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ORIGINAL ARTICLE

Glycosaminoglycans from fish swim bladder: isolation, structural characterization and bioactive potential

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Abstract The swim bladder of fish is an internal gas-filled organ that allows fish to control their buoyancy and swimming depth. Fish maws (the dried swim bladders of fish) have been used over many centuries as traditional medicines, tonics and a luxurious gourmet food in China and Southeast Asia. Little is known about the structural information of polysaccharides comprising this important functional material of fish tissue. In the present study, the total glycosaminoglycan (GAG) from fish maw was characterized. Two GAGs were identified, chondroitin sulfate (CS, having a molecular weight of 18–40 kDa) and heparan sulfate (HS), corresponding to 95% and 5% of the total GAG, respectively. Chondroitinase digestion showed that the major CS GAG was composed of ΔUA-1 → 3-GalNAc4S (59.7%), ΔUA-1 → 3-GalNAc4,6S (36.5%), ΔUA-1 → 3-GalNAc6S (2.2%) and ΔUA-1 → 3-GalNAc (1.6%) disaccharide units. ^{1}H–NMR analysis and degradation with specific chondroitinases, both CS-type A/C and CS-type B were present in a ratio of 1.4:1. Analysis using surface plasmon resonance showed that fibroblast growth factor (FGF)-2 bound to the CS fraction (K_D = 136 nM). These results suggest that this CS may be involved in FGF-signal pathway, mediating tissue repair, regeneration and wound healing. The CS, as the major GAG in fish maw, may have potential pharmacological activity in accelerating wound healing.

Keywords Glycosaminoglycans · Fish maw · Compositional analysis · Chondroitin sulfate · Heparan sulfate · Disaccharides · FGF-2

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Abbreviations

Ac	acetyl
AMAC	2-aminoacridone
CS	chondroitin sulfate
ΔUA	4-deoxy-β-L-*threo*-hex-4-enopyranosiduronic acid
DMMB	1,9-dimethylmethylene blue
ESI	electrospray ionization;
FGF	fibroblast growth factor
GAG	glycosaminoglycan
GalN	galactosamine
GlcA	glucuronic acid
GlcN	glucosamine
GPC	gel permeation chromatography
HPLC	high performance liquid chromatography
HS	heparan sulfate
IdoA	iduronic acid
KS	keratan sulfate
MS	mass spectrometry
MWCO	molecular weight cut-off

NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
RU	resonance units
S	sulfo
SEC	size exclusion chromatography
SPR	surface plasmon resonance
USP	United States Pharmacopeia

Introduction

The swim bladder is an internal, gas-filled organ that fish use to control their buoyancy and swimming depth (Fig. 1a). Fish maw (Fig. 1b) is the commercial term for dried swim bladders of large fish, such as croaker and sturgeon. The scale of fish maw production in Southeast Asia is quite large, particularly in Hong Kong and Southern China [1–3]. Fish maw is traditional Asian food delicacies and has been used as medicines and tonics in China and Southeast Asia for many centuries. Some traditional medical properties ascribed to fish maw include improving amnesia, insomnia, dizziness, anepithymia and weakness [4]. Researchers have also suggested that fish maw can decrease free radicals and ward off inflammation and cancer [5]. Recently, several reports have indicated that crude polysaccharide obtained from fish maw could rapidly heal cuts and prevent infection as well as thrombotic events [6–8]. However, little is currently known about the structure of polysaccharides from fish maw.

