoA) was not detected by this approach, and enzymatic degradation of the polysaccharide with B lyase disclosed the occurrence of this monosaccharide in trace amounts (3.0% of the total uronic acid content).

The identity of the polysaccharide was established on the basis of its chemical composition and via extensive nuclear magnetic resonance (NMR) studies, analyzing both the intact polymer and its depolymerization products.

ABC lyase treatment of chondroitin sulfate

Enzymatic digestion of the sample (60 mg) resulted in its depolymerization, and the fragments obtained were partially purified via SEC chromatography on Bio-Gel P2, yielding to two fractions: one less abundant and less retained from the column named Fr-1 (9 mg), and Fr-2 (47 mg). This last sample was a mixture of disaccharides capped with one unit of 4-deoxy-β-L-*threo*-hex-4-enosyl-uronate (ΔHexA) at the nonreducing end. The composition of this fraction was disclosed via both strong ion-exchange chromatography (SAX) and reverse phase ion-pairing (RPIP) HPLC analysis. The other fraction, Fr-1, contained the oligomers of the linkage region, which were separated via RPIP HPLC.

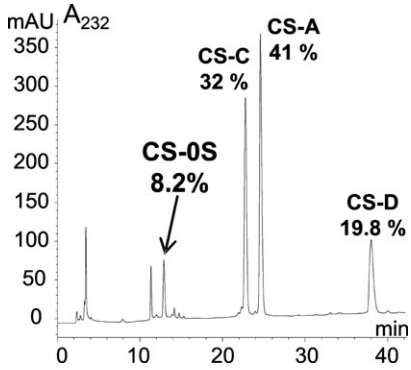

Fig. 2. SAX-HPLC profile, attributions, and integrations of Fr-2, the fraction containing the unsaturated disaccharides obtained by ABC lyase digestion of CS from *Scyliorhinus canicola*.

SAX-HPLC composition analysis of Fr-2 fraction

A chromatographic profile of this sample was obtained monitoring the eluate at 232 nm (Figure 2), and the occurrence of four, baseline separated, peaks was observed. The four peaks were assigned comparing their retention time with that of commercial standards and were assigned as: ΔHexA-GalNAc (CS-0S, 12.9 min), ΔHexA-GalNAc6S (CS-C, 22.8 min), ΔHexA-GalNAc4S (24.6 min, CS-A), and ΔHexA2S-GalNAc6S (CS-D, 38 min).

Their elution order was in agreement with reported data (Volpi 2004), and peak integration disclosed the following composition: CS-0S 8.2%, CS-A 41%, CS-C 32%, and CS-D 19.8% (Table I).

Spectroscopical analysis of intact chondroitin sulfate

The CS proton spectrum (Figure 3) presented broad-shaped signals (in agreement with the polysaccharide nature of the molecule) localized in the region between 4.8 and 3.3 ppm, with the exception of the methyl group of the *N*-acetyl residues resonating around 2 ppm (Mucci et al. 2000).

The occurrence of sulfate residue and their location was indirectly deduced by the displacement observed for both proton and carbon chemical shifts: this substituent induces the low field shift of geminal (up to 1 ppm) and vicinal protons (ca. 0.2 ppm);

Table I. Percentages of CS-A, CS-C, CS-D, and CS-0S motifs estimated via NMR and SAX chromatography

		CS-A	CS-C	CS-D	CS-0S
	Density (U_1)	83.5		16.5	–
NMR	Density (N_2)	45.6	54.4		–
	Composition	45.6	37.9[a]	16.5	–
HPLC		41	32	19.8	8.2

For the integration in the HSQC spectrum, the densities belonging to the anomeric protons of the GlcA units (U_1) and those of the protons in the position 2 of the GalNAc ones (N_2) were selected.
[a]NMR estimation of CS-C was possible combining the percentages of CS-A and CS-D from the two density sets, U_1 and N_2.

