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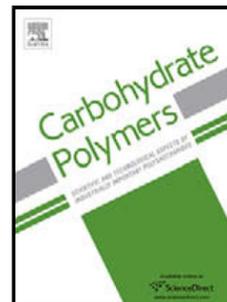
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Chondroitin Sulfate/Dermatan Sulfate from Corb (*Sciaena umbra*) Skin: Purification, Structural Analysis and Anticoagulant Effect

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Research Highlights

- Chondroitin sulfate/dermatan sulfate was purified from skin of corb (CSG).
- The chemical and structural analyses of CSG were studied.
- CSG has high sulfate content and a relatively low molecular mass.
- CSG exhibited significant anticoagulant effect.

Abstract

In this study, Chondroitin sulfate/dermatan sulfate was isolated and purified from the skin of corb (*Sciaena umbra*) (CSG) with a yield of 6.2%. Chemical and structural analysis showed that CSG consisted of high sulfate content 28.74% and an average molecular weight of 15.46 KDa. The separation of CSG by agarose-gel electrophoresis revealed the presence of DS and CS. Structural analysis of the purified CS/DS by means of SAX-HPLC after treatment with specific chondroitinases showed that this polymer was composed of nonsulfated disaccharide, monosulfated disaccharides and disulfated disaccharides in various percentages. The results also suggest that the percentage of CS and DS recovered in CSG were 24% and 76%, respectively. Anticoagulant activity *in vitro* was measured in plasma using classical anticoagulation tests: activated partial thromboplastin time (aPTT), thrombin time (TT) and prothrombin time (PT) tests. The findings thus indicated that the purified CS/DS exhibits a remarkably high anticoagulant effect.

Keywords: Fish skin; glycosaminoglycans; chondroitin sulfate; dermatan sulfate; chemical characterization; anticoagulant effect.

1. Introduction

Coagulation is a potential defence mechanism involved in the prevention of bleeding and hemorrhagic complications. The coagulation process consists of a series of stepwise that culminate in the formation of an insoluble clot. Nowadays, many strategies for preventing and treating

bleeding have focused on inhibition thrombin generation and blocking its activity (Dahlback, 2000).

Anticoagulants, such as heparin, are generally involved in preventing human body from thromboembolic diseases (Kreutz, 2014). Heparin is widely used as an anticoagulant and antithrombotic drug, which blocks the formation of thromboplastin, an important clotting factor in the blood. Otherwise, even though this drug showed to be benefic in some aspects, it can generate many healths problems for both humans (thrombocytopenia and thrombosis syndrome, hemorrhagic complications (Warkentin, 2006) and activations of platelets) and animal (Mad Cow disease). Therefore, considerable attention has recently been paid of the development of novel anticoagulants agents from natural sources with good safety and potency. In particular, epidemiological studies have indicated that sulfated polysaccharides could prevent bleeding disorders (Araújo, Nosedá, Cipriani, Goncalves, Duarte, & Ducatti, 2013; Li, Wu, Cai, & Ye, 2017). Indeed, both anticoagulant and antithrombotic activities are associated with the presence of sulfated groups, and their distribution along the polysaccharide chain.

Glycosaminoglycans (GAGs) are acidic, highly sulfated, complex and linear polysaccharides, which present in many varieties of organisms. The repeating unit consists of hexosamine residues (N-acetylgalactosamine (GalNAc) or N-acetylglucosamine) and uronic acid (glucuronic acid (GlcA) or its epimer the iduronic acid (IdoA)) with the exception of keratan sulfate). GAGs are generally divided into four groups: hyaluronic acid (HA), kertian sulfate (KS), chondroitin and dermatan sulfate (CS/DS) and heparin and heparin sulfate (HS) (Rodén, 1980).

GAGs are widespread in nature, occurring in a large variety of organisms such as vertebrates (Krichen et al., 2017b; Kaczmarek, Sionkowska, & Osyczka, 2017; Ben Mansour et al., 2010, Volpi, 2007; Volpi, 2009) and certain terrestrial or marine invertebrates (Sayari et al., 2016;

Abdelmalek et al., 2015; Okamoto, Higashi, Linhardt, & Toid, 2018; Yang, Wang, Yang, & Lv, 2018). Currently, several works indicated the presence of GAGs with heterogenous structures and biological properties of interest as well as anticoagulant activities mediated by physiological coagulation inhibitors, antithrombin (AT) and heparin cofactor II (HCII) (Barbbucci, Magnani, Lamponi, & Albanese, 1996; Athukorala, Lee, Kim, & Jeon, 2007).

Nowadays, a variety of analytical techniques, including agarose-gel electrophoresis, have been developed for the structural analysis of acidic polysaccharides with a high level of sensitivity. This approach is the most effective way of analysing GAGs in mixtures because it is a simple technique that offers a broad separation range. In fact agarose-gel electrophoresis has been applied for various purposes, such as to separate GAGs extracted from tissues, organs, biological fluids of invertebrates and vertebrates, to characterize radiolabelled sulfated polysaccharides from cells, to evaluate polyanions used as drugs and to control a purification step of a single GAGs species.

Therefore, the aim of this study was to evaluate the *in vitro* anticoagulant activity of glycosaminoglycans obtained from corb (*Sciaena umbra*) skin (CSG) and to investigate their structure characteristics.

2. Materials and method

2.1 Reagents

All chemicals and solvents were of analytical grade and used as received from commercial sources. Alcalase® 2.41 serine-protease from *Bacillus licheniformis* was purchased from Novozymes® (Bagsvaerd, Denmark). GAGs standard, heparan sulfate from bovine kidney, CS from bovine trachea and dermatan sulfate from porcine intestinal mucosa, were from Sigma-

Aldrich (St. Louis, MO, USA). Chondroitinase ABC, chondroitin ABC lyase, from *Proteus vulgaris* (EC 4.2.2.4), specific activity of 0.5-2 units/mg, and chondroitinase AC, chondroitin AC lyase, from *Flavobacterium heparinum* (EC 4.2.2.5), specific activity of 0.5-1.5 units/mg, were from Sigma–Aldrich. Unsaturated chondro/dermato disaccharides [Δ Di0S (Δ UA-[1 \rightarrow 3]-GalNAc), Δ Di4S (Δ UA-[1 \rightarrow 3]-GalNAc-4S), Δ Di6S (Δ UA-[1 \rightarrow 3]-GalNAc-6S), Δ Di2S (Δ UA-2S-[1 \rightarrow 3]-GalNAc), 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-[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 (Δ DitriS, Δ 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). All other reagents were of analytical grade.

