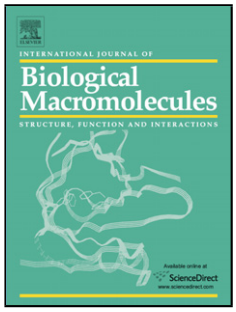

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Purification, Structural Characterization and Antiproliferative Properties of Chondroitin Sulfate/Dermatan Sulfate from Tunisian Fish Skins

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Highlights

- Chondroitin sulfate and dermatan sulfate were purified from Tunisian fish skins.
- Uronic acid content, disaccharide and trisaccharide compositions were determined.
- Chondroitin sulfate and dermatan sulfate may be useful for pharmacological applications.

Abstract

Chondroitin sulfate/dermatan sulfate GAGs were extracted and purified from the skins of grey triggerfish (GTSG) and smooth hound (SHSG). The disaccharide composition produced by chondroitinase ABC treatment showed the presence of nonsulfated disaccharide, monosulfated disaccharides ΔDi6S and ΔDi4S, and disulfated disaccharides in different percentages. In particular, the nonsulfated disaccharide ΔDi0S of GTSG and SHSG were 3.5% and 5.5%, respectively, while monosulfated disaccharides ΔDi6S and ΔDi4S were evaluated to be 18.2%, 59% and 14.6%, 47.0%, respectively. Capillary elecrophoresis analysis of GTSG and SHSG contained 99.2% and 95.4% of chondroitin sulfate/dermatan sulfate, respectively. PAGE analysis showed a GTSG and SHSG having molecular masses with average values of 41.72 KDa and 23.8 KDa, respectively. HCT116 cell proliferation was inhibited ($p < 0.05$) by 70.6% and 72.65% at 200 µg/mL of GTSG and SHSG respectively. Both GTSG and SHSG demonstrated promising antiproliferative potential, which may be used as a novel, effective agent.

Keywords: Glycosaminoglycans, chondroitin sulfate, dermatan sulfate, antiproliferative activity, fish skins.

1. Introduction

Glycosaminoglycans (GAGs) are linear, acidic, highly sulfated and complex polysaccharides. There are two main types of GAGs. Non-sulfated GAGs with hyaluronic acid (HA) and sulfated GAGs being chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS), heparin and heparan sulfate (HS) [1]. GAGs can be classified also in two types, galactosaminoglycans with CS and DS and glucosaminoglycans represented by heparin and HS.

GAGs are synthesized as polymers of disaccharide repeating units. The repeating regions are composed of uronic acid (D-glucuronic acid or L-iduronic acid) and amino sugar (D-galactosamine or D-glucosamine), with the exception of keratan sulfate which contains galactose instead. Furthermore, GAGs differ according to the type of hexuronic acid or hexosamine, hexose unit which they contain, thus the geometry of the glycosidic linkage between these units.

HA is very peculiar and neither modified but the other types of GAGs can be modified by the addition of O-sulfate groups on various hydroxyls (the three classes), removal of acetyl residues from some hexosamines replaced with N-sulfates (HS and heparin) and 5-epimerization of glucuronic acid residues to formid uronic acid residues (DS, HS and heparin). These modifications introduce heterogeneity within the chains and often have major roles in a wide variety of biological and pharmacological processes [2, 3].

GAGs are thought to be present in all animals. They can be extracted from terrestrial animal cartilages such as bovine, porcine and avian [4, 5] or from marine organisms such as ray skin [6], shark cartilage [7], sturgeon cartilage [8, 9] and bony fishes [10]. Recently, other group has reported the facile analysis of chondroitin sulfate in many bony fish [11].

Due to the structural heterogeneity, GAGs from different sources can be composed of repeating disaccharides having various sulfate groups located, in different percentages, inside the polysaccharide chains. These repeating disaccharide units can be monosulfated, disulfated or

trisulfated depending on the origin. As a consequence, GAGs with different charge densities may be produced from various sources possessing different proprieties and biological capacities.

Sulfated GAGs from marine sources possess important pharmacological activities such as antioxidant and neuroprotective actions [12], antiviral [13], anticoagulant and antithrombotic [14], antitumoral and anti-inflammatory [15], antiproliferative, anti-complementary, antipeptic and anti-adhesive activities [16]. These functions are attributed to the importance of the molecular size and some structural features required for biological activities, especially sulfate clusters to ensure interactions with cationic proteins [17, 18].

Furthermore, the global awareness of cancer as the second largest cause of death in people of various ages and background has led to so much research effort and clinical studies in the fight against the disease [19]. Most cytotoxic drugs used in cancer chemotherapy are highly toxic to a wide spectrum of normal tissues. In the last years, the search for natural antitumor compounds has gained considerable attention and the study of the antitumor activities of marine GAGs has become matters of great interest. Several investigations have reported that sulfated polysaccharides have antiproliferative activity in cancer cell lines in *vitro* [20] as well as inhibitive activity in tumors growing in mice [21]. Moreover, these polymers have been reported to induce apoptosis in several cancer lines and stimulate immune system cells to induce tumor cell death [22, 23].