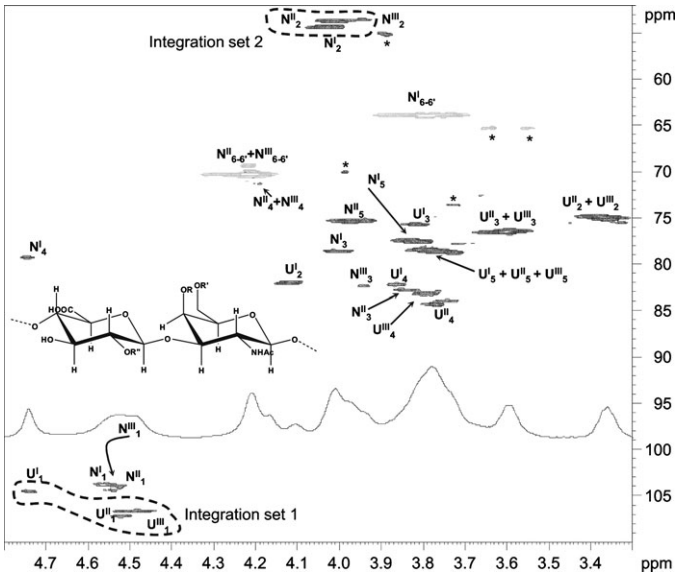

Fig. 3. (600 MHz, 288 K, D_2O, acetone as an internal reference) Chondroitin sulfate from lesser spotted dogfish: proton and HSQC spectra; attributions are indicated close to each density and peaks used for the integration are enclosed in the dotted circle. Integration set 1: H-1/*C*-1 cross peaks of GlcA residue $U^I{}_1$ represent the CS-D motif; $U^{II}{}_1$ and $U^{III}{}_1$ are nonsulfated uronic units and their integration represents the sum of CS-A and CS-C motifs. Integration set 2: H-2/*C*-2 cross peaks of GalNAc units; $N^I{}_2$ density diagnostic of CS-A unit; $N^{II}{}_2$ and $N^{III}{}_2$ densities were integrated together and represented CS-C and CS-D units, respectively. The structure of the different types of chondroitin disaccharide repeating unit is shown in the inset. CS-A: R = sulfate, R′ and R″ = H; CS-C: R′ = sulfate, R = R″ = H; CS-D: R′ = R″ = sulfate and R = H. Signals labeled with an asterisk were not assigned.

similarly, the sulfate bearing carbon chemical shift is increased by approx. 8 ppm, whereas that of the neighboring carbons is reduced by ca. 1–3 ppm.

Therefore, CS-D motif is characterized from the GlcA H-1 and H-2 signals at 4.74 ppm and 4.11 ppm, respectively, together with the GalNAc diastereotopic H-6s at 4.22 and 4.17 ppm; it is noteworthy that these last two signals are not distinctive of CS-D because they are presented from CS-C motif, as well. Finally, CS-A is identified from GalNAc H-4 proton at 4.74 ppm.

The combined use of the 2D NMR techniques made possible the complete interpretation of the spectra recorded (Table II): the occurrence of one broad peak was observed in the region between 4.49 and 4.56 ppm (Figure 3), and its left part was composed of the different GalNAc anomeric signals, whereas the right part arose from the GlcA units.

Table II. (600 MHz, 288 K) proton (plain) and carbon (italic) chemical shift attribution of the chondroitin sulfate isolate from the lesser spotted dogfish

	1	2	3	4	5	6
4-)-β-GlcA*2S*-(1→	4.74	4.11	3.81	3.86	3.78	–
U^I	*104.51*	*82.00*	*75.72*	*82.3*	*78.5*	–
4-)-β-GlcA-(1→	4.52	3.37	3.59	3.77	3.76	–
U^{II}	*107.18*	*72.2*	*76.5*	*84.17*	*78.7*	–
4-)-β-GlcA-(1→	4.49	3.38	3.59	3.79	3.76	–
U^{III}	*107.18*	*74.9*	*76.5*	*83.15*	*78.7*	–
3)-β-GalNAc*4S*-(1→	4.56	4.03	4.01	4.74	3.81	∼3.77[a]
N^I	*103.75*	*54.32*	*78.57*	*79.3*	*77.5*	*63.87*
3)-β-GalNAc*6S*-(1→	4.54	4.01	3.84	4.17	3.97	4.22–4.17
N^{II}	*103.75*	*53.63*	*82.81*	*∼71*[b]	*75.3*	*70.2*[b]
3)-β-GalNAc*6S*-(1→	4.54	3.94	3.94	4.22	3.97	4.22–4.17
N^{III}	*104.43*	*53.49*	*82.3*	*∼71*[b]	*75.3*	*70.2*[b]

