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Characterisation of hyaluronic acid and chondroitin/dermatan sulfate from the lumpsucker fish, *C. lumpus*

Charalampos G. Panagos[a], Derek Thomson[b], Claire Moss[b], Charles D. Bavington[b], Halldór G. Ólafsson[c], Dušan Uhrín[a,*]

[a] EaStCHEM School of Chemistry, Joseph Black Building, The King's Buildings, Edinburgh EH9 3JJ, UK
[b] GlycoMar Ltd, European Centre for Marine Biotechnology, Oban, Scotland PA37 1QA, UK
[c] Biopol, Einbúastíg 2, 545 Skagaströnd, Iceland

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ABSTRACT

The lumpsucker, *Cyclopterus lumpus*, a cottoid teleost fish found in the cold waters of the North Atlantic, and North Pacific, was identified as a possible source of GAGs. The GAGs present in the *C. lumpus* dorsal hump and body wall tissue were isolated and purified. Two fractions were analysed by NMR and their GAG structures determined as hyaluronic acid and CS/DS chains. The latter fraction contained GlcA (65% of the total uronic acids) and IdoA (the remaining 35%). All uronic acid residues were unsulfated, whilst 86% of the GalNAc was 4-sulfated and 14% was 6-sulfated. The presence of GlcA-GalNAc4S, IdoA-GalNAc4S and GlcA-GalNAc6S disaccharide fragments was confirmed. The isolated GAGs obtained from each tissue were biochemically characterised. The lumpsucker offers a high yield source of GAGs, which compares favourably with other sources such as shark cartilage.

1. Introduction

Glycosaminoglycans (GAGs) are widely distributed within the animal kingdom (Cássaro & Dietrich, 1977; Mathews, 1975; Medeiros et al., 2000). They are a family of polyanionic linear polysaccharides, with their structure described in terms of unique repeating disaccharide sequences. GAGs are generally divided into 4 groups: (i) the non-sulfated hyaluronic acid (HA), (ii) keratan sulfate (KS), (iii) chondroitin and dermatan sulfate (CS/DS), and (iv) heparin and heparin sulfate (HS). GAGs were initially identified as components of various structural and connective tissues (Comper & Laurent, 1978; Mathews, 1975) such as the skin (DS), cartilage (CS) and cornea (KS). In addition to their structural role, the presence of GAGs, in particular heparan sulfate, on the cell surface as proteoglycans predetermines their participation in a variety of cell regulatory and signalling pathways. Each tissue produces a specific repertoire of GAGs and these have been shown to bind and regulate a number of proteins, including chemokines, cytokines, defensins, growth factors, enzymes, proteins of the complement system and adhesion molecules (Asimakopoulou, Theocharis, Tzanakakis, & Karamanos, 2008; Coombe, 2008; Imberty, Lortat-Jacob, & Pérez, 2007; Vreys & David, 2007; Xu & Dai, 2010).

Many GAGs have anticoagulant, and anti-inflammatory properties. A typical example is heparin (Capila & Linhardt, 2002; Castelli, Porro, & Tarsia, 2004; Gandhi & Mancera, 2010), the most prominent and best understood member of the GAG family because of its widespread clinical use as an anticoagulant (Sasisekharan & Shriver, 2009). Another GAG with demonstrated medicinal properties is chondroitin sulfate, which is extensively used as supplement for joint health (Lovu, Dumais, & du Souich, 2008), although its degree of efficacy and the mechanism whereby it provides anti-inflammatory properties and contributes to cartilage repair are still a matter of controversy. Hyaluronic acid is used in the treatment of skin conditions and for wound repair, (Voigt & Driver, 2012), and in cosmetic skin care products. Dermatan sulfate (chondroitin sulfate B), also has a range of biological properties, although it has not yet been developed for therapeutic purposes (Volpi, 2010).

Like all polysaccharides, GAGs are not genetically encoded, but instead their structure is dependent on the action of multiple synthetic enzymes. This makes recombinant synthesis of GAGs extremely difficult and industry is still largely dependent on natural sources for GAG production. Pharmaceutical heparin is

Abbreviations: GAG, glycosaminoglycan; DS, dermatan sulfate; CS, chondroitin sulfate; HA, hyaluronic acid; KS, keratan sulfate; HS, heparan sulfate; GlcA, glucuronic acid; IdoA, iduronic acid; GalNAc, N-acetylated galactosamine.

* Corresponding author. Tel.: +44 0131 650 4742; fax: +44 0131 650 7155.
E-mail addresses: cpanagos@staffmail.ed.ac.uk (C.G. Panagos), derek@glycomar.com (D. Thomson), claire@glycomar.com (C. Moss), charlie@glycomar.com (C.D. Bavington), halldor@biopol.is (H.G. Ólafsson), dusan.uhrin@ed.ac.uk (D. Uhrín).

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almost exclusively derived from the mucosal tissues of mammals, like bovine lung and porcine gastric mucosa (Linhardt & Gunay, 1999). Chondroitin sulfate is extracted from bovine and porcine trachea, or from shark cartilage, although semi-synthetic fermentation methods are now also being commercialised (Cimini, De Rosa, & Schiraldi, 2012). Hyaluronic acid was originally extracted from rooster combs and human umbilical cord (da Rosa et al., 2012), but became the first GAG to be produced in large scale by bacterial fermentation technology (Liu, Liu, Li, Du, & Chen, 2011), generating a reliable and reproducible supply.