In this study, we investigated for the first time the antiproliferative activity of GAGs extracted and purified from the skins of grey triggerfish (GTSG) and smooth hound (SHSG), and their structure characterized was also evaluated.

2. Materials and Methods

2.1. Reagents

The chemicals and solvents used in the present study were purchased at the analytical grade or highest level of purity available. Alcalase® 2.4 L serine-protease from *Bacillus licheniformis* was obtained from Novozymes® (Bagsvaerd, Denmark). GAGs standard, HS from bovine kidney, CS from bovine trachea, DS from porcine intestinal mucosa, and HA (hyaluronan) from rooster comb, 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 were from Sigma–Aldrich. Unsaturated chondro/dermato disaccharides [ΔDi0S (ΔUA-[1→3]-GalNAc), ΔDi4S (ΔUA-[1→3]-GalNAc-4S), ΔDi6S (ΔUA-[1→3]-GalNAc-6S), ΔDi2S (ΔUA-2S-[1→3]-GalNAc), ΔDi2,4diS (ΔDi-diS B, ΔUA-2S-[1→3]-GalNAc- 4S), ΔDi2,6diS (ΔDi-diS D, ΔUA-2S-[1→3] GalNAc-6S), ΔDi4,6diS (ΔDi-diS E, ΔUA-[1→3]-GalNAc-4,6diS), and ΔDi2,4,6triS (ΔDitriS, ΔUA-2S-[1→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), 2-Aminoacridone (AMAC), sodium cyanoborohydride were from Sigma–Aldrich. QAE Sephadex® A-25 anion-exchange resin was from Pharmacia Biotech (Uppsala, Sweden). 2,5-diphenyl- tetrazolium bromide (MTT), cell culture medium (RPMI1640), foetal calf serum (FCS), phosphate buffer saline (PBS), trypsin-EDTA, penicillin and streptomycin mixture and L-glutamine (200 mM) were purchased from GIBCO-BCL (UK).

Fish by-products were obtained from the local fish market of Sfax, Tunisia. The samples were packed in polyethylene bags, placed in ice with a sample/ice ratio of approximately 1:3 (w/w), and transported to the research laboratory within 30 min. Upon arrival, the samples were washed twice with water and separated. Only the fish outer skin was collected and then stored in sealed plastic bags at −20 °C until further use for the extraction and analysis of GAGs.

2.2. Extraction of fish skin GAGs

GAGs were extracted according to a slightly modified version of the method described by Ben Mansour et al. [6]. In brief, the fish skins were cut into small pieces and homogenized using a Moulinex R62 homogenizer (Organotechnie, Courneuve, France). An amount of 5 g of sample was dissolved in 250 mL sodium acetate (0.1 M), EDTA (5 mM), cystein (5 mM) pH 6. Alacalase® was added, and the mixture was kept for 24 hours at 50 °C. The mixture was then left to cool down at room temperature and then filtered. The residue was washed with distilled water and filtered again. The filtrates were mixed, and GAGs were precipitated with cetylpyridinium chloride 10% (w/v). The mixture was kept for 24 hours at room temperature and centrifuged for 30 min at 5000 tr/min and 4 °C using a refrigerated centrifuge (Hettich Zentrifugen, ROTINA 380R, Germany). The pellet was washed with cetylpyridinium chloride 0.05% (w/v) and then dissolved in 200 mL NaCl solution in ethanol (100:15, v/v). An amount of 700 mL ethanol was added. GAGs containing solution was left for 24 hours at 4 °C and then centrifuged for 30 min at 5000 tr/min and 4 °C. The pellet was washed twice with ethanol 80% and then once with absolute ethanol. After that, the pellet was redissolved in desionised water and lyophilized in a freeze dryer (CHRIST, ALPHA 1-2 LD plus, Germany).

2.3. Purification of fish skins GAGs

The lyophilized GAGs were suspended in distilled water and then applied to a column (2 cm ×6 cm) packed with QAE Sephadex® A-25 anion-exchange resin equilibrated with NaCl 50 mM. GAGs were eluted with a linear gradient of NaCl from 50 mM to 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 [24] and agarose-gel electrophoresis [25, 26], and GAGs precipitated at 4 °C. After centrifugation at 10,000 g for 10 min, the pellet was dried at 40°C and solubilized in distilled water.

2.4. Agarose gel electrophoresis

Purified GAGs obtained from the skin of grey triggerfish (GTSG) and smooth hound (SHSG) were evaluated for the presence of other complex by agarose-gel electrophoresis performed in barium acetate/1, 2-diaminopropane. Agarose gel was prepared at a concentration of 0.5% in 0.04 M barium acetate buffer pH 5.8. A Pharmacia Multiphor II (from Pharmacia LKB Biotechnology, Uppsala, Sweden) electrophoretic cell instrument was used. Approximately 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 electrophoresis, 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 acetic acid–ethanol–water (0.1:5:5, v/v) for 30 min. Then destained with acetic acid–ethanol–water (0.1:5:5, v/v) [25, 26]. Plates were followed by the Stains-All staining procedure (25 mg in 500 mL ethanol–water 50:50 overnight in the dark and destained with water).