2.2 Materials

Corb (*Sciaena umbra*) were freshly purchased from the local fish market of Sfax, Tunisia. The biological materials were packed in polyethylene bags, placed in ice and transported to the research laboratory within 30 min. Upon arrival, the samples were washed twice with water and separated. Only the skin was collected and then stored in sealed plastic bags at -20°C until use for the extraction and analysis of GAGs.

2.3 Enzymatic extraction of glycosaminoglycans

GAGs were isolated according to a slightly modified version of the method of Ben Mansour et al. (2009). Firstly, the fish skin was cut into small pieces and homogenized using a Moulinex R62 homogenizer (Organotechnie, Courneuve, France). Five grams of sample was dissolved in 250 ml sodium acetate (0.1 M), EDTA (5 mM), cystein (5 mM) pH 6. Enzymatic hydrolysis was achieved by the addition of Alacalase®, and the mixture was kept for 24 h at 50°C. After the

required digestion time, the mixture was left to cool down at room temperature and then filtered. The residue was washed with distilled water and filtered again. The filtrate was mixed and then precipitated with cetylpyridinium chloride 2% (w/v) for 24 h at room temperature. Thereafter, the mixture was centrifuged for 30 min at 5869 g and 4°C. The pellet was washed with cetylpyridinium chloride 0.05% (w/v) and then blended with 200 ml NaCl solution (2M) in ethanol (100:15, v/v). After that, an amount of 700 ml ethanol was added. The polysaccharides containing solution were left for 24 h at 4°C and then centrifuged for 30 min at 5869 g and 4°C. The pellet was washed twice with ethanol 80% and then once with absolute ethanol. Finally, the pellet was redissolved in desionized water and freeze-dried using freeze-dryer (CHRIST, ALPHA 1–2 LD plus, Germany).

2.4 Purification of fish skin GAGs

The dried GAGs were dissolved in 5 ml of 10 mM NaCl. After centrifugation at 4000 rpm for 10 min, the supernatant was applied to a column (1.5 cm × 3 cm) packed with QAE-Sephadex® A-25 anion-exchange resin equilibrated in NaCl 10 mM. GAGs were eluted with a linear gradient of NaCl from 10 mM to 2.5 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 finally GAGs precipitated at 4°C. After centrifugation at 4000 rpm for 10 min, the pellet was dried at 40°C and solubilized in distilled water.

2.5 Chemical composition analysis

Total sugars were measured by the phenol-sulfuric acid method according to the method of Dubois et al. (1956). The sulfate content was performed by liquid-Ion Chromatography (HPLC) on a Metrohm chromatograph equipped with anion columns CI SUPER-SEP (100 mm×4.6 mm). 20

μl of samples or standard solutions were directly injected into the chromatographic system. The mobile phase consisted of phthalic acid (pH 3.8) and acetonitrile (95:10 v/v) was delivered at flow rate of 1.5 ml min^{-1} . The detection of the analytes was carried out using a conductivity detector. The test precision of the instrument was about $\pm 2\%$. Total uronic acid content was assessed colorimetrically according the method of Bitter & Muir (1962). All measurements were carried out in triplicates.

2.6 Determination of colour

The sample was placed between two steel dishes with a hole of 5.7 cm diameter. The colour of the films was determined by a tristimulus colorimeter (CHROMA METER CR-400/410. KONICA MINOLTA, Japan) using the CIE Lab scale ($C/2^\circ$), where L^* , a^* and b^* refer to the parameters measuring lightness, redness, and yellowness, respectively. A standard white plate with reflectance values of $L^* = 93.68$, $a^* = -0.69$, $b^* = -0.88$, was used as reference. The results were the average of five measurements taken at ambient temperature at different points on the samples.

2.7 Agarose-gel electrophoresis

Purified GAGs obtained from the skin of corb (CSG) were estimated for the presence of other complex by agarose-gel electrophoresis performed in barium acetate/1, 2-diaminopropane (Volpi, 1993; Volpi et al., 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. 10 μg of purified GAGs were applied to agarose gels and run in 0.05 M 1, 2-diaminopropane (pH 9.0) for 240 min at 100 mA. After migration, GAGs were fixed in the gel with 0.1% N-cetyl-N,N,N-trimethyl ammonium bromide in water for at least 6 h, dried and stained with 0.2% toluidine blue in ethanol–acetic acid-

water (100:2:98) for 30 min. Then destained with ethanol–acetic acid–water (100:2:98). The gel is also stained with the Stains-All, 12.5 mg/250 ml ethanol–water 50:50 overnight in the dark and destained with water.

2.8 Enzymatic treatment and disaccharide composition evaluation

To identify the species of GAGs extracted from corb skin, 50 μ l of the purified CS/DS was dried and blended in 40 μ l of 0.1 M ammonium acetate buffer pH 8. Thereafter, the mixture was centrifuged for 5 min at 4000 rpm and 4°C. Enzymatic treatment was achieved by the addition of 10 μ l of chondroitinase ABC (or chondroitinase AC) and the mixture was kept for 24 h at 37°C. After treatment of the purified CS/DS samples with these two lyases, the generated unsaturated disaccharides were separated and quantified by anion-exchange (SAX) by means of HPLC equipment from Jasco equipped with a 150 mm \times 4.6 mm stainless-steel column spherisorb 5-SAX (5 μ m, trimethylammoniopropyl groups Si-CH₂-CH₂-CH₂-N⁺(CH₃)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 4.0 for 5 min followed by a linear gradient from 5 to 60 min of 50 mM NaCl to 1.2 M NaCl pH 4.0, at a flow rate of 1.2 ml/min. Authentic unsaturated standard disaccharides were used for qualitative and quantitative purposes.

