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Short Paper

Isolation and Structural Characterization of Glycosaminoglycans from Heads of Red Salmon *(Oncorhynchus nerka)*

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Abstract

Glycosaminoglycans (GAGs) are linear, highly negatively charged polysaccharides. They are ubiquitous molecules exhibiting a wide range of biological functions with numerous applications in pharmaceutical, cosmetic, and nutraceutical industrials. The commercial fish-processing industry generates large quantities of solid waste, which can represent a potential resource for GAG production. In this study, we used a three-step recovery and purification scheme for isolation of GAGs from the heads of red salmon (*Oncorhynchus nerka*). The GAGs recovery yield was 6 to 7mg from 1 gram of salmon head powder. The recovered GAGs were structurally analyzed with polyacrylamide gel electrophoresis and by disaccharide composition analysis with reversed-phase ion-pair high-performance liquid chromatography. The analyses showed the major composition of the GAGs in red salmon head were chondroitin sulfate C and E.

Keywords: Glycosaminoglycans; Chondroitin Sulfate; Heparan Sulfate; Salmon; *Oncorhynchus nerka*

Abbreviations

GAG: Glycosaminoglycan; PAGE: Polyacrylamide Gel Electrophoresis; RPIP-HPLC: Reversed-Phase Ion-Pair High-Performance Liquid Chromatography; CHAPS: 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate

Introduction

Glycosaminoglycans (GAGs) are linear, negatively charged polysaccharides composed of a variable number of repeating disaccharide units. Based on the structure of their repeating disaccharides, GAGs can be classified into four families, heparan sulfate, chondroitin sulfate, hyaluronan, and keratan sulfate. GAGs mediate diverse patho-physiological 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, such as growth factors, enzymes, cytokines [1]. Because of intriguing biological activities of GAGs, they have numerous applications in the pharmaceutical, nutraceutical, and cosmetic industries [2-5]. For example, chondroitin sulfate has nutraceutical and pharmaceutical applications in arthritis, herpes virus infection, malaria, nervous tissue repair, and liver regeneration [2]. Traditionally, GAGs are commercially produced from animal tissues as a by-product of the meat and livestock industry, coming from rooster combs, bovine and swine tracheas/nasal tissues, bovine and porcine intestines and lungs [3], and bovine corneas [6]. Chondroitin sulfate is particularly abundant in bone, tendons, blood vessels, nerve tissue, and cartilage [7,8]. An increasing demand for GAGs has resulted in a shortage of appropriate source-tissues and the quality and identity of many of the currently used tissues are difficult to control. Therefore, it is necessary to broaden the tissue sources of commercial GAGs.

GAGs have been largely extracted and characterized from terrestrial vertebrates compared to aquatic and marine vertebrates [9]. Marine capture fisheries contribute over 50% of total world fish production and more than 70% of this production is processed [10]. The current discards from the world's fisheries exceeds 20 million tons which is about 25% of the total production of marine capture fisheries [11]. The commercial fish-processing industry generates large quantities of solid waste that contains viscera, fins, heads, skin and fish scraps [12], which can be a good resource of GAGs [13]. In the present study, we used a three-step recovery and purification scheme for the analysis of chondroitin sulfate GAGs from red salmon (*Oncorhynchus nerka*) head. The procedure relied on proteolysis, an ion-exchange spin column purification, and methanol precipitation. The recovered chondroitin sulfate GAGs were then determined using carbazole assay, polyacrylamide gel electrophoresis (PAGE), disaccharide analysis with RPIP-HPLC and detected by post-column fluorescence detection.

Materials and Methods

Materials

Two salmon (*Oncorhynchus nerka*) head powder samples (water soluble (SHS) and insoluble (SHI) were generously provided by Cherry Seime, University of Alaska Fairbanks.