Glycosaminoglycans (GAGs) are linear, negatively charged polysaccharides that are found ubiquitously in all animals. Based on their disaccharide composition, GAGs can be generally classified into four families that include heparan sulfate (HS), chondroitin sulfate (CS), hyaluronan (HA), and keratan sulfate (KS). HS is comprised of a repeating unit of *O*-sulfated →4) β-D-glucuronic acid (or α-L-iduronic acid) (1→4) α-D-*N*-acetylglucosamine (or *N*-sulfoglucosamine) (1→. CS is comprised of a repeating unit of *O*-sulfated →4) β-D-glucuronic acid (or α-L-iduronic acid) (1→3) β-D-*N*-acetylgalacatosamine (1→. HA is comprised of a repeating unit of →4) β-D-glucuronic acid (1→3) β-D-*N*-acetylglucosamine (1→ and contains no sulfo groups. KS has a primary backbone structure containing repeating disaccharide units of galactosyl (Gal) β1→4-*N*-acetylglucosaminyl (GlcNAc) β1→3 with one or both saccharide units containing 6-*O*-sulfo (S) groups. Minor amounts of fucose, sialic acid, and *N*-acetylgalactosamine are also often present in KS. GAGs play important roles in diverse physiological and pathophysiological processes including: blood coagulation, cell growth and differentiation, host defense and viral infection, lipid transport and metabolism, cell-to-cell and cell-to-matrix signaling, inflammation, angiogenesis and cancer through interacting with various proteins [9–15]. GAGs have numerous applications in the pharmaceutical, nutraceutical, and cosmetic industries [16–18]. For example, the highly sulfated HS, heparin, is widely used clinically anticoagulant [16], CS has nutraceutical and pharmaceutical applications in arthritis, herpes virus infection, malaria, nervous tissue repair, and liver regeneration [17] and HA is used in the treatment of eye and cartilage-based diseases [18]. In general, GAGs are produced from terrestrial vertebrates but recently marine vertebrates and invertebrates have become more important sources [19]. The commercial fish-processing industry generates large quantities of solid waste and represents an excellent resource for GAG production [19].

In the present study, the total GAG component was isolated from fish maw. The structure of the recovered was then determined by polyacrylamide gel electrophoresis (PAGE), disaccharide analysis and nuclear magnetic resonance (NMR) spectroscopy. The potential bioactivity was tested by protein binding analysis using surface plasmon resonance (SPR). These studies provide information of composition and structure of GAGs derived from the fish swim bladder and should improve our understanding of the biochemistry and function of these bio-macromolecules within this tissue.

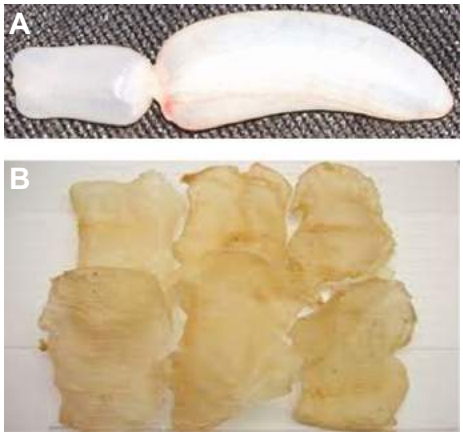

Fig. 1 Picture of fish swim bladder and commercial fish maw

Materials and methods

Materials

Commercial dried fish maw, "hudiejiao", was purchased from a local Asian supermarket. This fish maw was produced from the swim bladder of large, wild, deep-sea grouper. Reagents including 2-aminoacridone (AMAC), sodium cyanoborohydride ($NaCNBH_4$), urea and 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS), and acetic acid, were purchased from Sigma Aldrich (St. Louis, MO). Methanol (HPLC grade), acetonitrile (HPLC grade), ammonium acetate (HPLC grade), water (HPLC grade) and dimethyl

sulfoxide (DMSO) were from Fisher Scientific (Springfield, NJ). Actinase E was from Kaken Biochemicals (Japan). Heparin lyase I, II, III, chondroitin lyase ABC and chondroitin lyase AC were expressed in our laboratory in recombinant *Escherichia coli* strains, prepared as previously described [20, 21]. Chondroitinase B was purchased from Sigma Aldrich (St. Louis, MO). Unsaturated HS and CS disaccharide standards were purchased from Iduron Co (Manchester, UK). Q-Sepharose Fast Flow was from GE Healthcare Life Sciences (Marlborough, MA).