2.5. Enzymatic treatments and disaccharide composition evaluation

After treatment of the purified CS/DS samples with chondroitinase ABC, the generated unsaturated disaccharides were separated and quantified by anion-exchange (SAX) by means of HPLC equipment from Jasco equipped with a150 mm × 4.6 mm stainless-steel column spherisorb 5-SAX (5 μm, trimethylammoniopropyl groups Si-CH_2-CH_2-CH_2-$N^+(CH_3)_3$ in Cl^- form, from Phase Separations Limited, Deeside Industrial Park, Deeside Clwyd, U.K.) and detection at 232 nm. Isocratic separation was performed using 50 mM NaCl pH 4.00 for 5 min followed by a linear gradient from 5 to 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.

2.6. CS/DS molecular mass determination

The molecular mass of CS/DS obtained from the skins of GTSG and SHSG was determined by PAGE according to Edens et al. [27]. Twenty five micro gram of the purified CS/DS determined by uronic acid assay were layered on the gel. The related calibration curve was constructed by using oligosaccharide standards of known molecular mass prepared from CS [28]. 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.

2.7. Derivatization of CS/DS disaccharides with 2-Aminoacridone (AMAC)

Derivatization of Δ-disaccharides of GTSG and SHSG with AMAC was performed as described by Jackson [29] and modified by Kitagawa et al. [30] and Lamari et al. [31]. Thirty micro liter of the chondroitinase ABC treated samples were lyophilized and reconstituted with 20 µL of a 0.1 M AMAC solution in glacial acetic acid-DMSO (3:17, v/v) and 10 µL of a freshly prepared solution of 1 M sodium cyanoborohydride in water. After centrifugation, derivatization was performed by incubating at 45 °C for 4 h. The reaction was stopped by adding 30 µL of 50% v/v DMSO.

2.8. Capillary electrophoresis

AMAC derivatized GAGs disaccharides were analyzed using a Beckman HPCE instrument (P/ACE system 5000, Germany) equipped with a LIF detector. Separation and analysis were performed on an uncoated fused-silica capillary tube (50 µm I.D., 85 cm total length and 65 cm from the injection point to the detector) at 25 °C. The operating buffer was composed of 150 mM boric acid and 50 mM NaH_2PO_4 buffered at pH 7.0 with NaOH solution. The buffer was

degassed by vacuum filtration through a 0.2 µm membrane filter, followed by agitation in an ultrasonic bath. Before each run, the capillary tube was washed with 0.1 M NaOH for 1 min, double distilled water for 5 min, and then conditioned with the operating buffer for 5 min. The samples to be analyzed were injected automatically, using the pressure injection mode, in which the sample is pressurized for 10 s. The injection volume can be calculated with the Poiseuille equation as proposed by the manufacturer, giving an estimated volume of 6 nL per second of injection time. Electrophoresis was performed at 30 kV using normal polarity. Peak areas were recorded and calculated using the Beckman software system Gold V810.

2.9. Anti-proliferative activity of purified CS/DS

2.9.1. Cell culture and treatment

The tumor cells of human colon carcinoma HCT116 were obtained from the Faculty of pharmacy Chatenay malabry University Paris 11. HCT116 were cultured in DMEMF-12, supplemented with 10% FBS, 1% L-glutamine (200 mM), 1% of mixture penicillin (100 IU/mL) and streptomycin (100 mg/mL), at 37 °C with 5% CO_2.

The normal lymphocytes cells were isolated from heparinized human peripheral blood samples of healthy volunteers (15 mL) by using the Ficoll-Paque (specific gravity 1.077) gradient density method, as described previously [32]. Peripheral blood was diluted with phosphate-buffered saline (PBS; pH 7.2) in 1:2 ratio then centrifuged in a Ficoll-Histopaque (Sigma, USA) discontinuous gradient at 200 rpm at room temperature for 20 min in an ambient temperature (18–22 °C) for obtaining the characteristic layer containing the mononuclear cells. The lymphocytes were collected, washed with PBS and centrifuged (1500 rpm at room temperature for 5 min). Cells were suspended at a concentration of 1×10^7 cells/mL in PBS or RPMI-1640 medium (Sigma, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Gibco, Invitrogen Corporation, USA), 2 mM L-glutamine (Sigma, USA) and an antibiotic/antimycotic

cocktail (100 UI/mL penicillin G, 100 g/mL streptomycin and 250 ng/mL amphotericin B (Sigma, USA)). The human lymphocytes obtained were used for cell culture analysis as a normal control cells.