Marine organisms are a very useful source of sulfated polysaccharides, as their connective tissues (tunic of ascidians, cartilage, skin and body wall) can be rich in CS/DS polymers. Different species show a range of sulfation patterns, which alter binding characteristics and modify biological activity (Kozlowski, Pavao, & Borsig, 2011; Mansour et al., 2010; Mizumoto, Fongmoon, & Sugahara, 2013; Nandini, Itoh, & Sugahara, 2005; Pavao et al., 1998), conferring distinctive properties to the polysaccharide. CS/DS polymers are a major structural component, together with collagen, of the skeleton of cartilaginous fish such as sharks (Nandini et al., 2005) and are also found in the skin, the tunic or the body walls of some marine invertebrates (Ben Mansour et al., 2009; Mourao et al., 1996; Pavao, 1996). Moreover, unusual sulfated polysaccharides, such as branched fucosylated chondroitin sulfate isolated from sea cucumbers (Vieira, Mulloy, & Mourão, 1991; Yoshida, Minami, Nemoto, Numata, & Yamanaka, 1992), are unique to marine organisms and show interesting biological properties (Beutler et al., 1993; Borsig et al., 2007; Chen et al., 2011; Herencia et al., 1998).

CS/DS polysaccharides purified from marine sources have been used in human studies to further our understanding of their functions and therapeutic potential. In addition to their anticoagulant activity, CS/DS chains isolated from fish cartilage have been shown to have significant anti-inflammatory activity, but weak anticoagulant effects (Krylov et al., 2011; Lovu et al., 2008). CS/DS chains with different sulfation patterns purified from ray fish cartilage promote neurite outgrowth through their binding to the hepatocyte growth factor (HGF) (Hashiguchi et al., 2011) and those extracted from sturgeon have been shown to enhance the proliferation of fibroblasts in wound healing (Im, Park, & Kim, 2010). Purification of these polysaccharides is an expanding area of research as indicated by several publications during the past three years (Arima et al., 2013; Gargiulo, Lanzetta, Parrilli, & De Castro, 2009; Kakizaki, Tatara, Majima, Kato, & Endo, 2011; Maccari, Ferrarini, & Volpi, 2010; Murado, Fraguas, Montemayor, Vazquez, & Gonzalez, 2010; Zhao et al., 2013).

The lumpsucker, *Cyclopterus lumpus*, due to its unique body structure and physiology, was identified as a possible source of novel GAGs, in particular those associated with connective tissue and skin. *C. lumpus* is a cottoid teleost fish found in cold waters of the North Atlantic and North Pacific. The roe of these fish is currently used extensively in Scandinavian cuisine as an alternative to caviar, although the rest of the fish is often discarded. Despite having features similar to other coastal, bottom dwelling teleosts, the lumpsucker has a low-density cartilaginous skeleton and its body is surrounded by a subcutaneous jelly-like layer, which forms a prominent dorsal hump, contributing to its neutral buoyancy. This allows the fish to live in mid-water, without a swim bladder or the expenditure of large amounts of energy. Its tissue appears to be modified cartilage/mucochondroid, although there are few clear accounts in the literature (Benjamin, 1988; Davenport & Kjorsvik, 1986). The classification of teleost cartilage in general is still unclear and there appear to be a gradation of tissues and biochemical features in a range of teleosts (Witten, Huysseune, & Hall, 2010). Histological staining of these tissues has revealed the presence of GAGs in other teleosts, although there is little data for lumpsuckers (Benjamin, 1988).

In the current study the GAGs present in the female *C. lumpus* dorsal hump and body wall tissue were isolated and purified. They were characterised using biochemical and biological cell based assays, and their structure was determined by NMR.

2. Materials and methods

2.1. Extraction and purification of GAGs

Six female *C. lumpus*, all approximately 50 cm in length, were collected off the coast of Iceland, and their internal organs and roe were removed. Body tissues were separated into the dorsal hump (skin and tissue), or body wall (the head was removed leaving predominantly skin, body wall and muscle blocks). They were cut into approximately 3–5 cm sections to allow more effective extraction. Extraction was carried out by a modification of previous methods (Griffin et al., 1995). In brief, an equal volume of water was added to the tissue (750 g of dorsal hump, 7.5 kg of body) and it was proteolytically digested overnight using alcalase (2.5LDX Novozyme) 1:100 at pH 8–9, 60 °C with overhead stirring. The resultant liquor was filtered and mixed with 1/10 volume anion exchange resin (Lewatit VPOC1074/S6328 A 1:1 Lanxess) overnight. The resin was washed in water and bound material was eluted with 1 M or 5 M sodium chloride, and precipitated with 0.6 or 2 volumes (vol) of methanol. The precipitates were air-dried, resuspended, dialysed against water using 8 kDa MWCO tubing (Biodesign) and freeze-dried.

The 4 separated fractions were analysed by HPLC–size-exclusion chromatography (SEC) using a Waters Alliance 2695 system with refractive index (RI) and photodiode array (PDA) detection, and a Shodex SB806M column calibrated with dextran standards (Fluka 12, 25, 50, 80, 270, 670, 1400 kDa). They were also assayed by the carbazole reaction (Cesaretti, Luppi, Maccari, & Volpi, 2003) and by a modified sulfate assay (Terho & Hartiala, 1971). Based on this analysis the 5 M 2 vol fraction was further separated on a Sepacore preparative chromatography system (Buchi) using Q-sepharose (QS) anion exchange resin (GE). Gradient elution was carried out using 50 mM sodium chloride 50 mM Tris–HCl pH 7.5/2 M sodium chloride, 50 mM Tris–HCl pH 7.5, monitoring A214 nm, A280 nm and conductivity. Three peaks were collected for both dorsal hump and body wall preparations, dialysed (as above) and freeze-dried, before further biochemical and biological analysis.

2.2. Monosaccharide analysis by methanolysis, TMS derivatisation and GC–FID

Monosaccharide composition of the QS fractions was determined using a Shimadzu Gas chromatography 2014 system with flame ionisation detection (GC–FID) and ZB5-ms column. The samples (10 μl of a 10 mg/ml solution in water) were subjected to methanolysis by addition of 0.5 M methanolic-HCl (Supelco) at 85 °C for 4 h, followed by re-N-acetylation of free amines by addition of acetic anhydride. Samples were dried, washed in methanol and re-dried, before addition of tri-methylsilane (TMS) reagent (Supelco) and subsequent GC–FID analysis. Mixed monosaccharide standards (arabinose, xylose, rhamnose, fucose, mannose, glucose, galactose, glucuronic acid, galacturonic acid, N-acetyl-galactosamine, N-acetyl-glucosamine (all Sigma)) were run together with scyllo-inositol (Sigma) as an internal control, which was also added to all samples to enable calculation of a standard ratio.