Eight unsaturated CS/DS disaccharides standards (Di-0S, ΔUA-GalNAc (where ΔUA is 4,5-deoxy-L-*threo*-hex-4-enopyranosyl uronic acid); Di-4S, ΔUA-GalNAc4S; Di-6S, ΔUA-GalNAc6S; Di-UA2S, ΔUA2S-GalNAc; Di-di_{SB}, ΔUA2S-GalNAc4S; Di-diS_D ΔUA 2S-GalNAc6S; Di-diS_E, ΔUA-GalNAc4S6S; Di-triS, ΔUA2S-GalNAc4S6S) were obtained from Seikagaku Corporation (Japan). Actinase E was from Kaken Biochemicals (Tokyo, Japan). Chondroitin lyase ABC from *Proteus vulgaris* and chondroitin lyase ACII from *Arthrobacter aurescens* was from Seikagaku Corporation (Tokyo, Japan). Recombinant *Flavobacterial* heparin lyase I, II, and III were expressed in our laboratory using *Escherichia coli* strains, provided by Professor Jian Liu, University of North Carolina, College of Pharmacy, Chapel Hill, NC, USA). Vivapure MAXI QH columns were from Sartoriou Stedim Biotech (Bohemia, NY).

Isolation and purification of GAG

The salmon head samples (1g in 10ml water) were individually proteolyzed at 55° C with 1 % of Actinase E (20mg/ml) for 18 h. After the proteolysis, dry urea (8g) and dry CHAPS (0.36g) were added to each sample to afford 18ml solution (2 wt% in CHAPS and 8 M in urea). The resulting cloudy solutions were clarified by passing through a syringe filter containing a 0.2μm membrane. A Vivapure MAXI Q H spin column was equilibrated with 3ml of 8M urea containing 2% CHAPS (pH-8.3). The clarified filtered samples were loaded onto and run through the Vivapure MACI QH spin columns under centrifugal force (500×g). The columns were first washed with 3ml of 8 M urea containing 2% CHAPS at pH 8.3. The columns were then washed 5-times with 5ml of 200 mM NaCl. Chondroitin sulfate was released from the spin column by washing 3-times with 1 ml of 16% NaCl. Methanol (12ml) was added to afford an 80 vol% solution and the mixture was equilibrated at 4°C for 18h. The resulting precipitate was recovered by centrifugation (2500×g) for 15min. The precipitate was recovered by dissolving in 1.0ml of water and the recovered heparin was stored frozen for further analysis.

Quantification of GAGs by carbazole assay

The isolated GAGs were subjected to carbazole assay [14] to quantify the amount of GAG in each sample using heparin as the standard.

Polyacrylamide gel electrophoresis (PAGE) analysis

Gradient polyacrylamide gel electrophoresis (PAGE) was applied to analyze the molecular weight and polydispersity of each sample and sensitive to chondroitin lyases and heparin lyases. To each lane ~5μg samples of isolated GAGs, with or

without treatment by chondroitin lyases/heparin lyases, were subjected to electrophoresis against a standard composed of heparin oligosaccharides prepared enzymatically from bovine lung heparin, the gel was visualized with Alcian blue and then digitized with UN-Scan-it software (Silk Scientific, Utah) and MWavg was calculated [15].