2.9 CS/DS molecular mass determination

The molecular mass of CS/DS obtained from the skin of corb was determined by PAGE according to Edens et al. (Edens, al-Hakim, Weiler, Rethwisch, Fareed, & Linhardt, 1982). About 10 μ g of the purified CS/DS determined by uronic acid assay (Cesaretti et al., 2003) were layered on the gel. The related calibration curve was constructed by using oligosaccharide standards of known molecular masses prepared from CS (Buzzega, Maccari, & Volpi, 2010). After a run of 40 min at 100 V, the gel was stained with toluidine blue (0.1% in acetic acid 1%) for 30 min followed

by destaining in 1% acetic acid. Molecular masses 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.

2.10 *In vitro* anticoagulant activity of purified CS/DS

The action of CS/DS on haemostatic system was estimated by the evaluation of its anticoagulants activities in prothrombin time (PT), thrombin time (TT) and activated partial thromboplastin time (aPTT), using a semi-automatic line STA (Diagnostica Stago). The sample was dissolved in physiological serum. All analysis was carried out in triplicates and mean values were taken. Heparin sodium Choay (Sanofi-Aventis, France) was used as reference.

2.10.1 aPTT assay

The aPTT assay was determined by mixing normal citrated platelet poor plasma (PPP) (45 μ l) with 5 μ l of the purified CS/DS at different concentrations and incubated for 3 min at 37 °C. Afterward, 50 ml of aPTT reagent (CK-PREST) were added and the mixture was incubated for 3 min at 37 °C. The clotting time was immediately saved after the addition of 100 μ l of 25 mM CaCl₂. The clotting time is expressed in seconds and as ratio, with the average value of a normal subject less than 1.2. The enzyme activity control (C) is measured by replacing the 5 μ l of glycosaminoglucans with physiological serum.

2.10.2 PT assay

The prothrombin time assay was assessed by mixing 5 μ l of the purified CS/DS at different concentrations with 45 μ l of PPP. The mixture was incubated for 3 min at 37 °C. The clotting time was carried out after the addition of 100 μ l of Neoplastine[®] CI (DIAGNOSTICA-STAGO). The prothrombin time value is expressed in seconds.

2.10.3 TT assay

For the TT assay, 10 μ l of the purified CS/DS at different concentrations were incubated with 90 μ l of PPP for 3 min at 37 °C. The clotting time was determined after the addition of 100 μ l of thrombin (80 NIH). The thrombin time value is expressed in seconds.

2.11 Statistical analysis

All results were expressed as the mean standard deviation (SD). Data were analyzed using the SPSS statistic program (SPSS 10.0 for Windows, SPSS Inc., Chicago, IL).

Statistical differences between sample treatments were determined by one-way analysis of variance, and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1 Extraction, purification and chemical composition analysis

GAGs were extracted for the first time from the skin of corb (CSG) by digestion with Alcalase, followed by cetylpyridinium chloride treatment and absolute ethanol precipitation. After extraction by proteolytic treatment, CSG were fractionated on an anion-exchange resin and eluted with a linear NaCl gradient of increasing molarity.

The chemical compositions of CSG are shown in Table 1. Based on the dry weight, the CSG extraction yield was about 6.2%. The analysis of total sugars by the phenol/sulfuric acid method revealed that CSG had an average percentage of $47.25 \pm 0.044\%$, which could probably be ascribed to the efficiency of the extraction protocol. The findings also indicated that CSG had high uronic acid content ($44.9 \pm 1.53\%$). The sulfate content was estimated to be 28.74%, indicating a high polyanionic structure for CSG. This value was higher ($p < 0.05$) than glycosaminoglycans extracted from smooth hound and grey triggerfish (Krichen et al., 2015) and Norway lobster (Sayari et al., 2016). It may, therefore, be concluded that origin, nature of first matter, and type of extraction are responsible for the amount of sulfate. The colour values of glycosaminoglycans extracted from

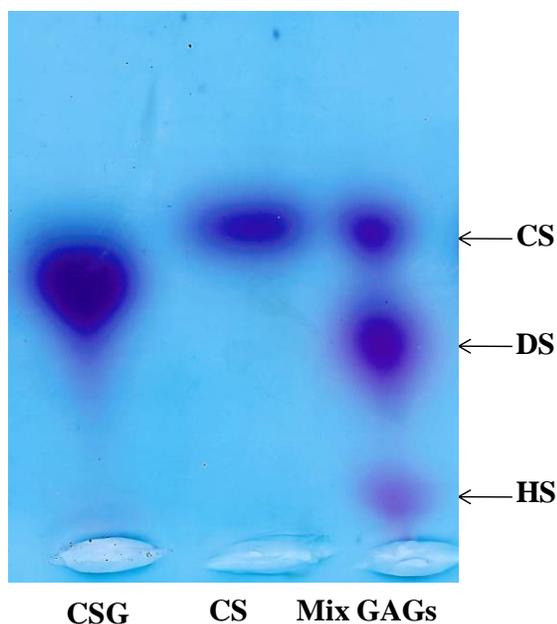
corb skin are also presented in Table 1. The lightness (L^*) value of CSG was 53.38% and indicated a dark colour. This value was lower than that previously described by Krichen et al. (2015) for the glycosaminoglycans extracted from gray triggerfish. The a^* (redness) and b^* (yellowness) values of CSG were 3.825 ± 0.07 and 2.29 ± 0.07 , respectively.

3.2 Agarose-gel electrophoresis

The separation of glycosaminoglycans in mixtures by agarose-gel electrophoresis and the recovery of single polysaccharide bands have been applied to the characterization of the purified CS/DS.

In the current study, agarose-gel electrophoresis specific for sulfated GAGs revealed the presence of a mixture of GAGs which are DS and CS (Figure. 1). In this context, previous results reported by Krichen et al. (2017a) indicated the presence of a mixture of GAGs which are DS, CS and HA by using agarose-gel electrophoresis.

Figure 1. Agarose-gel electrophoresis stained with toluidine blue and stain all of the GAGs purified from the skins of CSG.



3.3 Enzymatic treatments and disaccharide evaluation of CS/DS

It is noteworthy that GAGs are extremely difficult to analyze because of their negative charge, polydispersity and structural heterogeneity. Therefore, a classical strategy of detailed structural analysis of GAGs implicates the enzymatic digestion to obtain their disaccharide components.

