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Isolation and Characterization of Chondroitin Sulfates from the By-products of Marine Organisms

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Abstract By-products of marine organisms including salmon, skate, flatfish, and yellow goosefish were investigated to search for new source of chondroitin sulfate (CS). Agarose gel electrophoresis with chondroitinase depolymerization showed that purified chondroitin sulfate did not contain any other glycosaminoglycans. ^{1}H-nuclear magnetic resonance (NMR) spectra were acquired to confirm the structure and purity. The average molecular weight ranging from 22 to 64 kDa was determined by high performance size exclusion chromatography. Disaccharide compositions and purities were determined by strong anion exchange-high performance liquid chromatography (SAX-HPLC) after chondroitinase ABC depolymerization. SAX-HPLC data exhibited that the purity was from 81.7±1.3 to 114.2±2.5% and the yield was from 1.3 to 12.5%. All analytical results indicate that salmon cartilage, skate cartilage, and yellow goosefish bone could be promising sources of CS to substitute shark cartilage CS in commercial neutraceuticals.

Keywords: chondroitin sulfate, raw material, marine organism, characterization

Introduction

Glycosaminoglycans (GAGs) are highly sulfated linear polysaccharides that contain repeating disaccharide units composed of a hexosamine (D-glucosamine or D-galactosamine) and a uronic acid (D-glucuronic acid or L-iduronic acid); the exception is keratan sulfate which is mainly composed of D-glucosamine and D-galactose (1,2). There are several major classes of GAGs, including chondroitin sulfate (CS)/dermatan sulfate (DS), heparin/heparan sulfate, hyaluronic acid (HA), and keratan sulfate (KS) (3). With the exception of hyaluronic acid, other GAGs are synthesized in the form of proteoglycans that exist on the cell surfaces, of animal tissues, as well as in the extracellular matrix (4). Many researchers have uncovered and reported the physicochemical properties and biological function of proteoglycans (5-9).

CS is composed of repeating disaccharide units of [→4-D-glucuronic acid (GlcA) and β1→3 *N*-acetyl-D-galactosamine (GalNAc) β1→], which generally are sulfated at the C-4 and/or C-6 of GalNAc (10). However, CS can be substituted for *O*-sulfo groups at various positions (11). CS fulfils various biological functions in wound healing (12), the central nervous system (13), growth factor signaling (14), cell adhesion (15), cell division (16), and axonal regeneration (17). Most notably, CS has been regarded as a beneficial agent in the treatment of arthritis (18-20). CS has been isolated from various natural sources including mammalian species (bovine, porcine, and chicken cartilage) and marine species (shark, squid, and king crab) (21-24). Depending on the species, there are variations in molecular weight (Mw), chain length, and the position of sulfate groups. Mammalian CS generally is composed of monosulfated disaccharides such as ΔDi-4S (CS A), ΔDi-6S (CS C), and a small amount of other disaccharide units, whereas marine CS contains oversulfated disaccharides including ΔDi-2,6diS (CS D, shark cartilage), ΔDi-4,6diS (CS E, squid and salmon cartilage), and ΔDi-3,4diS (CS K, king crab) (25-27).

Although many suppliers have commercially obtained CS from mammalian species, which are more cost-effective than marine sources. However, frequent occurrences of animal epidemics including mad-cow disease, foot-and-mouth disease, and hog cholera have been a serious issue mitigating against use of CS from the former source (28). Hence, the search for safer sources is important in order to avoid potential biohazards. Thus, CSs have been purified from marine sources including whale (29), shark (30), skate (31), squid (32), salmon (33), king crab (34), and sea cucumber (35). Among them, shark cartilage has been the most commonly used as a commercial source of non-animal CS. Still, obtaining CS from shark cartilage might become problematic in the future, as the price of this raw material has been rising. Hence, it is prudent to investigate other marine organisms as alternative sources of abundant CS for the manufacture of CS-containing pharmaceuticals and nutraceuticals. Thus, the present study focused on the investigation of CS sources by using marine organism's by-products to substitute shark cartilage CS. In addition, CSs purified from selected by-products were characterized by using high performance size-exclusion chromatography (HPSEC), ^{1}H-nuclear magnetic resonance (NMR), strong anion-exchange high performance liquid chromatography (SAX-HPLC), and agarose gel-electrophoresis with a combination of chondroitinase digestion.