CD/DS disaccharide composition analysis

For chondroitinase digestion, 5µl of 0.2 M Tris-acetate buffer (pH 8.0) and 10µl of an aqueous solution containing chondroitinase ABC (50 mIU) and AC II was added to a 20µl portion of the sample solution and incubated at 37°C for 3h, followed by separation with Biomax (3500 nominal molecular weight limit, Millipore). The disaccharides products passing through the Biomax membrane were recovered and used for chondroitin/dermatan sulfate disaccharide analysis. Unsaturated disaccharides were determined by a reversed-phase ion-pair chromatography with sensitive and specific post column detection. A gradient was applied at a flow rate of 1.1ml/min on a Docosil column (4.6x150 mm) at 55°C. The eluents used were as follows: A, H_2O; B, 0.2 M sodium chloride; C, 10 mM tetra-n-butyl ammonium hydrogen sulfate; D, 50% acetonitrile. The gradient program was as follows: 0-10min, 1-4% eluent B; 10-11min, 4-15% eluent B; 11-20min, 15-25% eluent B; 20-22min, 25-53% eluent B; and 22-29min, 53% eluent B. The proportions of eluent C and D were constant at 12 and 17%, respectively. To the effluent were added aqueous 0.5% (w/v) 2-cyanoacetamide solution and 0.25 M NaOH at the same flow rate of 0.35ml/min by using a double plunger pump. The mixture passed through a reaction coil (diameter, 0.5mm; length, 10m) set in a temperature controlled bath at 125°C and a following cooling coil (diameter, 0.25mm; length, 3m). The effluent was monitored fluorometrically (excitation, 346 nm; emission, 410nm). The unsaturated disaccharides from chondroitin/dermatan sulfate, ΔUA-GclNAc, ΔUA2S-GlcNAc, ΔUA-GalNAc, ΔUA-GalNAc4S, ΔUA-GalNAc6S and ΔUA-GalNAc4S6S and ΔUA2S-GalNAc4S6S were used to prepare a standard curve for chondroitin sulfate analysis.

Results and Discussion

Quantification of isolated GAGs

We had been previously established a simple three-step procedure to quantitatively isolation of heparin from human plasma [16] and GAGs from zebrafish samples [17]. The isolation procedure involved protease digestion of the salmon head samples, strong-anion-exchange chromatography on a spin column followed by salt release and methanol precipitation. The isolated GAGs were subjected to carbazole assay to quantify the amount of GAG. The results showed the GAGs recovery yield was 6 to 7mg from 1 gram of salmon head powder (Table 1) samples. This GAG recovery yield is in good agreement with reported yields from different fish heads, ranging from 1.9 to 8.4mg GAGs/g dry sample [18].

Table 1 Quantification of isolated GAGs by carbazole assay

Sample	Soluble Salmon head powder **(SHS)**	Insoluble Salmon head powder **(SHI)**
Starting material	1.0 g	1.0 g
Isolated GAGs	7.0 mg	6.3 mg
MW based on PAGE analysis	12.5 KDa	12.8 KDa

Polyacrylamide gel electrophoresis (PAGE) analysis

GAGs isolated salmon heads were next analyzed by using PAGE with alcian blue staining (Figure 1 and 2). PAGE analysis with alcian blue staining confirmed that GAGs were present by a broad band of expected polydispersity. The gels were digitized with UN-Scan-it software (Silk Scientific, Orem, Utah) and the average MW of the GAGs was calculated based on the heparin oligosaccharide standard. The average MWs of GAGs from soluble and insoluble salmon head powder are 12.5 KDa and 12.8 KDa, respectively. The gels showed that the GAGs were only sensitive to chondroitin lyases, but not to heparin lyases, suggesting that primarily chondroitin sulfates were present.

Figure 1

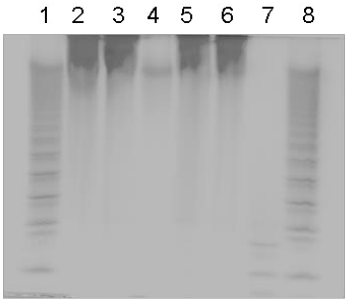

Figure 1. PAGE analysis on GAGs (digested by heparin lyases). Lane 1, Heparin oligosaccharide standards; Lane 2, GAG from SHS; Lane 3, GAG from SHI; Lane 4, heparin; Lane 5, heparin lyase treated GAG from SHS; Lane 6, heparin lyase treated GAG from SHI; Lane 7, heparin lyase treated heparin; Lane 8, heparin oligosaccharide standards.