Chemical shifts are expressed in δ relative to internal acetone (^{1}H at 2.225 ppm, ^{13}C at 31.5 ppm). Proton and carbon chemical shifts of the methyl of the *N*-acetyl group are 2.02 and 23.1 ppm, respectively.
[a]The two H-6 protons merge into one signal.
[b]*C*-4 and *C*-6 signals from GalNAc6S units are partially overlapped and possess different phases in the HSQC spectrum; as a result the less intense *C*-4 is almost entirely cancelled from that of *C*-6 and its chemical shift cannot be correctly appreciated (see the text and Figure 3).

In the case of each GalNAc residue, the COSY and TOCSY experiments led to the identification of the proton sequence from H-1 to H-4, whereas H-5 was individuated through the *intra* residue NOE contact with the anomeric proton; H-6s chemical shifts were deduced from the HSQC spectrum where they appeared in antiphase with respect to the other densities; additionally, each H-6 couple was assigned to a GalNAc residue on the basis of its *O*-4 sulfation pattern. This choice was based on Fr-2 SAX-HPLC analysis that disclosed the occurrence of minimal amounts of CS-0S and that each GalNAc unit was sulfated either at *O*-4 or at *O*-6, but never at both positions.

Accordingly, three different types of GalNAc residues were individuated: N^I, N^{II}, and N^{III} (Figure 3). The first one was sulfated at *O*-4 and belonged to the CS-A motif; the other two were sulfated at *O*-6, and comparison of their H-3 chemical shift with those published (Mucci et al. 2000) led their assignment to CS-C and CS-D motifs, respectively.

Similarly, GlcA residues (except U^I unit, Figure 3) showed all the ring proton resonances in the crowded high-field region of the spectrum; in most of the cases, the location of the H-5 proton was inferred by the aid of the NOESY spectrum. All attributions were in agreement with literature data (Mucci et al. 2000); U^I residue was attributed to a CS-D entity whereas it was not possible to ascribe U^{II} or U^{III} to any of the chondroitin pattern, CS-A and CS-C.

Signals related to unsulfated GalNAc or IdoA could not be detected, due to their low abundance.

Parallel to proton resonance attribution, the HSQC spectrum assignment was performed and some difficulties were found for *C*-4 of N^{II} and N^{III} units since these carbon signals were coincident with those from sulfated *C*-6, which were very intense and with opposite phases, leading to the almost complete cancellation of the C-4 intensity.

Additionally, the HSQC spectrum was used to determine the relative proportion among disaccharide subunits. This approach is based on the assumption that the signals to be compared display similar $^1J_{CH}$ and that a difference around 5–8 Hz from the

experimental set value does not cause a substantial variation of the integrated peak volumes. More importantly, within a specific density set, such as that composed of different anomeric GlcA signals, the volume measured scale with the $^1J_{CH}$ value used in the experiment, but their increase (or decrease) is affected to the same extent, so that the proportion between them is maintained even if their $^1J_{CH}$ is distant (i.e., 20 Hz) from the set experimental value (Guerrini et al. 2005).

Therefore, two different density sets were chosen: the first belongs to the *C*-1 carbons GlcA residues and the second to the *C*-2 carbons of the GalNAc units (Figure 3). Integration of set 1 (Table I) returned the percentage of CS-D motif (density U^I_1 in Figure 3) and the sum of CS-A and CS-C (density 2 in Figure 3); on the other hand, the percentage of CS-A was obtained analyzing the *C*-2 HSQC density of GalNAc N^I residue (Table I). Combining these sets of information, the relative proportions between CS-A, CS-C, and CS-D were obtained and were 45.6, 37.9 and 16.5, respectively. Signals related to the nonsulfated CS-0S unit were not detected in the spectrum, and no estimation of this motif was possible at this stage.