Methods

Extraction and separation of GAGs from fish maw

The dried fish maw (2.0 g) was soaked in distilled water until it became soft and swollen and then, cut into small pieces (<5 mm) with scissors. The tissue was suspended in 100 mL distilled water and proteolyzed at 55 °C with 10 mg/mL actinase E for 48 h and after centrifugation at 4000×g for 30 min, the supernatant was recovered and freeze-dried to obtain the crude fish maw GAGs. Crude GAGs were fractionated by a strong ion exchange chromatography using a Q-Sepharose Fast Flow column (1 × 10 cm), eluted with a step-wise gradient of NaCl (Fig. 2). The uronic acid contents of the fractions were determined by carbazole assay [22]. The total GAGs contained in the 1.4 M fraction Fig. 2, (40–60 mL) were collected, concentrated and precipitated at 4 °C overnight (in an explosion-proof refrigerator) by adding three volumes of methanol. The precipitated purified GAGs were recovered by centrifugation (4000×*g*) and dried under vacuum. Approximately 8.4 mg of purified GAGs were obtained from 2.0 g of dry fish maw.

Disaccharide analysis of GAGs in fish maw

A portion of the total GAG sample (20 μg) was dissolved in 200 L of 100 mM ammonium acetate containing 10 mM $CaCl_2$ (pH 7.4) and totally depolymerized by adding heparin lyase I, II, III (10 mU/each enzyme) and chondroitin lyase ABC (10 mU) at 37 °C for 24 h. Then, the products filtered using 3 K MWCO spin column and washed twice with distilled water. The filtrate, containing the HS and CS disaccharide products, was freeze-dried. The dried samples were AMAC-labeled according our previous method [23]. In brief, the dried sample was incubated with 10 μL of 0.1 M AMAC in DMSO/acetic acid (17/3,*v*/v) at room temperature for 10 min, followed by adding 10 μL of 1 M $NaBH_3CN$ for 1 h at 45 °C. A mixture containing all 17 disaccharide standards was similarly AMAC-labeled and used as an external standard.

LC was performed on an Agilent 1200 LC system at 45 °C using an Agilent Poroshell 120 ECC18 (2.7 μm, 3.0 × 50 mm) column. Eluent A was 50 mM NH_4OAc, and the eluent B was methanol. The mobile phase passed through the column at a flow rate of 300 μL/min. The gradient of eluent B increased from 5% to 30% in the first 20 min, and rose to 50% eluent B in the following 10 min, then to 100% eluent B in 1 min, and a 9 min flow of 100% eluent B was applied to elute all compounds. The column effluent entered the source of the electrospray ionization (ESI) mass spectrometry (MS) for continuous detection by MS. The electrospray interface was set in negative ionization mode with a skimmer potential of −40.0 V, a capillary exit of − 40.0 V, and a source of temperature of 325 °C, to obtain the maximum abundance of the ions in a full scan spectrum (150–1500 Da, 10 full scans/s). Nitrogen was used as a drying (5 L/min) and nebulizing gas (20 psi).

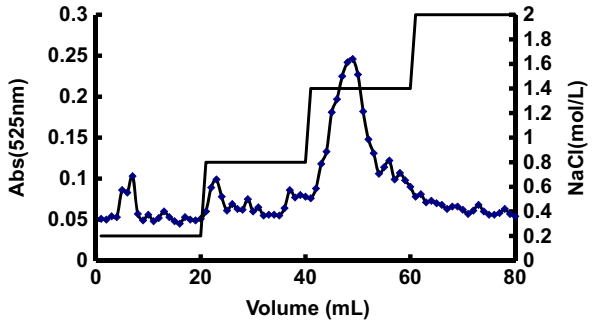

Fig. 2 Purification of GAGs from fish maw by using a Q Sepharose Fast Flow column

Recovery of CS from the total GAGs

A portion of the purified total GAG sample (5 mg) was completely digested (repeated digestion failed to give additional product) with heparin lyase I, II, III (40 mU/each enzyme) at 37 °C overnight to remove the trace HS. The CS component was recovered by centrifugal filtration using a 3-kDa molecular weight cut-off (MWCO) spin column.