2.9.2. Determination of cell mortality (MTT assay)

Cytotoxicity of CS/DS was defined using the colorimetric method described by Carmichael et al. [33]. This method assesses the ability of viable cells to form MTT formazan by the mitochondrial enzyme succinate dehydrogenase. The measurements reflect early cellular redox changes [34]. HCT-116 cells (2.5×10^5 cells/well in 96-well plates) and lymphocytes (6×10^4 cells / well in 96-well plates) were incubated at 37°C for 24 h with different concentrations of CS/DS (10 – 200 µg/mL). A negative control containing only cells was also evaluated. After treatment, the plates were incubated in the MTT solution (final concentration of 0.5 mg/mL) for 3 h. The dark-blue formazan crystals that formed in intact cells were dissolved with DMSO, and the absorbance at 570 nm was measured with a spectrophotometer microplate reader (Bioteck, Elx 800). The results were expressed as the percentage of MTT reduction relative to the absorbances measured from negative control cells. All assays were performed in triplicate.

2.10. Hemolytic activity

The hemolytic activities of the sulfated GAGs were determined by a slightly modified version method of Dathe et al. [35]. In brief, five milliliters of bovine blood were centrifuged at 3500 rpm for 10 min to isolate erythrocytes, which were then washed three times with 10 mM sodium phosphate, pH 7.5, containing NaCl 9 g/L (NaCl/Pi). The cell concentration of stock suspension was adjusted to 10^9 cells/mL. The cell suspension (12 mL), along with varying amounts of stock solution fractions and buffer, were pipetted into Eppendorf tubes to give a final volume of 50 mL. The Eppendorf tubes with 2.5×10^8 cells/mL were then incubated at 37 °C for 40 min. After centrifugation (5000 rpm, 5 min), 30 mL of supernatant were diluted in 500 mL

water. The absorbance of the diluted solution was measured at 420 nm. The absorbance obtained after treating erythrocytes with only NaCl/Pi and SDS (0.2%) was taken as 0 and 100%, respectively. The experiments were repeated three times to check reproducibility.

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 agarose gel electrophoresis of glycosaminoglycans

GAGs were extracted for the first time from the skins of GTSG and SHSG. Alcalase digestion followed by cetylpiridinium chloride precipitation was performed. Further absolute ethanol was used in second steps to precipitate GAGs and mineral ions. In a previous work [36], we have reported the chemical and physical characteristics of GTSG and SHSG. Fish sulfated GAGs extraction yields for GTSG and SHSG were 8.62% and 9.86%, respectively.

After proteolytic treatment, sulfated GAGs from GTSG and SHSG were purified on an anion-exchange resin. The agarose-gel electrophoresis confirmed the presence of a mixture of GAGs which are CS, DS and HA (Fig. 1). In another work, Maccari et al. [10] confirmed the presence of CS as a principal polysaccharide in cartilages of bony fishes by using agarose-gel electrophoresis.

In fact, agarose-gel electrophoresis has been applied for various purposes, such as to control a purification step of a single GAGs species [37, 38], 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 both qualitatively and

quantitatively [37, 39], to evaluate polysaccharide–protein interactions [40]. Agarose-gel electrophoresis is a useful technique to analyze GAGs in mixtures. Furthermore, the quantitative analyses of uronic acid by carbazole method showed that GTSG and SHSG contained 70% and 40%, respectively.

3.2 Enzymatic treatments and disaccharide evaluation of CS/DS

To characterize the structure of GAGs extracted from fish skins, the purified CS/DS were subjected to treatment with chondroitinase ABC, and unsaturated disaccharides produced were analyzed by SAX-HPLC (Fig 2.A and 2.B). Chondroitinase ABC, from *Proteus vulgaris*, is non-specific and cleaves all forms of CS. GAG lyase enzymes act through an eliminative mechanism to cleave a HexNAc-HexA bond, leaving a (4, 5)-unsaturated (termed Δ unsaturated) HexA residue at the new non-reducing terminus.

As shown in table 1, chondroitinase ABC produced various unsaturated disaccharides in different percentages from GAGs purified from fish skins. In particular, the nonsulfated disaccharide ΔDi0S of GTSG and SHSG were 3.5% and 5.5%, respectively, while monosulfated disaccharides ΔDi6S and ΔDi4S were evaluated to be 18.2%, 59.0% and 14.6%, 47.0%, respectively. Besides, the monosulfated disaccharides ΔDi6S and ΔDi4S showed in monkfish cartilages were 28.2% and 51.0%, respectively [10]. On the contrary, Hashiguchi et al. [41] reported that CS extracted from ray fish cartilages contains 61.9% of ΔDi6S and 27.0% of ΔDi4S. Interestingly, disulfated disaccharides were observed in both GAGs in different percentages. The disulfated disaccharide ΔDi2, 6 diS of GTSG and SHSG were 5.48% and 4.56%, respectively as well as ΔDi4,6 diS were similar for both fish CS/DS (5.66% for GTSG and 5.45% for SHSG), while the disulfated disaccharide ΔDi2,4 diS was found in elevated concentration in SHSG (20.1%). Maccari et al. [10] reported that the disulfated disaccharide ΔDi2,4 diS in CS purified from bony fishs was trace. Furthermore, the results revealed that both

GTSG and SHSG have little amounts in trisaccharide (1.0% and 0.4%, respectively). The presence of disulfated disaccharides produced a great overall charge density of GTSG and SHSG which were 1.17 and 1.26, respectively. These values were similar to that reported for CS from codofish and tuna cartilages [10].