These data indicate the strong prevalence of sulfation at position *O*-6 of the GalNAc residue, resulting from the sum of the CS-C and CS-D units, but the amount of the CS-A portion appears higher with respect to that reported for the shark cartilage (45.6% versus 27.7%) (Mucci et al. 2000).

RPIP-HPLC analysis of fraction Fr-2 and Fr-1

This separation technique has been successfully applied so far in the analysis of highly charged heparin (Thanawiroon et al. 2004), hyaluronic (Volpi 2007), and unsulfated chondroitin oligosaccharides (Volpi et al. 2008). In contrast with the eluents used in SAX chromatography, the mobile phase contains volatile ion-pairing reagents that provide a good resolution of the analytes and allows us to interface the HPLC system with a MS detector.

The separation of the disaccharide mixture present in Fr-2 was performed and led to the isolation of three components: ΔHexA-GalNAc6S (RT 12.5 min), ΔHexA-GalNAc4S (RT 13.2 min), and ΔHexA2S-GalNAc6S (RT 18.8 min).

The elution order of the three oligosaccharides appeared similar to that found for SAX chromatography, their spectroscopical attribution is reported in Table I of supporting information and, despite the presence of tributylamine (TBA) as counterion, their proton and carbon chemical shifts were found similar to that from literature data (Yamada et al. 1992).

RPIP-HPLC separation of Fr-1 led to the isolation of two glycopeptides belonging to the linkage region of the CS chains: Fr-1A (RT 5.1 min) and fr-1B (RT 6.8 min); the two glycopeptides differed just for the length of the chain, being Fr-1A made up of six monosaccharides and the other of eight residues, as visible in their proton spectra (Figure 4, structures in Figure 5).

The two oligosaccharides contained the canonical sequence of the linkage region of the CS-proteoglycans and were devoid of any inorganic substituent, such as sulfate or phosphate.

The proton spectrum of Fr-1A showed the H-4 and H-1 signals of the nonreducing unit, a ΔHexA residue, at 5.90 and 5.18 ppm, respectively; the anomeric signals of the other five residues were found in the region between 4.7 and 4.4 ppm and were assigned to one xylose (Xyl, 4.46 ppm), two galactose (Gal, 4.66 and 4.53 ppm), one GalNAc (4.53 ppm), and one GlcA (4.66 ppm)

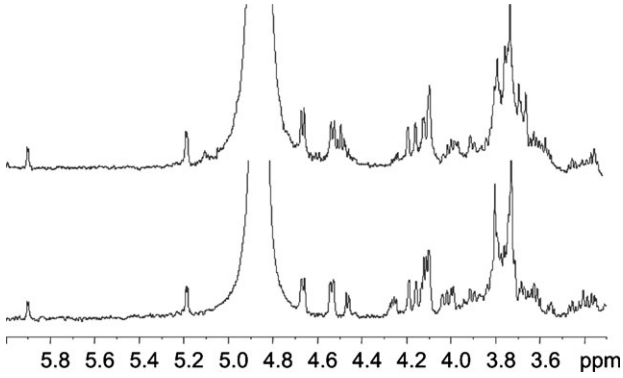

Fig. 4. (600 MHz, 293 K, D_2O) the proton spectrum of Fr-1A (below) and Fr-1B (above). Both compounds are in the salt form, with TBA as counterion.