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Materials and Methods

Materials Skate, yellow goosefish, and flatfish were obtained from a local seafood market in Seoul. Authentic shark (*Squatina oculata*), skate (*Dipturus flavirostris*), and salmon (*Oncorhynchus keta*) were obtained both from the Korea Food Research Institute and a local supplier. Control chondroitin sulfate (CS), originating from bovine trachea was purchased from New Zealand Pharmaceuticals (Palmerston North, New Zealand), and CS A was purchased from Calbiochem (San Diego, CA, USA). Authentic CS (20 kDa) from shark cartilage and 2 CSs (Mw 15 and 40 kDa) for use as Mw standards were obtained from Seikagaku Co. (Tokyo, Japan). Hyaluronic acids (100, 130, and 1,690 kDa) were purchased from Kibun Food Chemifa (Tokyo, Japan). Alcalase® (2.4 Anson units/g) from *Bacillus licheniformis* was purchased from Novozymes (Bagsvaerd, Denmark). Chondroitinase ABC from *Proteus vulgaris*, sodium carbonate, trichloroacetic acid solution, cetylpyridinium chloride, sodium chloride, tris hydroxymethyl aminomethane (Trizma® base), barium acetate, 1,2-diaminopropane, toluidine blue, and Stains-All were obtained from Sigma-Aldrich (St. Louis, MO, USA). The dialysis membrane (Spectra/Por® 1; MWCO 6-8 kDa) was purchased from Spectrum® Laboratories (Rancho Dominguez, CA, USA). Agarose was acquired from Cambrex Bio Science (Rockland, ME, USA). All of the reagents were of the analytical grade available.

Purification of CS from marine organisms Bones (yellow goosefish and flatfish), cartilage (shark, skate, and salmon), and head (flatfish) were individually cleaned, lyophilized, and ground. The dried powder (10 g) was suspended in 200 mL of 50 mM sodium carbonate buffer (pH 7.0) containing alcalase solution (final 5%, v/v) and incubated for 12 hr at 60°C and 200 rpm in a shaking incubator in order to depolymerize the proteins. After boiling for 10 min to inactivate the protease, the sample solution was filtered and cooled at 4°C. The trichloroacetic acid solution (6.1 M) was mixed with the filtrate to a final concentration of 5%(v/v), and the precipitated proteins were removed by centrifugation at 887×g for 30 min at 4°C. Then, after ethanol (80%, v/v) precipitation, centrifugation at 887×g for 30 min at 4°C was performed. After the precipitate was dried, dissolved in water, mixed with cetylpyridinium chloride solution (final 1%, w/v), and stored at room temperature for 1 hr, centrifugation was performed again at 887×g for 30 min at 4°C. The precipitate recovered was dissolved in 2.5 M of NaCl solution, again precipitated by ethanol (80%, v/v), and centrifuged once more at 887×g for 30 min at 4°C. The final precipitate was dissolved in water, dialyzed (MWCO 6-8 kDa) against water for 2 days at 4°C and freeze-dried.

Agarose gel-electrophoresis In order to confirm the absence of dermatan sulfate (CS B) in the CS samples, enzymatic depolymerization of the CSs with specific CS lyases (chondroitinase ABC and AC) followed by agarose gel-electrophoresis, were performed on a 0.5% gel prepared in 0.04 M of barium acetate buffer (pH 5.8), as previously described (36) but with minor modifications. Each lyophilized CS sample (1 μg) was depolymerized with 10 μL of chondroitinase ABC (0.1 mU/μL) and AC (0.1 mU/μL) dissolved in Tris-acetate buffer (50 mM Trizma® base and 60 mM sodium acetate, pH 8.0 with hydrochloric acid) at 37°C for 12 hr, respectively. After the reaction, 10 μL of 60% sucrose solution was mixed with sample solutions and then these preparations were loaded onto the gel. A constant current (50 mA) in 50 mM 1,2-diaminopropane (pH 9.0 with acetic acid) was applied for 60 min. After completing the electrophoresis, the gel was soaked in 0.2% cetylpyridinium chloride solution for 4 hr, dried at room temperature, initially stained with 0.2% toluidine blue solution in ethanol-water-acetic acid (50:49:1, v/v/v) for 30 min, and destained with ethanol-water-acetic acid (50:49:1, v/v/v). The gel was subsequently stained with 0.005% solution of Stains-All (in 50% EtOH) overnight in the dark and visualized after destaining with water.