Molecular weight determination of CS fraction

The molecular weight of CS fraction isolated from fish maw was determined by gel permeation chromatography (GPC) high performance liquid chromatography (HPLC) using United States Pharmacopeia (USP) against a heparin standard. A TSK SWXL 4000 column (7.8 mm × 30 cm) and a TSK SWXL 3000 column (7.8 mm × 30 cm) were in series with a mobile phase composed of 0.1 M ammonium acetate. The temperature was set at 30 °C. The flow rate was 0.6 mL/min. Refractive index detection was used in detection. Data were analyzed using size exclusion chromatography (SEC) specialist (Shimadzu LC solution GPC software, Japan).

Polyacrylamide gel electrophoresis (PAGE) analysis

The CS fraction isolated from total GAGs digested separately with chondroitinase AC and chondroitinase B and analyzed by native PAGE using 0.75 mm × 6.8 cm × 8.6 cm mini gels cast from 15% T resolving gel monomer solution and 5% T stacking gel monomer solution. In this experiment, CS-A and CS-B (dermatan sulfate) standards were also digested separately with chondroitinase AC and chondroitinase B. The mini-gels were subjected to electrophoresis at a constant 200 V for 30 min and stained with 0.5% (*w*/*v*) alcian blue.

NMR analysis

After removing the trace HS from the total GAG isolated from fish maw with heparin lyase I, II, III, the CS was analyzed by ^{1}H–NMR spectroscopy to characterize its structure. The sample was dissolved in 0.5 mL D_2O (99.996%, Sigma, Co.) and freeze-dried repeatedly to remove the exchangeable protons. The sample was then re-dissolved in 0.4 ml $^{2}H_2O$. The ^{1}H–NMR was performed on 800 MHz Bruker 800 spectrometer with Topsin 2.0 software.

Biotinylation of CS fraction and immobilization on a SPR chip

A purified CS fraction (2 mg), amine-polyethylene glycol (PEG)3-biotin (2 mg), and $NaCNBH_3$ (10 mg) were dissolved in 180 μL of H_2O, and 20 μL of acetic acid was added. The reaction mixture was heated at 70 °C for 24 h. After 24 h, an additional 10 mg of $NaCNBH_3$ was added to the reaction mixture and the mixture heated under the same conditions for an additional 24 h. After cooling to room temperature, the reaction mixture was desalted using a 3 kDa spin column, and the biotinylated CS was collected and lyophilized.

Biotinylated CS was immobilized on the carboxymethylated dextran streptavidin sensor chip. The sensor chip was conditioned with 1 M NaCl in 50 mM NaOH at a flow rate of 10 μL/min, and biotinylated CS (10 μL) were then injected onto the flow channel. The control flow channel was prepared with a saturated solution of biotin. Successful immobilization was confirmed by observing a resonance unit (RU) of ≥100.

Binding assay for assessing interactions between fibroblast growth factor (FGF)-2 and CS using SPR.

Various concentrations of FGF-2 (63, 125, 250, 500, and 1000 nM) were prepared in HBS-EP buffer [0.01 M HEPES, 0.15 M NaCl, 3 mM EDTA, and 0.005% surfactant P20 (pH 7.4)] and used in these studies for the CS sensor chip. FGF-2 was injected over the surface of appropriate sensor chips at a flow rate of 30 μL/min. After sample injection, the surface of the sensor chip was dissociated by being washed with 90 μL of HBS-EP buffer, followed by washing with 30 μL of 2 M NaCl for regeneration. RU was monitored as a function of time (sensorgram) at 25 °C.

