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Functional chondroitin sulfate from *Enteroctopus dofleini* containing a 3-*O*-sulfo glucuronic acid residue

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ABSTRACT

There are several reports that chondroitin sulfate containing K-type units [GlcA (3S)-GalNAc (4S)] exhibiting similar levels of neurite outgrowth promoting activities as CS having high amounts of B-, D- and E-type disulfated disaccharides. Although CS containing K-type units possess important biological activities, there are only few sources, such as king crab cartilage, squid cartilage or sea cucumber. In this study, CS containing 13.9% of K-type units was found in octopus (*Enteroctopus dofleini*) cartilage using different substrate specificities of chondroitinases. The 2D NMR spectra showed cross-peaks assigned to protons on sugar ring of GlcA (3S), demonstrating the presence of K-type units in octopus CS. Furthermore, proportion of fucosylated disaccharide units in octopus CS was very low. Octopus CS showed high affinity for growth factors and stimulated neurite outgrowth of hippocampal neurons, similar to the activity of squid CS-E. These results strongly suggest that octopus cartilage is a rich source of CS-K and has important biological activities.

1. Introduction

Chondroitin sulfate (CS) is a glycosaminoglycan, the carbohydrate moieties of CS proteoglycans (PGs) found on the cell surface and in the extracellular matrix. CS is a linear, sulfated polysaccharide containing a repeating disaccharide unit, [→4)-β-D-GlcA-(1→3)-β-D-GalNAc-(1→]$_n$, where GlcA is glucuronic acid and GalNAc is *N*-acetylgalactosamine (Roden, 1980). A disaccharide unit containing iduronic acid (IdoA) in place of GlcA is commonly found in dermatan sulfate (DS), a stereoisoform of CS that differs in the C-5 configuration of the hexuronic acid moieties (Malmström, Bartolini, Thelin, Pacheco, & Maccarana, 2012). The disaccharide units of CS found in natural products are commonly classified into five groups, based on their characteristic disaccharide units and include GlcA-GalNAc (4S) (A-type unit: CS-A), IdoA (2S)-GalNAc (4S) (B-type unit: DS), GlcA-GalNAc (6S) (C-type unit: CS-C), GlcA (2S)-GalNAc (6S) (D-type unit: CS-D) and GlcA-GalNAc (4S, 6S) (E-type unit: CS-E) (Higashi, Okamoto, Mano, Wada, & Toida, 2014).

There have been many reports of diverse biological processes of CS having high content of disulfated disaccharides (B-, D- and E-type units), including differentiation, migration, tissue morphogenesis, immune response and wound repair through the binding of the growth factors in mammals (Maeda, 2015; Pomin, 2015; Sugahara et al., 2003; Toida, Sakai, Akiyama, & Linhardt, 2006). Interestingly, marine organisms are known to represent rich sources of highly sulfonated polysaccharides having rare structures. For instance, K-type units [GlcA (3S)-GalNAc (4S)] were identified from the CS derived from king crab (*Tachypleus tridentataus*) cartilage, sea cucumber (*Ludwigothurea grisea*) and squid cartilage (Kinoshita et al., 1997; Seno, Yamashiro, & Anno, 1974; Vieira, Mulloy, & Mourão, 1991). It should be noted that CS derived from king crab cartilage and sea cucumber are also fucosylated at the 3-OH position of GlcA (Fongmoon et al., 2007; Vieira et al., 1991). Furthermore, fucopyranosyl units linked to GlcA in sea cucumber are also sulfated (Vieira et al., 1991)

It is of interest to note that CS, having a fucosylated or a sulfated 3-OH position in its GlcA residue, shows different susceptibility to chondroitinases compared with other types of CS. For example, fucosylated CS from sea cucumber exhibits resistance to chondroitinase ABC (Chase ABC) and ACII and partial acid hydrolysis is needed to prepare the disaccharide components of CS from sea cucumbers (Vieira et al., 1991). In the case of CS-K from king crab

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cartilage, K-type units containing 3-*O*-sulfo GlcA residues show resistance to Chase ACII, however, they are sensitive to Chase ABC (Sugahara et al., 1996). Consequently, (1–3)-linkage of K-type units [GlcA (3S)-GalNAc (4S)] and (1–4)-linkage of disaccharides are completely digested by Chase ABC, resulting in GalNAc (4S) residues being produced from K-type units (Fongmoon et al., 2007).