2.3. Disaccharide analysis by enzymatic depolymerisation

Disaccharide analysis was carried out on the fractions by digestion of 1 mg of sample (100 μl of a 10 mg/ml solution in water) using either chondroitinase ABC lyase, chondroitinase B or heparinase II (Grampian Enzymes) under recommended buffer conditions. The resulting digest was analysed by HPLC Ion exchange chromatography (IEC) using Waters Alliance 2695 system with a ProPac PA1 column (4 mm × 250 mm Dionex), running a water (pH 3.5)/2 M sodium chloride (pH 3.5) gradient elution and PDA detection at 232 nm (Turnbull, 2001). Chondroitin (Di-0S, Di-4S, Di-6S, UA2S, Di-Se, Di-Sd, Di-Sb, TriS) or heparin (IVA, IVS, IIA, IIIA, IIS, IIIS, IA, IS) disaccharide standards (Dextra Labs) were used, and chondroitin sulfate (Sigma), dermatan sulfate (Celsus Laboratories) or heparin (Sigma) were run as sample controls. Samples were also analysed by HPLC–SEC using Shodex SB806M column as described above, to determine the extent of the digestion and the molecular weight profile of the generated products.

2.4. NMR spectroscopy

Ethylenediaminetetraacetic acid (EDTA) and trimethylsilyl propionate (TSP) were purchased from Goss Scientific Instruments Ltd and Aldrich, respectively. The samples (20 mg) were dissolved in 99.9% D_2O (Aldrich, 540 μl) containing deuterated $NaH_2PO_4 + Na_2HPO_4$ buffer (10 mM, pH 7.2). A stock solution (10 μl) of EDTA and TSP was added. The stock solution was prepared by dissolving EDTA (4 mg) and TSP (9 mg) in the phosphate buffer (200 μl). The pH was adjusted to 7.2 by adding few drops of a concentrated solution of NaOH in D_2O. All spectra were acquired at 50 °C on an 800 MHz Avance I Bruker NMR spectrometer equipped with a z-gradient triple-resonance TCI cryoprobe. The spectra were referenced (0 ppm) using the ^1H and ^{13}C signals of TSP.

1D ^{13}C NMR spectra were acquired using relaxation and acquisition times of 2 and 0.34 s, respectively; 24,576 scans per spectrum were accumulated. The FIDs were zero filled once and a 2 Hz exponential line broadening was applied prior to Fourier transformation. 2D ^1H, ^{13}C HSQC spectra were acquired using t_1 and t_2 acquisition times of 15 and 106 ms, respectively; 32 scans were acquired into each of 512 F_1 complex data points resulting in the total experimental times of 12 h per sample. The standard 2D HSQC-TOCSY BRUKER pulse sequence was modified by appending a ^1H spin-echo of overall duration of $1/^1JCH$ (optimised for a $^1J_{CH}$ = 150 Hz) after the TOCSY spin-lock and two 2D ^1H,^{13}C HSQC-TOCSY spectra were acquired in an interleaved manner. The first one with and the second one without a 180° ^{13}C pulse applied simultaneously with the 180° ^1H pulse of the final spin-echo. This resulted in a difference in sign of one-bond cross peaks between the two spectra. Addition of the two 2D matrices prior to processing yielded a 2D HSQC-TOCSY spectrum with substantially reduced one-bond crosses peaks. Such treatment has facilitated identification of weak TOCSY cross peaks. The subtraction of matrices yielded a regular 2D ^1H, ^{13}C HSQC spectrum. Each 2D HSQC-TOCSY spectrum was acquired using t_1 and t_2 acquisition times of 30 and 106 ms, respectively; 1024 complex data points using 40 scans were collected in F_1 in each experiment; the total duration of the experiment was 70 h. A DIPSI-2 mixing sequence was applied for 20 ms. The relative content of the GlcA and IdoA in the CS/DS fractions was determined by the integration of 1D and 2D spectra in the MNOVA (Mestrelab Research). The integration was done using ^{13}C signals of C1 and C4 of respective residues and further verified by integrating the H2/C2 cross peaks in the ^1H, ^{13}C HSQC spectrum.

2.5. Biological activity

The effect of the fractions on cell viability was measured using a BHK cell line (hamster kidney fibroblast, ECACC 85011433) cultured in GMEM (Sigma) with tryptose (Sigma), glutamine (PAA) and FCS (Sigma). Triplicate wells of cells and samples (0.1 mg/ml) were incubated overnight at 37 °C, in a 96 well plate, and metabolic activity measured using Cell Titre Glow luminescent reagent (Promega) and a Synergy II plate reader (Biotek). Fucoidan (Marinova) and doxorubicin (Sigma) were run as assay controls, and % cell viability was calculated based on comparison to an untreated control.

The effect of fractions on neutrophil elastase activity was measured by incubation with freshly isolated human neutrophils. The samples and a fucoidan control (Marinova) (0.1 mg/ml) were mixed with freshly isolated neutrophils, 5 μg/ml cytochalasin B (Sigma) and 10 ng/ml TNFα (Merck), and incubated for 30 min at 37 °C. 100 ng/ml fMLP (Sigma) was then added and cells further incubated for 45 min. Cells were removed by centrifugation and the supernatants mixed with elastase substrate (Merck) in triplicate wells of a 96-well microplate. A kinetic read was carried out on a Powerwave HT plate reader (Biotek) with measurements taken at 405 nm every 5 min for 1 h, and VMax calculated for each sample. % elastase activity was calculated by comparison to an untreated control.