Table III. (600 MHz, 293 K, D_2O) proton (plain) and carbon (italic) chemical shift attribution of Fr-1A (structure in Figure 5) analyzed in its salt form with TBA as counterion

	1	2	3	4	5	6
t-ΔHexA-(1→	5.18	3.79	4.09	5.90	–	–
	102.15	*70.8*	*66.9*	*108.6*	n.d.[a]	n.d.[a]
4-)-β-GlcA-(1→	4.66	3.45	3.62	3.77	3.71	–
	105.2	*74.2*	*75.0*	*80.9*	*77.6*	n.d.[a]
3-)-β-Gal-(1→; II	4.66	3.74	3.80	4.15	3.68	3.77[b]
	105.2	*71.3*	*83.9*	*69.5*	*76.2*[b]	*62.41*[b]
3-)-β-Gal-(1→; I	4.53	3.67	3.82	4.19	3.73[b]	3.77[b]
	102.5	*71.2*	*83.4*	*69.8*	*76.3*[b]	*62.41*[b]
3)-β-GalNAc-(1→	4.53	4.00	3.90	4.09	3.73[b]	3.77[b]
	102.5	*52.4*	*81.2*	*68.8*	*76.3*[b]	*62.41*[b]
3)-β-Xyl-(1→	4.46	3.37	3.61	3.87	4.11–3.40	–
	103.9	*73.9*	*75.0*	*77.7*	*64.3*	–
	H_α	H_β				
Ser	3.99	4.02–4.26	–	–	–	–
	55.84	*69.2*	–	–	–	–

[a]Carbon chemical shift not determinable from the HSQC spectrum.
[b]H-5/*C*-5 and H-6/*C*-6 signals for Gal and GalNAc units are overlapped and any punctual attribution is not possible.

residues, all in the β configuration on the basis of their chemical shift and of their the $^3J_{H1,H2}$ coupling constants (ca. 8 Hz).

The spectra collected were interpreted according to the general strategy adopted for CS polysaccharide, leading to the complete attribution of all the signals (Table III) and to establishment of the sequence between the different units.

As a result, Fr-1A contained the canonical sequence of the linkage tetrasaccharide elongated with one CS repeating unit at its nonreducing end (Figure 3).

Although this molecule was studied as the TBE salt form, its proton chemical shifts were in agreement with the published data (Sugahara et al. 1988); additionally, in this case, it was possible to determine the carbon chemical shifts as well, which is so far reported only for the alditol form of the molecule.

In contrast with the proton spectrum of Fr-1A that recorded for Fr-1B (Figure 4, structure in Figure 5) showed a more crowded carbinolic region due to the presence of two additional sugar units, identified as a GlcA and a GalNAc moiety.

Due to the low abundance of this compound, it was possible to attribute its proton chemical shift (Table IV) and the sequence among the residues, resulting on the structure reported in Figure 5.

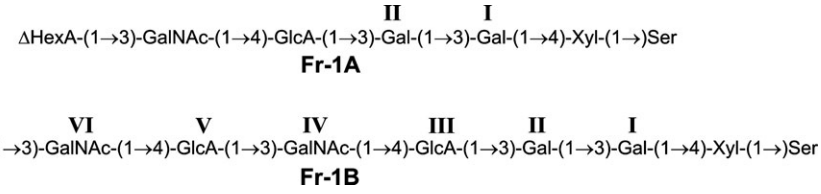

Fig. 5. Structure of the two oligosaccharides, Fr-1A and Fr-1B, isolated from RPIP-HPLC chromatography. The configuration of all glycosidic linkages is β and omitted for clarity.

Table IV. (600 MHz, 293 K, D_2O) proton chemical shift attribution of Fr-1B (the structure in Figure 5) analyzed in its salt form with TBA as counterion

	1	2	3	4	5	6
t-ΔHexA-(1→	5.18	3.79	4.09	5.90	–	–
4-)-β-GlcA-(1→; III	4.66	3.45	3.63	3.77	3.71	–
3-)-β-Gal-(1→; II	4.66	3.74	3.80	4.12	3.69	∼3.75[a]
3-)-β-Gal-(1→; I	4.53	3.67	3.83	4.19	3.73	∼3.75[a]
3)-β-GalNAc-(1→; IV	4.53	4.00	3.90	4.10	3.72	∼3.75[a]
3)-β-GalNAc-(1→; VI	4.50	3.99	3.80	4.16	3.69	∼3.75[a]
4-)-β-GlcA-(1→; V	4.48	3.37	3.57	3.74	3.69	–
3)-β-Xyl-(1→	4.46	3.37	3.61	3.87	4.12–3.40	–
	H_α	H_β				
Ser	3.98	4.02–4.26				

[a]H-6 signals for Gal and GalNAc units are overlapped and any punctual attribution is not possible.