To identify the species of GAGs isolated from corb skin, the purified CS/DS was subjected to specific treatment with two lyases: chondroitinase ABC and AC. The unsaturated disaccharides produced were also quantitatively and qualitatively evaluated by strong anion-exchange SAX-HPLC (Figure2). Chondroitinase ABC, from *Proteus vulgaris*, is non specific and cleaves all forms of CS. In fact, this enzyme catalyzes the eliminative degradation of polysaccharides containing (1-4)- β -D-hexosaminy and (1-3)- β -D-glucuronosyl or (1-3)- α -L-iduronosyl linkages to disaccharides containing 4-deoxy- β -D-gluc-4-enuronosyl groups. It acts on chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate, and acts slowly on hyaluronate.

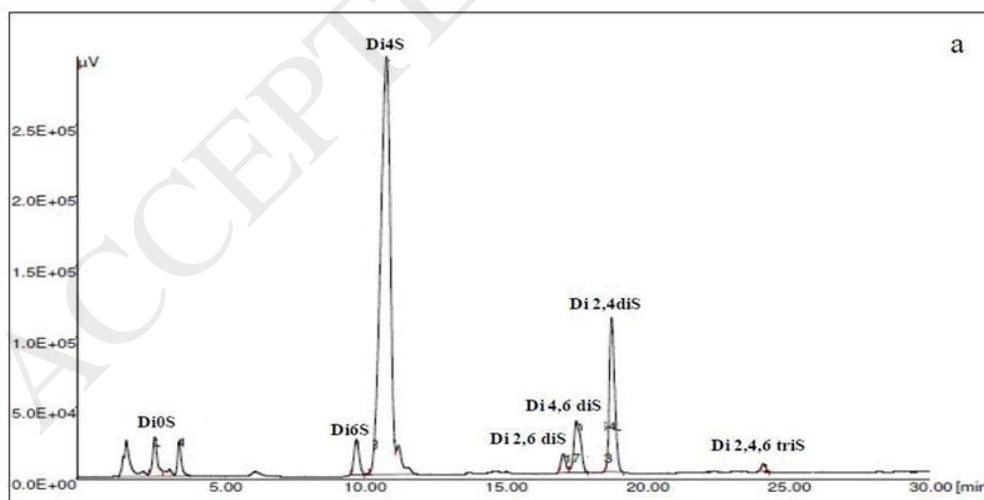
After treatment of the purified CS/DS with chondroitin ABC lyase, various unsulfated disaccharides were obtained in different percentages (Table 2). Obviously, the nonsulfated disaccharide $\Delta\text{Di}0\text{S}$ of CSG was 3.3 %, while monosulfated disaccharides $\Delta\text{Di}6\text{S}$ and $\Delta\text{Di}4\text{S}$ were estimated to be 3.2% and 70.9% respectively. Besides, the disulfated disaccharides $\Delta\text{Di}2, 6 \text{ diS}$ and $\Delta\text{Di}4,6 \text{ diS}$ of the purified CS/DS were about 1.58% and 6.08%, respectively, whereas the obtained results indicate that the disulfated disaccharide $\Delta\text{Di}2,4 \text{ diS}$ was found in elevated amounts in CSG (14.37%). In this respect, Maccari, Galeotti, & Volpi. (2015) suggested that the disulfated disaccharide $\Delta\text{Di}2,4 \text{ diS}$ in CS purified from bony fishes was trace. The findings showed also that CSG have low concentration in trisaccharides (0.550%). These values were quite similar to those previously reported in the work of Krichen el al, 2017a. Moreover, the presence of disulfated disaccharides produced a great overall charge density of CSG which was 1.2. Various investigations have reported that the degree and position of sulfation, inside the polysaccharides chains, contain different proprieties and biological activities. Indeed, the analysis by SAX-HPLC proved that the 4-sulfated disaccharide content in the purified CSG is higher than sulfated disaccharide in position 6 producing a 4S/6S ratio of 22.12.

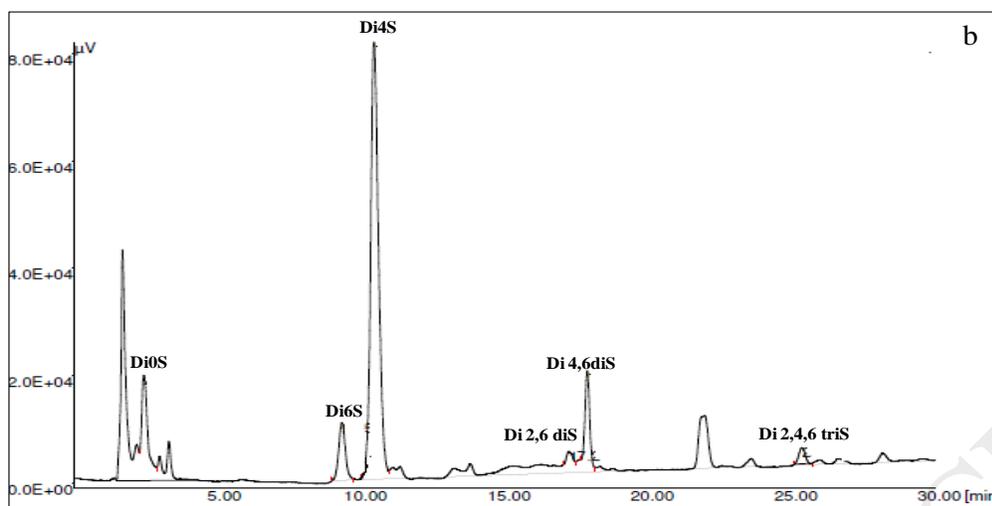
On the other hand, Chondroitinase AC, from *Flavobacterium heparinum*, is an enzyme that cleaves sulfated and non-sulfated polysaccharide chains with (1-4) linkages between hexosamines and glucuronic acid residues, by an elimination mechanism. The resulting oligosaccharide products are mainly disaccharides with unsaturated uronic acids. Chondroitinase AC specifically degrades chondroitin sulfates A and C, but not chondroitin sulfate B (dermatan sulfate). In fact, according to the results (Table 2), the chondroitinase AC produced high disulfated disaccharide in position 2 and 6 compared to chondroitinase ABC (3% and 1.585%, respectively). Otherwise, the disulfated disaccharide in position 2 and 4 ($\text{Di}2, 4 \text{ diS}$) was most generated by chondroitinase ABC than AC

(14.37% and 9.68%, respectively). In summary, our results suggested that the CS is sulfated, especially in positions 6 and positions 2 and 6 as opposed to 4S and 2,4 diS species belonging to DS (Malavaki, Mizumoto, Karamanos, & Sugahara, 2008).