The effects of fractions on blood coagulation were measured using an activated Partial Thromboplastin Time (APTT) assay using an ACL9000 automatic coagulometer (Instrumentation Laboratories). This assay was used to measure effects of saccharides on both the intrinsic (involving Factors XII, XI, IX, VIII) and common (Factors X, V, II, fibrinogen) blood coagulation pathways. The ACL9000 was calibrated daily using a normal control, and abnormal control plasma (Instrumentation Laboratories). A standard curve was prepared using 5th International heparin standard (NIBSC) at 0.5–5 units/ml in water, in 0.5 unit increments. 30 μl of test saccharides at 1 mg/ml in water, a water blank, a heparin control (H3393 Sigma) at 0.01 mg/ml in water, and heparin standards, were pipetted into reaction vessels. 270 μl of human plasma (TCS reagents), which had been previously thawed and filtered at room temperature, was added to each reaction vessel. The reaction vessels were placed in the carousel of the ACL9000. The APTT programme was selected, with APTT-SP reagent (colloidal silica dispersion with phospholipids to stimulate contact activation, Factor XII production, Factor X and prothrombin activation) and calcium, being added automatically to the samples. The time to clotting (from the addition of calcium to thrombus formation, measured by optical density) in seconds was displayed. Any samples out of the standard curve range were further diluted in water and rerun. A standard curve was generated by plotting time to clot against heparin standard concentration (IU), and the estimated APTT value (IU/mg) for the test saccharides was calculated.

3. Results

3.1. Purification of GAGs from C. lumpus tissues, their monosaccharide composition and sulfate analysis

In both preparations the majority of the extract was recovered in the 1 M 2 vol (~3% yield from wet starting mass) or 5 M 2 vol fractions (~20% yield from body tissue, ~40% yield from dorsal hump). Analysis of these crude fractions indicated that the 5 M 2 vol fraction contained sulfated material. Consequently the 5 M 2 vol fractions were further fractionated by preparative ion exchange chromatography, which yielded three further separate fractions, **I–III**. The corresponding peaks occurred at the same elution times for both dorsal hump (**D**) and body wall (**W**) preparations, but there

Table 1
Biochemical characteristics of QS fractions from *C. lumpus* extracts.

Sample	Description	% Overall yield from starting mass	% Sulfate (by MW)	Uronic acid content (mg/ml)	MW by Shodex SB806M (RI/kDa)	Peak area%
Dorsal hump						
DI	QS 36–41 min	4	<3.8	0.63	>1400, 22.8, 4.4	90.2, 9, 0.8
DII	QS 42–49 min	1.4	<3.8	0.4	>1400, 22.2, 3.3	57.7, 40.9, 1.4
DIII	QS 50–58 min	24	16	0.78	151.8	100
Body wall and muscle						
WI	QS 37–41 min	1	<3.8	0.45	>1400, 158.3, 21	30.9, 22.6, 46.6
WII	QS 42–49 min	2.5	<3.8	0.5	17.2	100
WIII	QS 50–58 min	14.5	16	0.72	120	100

were differences in recovery, probably due to contamination of the body wall preparation by nucleic acid from the muscle tissue.

The three fractions obtained for each tissue were biochemically characterised (purity, sulfate, uronic acid and monosaccharide). The results indicated that the dorsal hump and body wall were similar in composition, with a non-sulfated high molecular weight polymer dominating the earlier eluting fractions, and a sulfated 120–150 kDa polymer present in the late eluting peak (Table 1).

Monosaccharide analysis indicated that the fractions varied in their heterogeneity (Table 2). Fractions **DI** and **WI**, contain mainly N-acetylated glucosamine (GlcNAc) and glucuronic acid (GlcA). This data, combined with undetectable sulfate content (<3.8% Table 1), suggest that fraction **I** contains mainly hyaluronic acid, which is particularly enriched in the dorsal hump preparation (**DI**).

Fraction **II** of both extracts showed a much more mixed composition, with GlcNAc, GlcA and N-acetylated galactosamine (GalNAc) all present in large amounts, probably derived from the earlier and later eluting peaks (Table 2). Also, evidence from HPLC-PDA indicated absorbance at A260 (not shown), in particular in the body wall sample, suggesting this was likely to be associated with nucleic acid, and A280 (not shown) from QS also indicated some non-sugar material in this peak.

Fractions **DIII** and **WIII**, which represented very discrete late eluting peaks by QS, contained mainly GalNAc and iduronic acid (IdoA) (Table 2). These data, together with a sulfate value of ~16% (by MW), suggests that fraction **III** contains mainly CS/DS chains.

It is important to note that most monosaccharide analysis methods can result in underestimation of the uronic acid content, due either to the resistance of these molecules to hydrolysis, or their potential destruction once liberated. Resistance is particularly the case when the adjacent units are N-sulfated or N-acetylated hexosamines, making GAGs some of the most difficult molecules to analyse. The described conditions were selected to give the best balance between preservation of side groups and liberated monosaccharides, as well as representative hydrolysis of the native polysaccharide. However, the reported ratio of hexosamine sugar to uronic acid is still not 1:1, as would be expected in a GAG, most likely due to poor recovery. Consequently, the results of the monosaccharide analysis were only used qualitatively and their major purpose was to aid in the selection of 'GAG-like' fractions which were then subjected to NMR analysis.

The most highly charged fractions **DIII** and **WIII** were also analysed for their disaccharide composition. Whilst both these samples were resistant to digestion using heparinase II, digestion of both

Table 2
Monosaccharide analysis by methanolysis TMS derivatisation and GC–FID of the polysaccharides isolated from *C. lumpus*.