CS was composed of repeating disaccharides having various sulfate groups located, in different percentages, inside the polysaccharide chains. The degree and position of sulfation possess different proprieties and biological capacities. In fact, the analysis by SAX-HPLC showed that the 4-sulfated disaccharide content in GTSG and SHSG is higher than sulfated disaccharide in position 6 producing a 4S/6S ratio of 3.3 and 3.2 respectively quite similar to the 4S/6S ratio amount previously discribed by Volpi [4, 5] for CS purified from terrestrial cartilages. Maccari et al. [10] also reported that CS from codfish and tuna cartilages have a 4S/6S ratio equal to 2.19 and 2.23 respectively.

3.3. CS/DS molecular mass determination

It is well known that GAGs from different sources have variable structures and properties. In particular, they can have repeating disaccharides with various numbers and position of sulfate groups, as well as their amount. Furthermore, GAGs may also possess various molecular masses and polydispersities depending on the source. Fig. 3 shows the PAGE analysis of CS/DS purified from fish skins calculated on a calibration curve of CS fractions of known molecular masses. SHSG shows a larger polydispersity and a higher molecular mass value (41.72 KDa) than GTSG (23.8 KDa) (Table 1). In the same contexte, Chatziioannidis et al. [42] and Ben Mansour et al. [43] isolated DS with 31.2 kDa and 33 kDa from the skin of the rays *Raja clavata* and *Raja radula*, respectively. Moreover, Maccari et al. [10] confirmed that molecular mass values of CS from bony fishes range from 13.46 to 48.68 KDa. On the contrary, CS from cartilaginous fishes, shark and raja, has higher molecular weight values, 50 and 70 KDa, respectively [4, 5].

3.4. Capillary electrophoresis

Quantitative disaccharide compositional analysis is one of the most important strategies for structural characterization of GAGs and it is directly related to their biological and functional studies. Many techniques are utilized to determine the structural characterization of GAGs such as HPLC, thin-layer chromatography/densitometry [44], UV spectrophotometry after derivatization with ninhydrin [45], FACE analysis [46] and capillary electrophoresis (CE) [47].

2-Aminoacridone (2-AMAC) is a highly fluorescent hydrophobic molecule, which contains a primary amine group that reacts with acidic GAGs-derived di- and oligosaccharides [48, 49]. Quantitative derivatization is generally obtained with a 100-fold excess of this reagent. AMAC is used as an attractive labeling reagent for analysis of both charged and neutral oligosaccharides with electrophoretic (FACE and capillary electrophoresis) and chromatographic techniques. Fig.4 shows the CE-LIF separation of Δ-disaccharides of GTSG and SHSG fluorotagged with AMAC. The CE-LIF can distinguish the HA and the nonsulfated CS. However, the percentage recoveries of CS/DS calculated using the Beckman software system Gold V810 of GTSG and SHSG were 99.2% and 95.4% respectively, wheras the percentages of HA recovered in GTSG and SHSG were 0.8% and 4.6% respectively. This data showed that both GAGs extracts were composed of CS/DS with trace amounts of HA.

3.5. Antiproliferative activity of GTSG and SHSG

GAGs are the most therapeutically explored carbohydrates of the pharmaceutical market [50]. They belong to a class of sugars named sulfated glycans. Among various types, the commonly used GAGs in medicine are heparin, chondroitin sulfate and keratan sulfate. Although composed of disaccharide units and heavily sulfated, GAGs are very complex and heterogeneous in terms of structure.

The biological functions of GAGs are highly diversified, ranging from relatively simple mechanical support functions to more intricate effects on various cellular processes such as cell adhesion, proliferation and differentiation. Rapid cell proliferation is an important characteristic of malignant transformation. There is ample evidence for a role of GAGs and proteoglycans in controlling cell proliferation [51]. In this context, antiproliferative activity of the GAGs was investigated.