Chemical compositions and purities of CSs by SAX-HPLC The ÄKTA purifier system (Amersham Pharmacia, Uppsala, Sweden) equipped with a P-900 pump, a UV detector, and a fraction collector was used for strong anion-exchange high performance liquid chromatography (SAX-HPLC) and high performance size exclusion chromatography (HPSEC) analyses (28). UNICORN software version 5.01 (Amersham Pharmacia) was used to control the apparatus and to collect the data. The mobile phases used in the HPLC analysis were filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, USA) and degassed under vacuum before use. The chemical composition and purities of the CSs were determined by the validated disaccharide analytical method after the treatment of chondroitinase ABC, as previously described (37). One-hundred μL of control CS solution (10 mg/mL) and CS test sample solutions (10 mg/mL) were mixed with 800 μL of Tris-acetate buffer (50 mM Trizma® base and 60 mM of sodium acetate, pH 8.0), respectively. The samples were depolymerized with 100 μL of chondroitinase ABC (1 mU/μL) overnight at 37°C. After heating for 5 min and filtering on 0.2-μm syringe filters (PALL Life Sciences, Ann Arbor, MI, USA), reaction mixtures were injected (100 μg/μL) into a SAX-HPLC using a Hypersil SAX column (4.6×250 mm, 5 μm) from Thermo Hypersil-Keystone (Bellefonte, PA, USA). After the sample injection, the column was washed with water (pH 3.5) corresponding to 1 column volume (CV) for 4 min, and a linear gradient of 0-1.0 M of NaCl (pH 3.5, 10 CV) was used. The absorbance was monitored at 232 nm and the flow rate was 1.0 mL/min. The purities of the CS samples were determined by comparing the total peak area of the disaccharides (ΔDi-0S, ΔDi-6S, ΔDi-4S, ΔDi-2,6diS) and that of the control CS (37).

Determination of average Mw of CSs by HPSEC HPSEC equipped with a TSK G6000 PWXL column (7.5×300 mm, Tosoh, Tokyo, Japan) and a TSK guard column (7.5×75 mm, Tosoh), using Mw standard samples, was performed to determine the average Mw of the CS test sample (28,38). The mobile phase was 0.1 M of NaCl and the flow rate was 1.0 mL/min. The UV absorption was measured at 206 nm. Three kinds of hyaluronic acids (average Mw 1,690, 130, and 100 kDa) and 2 CSs (average Mw 40 and 15 kDa) as Mw-standard samples were individually injected (500 μg/100 μL) into the HPLC in

order to derive a Mw calibration curve, which was established by plotting the logarithm of the Mw vs. each retention time of the Mw standards (28). The average Mw of the CS test sample were determined through the Mw calibration curve.

^{1}H-NMR spectroscopy The structural integrity of the CS test sample was observed by ^{1}H-NMR spectroscopy. Ten mg of each sample was exchanged with 1 mL of D_2O (Sigma-Aldrich) followed by lyophilization. The dried samples were re-dissolved in 600 μL of D_2O and analyzed using a 500 MHz Bruker (Karlsruhe, Germany).

Results and Discussion

Preparation of CSs from marine organisms The CS samples from the shark (cartilage), skate (cartilage), yellow goosefish (bone), salmon (cartilage), and flatfish (bone and head) were purified as a white powder. Their yields (%) and purities (%) were determined by SAX-HPLC analysis of CS disaccharides derived by enzymatic depolymerization which we previously reported (37). The purity (%) of CS from marine organisms ranged from 81.7±1.3 to 114.2 ±2.5% (Table 1). Although CSs isolated from salmon cartilage and yellow goosefish bone exhibited more than 100% purity compared with the control CS, their yields were less than 4% (3.5 and 2.6%, respectively). In case of flatfish head and bone CSs, they were inadequate to be a high-quality CS raw material because of their lower purity (<85%) and yield (<1.5%). Shark cartilage, which is a commercial CS source, was used as a raw material to compare with the purity and the yield of CSs purified from by-products of the other marine organisms.