Monosaccharide%	**DI**	**DII**	**DIII**	**WI**	**WII**	**WIII**
Arabinose	0	0	0	0	0	0
Rhamnose	0	2.1	0	0	25.3	0
Fucose	0	0	0	0	0	0
Xylose	0	2.6	0.9	1.4	3.9	1
Iduronic acid	0	5.6	40.3	0	0	30.5
Galacturonic acid	4.2	0	0	3.3	0	0
Mannose	4	2	0	10.5	0	0
Galactose	4	4.5	2.4	10.7	7	2.2
Glucose	0	0	0	1.3	0	0
Glucuronic acid	28.0	22.5	6.4	22.4	13.8	6.6
N-acetyl Galactosamine	0	28.6	47.6	0	34.8	59.7
N-acetyl Glucosamine	59.8	32.1	2.4	50.4	15.2	0

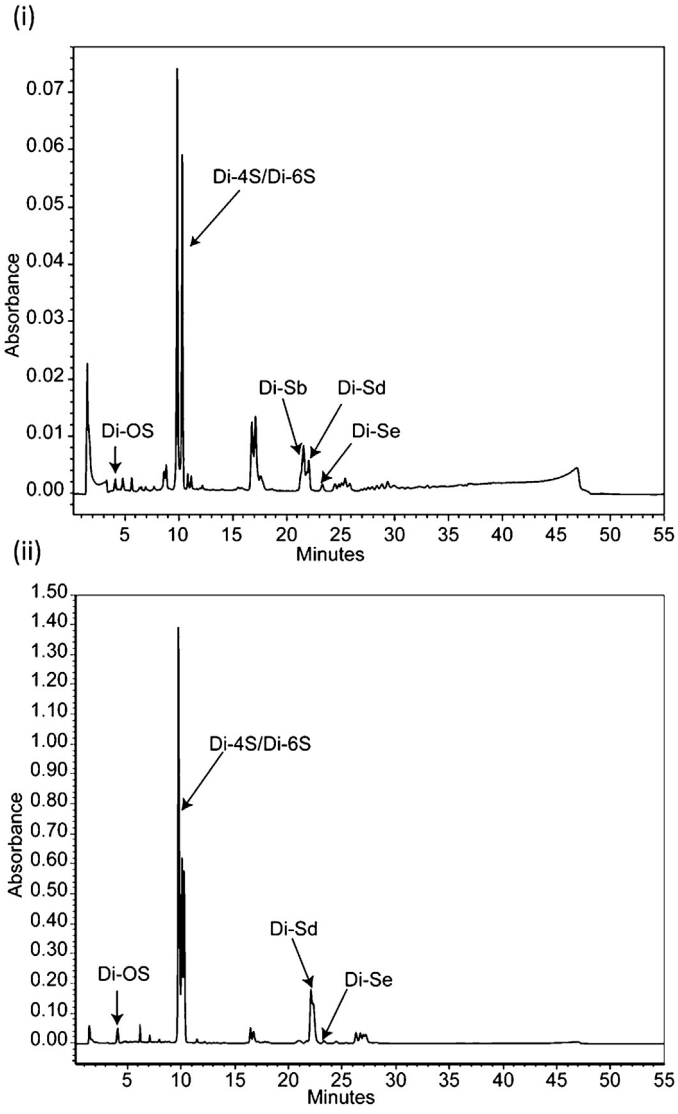

Fig. 1. Disaccharide analysis of (i) fraction **DIII** and (ii) chondroitin sulfate (Sigma) after digestion with chondroitinase ABC lyase. Chondroitin-like disaccharide peaks corresponding to standards are observed in both chromatograms: (Di-OS = unsulfated; Di-4S = ΔUA → GalNAc4S; Di-6S = ΔUA → GalNAc6S; Di-Sb = ΔUA-2S → GalNAc4S; Di-Sd = ΔUA-2S → GalNAc6S; Di-Se = ΔUA → GalNAc4S,6S).

samples was observed with chondroitinase ABC lyase. Whilst the peak signals were weak, indicating only partial hydrolysis, they were clearly present when compared to the chondroitin sulfate control (Fig. 1) and standards.

The major peaks at ~9.8–10 min in both chromatograms represent the GalNAc4S (CS-A) and GalNAc6S (CS-C) containing disaccharides. The peaks at 17 min do not correlate with any of the CS disaccharide standards and are likely to be tri- or tetrasaccharides, indicating incomplete digestion. Minor peaks indicating CS-B, CS-D, CS-E and unsulfated disaccharides are also present (Fig. 1).

DIII and **WIII** were also successfully digested by chondroitinase B (data not shown). The HPLC–SEC analysis of the chondroitinase B digests indicated that the overall molecular weight of the sample was reduced and only much smaller polymer and oligosaccharides remained, suggesting that the polymer was comprised of both CS and DS containing elements, rather than a mixture of two separate polysaccharides.

Based on the monosaccharide and disaccharide data, fractions **DI**, **DIII** and **WIII** were selected for NMR analysis to further elucidate their structures.

3.2. NMR analysis of C. lumpus GAGs

Based on the GC analysis, sample **DI** was believed to be a hyaluronic acid. Indeed, both the ^{13}C and the ^{1}H, ^{13}C HSQC spectrum (Fig. 2) show a clean homogenous sample, characteristic of a non-sulfated GAG. A 2D ^{1}H, ^{13}C HSQC-TOCSY spectrum with a mixing time of 20 ms showed correlations between all vicinal protons of the sugar rings, as would be expected due to their axial-axial arrangement. The derived complete assignment of ^{1}H and ^{13}C resonances (Table 3) is in good agreement with the proton and carbon chemical shifts of hyaluronan oligosaccharides (Toffanin et al., 1993).

The NMR spectra of the CS/DS fractions (**DIII** and **WIII**) of the *C. lumpus* were essentially identical and therefore only the latter sample was analysed in depth. This was not as straightforward as the analysis of the hyaluronic acid. Differences in the sulfation and epimerisation of GlcA to IdoA created heterogeneity that is evident from the ^{13}C and 2D ^{1}H, ^{13}C HSQC spectrum of **WIII** (Fig. 3).