Due to the incapacity of chondroitinase AC to degrade chondroitine sulfate B (dermatan sulfate), the percentages of CS and DS were performed by calculating the proportion between the total area of the chromatogram of AC-samples and the total area of ABC-samples. Therefore, our results suggest that the percentage of CS and DS recovered in CSG were 24% and 76%, respectively.

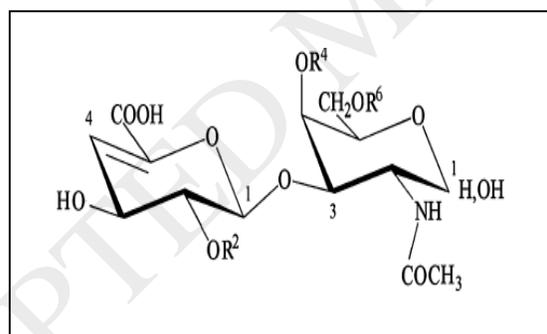
Figure 2. SAX-HPLC separation of the unsaturated disaccharides from CS/DS purified from corb skins and treated with chondroitinase ABC (a) and chondroitinase AC (b). Δ Di0S (Δ UA-GalNAc), Δ Di6S (Δ UA-GalNAc 6S), Δ Di4S (Δ UA-GalNAc 4S), Δ Di2, 6diS (Δ UA2S-GalNAc 6S) Δ Di4, 6diS (Δ UA GalNAc4, 6diS), Δ Di2, 4diS (Δ UA2S-GalNAc4S). The identity of disaccharide species was assured by coelution with purified standards (Seikagaku Co. /Sigma–Aldrich).





With all of the information combined, the hypothetical structures for CS/DS were determined as shown in figure 3.

Figure 3. Structures of repeating disaccharide units forming CS/DS.

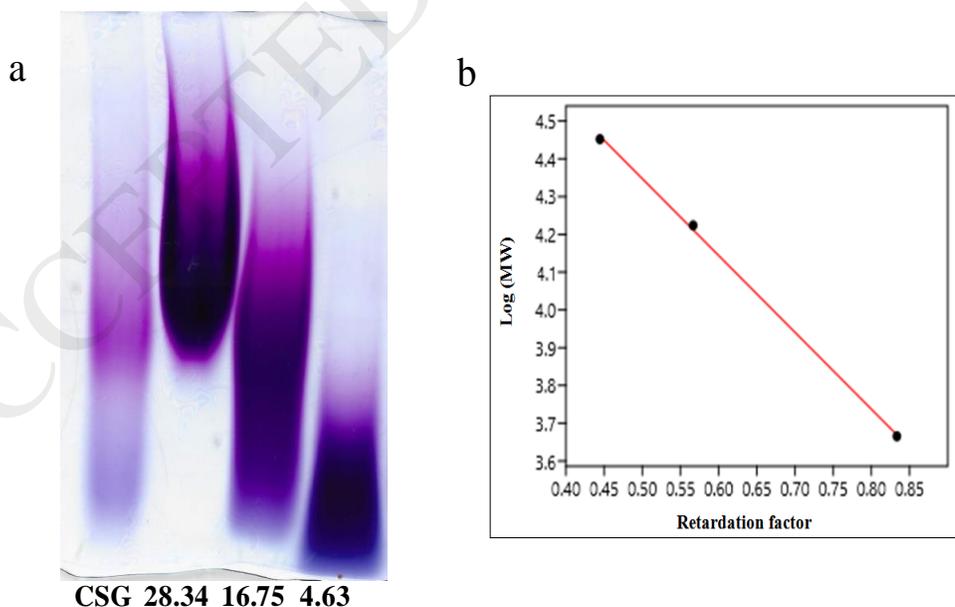


3.4 CS/DS molecular mass determination

Glycosaminoglycans (GAGs), from different sources, are glycans of various properties and complex structures. They are synthesized as polymers of repeating disaccharides with diverse numbers and position of sulfate groups, as well as their amount. In particular, different degrees of glycosaminoglycans sulfation result in their different charge densities. The charge density differences impact their migration behavior in polyacrylamide gel electrophoresis, one of the most

methods for determining relative molecular masses of glycosaminoglycans. Figure. 4a, shows the PAGE analysis of the skin fish CS/DS calculated on a calibration curve of CS fractions of known molecular masses. Consequently, based on the calibration curve (Figure. 4b), which given as follow $\log Mw = y = -2.0348x+5.365$ ($r=-0.99965$), the purified CS/DS indicate an average molecular mass of 15.46 KDa. In this context, Maccari et al. (2015) confirmed that molecular masses values of CS from bony fishes range from 13.46 to 48.68 KDa. More recently, Ben mansour et al. (2017) isolated a fucosylated polysaccharide from sea cucumber with an average molecular weight of 45.8 KDa.

Figure 4. (a) PAGE analysis of chondroitin sulfate/dermatan sulfate GAGs purified from the skin of CSG and (b) the related calibration curve. The calibration curve was constructed by using chondroitin sulfate standards of known molecular mass prepared from chondroitin sulfate and having masses of 28.340 KDa, 16.750 KDa and 4.630 KDa.