Agarose gel electrophoresis The CS test samples were treated with two kinds of CS lyases (chondroitinase ABC and AC), and the results indicate that there are no other GAGs, that is, no heparin, heparan sulfate, hyaluronic acid, or dermatan sulfate (Fig. 1).

Chemical composition of CSs from marine organisms In order to determine the chemical compositions and the purities of the CS test samples, the CS disaccharides produced by chondroitinase ABC mediated-enzymatic depolymerization were analyzed by SAX-HPLC and compared with those of various other kinds of CSs. The chemical compositions of the CS test samples are shown in Fig. 2. The CS obtained from yellow goosefish bone consists of monosulfated disaccharides ΔDi-0S (28.2%), ΔDi-6S (56.5%), and ΔDi-4S (14.5%) and a small amount of disulfated disaccharides ΔDi-2,6diS (0.4%) and ΔDi-2,4diS (0.1%) (Table 1). Even though yellow goosefish is one of the marine organisms, it has mainly monosulfated disaccharides, unlike shark, skate, salmon, and flatfish (Fig. 2). However, it contains plentiful monosulfated disaccharides (71.3%, ΔDi-6S and ΔDi-4S), and the ratio of unsulfated disaccharide was higher than that of the control CS. And yet, the quantity of high-sulfated disaccharides (ΔDi-2,6diS, ΔDi-4,6diS, ΔDi-2,4diS, and ΔDi-2,4,6triS) was only 0.7% (Table 1). With the exception of the CSs obtained from yellow goosefish bone and salmon cartilage, the other marine CSs were found to contain high-sulfated disaccharides. The disaccharide compositions of the CSs obtained from shark and skate cartilage were very similar to each other (Fig. 2B, 2C, and Table 1). In the case of the CSs obtained from the flatfish (head and bone), only small amounts of ΔDi-4,6diS and ΔDi-2,4diS were detected with ΔDi-2,6diS, according to the HPLC chromatograms (data not shown). GAGs play many important roles in the biological systems and distributed throughout the animal kingdom from the phylogenetic tree in both vertebrates and invertebrates (39). For example, CS from the body wall of sea cucumber was composed of 11.2% ΔDi-0S, 10.4% ΔDi-4S, 56.0% ΔDi-6S, and 22.4% ΔDi-4,6diS (43). They suggested that the unique CS structure may be associated with the phenomenon

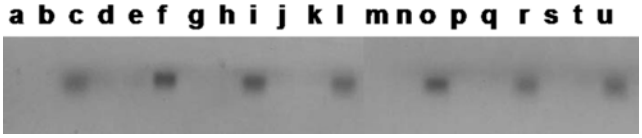

Fig. 1. Agarose gel electrophoresis. (a) control CS+CSL ABC; (b) control CS+CSL AC; (c) control CS; (d) shark cartilage CS+CSL ABC; (e) shark cartilage CS+CSL AC; (f) shark cartilage CS; (g) skate cartilage CS+CSL ABC; (h) skate cartilage CS+CSL AC; (i) skate cartilage CS; (j) yellow goosefish bone CS+CSL ABC; (k) yellow goosefish bone CS+CSL AC; (l) yellow goosefish bone CS; (m) salmon cartilage CS+CSL ABC; (n) salmon cartilage CS+CSL AC; (o) salmon cartilage CS; (p) flatfish head CS+CSL ABC; (q) flatfish head CS+CSL AC; (r) flatfish head CS; (s) flatfish bone CS+CSL ABC; (t) flatfish bone CS+CSL AC; (u) flatfish bone CS. CSL is the chondroitin sulfate lyase (chondroitinase).

Table 1. Percentage of disaccharide composition of marine and authentic chondroitin sulfates