Results and discussion

Isolation and quantification of total GAGs from fish maw

The commercial dried fish maw was very tough, thus, it was soaked in distilled water to become soft and then cut into small pieces before enzymatic hydrolysis. After protease digestion, the crude GAG extract was purified using a Q-Sepharose Fast Flow column eluted with stepwise NaCl gradient, according to previous studies. Most protein was removed with low concentration of NaCl. The major GAG peak eluted at 1.4 M NaCl and was detected using carbazole assay for uronic acid (Bitter and Muir, 1962) and was collected (Fig. 2, 40–60 mL). It is difficult to separate different GAGs (i.e. HS, CS), using only anion exchange chromatography. Since these are polydisperse mixtures containing variable levels of sulfation. The total purified GAG content of the dried sample was 0.4 wt.% (purified GAG dry wt./dry wt. fish maw).

Disaccharide analysis of GAGs in fish maw

The compositional analysis of GAG disaccharides gives important structural information and is an efficient method for measuring the variation of GAG structures coming from HA, HS and CS GAGs. GAGs are enzymatically digested with heparin lyase I, II, III and chondroitin lyase ABC (chondroitinase ABC digests all types of CS including dermatan sulfate as well as HA) to obtain HS, CS and HA disaccharides. These disaccharides were AMAC-labeled by reductive amination and analyzed by reversed-phase HPLC-MS/MS. The total GAGs were mainly composed of CS (94.9%) and a small amount of HS (5.1%) containing ΔUA (1→4) GlcNAc (HS0S) and ΔUA (1→4) GlcNS (HSNS), where ΔUA is 4-deoxy-β-L-*threo*-hex-4-enopyranosiduronic acid, GlcN is glucosamine, Ac is acetyl, and S, is sulfo (Fig. 3).

The total GAG purified content of fish maw could be separated into the heparin/HS family and CS/HA families by selective treatment with polysaccharide lyases (Linhardt, 1994). The total GAGs were then treated with a mixture of heparin lyases, which converted the heparin/HS into disaccharide and tetrasaccharide products that could be removed from the CS/HA GAGs using a 3 kDa MWCO spin column. The disaccharides composition was also analyzed. The results show that on treatment of the major component in fish maw, CS, with chondroitinase ABC, it was mainly composed of CS-A/CS-B (ΔUA (1→3) GalNAc4S, 59.7%) and CSE (ΔUA (1→3) GalNAc4S6S, 36.5%), where GalN is