The resonance assignment achieved by a combined analysis of 2D ^{1}H, ^{13}C HSQC and 2D ^{1}H, ^{13}C HSQC-TOCSY spectra (Table 4) identified GlcA-GalNAc4S and IdoA-GalNAc4S but also GlcA-GalNAc6S disaccharide fragments. The integration of the ^{13}C spectrum found GlcA to comprise 65% of the total uronic acids, whereas IdoA accounted for the remaining 35%. All uronic acid residues were unsulfated. GalNAc4S accounted for the 86% and GalNAc6S for the remaining 14% of the total GalNAc content.

The presence of both GlcA-GalNAc4S and IdoA-GalNAc4S disaccharides resulted in the appearance of two sets of signals attributed to GalNAc4S, since the different uronic acids created slightly different chemical environment for the GalNAc nuclei. The peaks assigned to the IdoA-GalNAc4S disaccharide comprised 34% of the total GalNAc, a figure in close agreement with the analysis of the ^{13}C spectrum, which further verifies the assignment. Only one set of peaks for GalNAc6S was found belonging to the GlcA-GalNAc6S disaccharide, either because the signals of the IdoA-GalNAc6S disaccharides were too weak to be detected or because there is no such disaccharide in the sample. The assignment of resonances agreed well with the existing literature data (Bossennec, Petitou, & Perly, 1990; Huckerby et al., 2001; Pavao et al., 1998; Sanderson, Huckerby, & Nieduszynski, 1989).

3.3. Anti-inflammatory and anticoagulant properties

The polysaccharides isolated from *C. lumpus* and identified as GAGs by NMR were also tested for their effects in cell-based assays and on blood haemostasis (Table 5).

The fractions containing predominantly hyaluronic acid (**DI** and **WI**) did not have any effect on BHK cell viability, illustrating no or minimal cytotoxic effects. They showed no or minimal effects on neutrophil elastase, and did not possess any significant anticoagulant activity (based on the APTT assay) (Table 5). This suggested that the HA isolated did not have strong anti-inflammatory properties, which have previously been shown to be polymer length dependent and highly variable. This profile of responses is supportive that HA is the dominant polysaccharide present in these fractions.

Fractions **DII** and **WII** were mixed in composition. They had no effect on BHK cell viability or on blood coagulation, but did appear to inhibit the elastase enzyme (Table 5). Whilst it cannot be confirmed, this is most likely due to the nucleic acid contamination present in the sample (see Section 2.1), which is know to inhibit the elastase enzyme.

Fractions **DIII** and **WIII** containing predominantly CS and DS chains, showed some effects on elastase enzyme (15–20% inhibition), and very minor effects on blood coagulation (2–3 IU/mg). This profile is in keeping with the comparatively low level of sulfate of these polysaccharides which was 16% by MW, and determined as a monosulfated disaccharide unit by NMR. CS and DS compounds with higher levels of sulfation have been shown to have much greater inhibitory effects on elastase enzyme activity and act as weak anti-coagulants.

4. Discussion

The NMR analysis of extracts obtained from the *C. lumpus* shows that both the body wall and the dorsal hump of the fish contain hyaluronic acid and chondroitin sulfate (65%)/dermatan sulfate (35%) chains. The main types of chondroitin sulfate were identified as CS-A (GlcA-GalNAc4S) and CS-C (GlcA-GalNAc6S), with CS-A being the majority. Dermatan sulfate (CS-B) was present as IdoA-GalNAc4S. NMR was unable to determine whether fractions **DIII** and **WIII** contained a mixture of CS and DS polysaccharides or whether they represented hybrid CS/DS chains. However, digestion of the two fractions by chrondroitinase B, which is specific for dermatan chains, resulted in an overall reduction in the molecular weight of the polymer, with only lower molecular weight polymer and oligosaccharides remaining. This suggested that the polysaccharide contains hybrid CS and DS chains, as reported in the literature for other CS/DS molecules (Bielik & Zaia, 2010; Radhakrishnamurthy, Jeansonne, & Berenson, 1986; Zamfir et al., 2012), rather than a mixture of separate CS and DS polysaccharides.

Disaccharide analysis of the GAG-containing fractions revealed several very minor components (CS-B, CS-D, CS-E and unsulfated disaccharide) in **WIII**, similar to the commercial CS (Fig. 1). However, the enzymatic digestion method was not quantitative in this study. The subsequent NMR analysis indicated that numerous very week signals were present in the NMR spectrum. Their intensity was however below the level where positive NMR identification could be attempted in the presence of the dominant CS-A and CS-C signals.

There seem to be no differences in the GAG content of the extracts from the dorsal lump and the rest of the body although there is some variation in comparative yields. The dorsal hump contains a higher percentage of HA and CS/DS, which was consequently easier to purify. This is to be expected due to the much less complex nature of the dorsal hump compared to the body wall/muscle