Figure 2

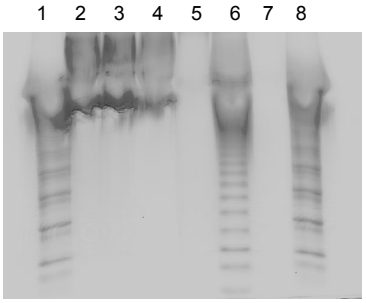

Figure 2 PAGE analysis on GAGs (digested by chondroitin lyases). Lane 1, heparin oligosaccharide standards; Lane 2, GAG from SHS; Lane 3, GAG from SHI; Lane 4, chondroitin sulfate C; Lane 5, chon-

droitin lyase treated GAG from SHS; Lane 6, chondroitin lyase treated GAG from SHI; Lane 7, chondroitin lyase treated chondroitin sulfate C; Lane 8, Heparin oligosaccharide standards.

Disaccharide composition analysis of isolated GAGs

Chondroitin sulfate is composed of a repeating disaccharide motif of glucuronic acid (GlcA) and N-acetylgalactosamine (GalNAc). We previously developed RPIP-HPLC with post-column fluorescence detection method to analyze the disaccharide composition of GAGs [19]. The RPIP-HPLC method gave good resolution of eight standard chondroitin sulfate disaccharides (Figure 3A and B) and working well with the chondroitin sulfate C (as positive control) disaccharide analysis. The results of chondroitin sulfate disaccharide analysis of the isolated GAGs are showed in Table 2 and Figure 4. Composition analysis of disaccharides showed a variation of structures of chondroitin sulfate derived from the two salmon head different samples. The major composition of the chondroitin sulfate was chondroitin sulfate C in both salmon head samples, while much high chondroitin sulfate E composition was discovered in the insoluble sample.

Figure 3

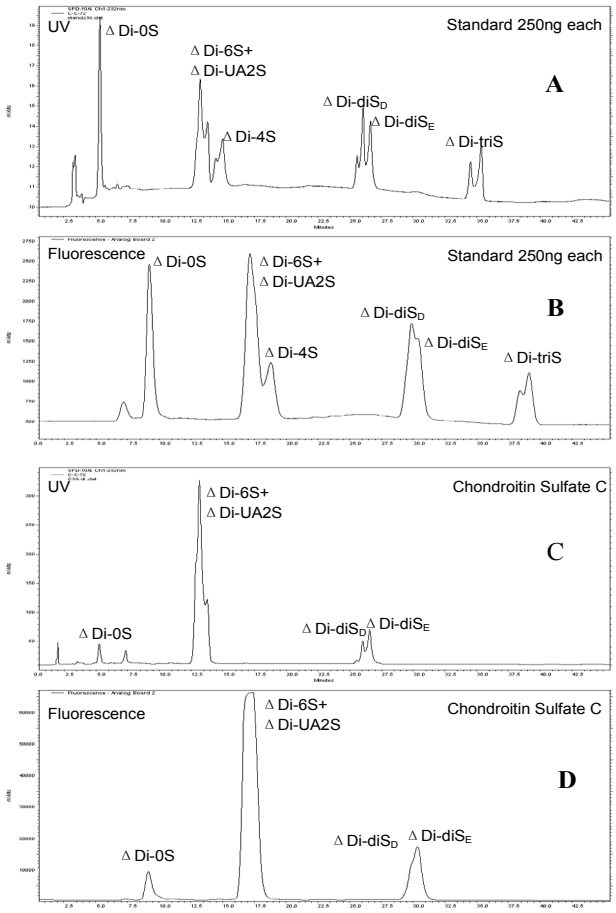

Figure 3. Chromatograms of disaccharide analysis of chondroitin sulfate standard (panel A, UV detection and panel B, fluorescence detection) and chondroitin sulfate C (panel C, UV detection and panel B, fluorescence detection) by RPIP-HPLC.