3.5 *In vitro* anticoagulant activity

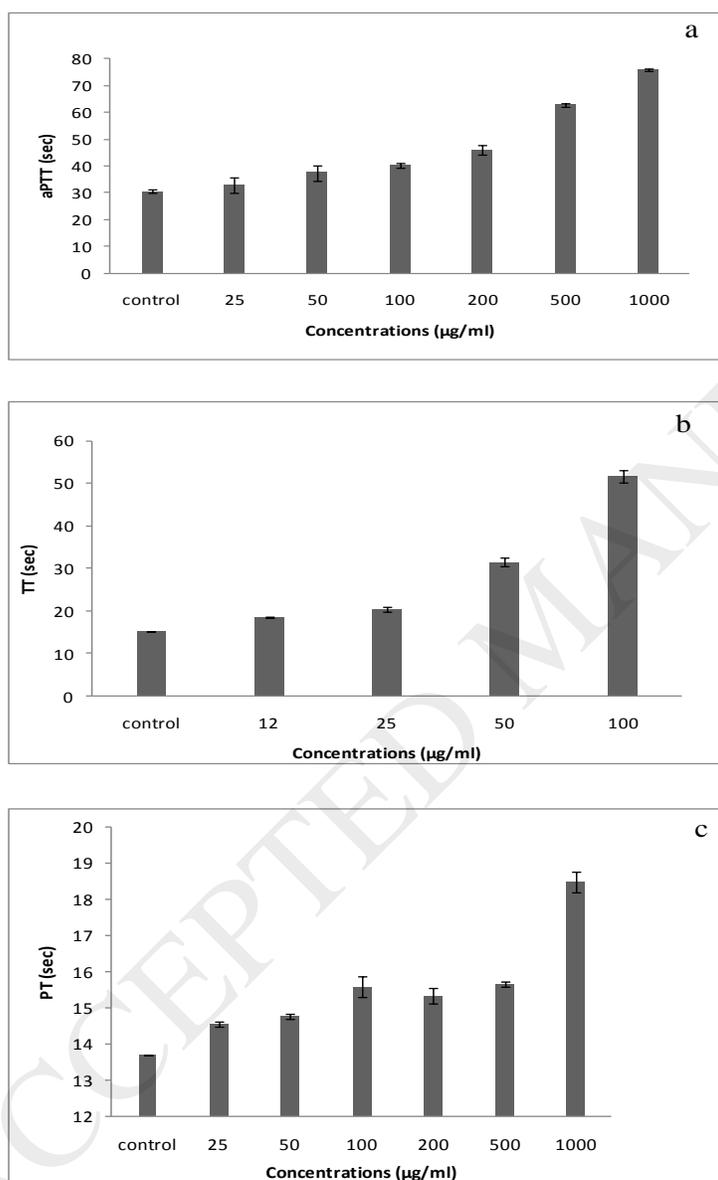
It is well known that the blood coagulation system consists of intrinsic, extrinsic and common pathways (Cheng, Sun, & Zhao, 2014). The anticoagulant activity is commonly estimated by the classical coagulation assays including aPTT, PT and TT. Heparin was used as positive control. (Xiang, Wang, Qin, Xiang, Su, & Zhao, 2015). In fact, aPTT and PT is related to the intrinsic coagulation pathways and extrinsic pathways, respectively, while TT is considered as an indicator of the content and coagulation activity of fibrinogen in plasma in the common phase of coagulation process (Cheng et al., 2014).

The anticoagulant activity of the purified CS/DS was first investigated by aPTT assay. As can be seen in Figure. 5a, the anticoagulant effect of this polymer was concentration dependent, the values increased with increasing concentrations of sample. Indeed, the results indicated that the addition of 1000 $\mu\text{g/ml}$ of the purified CS/DS caused a significant prolongation of activated partial thromboplastin time (aPTT). The prolongation of the clotting time for CS/DS was about 2.48 times ($p < 0.05$) greater than that of the control (75.95 and 30.55 s, respectively at 1000 $\mu\text{g/ml}$). Moreover, results revealed that the use of 7 $\mu\text{g/ml}$ of heparin caused a significant prolongation of activated partial thromboplastin time (aPTT). At this concentration the clotting time of heparin was 120 s which can induce a high risk of bleeding. Consequently, the prolongation of the aPTT suggests inhibition of the intrinsic and/or the common pathways of coagulant and thus this CS/DS represents a new natural antithrombic agent (Leadley, Chi, Rebello & Gagnom, 2000). Similarly, many reports revealed that sulfated polysaccharides isolated from different sources, such as green seaweed (*Monostroma angicava*) (Li, Liu, He, Wang, Cao, Xia et al, 2017) and sea cucumber (Ben Mansour et al., 2017) are used as antithrombotic agent. Furthermore, TT was effectively prolonged by the purified CS/DS with increasing concentration of sample and the clotting time was 51.7 s at

100 $\mu\text{g/ml}$ (Figure. 5b). To conclude, an increase in TT suggests the thrombin inhibition or fibrin polymerization as thrombin inhibition-dependent clotting time. In the other hand, the obtained data showed that the use of 7 $\mu\text{g/ml}$ of heparin increase the clotting time to more than 120 s. Moreover, anticoagulant activity of CS/DS was also proven by their capacity to prolong the prothrombin time (PT). As illustrated in Figure. 5c, the PT determined with the presence of the purified CS/DS, at different concentrations, was effectively higher than the control clotting time (13.7 s). Obviously, PT was significantly prolonged to 18.5 s at 1000 $\mu\text{g/ml}$ of CS/DS. Overall, the obtained results demonstrated that this polymer showed significant anticoagulant activity on the extrinsic pathway of coagulation.

To summary, our results suggested that the purified CS/DS isolated from the skin of corb exhibit a remarkably higher anticoagulant activity. This powerful effect may be due to its mechanism of action for thrombin inhibition. Importantly, the anticoagulant effect of the marine glycosaminoglucans were related to divers structural parameters, such as the degree of sulfation, the sulfation position, the molecular weight, type of sugar and glycosidic branching. Therefore, the high sulfated content in particular the double sulfated residues were essential for the anticoagulant activity. In this context, Pereira, Melo, & Mourão. (2002) reported that the monosulfated disaccharide in position 4 and / or disulfated disaccharide in positions 2 and 4 are required for thrombin inhibition by activating cofactor II of heparin and antithrombin, respectively. These studies were confirmed by results obtained with enzymatic treatment using specific lyases. More recently, Liang et al. (2018) confirmed that greater anticoagulant activities of sulfated polysaccharides could be obtained when molecular weight maintained in a moderate range and DS remained higher.

Figure 5. Anticoagulant activity of the purified CS/DS extracted from the skin of corb (CSG) at different concentrations evaluated by the measurement of (a) the activated partial thromboplastin time (aPTT), (b) time of thrombin and (c) prothrombin time.