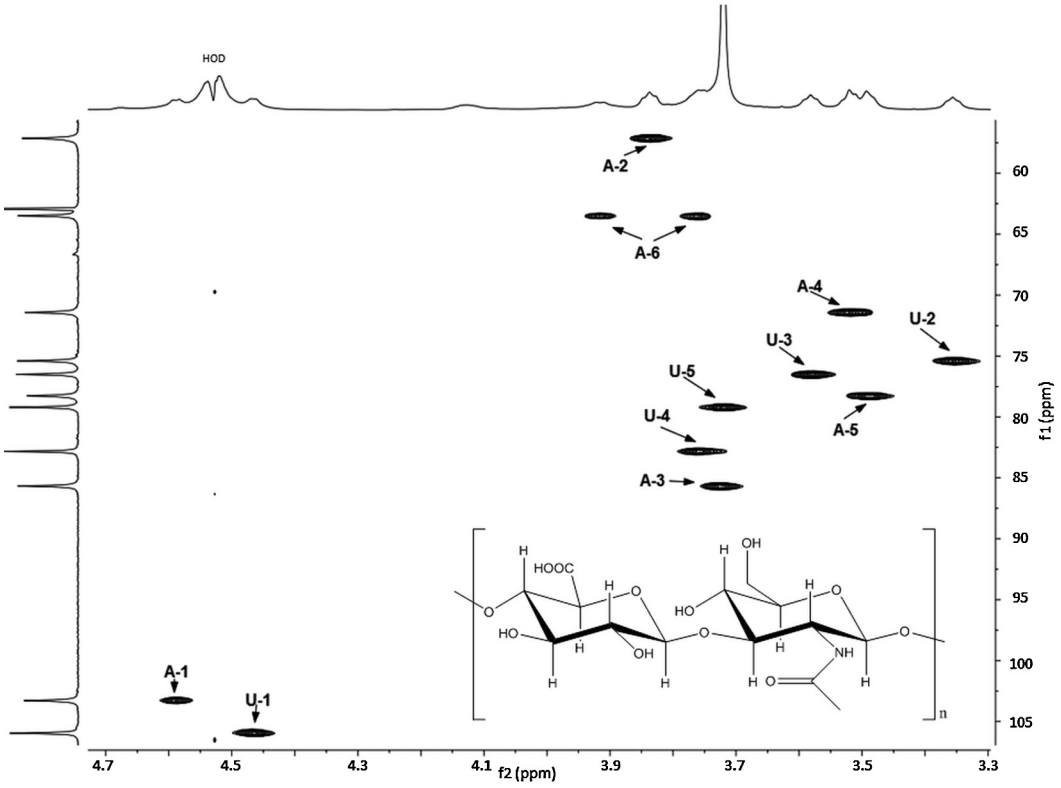

Fig. 2. 2D ^{1}H ^{13}C HSQC spectrum of fraction **DI**. The 1D ^{1}H and ^{13}C spectra are shown on the sides. The inset shows the structure of HA.

Table 3
The ^{1}H and ^{13}C chemical shifts (ppm) of **DI** fraction and hyaluronic acid oligosaccharides.[a,b]

	GlcA		GlcNAc	
	DI	Lit[a]	DI	Lit[a]
H1/C1	4.47/105.88	4.48/103.80	4.59/103.21	4.74/101.40
H2/C2	3.35/75.37	3.34/73.29	3.84/57.09	3.81/55.06
H3/C3	3.58/76.56	3.51/74.47	3.72/85.62	3.73/83.86
H4/C4	3.76/82.84	3.51/80.83	3.52/71.43	3.54/69.36
H5/C5	3.72/79.22	3.73/77.21	3.49/78.25	3.48/76.23
H6/H6	–/177.68	–/175.01	3.76–3.92/63.50	3.76–3.90/61.37

[a] Taken from Toffanin et al. (1993).
[b] The average pairwise difference between the current and literature ^{13}C chemical shifts of 2.06 ± 0.2 ppm indicates different referencing used.

Table 4
The ^{1}H and ^{13}C chemical shifts (ppm) of **WIII** fraction and the corresponding literature data.

	GlcA-GalNAc 4S		GlcA-GalNAc 6S		IdoA-GalNAc 4S	
	GlcA	GalNAc 4S	GlcA	GalNAc 6S	IdoA	GalNAc 4S
H1/C1	4.47/106.5	4.59/103.7	4.49/106.99	4.57/103.59	4.90/105.95	4.68/104.91
H2/C2	3.39/75.15	4.03/54.40	3.36/75.41	4.02/53.83	3.54/72.18	4.05/54.84
H3/C3	3.58/76.47	4.02/78.43	3.62/76.62	3.85/82.90	3.92/74.01	4.02/78.2
H4/C4	3.79/83.32	4.75/79.37	3.74/83.93	ND	4.11/83.05	4.66/78.94
H5/C5	3.69/79.57	3.83/77.49	3.69/79.57	3.96/75.54	4.72/72.25	3.83/77.49
H6/C6	–/177.13	3.80/63.81	–/177.18	4.23/70.40	–/176.68	3.80/63.81

Literature data

	GlcA-GalNAc 4S[a]		GlcA-GalNAc 6S[a]		IdoA-GalNAc 4S[b]	
	GlcA	GalNAc 4S	GlcA	GalNAc 6S	IdoA	GalNAc 4S
H1/C1	4.63/105.94	4.67/103.59	4.55/105.56	4.66/104.28	4.90/105.7	4.72/105.3
H2/C2	3.47/75.86	4.08/54.94	3.45/75.85	4.03/53.93	3.53/72.0	4.02/54.2
H3/C3	3.64/76.78	4.16/78.62	3.63/77.08	3.95/82.68	3.95/73.7	4.02/77.8
H4/C4	3.80/83.07	4.63/79.12	3.76/84.26	4.19/70.17	4.10/82.7	4.65/78.5
H5/C5	3.78/79.09	3.87/77.69	3.76/78.83	4.01/75.70	4.72/72.0	3.78/77.0
H6/C6	–	3.80/63.98	–	4.24/70.36	–	3.78/63.5

[a] Huckerby et al. (2001).
[b] Pavao et al. (1998).

Table 5
Biological activity of QS fractions from *C. lumpus* extracts.

Sample	Description	% Neutrophil elastase activity	% BHK cell viability	Estimated APTT (IU/mg)
Dorsal hump				
DI	QS 36–41 min	114.8	103	0.9
DII	QS 42–49 min	59.2	101	0.7
DIII	QS 50–58 min	81	102.7	2.6
Body wall and muscle				
WI	QS 37–41 min	86.6	102.9	1.1
WII	QS 42–49 min	44.8	90.8	1.1
WIII	QS 50–58 min	84.3	103.5	2.7
Control	Fucoidan	11.6	92.5	–

preparation. However, yield of the CS/DS chains relative to HA was still high from the body wall preparation.