The amounts and distribution of sulfate groups on both sugar residues of the GAGs chain are the major determinant of antiproliferative activity. In this study, the effect of purified CS/DS on cell viability was investigated by the MTT assay. The viability of HCT116 cells treated with increasing concentrations of CS/DS ranging from 10-200 µg/mL for 24 h was determined using a colorimetric MTT-based assay (Fig. 5A and 5B). The sulfated GAGs from fish skins showed significant ($p < 0.05$) antiproliferative activity at all concentrations tested. Interestingly, GTSG and SHSG were most active at 200 µg/mL with 70.6% and 72.65% of cell proliferation inhibition. The IC_{50} values of 57 µg/mL and 65 µg/mL were obtained for GTSG and SHSG, respectively. All tested CS/DS concentrations showed no cytotoxicity against the normal lymphocytes. In the same context, Zhang et al. [52] reported that the sulfated polysaccharide from the sea cucumber (Stichopus japonicas) stimulated the proliferation of neural stem/progenitor cells. Kunou and Hatanaka [53] found that a synthetic (1 → 6)-α-D-mannopyranan sulfate could stimulate 3T3-L1 cell proliferation and that the degree of sulfation was an important factor in this process. Our results showed that CS/DS, which possess relatively higher sulfate content, exhibited the strongest proliferation enhancement and suggest that the sulfate content is probably the major factor that affects the cell proliferation in this study. Antiproliferative activity of many sulfated polysaccharides has been reported. Ulvans inhibits human epithelial colorectal adenocarcinoma cells (CACO-2) proliferation [53]. Fucans were

effective against several tumor cells, like as HL60 cells [54], sarcoma 180 [20], murine colon cancer cell line (CT-26), mouse melanoma cell line (B-16), human leukemia cell line (U-937) [55].

3.6. Hemolytic activity of CS/DS

The clinical use of heparin is limited by some undesirable effects as hemorrhagic complications due to its capacity to interfere in the hemostatic balance. Thus, it is of great importance to investigate the effect of the CS/DS from fish skins on haemostasia. The hemolytic activity of GTSG and SHSG was tested on bovine erythrocytes. Several concentrations for samples were tested (10 - 200 µg/mL). For all concentrations, no hemolysis was observed. These results show that both CS/DS would be non-toxic even if used at high concentrations.

4. Conclusions

GAGs were purified for the first time from the skins of GTSG and SHSG and described in the present study with their structural disaccharide composition and properties. Furthermore, on the basis of the data collected, CS/DS from fish skins showed high percentages of disulfated disaccharides producing anoverall great charge density. Finally, these newly identified sources of CS/DS might be potentially useful for cosmotological and pharmacological application.

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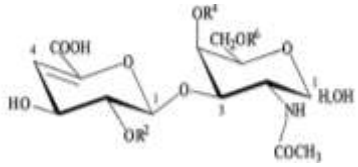
Table.1. Amount, disaccharide composition, charge density and molecular mass values of CS/DS GAGs purified from fish skins



The scheme illustrates the CS/DS unsaturated disaccharides produced by the action of chondroitinase lyases. CS, chondroitin sulfate; DS, dermatane sulfate,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.

	GTSG	SHSG
ΔDi0S (ΔUA-GalNAc) (%)	3.51	5.525
ΔDi2S (ΔUA-GalNAc 2S) (%)	-	2.42
ΔDi6S (ΔUA-GalNAc 6S) (%)	18.24	14.551
ΔDi4S (ΔUA-GalNAc 4S) (%)	59.043	47.045
ΔDi2, 6S (ΔUA2S-GalNAc 6S) (%)	5.483	4.563
ΔDi4, 6S (ΔUA 4S-GalNAc 6S) (%)	5.669	5.455
ΔDi2, 4S (ΔUA2S-GalNAc 4S) (%)	7.034	20.066
ΔDi2, 4,6,Tris (ΔUA2S-GalNAc 4,6 diS) (%)	1.012	0.375
4S/6S	3.34	3.2
Charge density	1.17	1.26
Molecular mass (kDa)	23.8	41.72

Fig.1. Agarose-gel electrophoresis stained with toluidine blue and stain all of the GAGs purified from the skins of GTSG and SHSG.