In conclusion, chondroitin sulfate was extracted from *Scyliorhinus canicula* cartilage and analyzed with the perspective to use this specimen as a source of this polysaccharide.

Analyses were focused to determine its chemical composition, with special regard to its sulfation pattern, a feature of special relevance since not all the known sulfation patterns are compatible with the use of this compound as a drug.

Chemical analyses revealed the presence of just the typical sugars belonging to chondroitin sulfate polysaccharide, with a minimal amount of iduronic acid (ca. 3%), and its linkage region; monosaccharides such as fucose and glucose were not detected, so rare motifs, such as those found in the CS from sea cucumber (Vieira et al. 1991) or squid (Habuchi et al. 1977), were excluded.

NMR investigation on the intact polymer was of importance because it ruled out the occurrence of rare sulfation patterns, such as those presenting GlcA sulfated at *O*-3, and/or oversulfated repeating units.

These two motifs can be easily detected only via NMR since the first is digested and destroyed during ABC lyase treatment (Sugahara et al. 1996), whereas the second is not cleaved by the enzyme and therefore not seen in SAX chromatography.

Therefore, combining the results from the chemical and spectroscopical procedures employed, it turned out that this chondroitin sulfate polysaccharide was devoid of rare and potentially toxic motifs and composed of CS-A, CS-C, CS-D, and CS-0S motifs in the ratio of 41, 32, 19.8, 8.3, respectively, as estimated via SAX chromatography.

This chemical composition is rather close to that reported for shark cartilage, but the amount of CS-A disaccharides is slightly higher.

Besides the structure elucidation of the polysaccharide, it was possible to gain information regarding its oligosaccharides in the linkage region (Sasisekharan et al. 2006): Fr-1A and Fr-1B (Figure 5), which displayed the classical tetrameric sequence β-GlcA-(1→3)-β-Gal-(1→3)-β-Gal-(1→4)-β-Xyl-(1→*O*-Ser, without any inorganic substituent, elongated with one (Fr-1A) or two (Fr-1B) chondroitin repeating units.

This last part of the work has also led to the carbon chemical shift assignment of Fr-1A glycopeptide, so far not reported, and it has established RPIP-HPLC as a valuable tool to separate and analyze, via NMR, highly charged oligosaccharides, showing that signals belonging to the ion-pairing reagent did not interfere with the signals of CS oligosaccharides, making any further purification procedure unnecessary.

On the basis of the data collected, it is reasonable to assume that chondroitin isolated from *Scyliorhinus canicula* might be compatible for scientific and pharmacological applications, making this small bottom living shark, a useful source of this compound.

Material and methods

Isolation of the chondroitin sulfate from fresh cartilage

CS was isolated from the fresh cartilage of the lesser spotted dogfish (16 exemplars, ca. 300 g each) as described (Heinergard and Sommarin 1987). Briefly, the diced tissue (180 g) was suspended in a buffer solution (0.1 M of sodium acetate, 10 mM of cysteine-HCl, and 2.4 mM EDTA, pH 6.8, 400 mL) and digested with papain (1 U for 100 mg of tissue, 18 h at 65°C, papain from papaya latex, crude powder 2.2 units/mg solid, P3375 Sigma-Aldrich, St. Louis, MO). The digestion was terminated denaturing the enzyme at 100°C for 15′, the unreacted solid was removed by centrifugation and the supernatant treated with 4 volumes of ethanol to allow the precipitation of the polysaccharide components (GAGs). The solution was cooled and left at 4°C overnight. The sediment was collected by centrifugation (5000 rpm, 30 min, 4°C) suspended in 50 mM sodium acetate (10 mL), precipitated upon the addition of two volumes of ethanol, centrifuged and the resulting solid was precipitated a second time under the same conditions and freeze-dried (9 g, yield respect to the cartilage tissue: 5.0%). All the supernatants were pooled and were not further considered.