The types of CS/DS chains present in *C. lumpus*, and the molecular weight of the polymer, are in keeping with other studies on fish glycosaminoglycans (Arima et al., 2013). CS-A and CS-C appear to be the more common forms, although there is a wider variation in the degree of epimerisation to DS chains. Different tissues and species have varying compositions, which do not appear to conform to any trends in terms of tissue or species classification. In the case of the lumpsucker, the MW of the CS/DS isolated from the body wall appeared to be slightly smaller than that present in the dorsal hump, but there were no other differences between tissues.

The presence of glycosaminoglycans in the dorsal hump and body wall tissues support the theory that these tissues contribute to buoyancy in the female lumpsucker. The high percentage composition of lumpsucker tissue of hyaluronic acid and CS/DS are consistent with other types of cartilaginous tissues (Carney & Muir, 1988), which can be described as viscoelastic materials consisting of a strong collagen fibrillar network enmeshed with proteoglycan macromolecules, a fluid phase, which is water, and an ionic phase of counter ions (Lu & Mow, 2008). Although it should be noted that the presence of collagen in lumpsucker tissue was not evaluated in this study, it has been previously identified by others (Benjamin, 1988). The sulfate and carboxylate groups present in cartilage give rise to a high fixed charge density which results in a high Donnan osmotic potential. We propose that the gelatinous tissues of the lumpsucker may have an unusually high GAG content in order to increase the water content required to achieve low density; the dorsal hump tissue was reported to be less dense than sea water (Davenport & Kjorsvik, 1986), contributing to the fish's low overall body density. It has also been suggested that this layer provides some rigidity, to help compensate for the reduced internal skeleton and watery muscle blocks of the female (Davenport & Kjorsvik, 1986).

The CS/DS polysaccharides showed minor anti-inflammatory activity in the selected elastase assay, and no anticoagulant activity. This is in keeping with other literature and could be attributed to their low sulfation. We acknowledge that the APPT dose-response relationship for the fish GAG is likely to be different to standard heparin, but the estimated results are a useful semi-quantitative result, which supports the biological and chemical characterisation

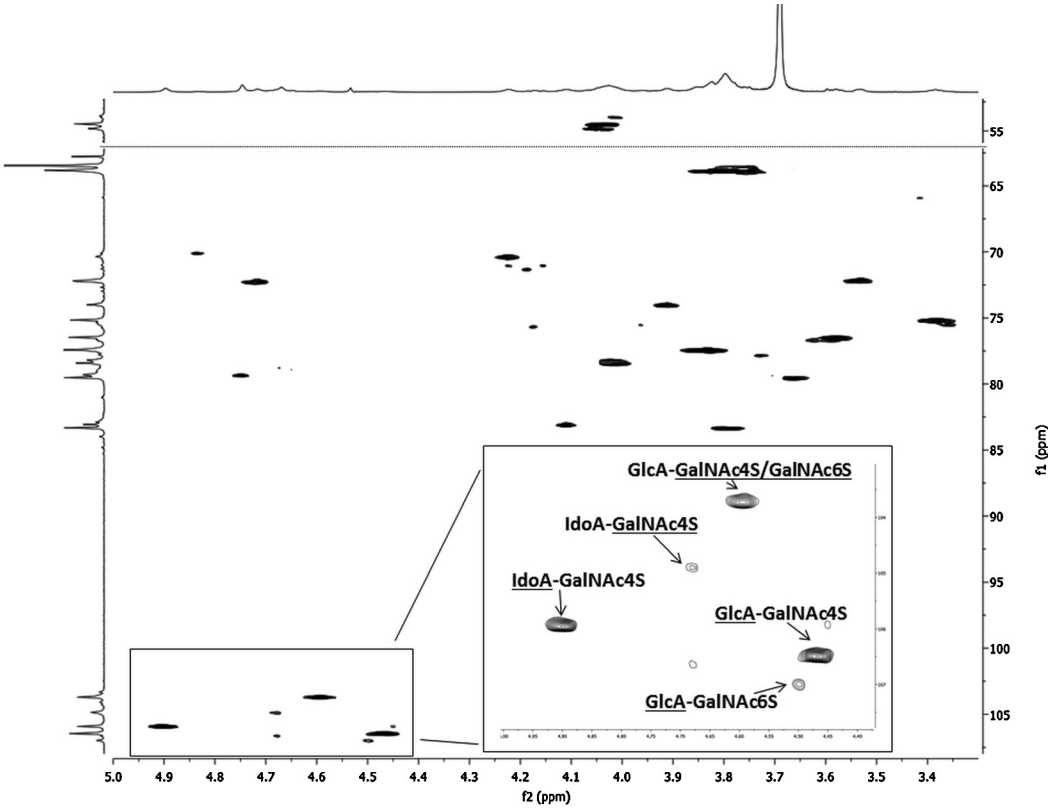

Fig. 3. 2D ^{1}H, ^{13}C HSQC spectrum of the CS/DS fraction (**WIII**) of *C. lumpus*. The 1D ^{1}H and ^{13}C spectra are shown on the sides. The insert shows an expansion of the anomeric region.

of these GAGs. However, they are likely to show effects in other biological assays, as indicated by the numerous reports on the properties of these polysaccharides (Krylov et al., 2011; Lovu et al., 2008). Chondroitin sulfate is used widely as a dietary supplement for osteoarthritis and joint pain. The lumpsucker could provide another alternative source of CS due to the yields present in the selected starting material, and the availability of tissue as a by-catch from the Icelandic lumpsucker fish roe industry, which could produce 4000 tonnes per annum of raw material.

5. Conclusions

This study has demonstrated that, although not structurally or functionally novel, the lumpsucker offers a high yield source of GAG, which compares favourably with sources such as shark cartilage (1000–2000 mg per 100 g dry defatted tissue (Arima et al., 2013)). Moreover the availability of shark cartilage is declining due to over fishing and subsequent protection by international treaties. The potential commercial exploitation of the lumpsucker by-catch as new source of HA and CS is the subject of further evaluation.

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