CS source[1)]		Purity (%)	Yield (%)	Dissaccharide ratio[2)] (%)					
				ΔDi-0S:	-6S:	-4S:	-2,6diS:	-4,6diS:	-2,4diS:
Control CS		100	X	8.2±0.2	39.6±0.2	51.3±0.1	0.6±0.2	0.4±0.1	0
Salmon	Cartilage	114.2±2.5	3.5	16.1±0.1	55.6±0.1	27.1±0.1	1.0±0.0	0	0
Shark	Cartilage	94.5±3.3	9.7	2.4±0.0	39.3±0.5	35.4±0.8	21.0±0.3	0.2±0.0	0.8±0.0
Skate	Cartilage	93.0±0.5	12.5	2.5±0.1	40.4±0.1	35.4±0.2	20.9±0.2	0.1±0.0	0.6±0.0
Flatfish	Head	84.1±2.9	1.4	9.8±0.1	37.2±0.5	42.0±0.6	6.9±0.4	1.7±0.6	2.5±0.4
Flatfish	Bone	81.7±1.3	1.3	12.4±0.2	40.5±0.8	35.9±0.7	6.9±0.6	2.0±0.8	2.4±0.4
Yellow goosefish	Bone	110.5±8.5	2.6	28.2±0.2	56.5±0.3	14.5±0.1	0.4±0.3	0	0.1±0.0

[1)]Purified CS test samples and control CS were digested with chondroitin sulfate lyase ABC and then analyzed by SAX-HPLC.
[2)]In regular sequence Di-0S: Di-6S: Di-4S: Di-2,6diS: Di-4,6diS: Di-2,4diS by percentage (%).