4. Conclusion

In this paper, CS/DS have been isolated from the skin of corb and described with respect to chemical, structural characterization and anticoagulant effect. PAGE analysis showed that CSG having relatively low molecular mass (15.46 KDa). Additionally, the extracted CS/DS was also noted to contain elevated percentages of disulfated disaccharides, producing an overall great charge density. Moreover, the percentage of CS and DS recovered in CSG were 24% and 76%, respectively. Our results also indicated that the purified CS/DS from skin of corb exhibited potential anticoagulant activities, which improves with increasing of DS. These data also revealed that better anticoagulant activities could be obtained when molecular weight maintained in a moderate range. Further, the presence of monosulfated disaccharide in position 4 and / or disulfated disaccharide in positions 2 and 4 are required for thrombin inhibition by activating cofactor II of heparin and antithrombin, respectively. Also, the elevated sulfate content of CS/DS is one of the most important factors for its anticoagulant effects. Finally, this new CS/DS, with their particular structural properties, might be potentially useful for pharmacological applications.

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References

- Abdelmalek, B.E., Sila, A., Krichen, F., Karaoud, W., Martinez-Alvarez, O., Ellouz-Chaabouni, S., Ayadi, MA & Bougatef, A. (2015). Sulfated polysaccharides from *Loligo vulgaris* skin: Potential biological activities and partial purification. *International Journal of Biological Macromolecules*, 72, 1143-1151.

- Araújo, C.A., Nosedá M.D., Cipriani, T.R., Gonçalves A.G., Duarte, M.E & Ducatti, D.R.B. (2013). Selective sulfation of carrageenans and the influence of sulfate regiochemistry on anticoagulant properties. *Carbohydrate Polymers*, 91, 483-91.
- Athukorala, Y., Lee, K.W., Kim, S.K & Jeon, Y.J. (2007). Anticoagulant activity of marine green and brown algae collected from Jeju Island in Korea. *Bioresource Technology*, 98, 1711-6.
- Barbbucci, R., Magnani, A., Lamponi, S & Albanese, A. (1996). Chemistry and biology of glycosaminoglycans in blood coagulation. *Polymers for Advanced Technologies*, 7, 675-85.
- Ben Mansour, M., Balti, R., Ollivier, V., Ben Jannet, H., Chaubet, F & Maaroufi, R.M. (2017). Characterization and anticoagulant activity of a fucosylated chondroitin sulfate with unusually procoagulant effect from Sea cucumber. *Carbohydrates Polymers*, 174, 760-771.
- Ben Mansour, M., Majdoub, H., Bataille, I., Roudesli, M.S., Hassine, M., Ajzenberg, N., Chaubet, F & Maarouf, R.M. (2009). Polysaccharides from the skin of the ray *Raja radula*. Partial characterization and anticoagulant activity. *Thrombosis Research*, 123, 671-678.
- Ben mansour, M; Dhahri, M; Hassine, M; Ajzenberg, N; Venisse, L; Ollivier et al. (2010). Highly sulfated dermatan sulfate from the skin of the ray *Raja montagui*: anticoagulant activity and mechanism of action. *Comparative Biochemistry and Physiology*, 156,206-215.
- Bitter, T & Muir, H.M. (1962). *Analytical Biochemistry*. 4, 330-334.
- Buzzega, D., Maccari F & Volpi, N. (2010). Determination of molecular mass values of chondroitin sulfates by fluorophore-assisted carbohydrate electrophoresis (FACE). *Journal of Pharmaceutical and Biomedical Analysis*, 51, 969-972.
- Cesaretti, M., Luppi, E., Maccari, F & Volpi, V. (2003). A 96-well assay for uronic acid carbazole reaction. *Carbohydrate Polymers*. 54, 59-61.

- Cheng, C., Sun, S.D & Zhao, C.S. (2014). Progress in heparin and heparin-like/mimicking polymer-functionalized biomedical membranes. *Journal of Materials Chemistry*, 2, 7649-7672.
- Dahlback MD, B. (2000). Blood coagulation. *The Lancet*, 355, 1627-1632.
- Dubois, M., Gilles, K.A., Hamilton, J.K & Rebers, P.A & Smith, F. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry*. 28, 350-356.
- Edens, R. E., al-Hakim, A., Weiler, J. M., Rethwisch, D. G., Fareed, J., & Linhardt, R. J. (1982). Gradient polyacrylamide gel electrophoresis for determination of molecular weights of heparin preparations and low-molecular-weight heparin derivatives. *Journal of Pharmaceutical Sciences*, 81, 823-827.
- Kaczmarek, B., Sionkowska, A & Osyczka, A.M. (2017). Collagen-based scaffolds enriched with glycosaminoglycans isolated from skin of *Salmo salar* fish. *Polymer Testing*, 62,132-136.
- Kreutz, R. (2014). A clinical and pharmacologic assessment of once-daily versus twicedaily dosing for rivaroxaban. *Journal of Thrombosis and Thrombolysis*, 38,137-149.
- Krichen, F., Ghilissi, Z., Ben Amor, I., Sayaria, N., Kallel, R., Gargouri, J., Sahnoun, Z., Boudawara, T & Bougatef, A. (2017b). In vitro and in vivo anti-coagulant activity and toxicological studies of marine sulfated glycosaminoglycans. *Experimental and Toxicologic Pathology*, 69, 45-63.
- Krichen, F., Karoud, W., Sila, A., Abdelmalek, B.E., Ghorbel, R., Ellouz-Chaabouni, S & Bougatef, A. (2015). Extraction, characterization and antimicrobial activity of sulfated polysaccharides from fish skins. *International Journal of Biological Macromolecules*, 75,283-289.