It should be noted that physiological function of both the fucosyl and sulfo groups substituted at the 3-OH position of GlcA remains unclear. For instance, neurite outgrowth promotion (NOP) activity of fucosidase-treated king crab CS-K was higher than that of fucosylated CS-K (Fongmoon et al., 2007). In contrast, fucosylated CS can potentiate the thrombin inhibition activity of antithrombin and heparin cofactor II (Chen et al., 2011; Mourão et al., 1996). Therefore, it is important to identify new sources of CS-K with biological activities to better understand the physiological function of 3-OH modified GlcA.

We have purified CS (or DS) from a variety of animal tissues and characterized their structures to better understand their biological distribution and to explore new sources of polysaccharides as functional foods, nutraceuticals, cosmetics and drugs (Ha et al., 2005; Higashi et al., 2015; Kim et al., 1998; Sakai et al., 2003; Warda, Gouda, Toida, Chi, & Linhardt 2003; Warda, Mao, Toida, & Linhardt, 2003). In the present study, we have identified a CS containing 3-*O*-sulfo group-substituted GlcA isolated from octopus (*Enteroctopus dofleini*) cartilage. Furthermore, this octopus CS-K stimulated neurite outgrowth of hippocampal neurons at a similar level as squid CS-E. These results demonstrate that octopus cartilage is a rich source of CS-K having important biological activities.

2. Experimental

2.1. Chemicals

The cartilage of *E. dofleini* was purchased from Karaku Co., Hokkaido, Japan. Actinase E was from Kaken Pharmaceutical Co., Ltd., Tokyo, Japan. Chondroitinase ABC (ChaseABC) from *Proteus vulgaris*, chondroitinase ACII (Chase ACII) from *Arthrobacter aurescens*, chondro-4-sulfatase from *P. vulgaris*, chondro-6-sulfatase from *P. vulgaris*, unsaturated disaccharides (ΔDi-0S, ΔDi-4S, ΔDi-6S, ΔDiUA-2S, ΔDi-diS_E, ΔDi-diS_B, ΔDi-diS_D, ΔDi-TriS), CS-A (6.3% of ΔDi-0S, 74.2% of ΔDi-4S, 19.5% of ΔDi-6S, 0.3% of ΔDi-diS_E) from whale cartilage, CS-C (1.8% of ΔDi-0S, 13.7% of ΔDi-4S, 70.8% of ΔDi-6S, 2.9% of ΔDi-diS_E, 10.8% of ΔDi-diS_D) from shark cartilage, CS-E (10.0% of ΔDi-0S, 17.0% of ΔDi-4S, 8.31% of ΔDi-6S, 63.6% of ΔDi-diS_E, 1.08% of ΔDi-diS_D) from squid cartilage, DS (6.5% of Δdi-0S, 83.2% of ΔDi-4S, 1.2% of ΔDi-6S, 9.1% of ΔDi-diS_B) from porcine intestine were purchased from Seikagaku Corp., Tokyo, Japan (Higashi et al., 2014). Heparan sulfate and heparin were obtained from Celsus Inc., Ohio, USA. Hyaluronic acid was purchased from Kikkoman Corp., Chiba, Japan. Tetrasaccharides and hexasaccharides prepared from CS were obtained from Iduron, Manchester, UK. The other analytical reagents used were of analytical grade.