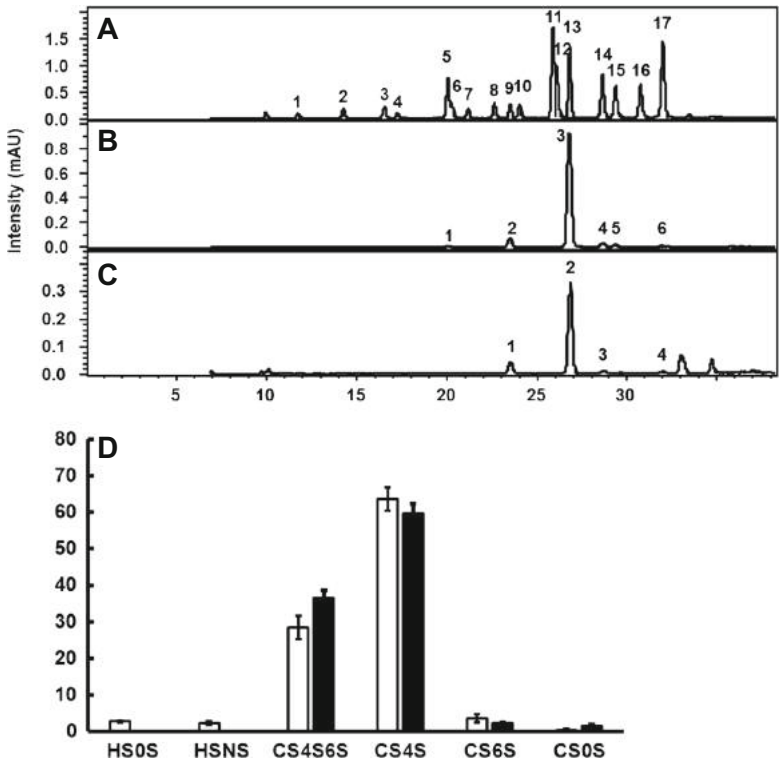

Fig. 3 Disaccharide analysis. **a** 17 disaccharides standards (1. ΔUA2S (1→4) GlcNS6S, TriSHS; 2. ΔUA (1→4) GlcNS6S, NS6SHS; 3. ΔUA2S (1→4) GlcNS, NS2SHS; 4. (ΔUA2S (1→3) GalNAc4S6S, Tri SCS; 5. ΔUA (1→4) GlcNS, NSHS; 6. (ΔUA2S (1→3) GalNAc4S SBCS, 7. ΔUA2S (1→4) GlcNAc6S, 2S6SHS; 8. (ΔUA2S (1→3) GalNAc6S, SDCS; 9. ΔUA (1→4) GlcNAc6S, 6SHS; 10. (ΔUA (1→3) GalNAc4S6S, SECS; 11. ΔUA2S (1→4) GlcNAc, 2SHS; 12. (ΔUA2S (1→3) GalNAc4S, 2SCS; 13. (ΔUA (1→3) GalNAc4S, 4SCS; 14. ΔUA (1→4) GlcNAc, 0SHS; 15. (ΔUA (1→3) GalNAc6S, 6SCS; 16. (ΔUA (1→3) GlcNAc, 0SHA; and 17. (ΔUA (1→3) GalNAc, 0SCS.); **b** disaccharide analysis of total GAGs from fish maw; **c** disaccharide analysis of CS fraction from fish maw; **d** disaccharide composition of total GAGs and CS fraction

galactosamine (Fig. 3). CSE as a highly sulfated CS, is known to serve a similar function as heparin in invertebrate animals. However, CS-A (→4) GlcA (1→3) GalNAc4S (1→) and CS-B (dermatan sulfate) (→4) IdoA (1→3) GalNAc4S (1→) are not differentiated by disaccharide analysis and required ^{1}H–NMR analysis and application of selective chondroitinases.

Molecular weight determination of CS fraction

The purified CS fraction showed two partially overlapped peaks in the size-exclusion chromatogram having a molecular weight range from 18 to 40 kDa, as determined using the USP calibration method with heparin as the molecular weight standard (Fig. 4).