Chromatographic purification

An aliquot of the solid (450 mg) was purified via anion-exchange chromatography using Q-Sepharose fast flow as adsorbent (GE Healthcare, Hercules, CA, column size: 2.5 × 18 cm,

3 mL/min). Gel was equilibrated with 10 mM NaCl and the sample was eluted increasing stepwise NaCl concentration (100, 200, 400, 700, and 1000 mM), each solution covered twice the column volume and the eluate was monitored reading the absorbance at 220 nm. Peaks containing carbohydrate material were visualized according to the phenol assay (Taylor 1995) and were found at high ionic strength; the most abundant fraction was eluted with the 700 mM NaCl buffer, and a minor one was found in the 1 M eluate. Fractions from the 700 mM elution were pooled accordingly, dialyzed, and freeze-dried, yielding to 150 mg of chondroitin sulfate polysaccharide (yield scaled with respect to the total cartilage tissue: 1.5%).

Chemical composition analysis

Monosaccharides were analyzed as acetylated *O*-methylglycoside derivates as reported (Holst 2000). Gas liquid chromatography mass spectrometry (GC-MS) analysis was performed with an Agilent 5973 instrument (Agilent, Santa Clara, CA), using a SPB-5 capillary column (Supelco, St. Louis, MO, 30 m × 0.25 i.d. flow rate 0.8 mL/min, He as carrier gas). Mass spectra were recorded at ionization energy of 70 eV and an ionizing current of 0.2 mA. The temperature program used for the analysis of the mixture of acetylated *O*-methylglycoside derivates was: 150°C for 3 min, 150→300°C at 10°C/min, 300°C for 12 min.

Depolymerization of GAGs

CS (60 mg) was dissolved in the Tris-HCl buffer (0.1 M, pH 8.0, 20 mg/mL) and depolymerized with ABC lyase (1 U/100 mg chondroitin sulfate, ABC lyase from *Proteus vulgaris* lyophilized, stabilized with BSA, 0.85 units/mg solid, C2905 Sigma-Aldrich, St. Louis, MO) at 37°C for 18 h; ABC lyase was then heat inactivated (100°C, 15 min) and the solution treated with trypsine to degrade ABC lyase and other proteic components eventually present. Finally, trypsine was heat inactivated as above, and the solution cooled and freeze-dried. The corresponding mixture of oligosaccharides was purified via SEC chromatography on a Bio-Gel P2 (1.5 × 116 cm, Bio-Rad, Hercules, CA) using 50 mM ammonium acetate as a buffer at a flow rate of 0.2 mL/min. The column eluate was monitored in continuous with a refractive index refractometer (K-2310 Knauer, Berlin, DE) and two main carbohydrate containing fractions were identified with the phenol test (Fr-1: 9 mg, and Fr-2: 47 mg).

Iduronic acid enzymatic determination

CS (10 mg) was dissolved in the Tris-HCl buffer, as above, and treated with B lyase (1 U/10 mg, B lyase from *Flavobacterium heparinum*, Sigma C8058, St. Louis, MO) or ABC lyase (1 U/10 mg), at 37°C. Reaction was monitored reading UV absorbance at 232 nm and an aliquot of each sample was appropriately diluted with the same buffer, in order to have an UV reading in the range of the linear response of the instrument ($0.3 < A_{232} < 0.8$) and read versus the buffer solution. Each absorbance was corrected with the value measured for a solution containing the same polysaccharide, but without enzyme; each determination was performed in duplicate and the final value reported is the average of the two measurements.

The reaction with ABC lyase was already complete after 2.5 h since no significant absorbance increase was observed afterward; although the B lyase reaction ran parallel, it did not produce a net increase in UV absorbance and IdoA percentage was calculated on the basis of the UV reading at the same time point selected for ABC lyase reaction, 5 h.

HPLC purification

Analyses were performed on an Agilent 1100 HPLC instrument, equipped with a binary pump and a UV/VIS detector.