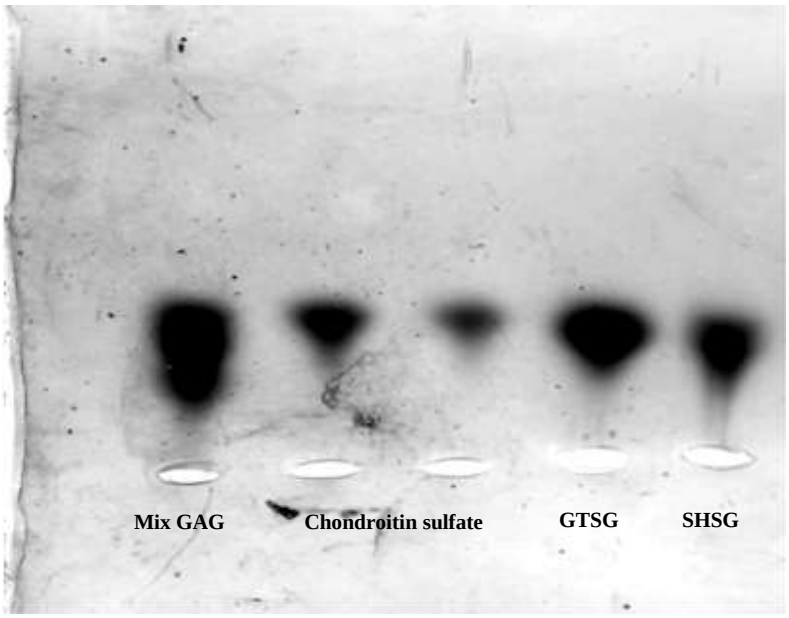


Fig.2. SAX-HPLC separation of the unsaturated disaccharides produced by chondroitin sulfate/ dermatan sulfate purified from various fish skins and treated with chondroitin ABC lyase. (A) GTSG and (B) SHSG. ΔDi0S (ΔUA-GalNAc), ΔDi6S (ΔUA-GalNAc 6S), ΔDi4S (ΔUA-GalNAc 4S), ΔDi2, 6diS (ΔUA2S-GalNAc 6S) ΔDi4, 6diS (ΔUA GalNAc4, 6diS), ΔDi2, 4diS (ΔUA2S-GalNAc4S). The identity of disaccharide species was assured by co-elution with purified standards (Seikagaku Co./Sigma–Aldrich).

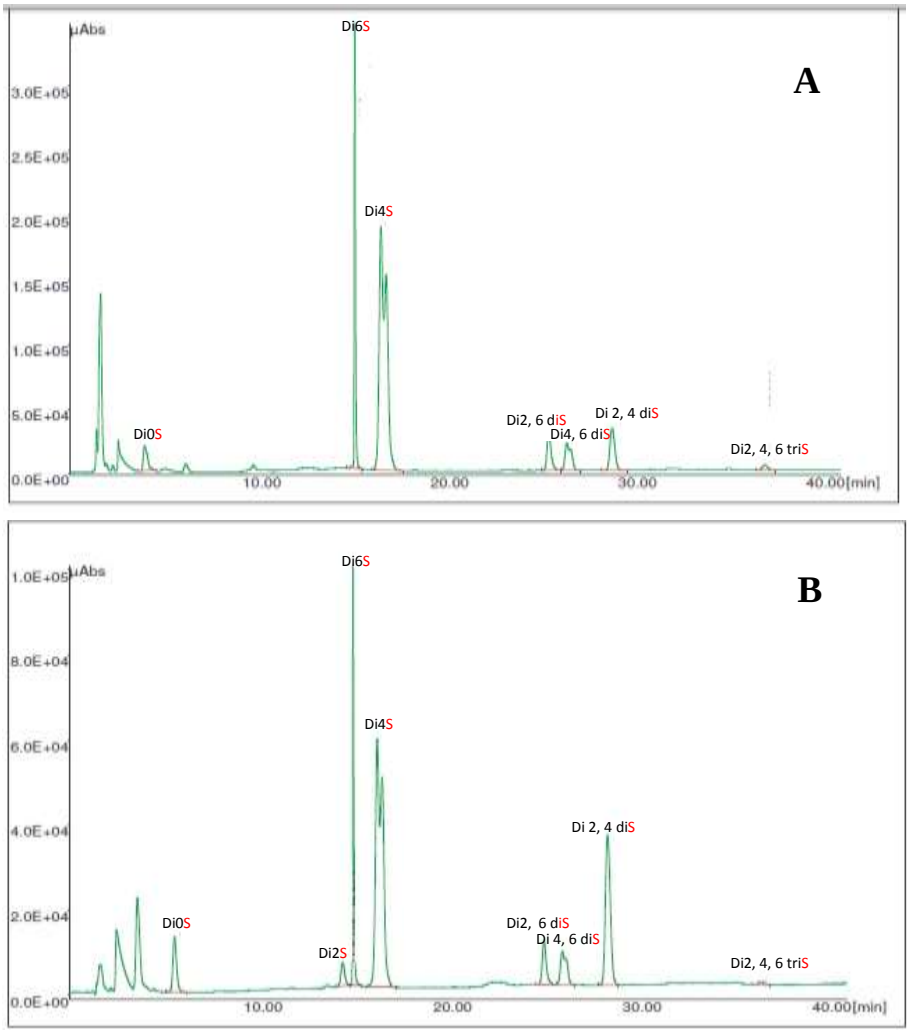


Fig.3. PAGE analysis of chondroitin sulfate/dermatan sulfate GAGs purified from the skins of GTSG and SHSG. The calibration curve was constructed by using chondroitin sulfate standards of known molecular mass prepared from chondroitin sulfate and having masses of 32.74 KDa, 14.5 KDa and 4.735 KDa.