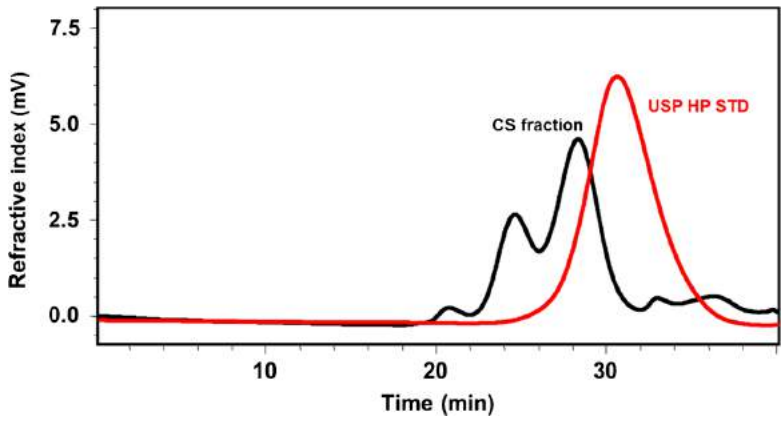

Fig. 4 HPLC-GPC chromatogram of CS fraction form fish maw

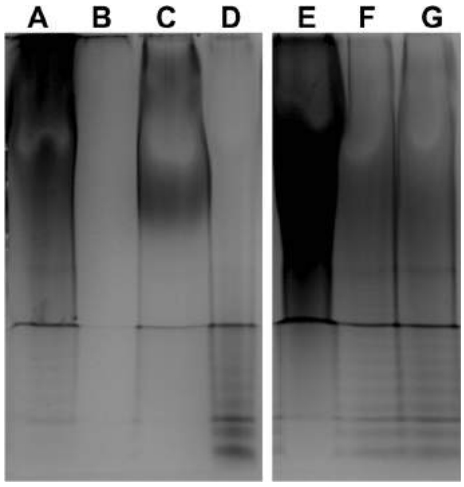

Fig. 5 PAGE analysis of fish maw CS. Lane A. chondroitin sulfate A standard; Lane B. chondroitin sulfate A standard totally treated with chondroitinase AC; Lane C. CS fraction from fish maw; Lane D. CS fraction from fish maw totally treated with chondroitinase AC; Lane E. chondroitin sulfate B standard; Lane F. chondroitin sulfate B standard totally treated with chondroitinase B; Lane G. CS fraction from fish maw totally treated with chondroitinase B

PAGE analysis

Chondroitinase AC specifically depolymerizes CS-A and CS-C, but not CS-B (dermatan sulfate) producing mainly disaccharides. Chondroitinase B only depolymerizes CS-B, usually producing oligo-saccharides (CS-B often contains resistant type-A and -C domains) and 4-sulfated disaccharides. Commercial CS-A standard and CS-B standards were used as positive controls. No bands in lane 2 (CS disaccharides contain insufficient negative-charge to stain with alcian blue) indicating that CS-A standard was exhaustively depolymerized by chondroitinase AC (Fig. 5). However, there were still obvious bands present in lane 4 indicating that in addition to CS-A or –C, the purified CS fraction from fish maw is another type CS (Fig. 5). On treatment with chondroitinase B, the CS-B standard and purified CS fraction from fish maw showed a similar distribution of bands in lanes F and G. This suggests that the fish maw was also CS susceptible to chondroitinase B (Fig. 5). Thus, the results of PAGE analysis clearly demonstrate the presence of CS-A/C and CS-B in fish maw.

^{1}H–NMR spectra

The ^{1}H–NMR spectrum of the purified CS fraction from fish maw was used to determine the ratio of CS-A, CS-E and CS-B (Fig. 6). In the spectrum shown in Fig. 6, the peaks at 4.98, 4.44, 3.34 and 3.56 ppm represent the 1H–IdoA, 1H- GlcA, 2H–IdoA and 2H–GlcA. These assignments are based on previous literature [24]. Integration of the peak area for 1H–IdoA and 1H–GlcA affords a ratio of CS-B to CS-A of 1:1.4.

Binding of FGF-2 to CS fraction from fish maw

Growing evidence suggests CS plays an important role in the wound healing process [17, 25, 26]. A number of recent studies have used CS as a biomaterial in cartilage repair, tissue engineering and growth factor release [17]. Recent evidence also suggests that CS has crucial biological functions in wound repair [26]. There is limited information on the structural features of CS that impact its wound healing activities.

Based on previous studies, one important pharmacological value of fish maw is to improve wound healing and in tissue regeneration [6, 7]. Furthermore, this activity has been ascribed to its polysaccharide component [7]. In present study, we isolated CS from fish maw and found it to be the major GAG in fish maw. Moreover, fish maw CS was primarily comprised of CS-A (→ 4) GlcA (1 → 3) GalNAc4S(1→), CS-B (→4) IdoA (1 → 3) GalNAc4S (1→), and CS-E (→4) GlcA (1 → 3) GalNAc4S, 6S (1→).