SAX chromatography: fraction Fr-2 was analyzed by strong anion-exchange chromatography (Volpi 2004), using Supelco LC-SAX (4.6 × 250 mm) column equilibrated with 10 mM NaCl; oligosaccharides were eluted with a linear NaCl gradient (5 min at 10 mM NaCl and then to 200 mM NaCl over 30 min, additional 15 min in isocratic at 200 mM) and the elution profile was monitored reading at 232 nm.

RPIP separations (Volpi et al. 2008) were performed on a 5 μm Supelco RP-C8 column (4.6 × 250 mm); eluate was monitored at 232 nm, and solvent gradient was optimized for each sample.

Fr-1: eluent A was water/acetonitrile (9:1) and the eluent B was water/acetonitrile (65:35); TBA (15 mM) and ammonium acetate (50 mM) were added to both eluents, and the pH was adjusted to 7.0 with acid acetic. Sample (10 mg/mL, 100 μL each injection) was separated varying buffer B (flow rate 0.8 mL/min) as follows: from 0 to 50% in 15 min, and then 100% for 5 min.

Fr-2: eluents were the same as mentioned above except that acetonitrile in buffer A was 2%. Sample (10 mg/mL, 100 μL each injection) was separated varying buffer B as follows: from 0 to 30% in 15 min, from 30% to 60% in 5 min, holding at 60% for other 5 min at a flow rate of 0.8 mL/min for elution.

Peaks were collected from RPIP chromatography, and acetonitrile was removed with a rotary evaporator and the solution was freeze-dried.

NMR spectroscopy

^{1}H and ^{1}H-^{13}C NMR experiments of the intact chondroitin sulfate and of the two glycopeptides (Fr-1A and Fr-1B) were carried out on a Bruker DRX-600 equipped with a cryogenic probe; spectra were calibrated with respect to internal acetone (δ_h = 2.225 ppm; δ_C = 31.45 ppm). For all the homonuclear spectra, experiments were measured with data sets of 2048 × 512 points; a mixing time of 200 and 120 ms was employed for NOESY and TOCSY, respectively. Each data matrix was zero-filled in both dimensions to give a matrix of 4096 × 2048 points and was resolution-enhanced in both dimensions by a shifted sine-bell function before Fourier transformation.

The HSQC experiment was measured using a data set of 2048 × 512 points; 64 scans were acquired for each t_1 value, and the pulse sequence was optimized for a 150 Hz coupling constant. During processing, the matrix was extended to 4096 × 1024 points by forward linear prediction extrapolation. All NMR spectra were acquired, transformed, and analyzed with Topspin 2.1 program.

^{1}H and ^{1}H-^{13}C NMR experiments of the unsaturated disaccharides were carried out on a Bruker DRX-600 equipped with a *z*-gradient reverse probe under the same conditions listed above. HMBC spectra were measured using a data set of 2048 × 512 points; 64 scans were acquired for each t_1 value, and during

processing, the matrix was extended to 4096 × 1024 points by forward linear prediction extrapolation.

Supplementary Data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest statement

None declared.

Abbreviations

COSY, correlation spectroscopy; CS, chondroitin sulfate; CS-0S, nonsulfated chondroitin; CS-A, chondroitin 4-sulfate; CS-C, chondroitin 6-sulfate; CS-D, chondroitin 2,6-disulfate; CS-E, chondroitin 4,6-disulfate; ΔHexA, 4-deoxy-β-L-*threo*-hex-4-enosyl-uronate; Gal, galactose; GalNAc, *N*-acetylated galactosamine; GC-MS, gas liquid chromatography mass spectrometry; GlcA, glucuronic acid; HMBC, heteronuclear multiple bond correlation; HSQC, heteronuclear single quantum correlation; IdoA, iduronic acid; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; RPIP-HPLC, reverse phase ion-pairing high performance liquid chromatography; SAX, strong anion exchange; TBA, tributylamine; TOCSY, total correlation spectroscopy; Xyl, xylose.

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