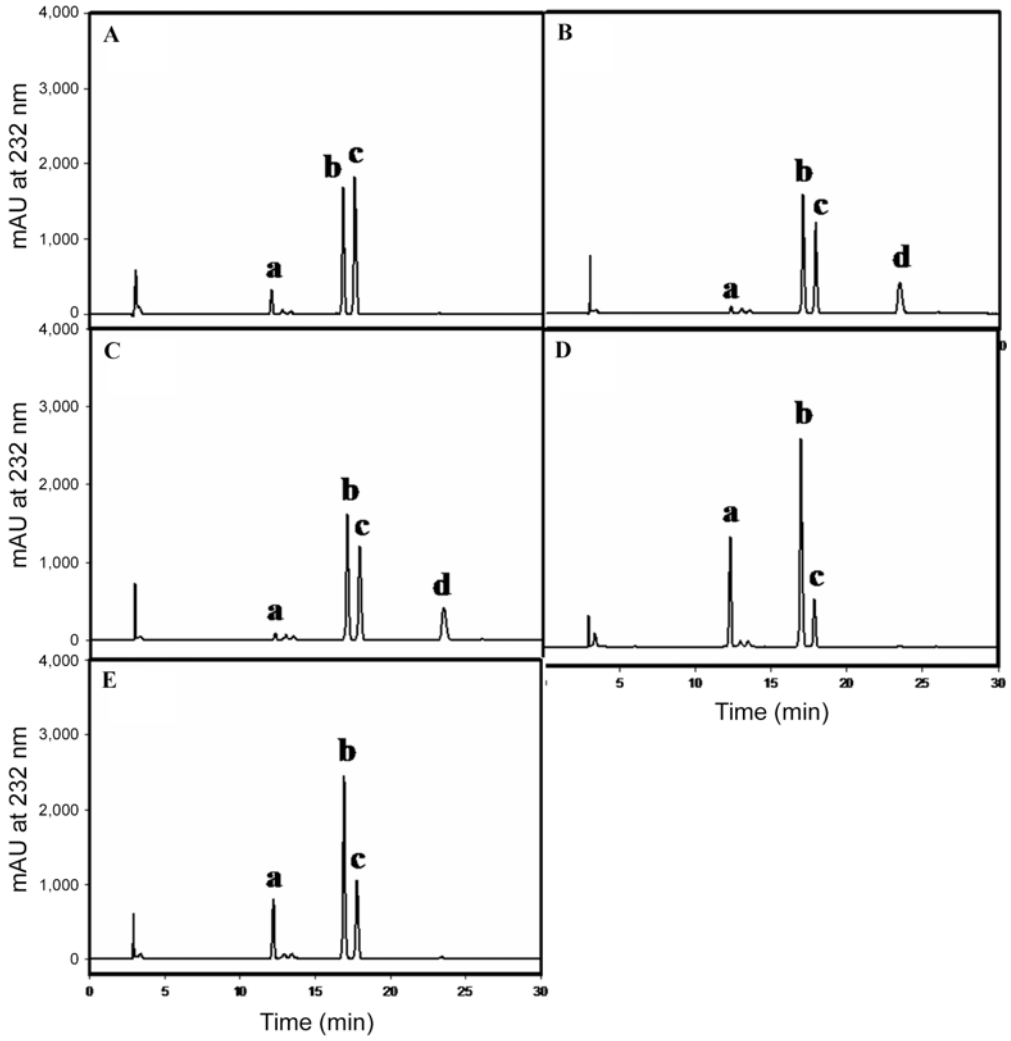

Fig. 2. SAX-HPLC analysis of CS disaccharides produced by enzymatic depolymerization. (A) control CS; (B) shark cartilage CS; (C) skate cartilage CS; (D) yellow goosefish bone CS; (E) salmon cartilage CS. (a) ΔDi-0S (ΔUA-[1→3]-GalNAc); (b) ΔDi-6S (ΔUA-[1→3]-GalNAc-6S); (c) ΔDi-4S (ΔUA-[1→3]-GalNAc-4S); (d) ΔDi-2,6diS (ΔUA-2S-[1→3]-GalNAc-6S); (e) ΔDi-4,6diS (ΔUA-[1→3]-GalNAc-4,6diS). Control CS originated from bovine trachea was purchased from New Zealand Pharmaceuticals.

called 'connective tissue catch' where sea cucumber body wall becomes tough by stimulation. Nader *et al.* (42) reported that CS exhibits different structural features among 3 molluscs species and the possible role of CS may be related to cell recognition and/or adhesiveness. Therefore, the variation of CS disaccharide composition of each sample investigated in this study may suggest the different biological roles of CS in each marine organism.

Determination of average Mw of marine organism CSs by HPSEC The Mw-standard samples and the CS test samples were analyzed by HPSEC (Fig. 3). The average Mw of the CSs obtained from shark cartilage, skate cartilage, yellow goosefish bone, salmon cartilage, flatfish head, and flatfish bone were found to be 64, 55, 54, 51, 24, and 22 kDa, respectively. Among them, the CSs obtained from the flatfish head and bone contained low Mw impurities, which were detected at 206 nm of UV.

^{1}H-NMR spectroscopy ^{1}H-NMR spectroscopy was performed to confirm the disaccharide composition and the structural integrity of the CS samples, and the results were compared to those of the control CS (Fig. 4). A characteristic signal at 4.7 ppm was assigned as H4 of the *O*-sulfate substituted on the C4-position of GalNAc (40, 41) whereas the CS sample from skate shown in Fig. 4B represent a characteristic signal at 4.2 ppm, which was assigned as H6 of *O*-sulfate group and this is also shown in Fig 4A and 4C. The CS from yellow goosefish contains a less sulfate group on C4-postion of GalNAc, which shows a downfiled signal at 4.7 ppm in Fig. 4C. This result is also consistent with the composition analysis of disaccharide using HPLC (Fig. 2). When we compared with HPLC analysis, CS from flatfish bone contains an almost equal content of 4-*O*- and 6-*O*-sulfate groups. The signal intensity of 2 overlapped peaks indicating methyl groups between 1.95 and 2.05 ppm confirmed the above result.

Recently, production cost and the safety of CS have become an important issue to suppliers and consumers,