Table 2. Disaccharide composition of GAGs

	ΔDi-0S (%)	ΔDi-6S/ΔDi-UA2S (%)	ΔDi-4S (%)	ΔDi-diS$_D$ (%)	ΔDi-diS$_E$ (%)	ΔDi-triS (%)
CSC	3.7	81.6	0	5.1	9.6	0
SHI	1.9	64.4	0	0	33.7	0
SHS	17.5	81.1	0	0.5	0.9	0

Figure 4

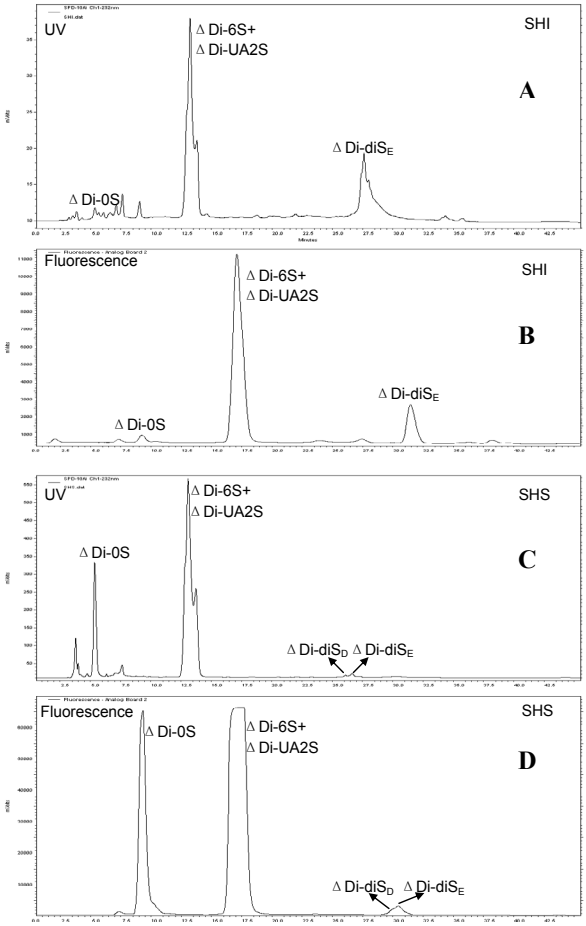

Figure 4. Chromatograms of disaccharide analysis of chondroitin sulfate from salmon head samples by RPIP-HPLC. Panel A, chondroitin sulfate from SHI with UV detection and panel B, chondroitin sulfate from SHI with fluorescence detection; Panel C, chondroitin sulfate from SHS with UV detection and panel D, chondroitin sulfate from SHS with fluorescence detection.

Chondroitin sulfate has been implicated in various physiological functions including cell division and morphogenesis [20, 21] central nervous system development [22] and signal

transduction [23, 24]. It is particularly interesting to find a large portion of chondroitin sulfate E in water insoluble salmon head sample, while water soluble salmon head sample was rich in chondroitin sulfate C. Chondroitin sulfate E is a relatively rare form of chondroitin sulfate that was originally isolated from squid cartilage [25]. It has a highly sulfated structure of [4)-β-D-GlcA-(1→3)-β-D-4, 6-O-disulfo-GalNAc-(1→]n. Chondroitin sulfate E is also found in bone marrow-derived mast cells and mucosal mast cells [26]. Since chondroitin sulfate E is highly sulfated, it interacts with many proteins, and plays several important biological roles in neurite elongation [7]; bone formation and biomineralization [26]; and blocking herpes simplex virus invasion of cells at substantially low concentrations [27].

Since salmon head is rich in both chondroitin sulfate C and chondroitin sulfate E, its use as a new source material should be of interest to workers in the pharmaceutical, nutraceutical, and cosmetic industries. Moreover, the three-step recovery and purification process described, involving proteolysis, ion-exchange chromatography, and alcohol precipitation is readily scalable.

Conclusion

We used a three-step recovery and purification scheme for isolation of GAGs from red salmon (*Oncorhynchus nerka*) head. The GAGs recovery yield was 6 to 7mg from 1 gram of salmon head powder. The structure analysis showed the major composition of GAG in red salmon head is chondroitin sulfate C and chondroitin sulfate E. This study broadens the tissue source for commercial GAGs production to include a plentiful waste product in the fish processing industry.

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