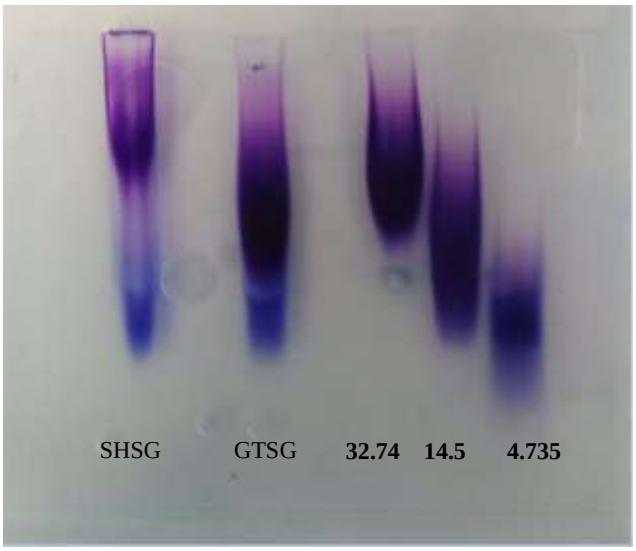


Fig.4. Electropherograms of analyses performed at 30 KV with detector LIF of CS/DS GAGs purified from fish skins. (A) GTSG and (B) SHSG.

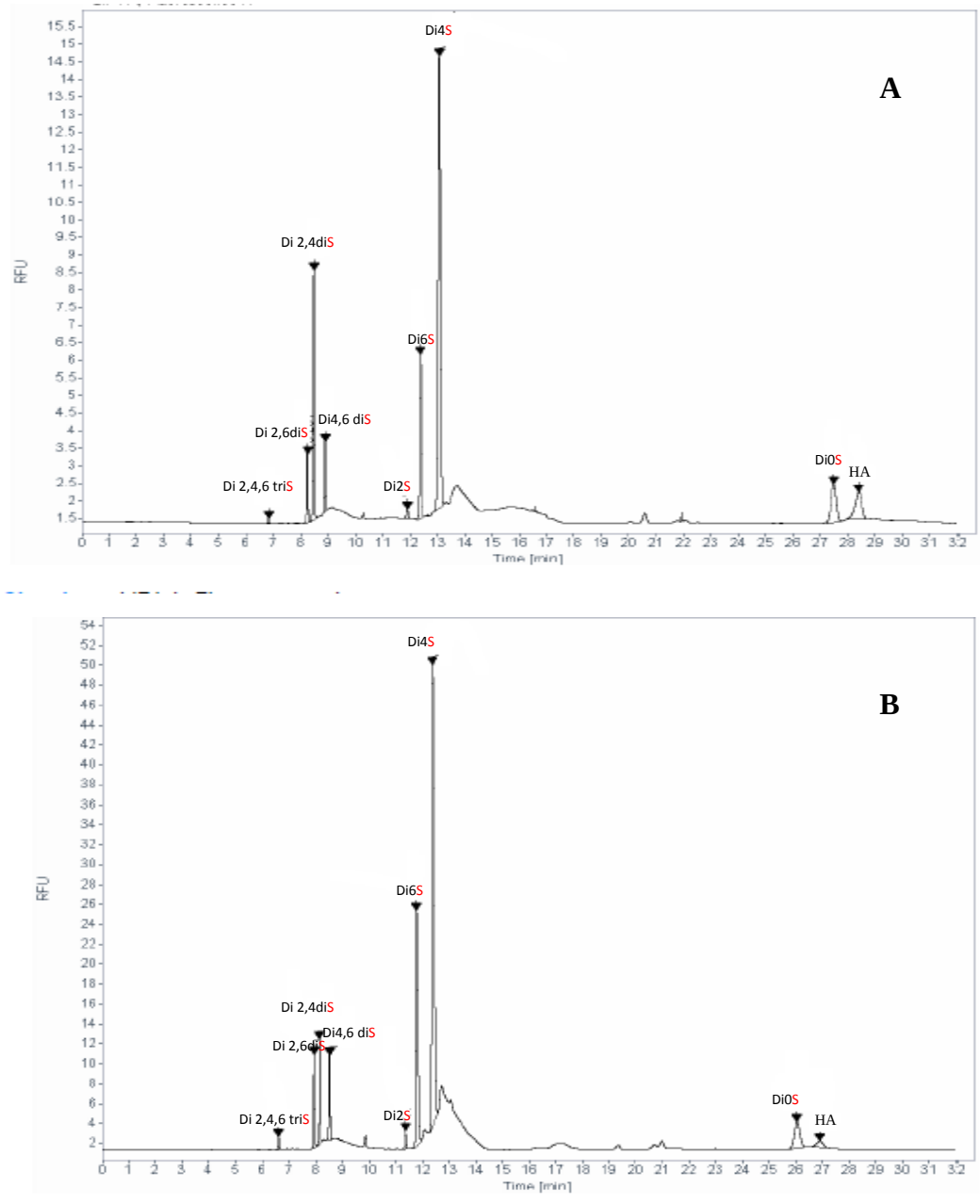


Fig.5. Inhibition of HCT116 human colon cell line and cytotoxicity against lymphocytes and erythrocytes by the purified CS/DS from fish skins (A) SHSG (B) GTSG.