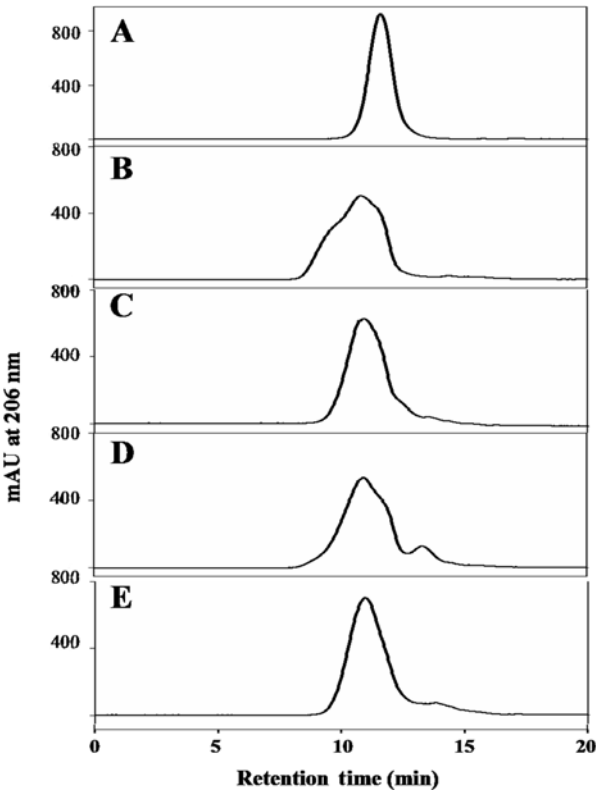

Fig. 3. Determination of average molecular weights of CS samples obtained from marine organisms using HPSEC. (A) control CS; (B) shark cartilage CS; (C) skate cartilage CS; (D) yellow goosefish bone CS; (E) salmon cartilage CS.

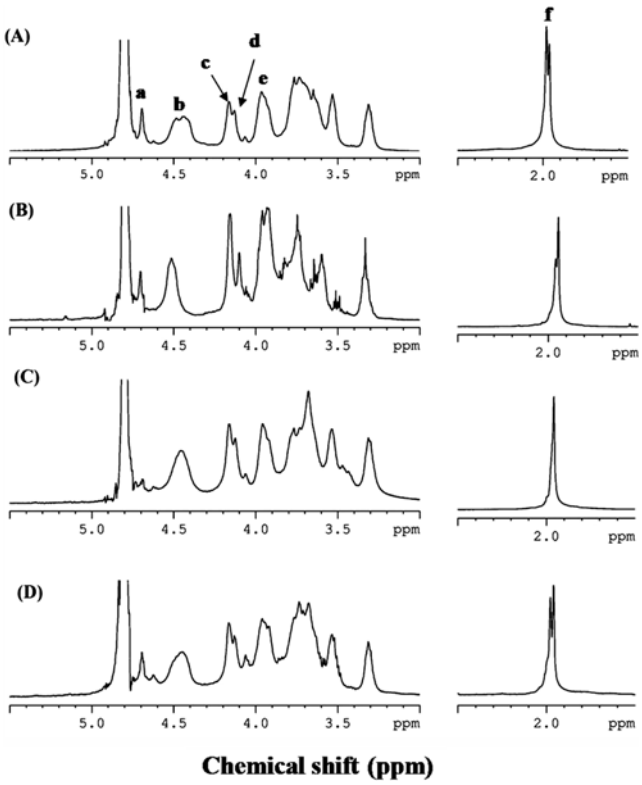

Fig. 4. ^{1}H-NMR spectra of CS samples obtained from marine organisms. (A) control CS from bovine cartilage; (B) skate cartilage CS; (C) yellow goosefish bone CS; (D) flatfish bone CS. Signals: (a) H-4 of GalNAc; (b) H-1 of GalNAc; (c) H-6 of GalNAc6S; (d) H-4 of GalNAc6S; (e) H-2 of GalNAc; (f) *N*-acetyl signals of GalNAc4S and GalNAc6S.

Table 2. Retention time and average molecular weight of chondroitin sulfates

CS source		Retention time (min)	Average Mw (kDa)
Control CS		11.6	14
Salmon	Cartilage	11.0	51
Shark	Cartilage	10.8	64
Skate	Cartilage	10.9	55
Flatfish	Head	11.3	24
Flatfish	Bone	11.4	22
Yellow goosefish	Bone	10.9	54

given that CS has been widely used as a raw material for applications in cosmetics, pharmaceuticals, and nutraceuticals. Thus, in order to investigate new commercial sources of CS, the present study focused on the by-products of marine organisms such as skate, salmon, flatfish, and yellow goosefish. Because CS derived from marine organisms is free from the potential risks associated with animal diseases, it is safer than that of bovine and porcine tissues. Additionally, it is more cost-effective than CS purified from shark cartilage, which is the most common sources of CS for pharmaceuticals and nutraceuticals. Although several previous reports have shown the presence of CS in marine sources including species of molluscs (42), the body wall of sea cucumber (43), squid (32, 44), and king crab (34), the yields of CS from their dry tissues were very low and, furthermore, those kinds of CS sources are expensive. Based on our results, we conclude that several by-products obtained from marine organisms including skate, salmon, and yellow goosefish could be the valuable sources of pharmaceutical-grade CS as their purities and yields are comparable with shark cartilage CS.

Acknowledgments

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