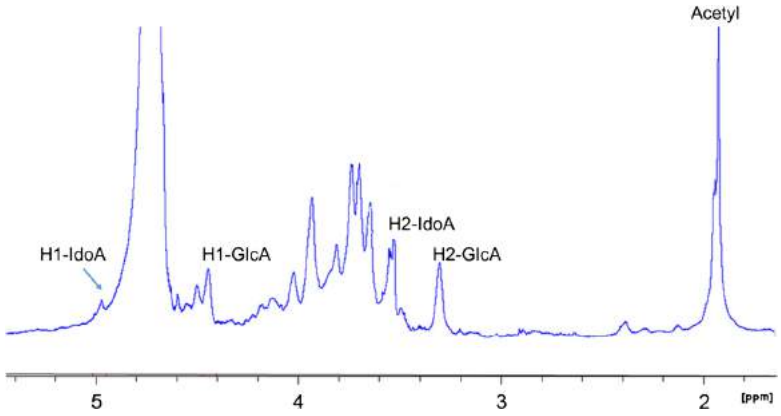

Fig. 6 ^{1}H–NMR of purified CS fraction from fish maw

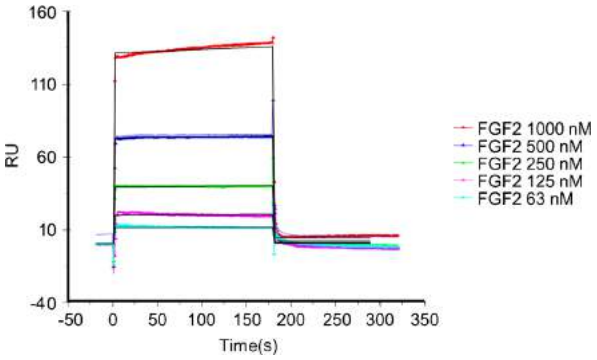

Fig. 7 Sensorgrams of interactions between FGF-2 protein and purified CS from fish maw. The concentrations of FGF-2 were 63, 125, 250, 500, and 1000 nM. The black curves are the fitting curves using models from BIA evaluation version 4.0.1

Fibroblast growth factor 2 (FGF2) is a mesenchyme-derived growth factor that displays mitogenic, migratory, and morphogenic functions and is also known to play role in angiogenesis, organ development, organ regeneration, and wound healing [15]. With respect to wound healing, FGF2 has been studied as a potential therapeutic anti-scarring agent [27]. CS-B, CS-E, and CS-A have been found to promote FGF2 signaling through FGF receptor 3C at 74%, 34% and 6% of the level observed for heparin-based signaling [13].

We next preliminarily investigated the interactions between CS fraction from fish maw and FGF-2 using SPR. On this basis, we began by examining interaction of CS with FGF-2 by a real time SPR binding assay to determine binding kinetics. FGF-2 (63–1000 nM) was injected over the surface of a CS immobilized sensor chip. Sensorgrams were fit globally to generate kinetic constants under a standard Langmuir kinetic model (Fig. 7), and observed kinetic K_D were 136 nM. The sensorgrams obtained showed the RU increased in a concentration-dependent manner. These results may shed a clue that the role of fish maw in improving wound healing suggesting that its CS content promotes FGF-2 mediated cell growth.

In summary, the total GAGs from fish maw was isolated and characterized. The GAG composition analysis showed that chondroitin sulfate was the major (95%) and HS was the minor (5%) component. CS-type A and CS-type B were present in a ratio of 1.4:1. SPR analysis showed FGF-2 bound to the CS fraction with high affinity (K_D = 136 nM), suggesting that the CS could be involved in FGF-signal pathway, mediating tissue repair, regeneration and wound healing. This information should improve the knowledge of the biochemistry and function of these bio-macromolecules in fish swim bladder.

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Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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