- Krichen, F., Volpi, V., Sila, S., Maccari, F., Mantovani, V., Galeotti, F., Ellouz-Chaabouni, S Bougatef, A. (2017a). Purification, Structural Characterization and Antiproliferative Properties of Chondroitin Sulfate/Dermatan Sulfate from Tunisian Fish Skins. *International Journal of Biological Macromolecules*, 95, 32-39.
- Leadley, R.J., Chi, L., Rebello, S.S & Gagnon, A. (2000). Contribution of in vivo models of thrombosis to the discovery and development of novel antithrombotic agents. *Journal of Pharmacological and Toxicological Methods*, 43, 101-116.
- Li, N., Liu, X., He, X., Wang, S., Cao, S., Xia, Z., Xian, H., Qin, L & Mao, W. (2017). Structure and anticoagulant property of a sulfated polysaccharide isolated from the green seaweed *Monostroma angicava*. *Carbohydrate Polymers*, 159, 195-206.
- Li, R., Wu, G., Cai, X & Ye, Y. (2017). In vitro anticoagulant activity of polyanionic graft chains modified poly(vinyl alcohol) particles. *Radiation Physics and Chemistry*, 134, 27-32.
- Liang, L; Ao, L; Ma, T; Ni, Y; Liao, X; Hu, X & Song, Y. (2018). Sulfated modification and anticoagulant activity of pumpkin (*Cucurbita pepo*, Lady Godiva) polysaccharide. *International Journal of Biological Macromolecules*, 106,447-455.
- Maccari, F & Volpi, N. (2003). Detection of submicrogram quantities of *Escherichia coli* lipopolysaccharides by agarose-gel electrophoresis. *Analytical Biochemistry*, 32, 185-189.
- Maccari, F., Galeotti, F & Volpi, N. (2015). Isolation and structural characterization of chondroitin sulfate from bony fishes. *Carbohydrates Polymers*, 123, 143-147.
- Mlavakia, C.J., Asimakopoulou, P.A., Lamari, F.N., Theocharisa, A.D., Tzanakakisc, G.N & Karamanosa, N.K. (2008). Capillary electrophoresis for the quality control of chondroitin sulfates in raw materials and formulations. *Analytical Biochemistry*, 374, 213-220.

- Okamoto, Y., Higashi, K., Linhardt, R.J & Toid, T. (2018). Comprehensive analysis of glycosaminoglycans from the edible shellfish. *Carbohydrates Polymers*, 184,269-276.
- Pereira, M.S., Melo, F.R & Mourão P.A.S. (2002). Is there a correlation between structure and anticoagulant action of sulfated galactans and sulfated fucans? *Glycobiology*. 12, 573-580.
- Roden, L., (1980). Structure and Metabolism of Connective Tissue Proteoglycans. *The biochemistry of Glycoproteins and Proteoglycans*, 267-371.
- Sayari, N., Balti, R., Ben Mansour, M., Ben Amor, I., Graiet, I., Gargouri, J & Bougatef A. (2016). Anticoagulant properties and cytotoxic effect against HCT116 human colon cell line of sulfated glycosaminoglycans isolated from the Norway lobster (*Nephrops norvegicus*) shell. *Biomedicine & Pharmacotherapy*, 80, 322-330.
- Volpi, N & Maccari, F. (2002). Detection of submicrogram quantities of glycosaminoglycans on agarose-gels by sequential staining with toluidine blue and stains-all. *Electrophoresis*, 23, 4060-4066.
- Volpi, N & Maccari, F. (2005). Glycosaminoglycan Composition of the Large Freshwater Mollusc Bivalve *Anodonta anodonta*. *Biomacromolecules*, 6, 3174-3180.
- Volpi, N. (1993). “Fast moving” and “slow moving” heparins, dermatan sulfate, and chondroitin sulfate: Qualitative and quantitative analysis by agarose-gel electrophoresis. *Carbohydrate Research*, 247, 263-278.
- Volpi, N. (2007). Analytical aspects of pharmaceutical grade chondroitin sulfates. *Journal of Pharmaceutical Sciences*. 96,3168-3180.
- Volpi, N. (2009). Capillary electrophoresis determination of glucosamine in nutraceutical formulations after labeling with anthranilic acid and UV detection. *Journal of Pharmaceutical and Biomedical Analysis*, 49,868-871.

- Warkentin TE. (2006). HIT: lessons learned. *Pathophysiology of Haemostasis and Thrombosis*, 35,50-7.
- Xiang, T., Wang, R., Qin, H., Xiang, H., Su, B.H & Zhao, C.S. (2015). Excellent biocompatible polymeric membranes prepared via layer-by-layer self-assembly. *Journal of Applied Polymer Science*, 132, 41245.
- Yang, L., Wang, Y., Yang, S & Lv, Z. (2018). Separation, purification, structures and anticoagulant activities of fucosylated chondroitin sulfates from *Holothuria scabra*. *International Journal of Biological Macromolecules*, 108,710-718.

Table 1: Chemical composition of glycosaminoglycans extracted from corb (*Sciaena umbra*) skin. Values are given as mean \pm SD from triplicate determinations. Physico-chemical composition was calculated based on dry matter.

Table 2: Amount, disaccharide composition and charge density values of CS/DS GAGs purified from fish skin.

Δ UA, 4,5-unsaturated uronic acid; GalNAc, N-acetyl-galactosamine; S, sulfate group. The percentage of each identified disaccharide was determined by purified standards (Seikagaku Co./Sigma–Aldrich) and reported as weight percent. Charge density was calculated by considering the number of sulfated groups per disaccharide unit.

Table 1: Chemical composition of glycosaminoglycans extracted from corb (*Sciaena umbra*) skin. Values are given as mean \pm SD from triplicate determinations. Physico-chemical composition was calculated based on dry matter.

Composition (%)	CSG
Sulfate	28.74 \pm 0.74
Total sugars	47.2 \pm 0.044
Uronic acid	44.9 \pm 1.53
Colour	
L*	53.38
a*	3.825 \pm 0.07
b*	2.29 \pm 0.07
Yield	6.2 \pm 0.67

Table 2: Amount, disaccharide composition, charge density and molecular mass values of CS/DS GAGs purified from fish skin.

	Chondroitinase ABC	Chondroitinase AC
Δ Di0S (Δ UA-GalNAc) (%)	3.301	12.966
Δ Di6S (Δ UA-GalNAc 6S) (%)	3.205	7.262
Δ Di4S (Δ UA-GalNAc 4S) (%)	70.904	65.212
Δ Di2, 6S (Δ UA2S-GalNAc 6S) (%)	1.585	3.002
Δ Di4, 6S (Δ UA 4S-GalNAc 6S) (%)	6.082	9.687
Δ Di2, 4S (Δ UA2S-GalNAc 4S) (%)	14.373	-
Δ Di2, 4,6Tris (Δ UA2S-GalNAc 4,6 diS) (%)	0.550	1.872
Charge density	1.2	1.03

Δ UA, 4,5-unsaturated uronic acid; GalNAc, N-acetyl-galactosamine; S, sulfate group. The percentage of each identified disaccharide was determined by purified standards (Seikagaku Co./Sigma–Aldrich) and reported as weight percent. Charge density was calculated by considering the number of sulfated groups per disaccharide unit.