2.2. Isolation of crude glycosaminoglycans from octopus cartilage

Dried octopus cartilage was cut into small pieces that were homogenized by a Waring blender and defatted with 3-volumes of acetone for overnight. The precipitates were obtained by centrifugation and dried over silica gel in a desiccator. The residual dry powder of octopus cartilage was proteolyzed at 45 °C with actinase E (10 mg/g dry powder) in 50 mM Tris acetate (pH 8.0) for 18 h. After the proteolysis, the β-elimination reaction on the reducing termini of peptidoglycan chains was performed with 0.5 mol/L NaOH containing 0.3 M sodium borohydride (20 mL/g dry sample) at 4 °C for 18 h. The reaction mixture was then neutralized with 1.0 M HCl. Next, the resulting GAG chains were precipitated by the addition of 5% cetylpyridinium chloride (CPC; final concentration 0.1%) containing 0.03 mol/L NaCl at 4 °C for 3 h. The GAG-CPC complex was collected by centrifugation at 2300 × *g* for 15 min. The GAG chains were extracted from the GAG-CPC complex by the addition of 2.5 M NaCl, and the mixture was centrifuged at 2300 × *g* for 15 min. The GAG chains were precipitated from the supernatant by the addition of 11 volumes of 85% ethanol at 4 °C for 16 h and were collected by centrifugation at 2300 × *g* for 15 min. The GAG chains were then isolated through dialysis against distilled water at room temperature for 16 h followed by lyophilization to afford partially purified GAG.

The crude GAG sample (approximately 5 mg of dry powder) in 2 mL water was applied at a flow rate 5 mL/min on a HiPrep DEAE FF (16 mm i.d. × 100 mm, obtained from GE Healthcare Europe GmbH) and fractionated to prepare the highly sulfated CS oligosaccharides. The eluents were (A) 50 mM sodium phosphate, (B) 2.0 M NaCl in 50 mM sodium phosphate, respectively. The gradient program was 0–20 min (5% B), 20–100 min (5–100% B), and 100–120 min (100% B). Fractionated samples were collected at 20 min-intervals, dialyzed, freeze-dried and kept stored at 4 °C.

2.3. HPLC analysis for CS oligosaccharides obtained by Chase ACII treatment

Crude GAGs (5 μg) were incubated in a reaction mixture (35 μL) containing 28.6 mM Tris acetate (pH 8.0), 50 mIU of Chase ACII. After 16 h at 37 °C, samples containing CS oligosaccharides were boiled and evaporated, re-suspended in 10 μl of 50% of acetonitrile. The HPLC system was constructed with Elite LaChrom Pump L-2130, Elite LaChrom UV Detector L-2400, a chromatointegrator D-2500 (Hitachi High-Technologies Corp., Tokyo, Japan) and a sample injector with a 20 μL loop (Model 7725i; Reodyne, CA). The isocratic tandem-mode HPLC system, in which TSKgel amide-80 (4.6 mm I.D. × 15 cm; Tosoh Corporation, Tokyo, Japan) and Asahipak GS320H (7.6 mm I.D. × 25 cm; Shodex, Tokyo, Japan) were connected in series. The isocratic elution conditions were as follows: eluent, 65% acetonitrile and 8.75 mM sodium phosphate (pH 5.2); flow rate, 0.6 mL/min. The effluent was monitored at 204 nm. Disaccharide composition analysis of CS and HS was performed as described previously (Higashi et al., 2015). Hyaluronan (HA) contents in crude GAGs were analyzed according to the method of Toyoda, Muraki, Imanari, and Kinoshita-Toyoda (2011).

2.4. ^{1}H NMR spectroscopy

^{1}H NMR spectra of the purified oligosaccharides were recorded on a JEOL JNM-ECA600 (600 MHz) spectrometer in the FT mode. Each oligosaccharide (approximately 6 mg) for NMR analysis was dissolved in deuterium oxide (D_2O, 99.90% D) and lyophilized three-times to replace exchangeable protons with deuterons. Then the lyophilized samples were dissolved in 0.6 mL of D_2O and transferred to the NMR tube. The one and two dimensional (1D and 2D) NMR experiments were recorded with a relaxation time of 1.5 s at a probe temperature of 25 °C. 1D ^{1}H NMR spectra were recorded with an acquisition time of 1.45 s and a number of 300 scans. 2D ^{1}H/^{1}H COSY (correlation spectroscopy) and TOCSY (total correlated spectroscopy) spectra were recorded with a number of 112 and 148 scans, respectively. 2D TOCSY spectra were run with a mixing time of 100 ms. The fucose and sulfate compositions of oligosaccharides were analyzed by NMR based on integration area of the each proton attaching to the sulfonated C atom, and the proton signals in the 1D spectra were assigned using the 2D ^{1}H/^{1}H COSY and TOCSY spectra.

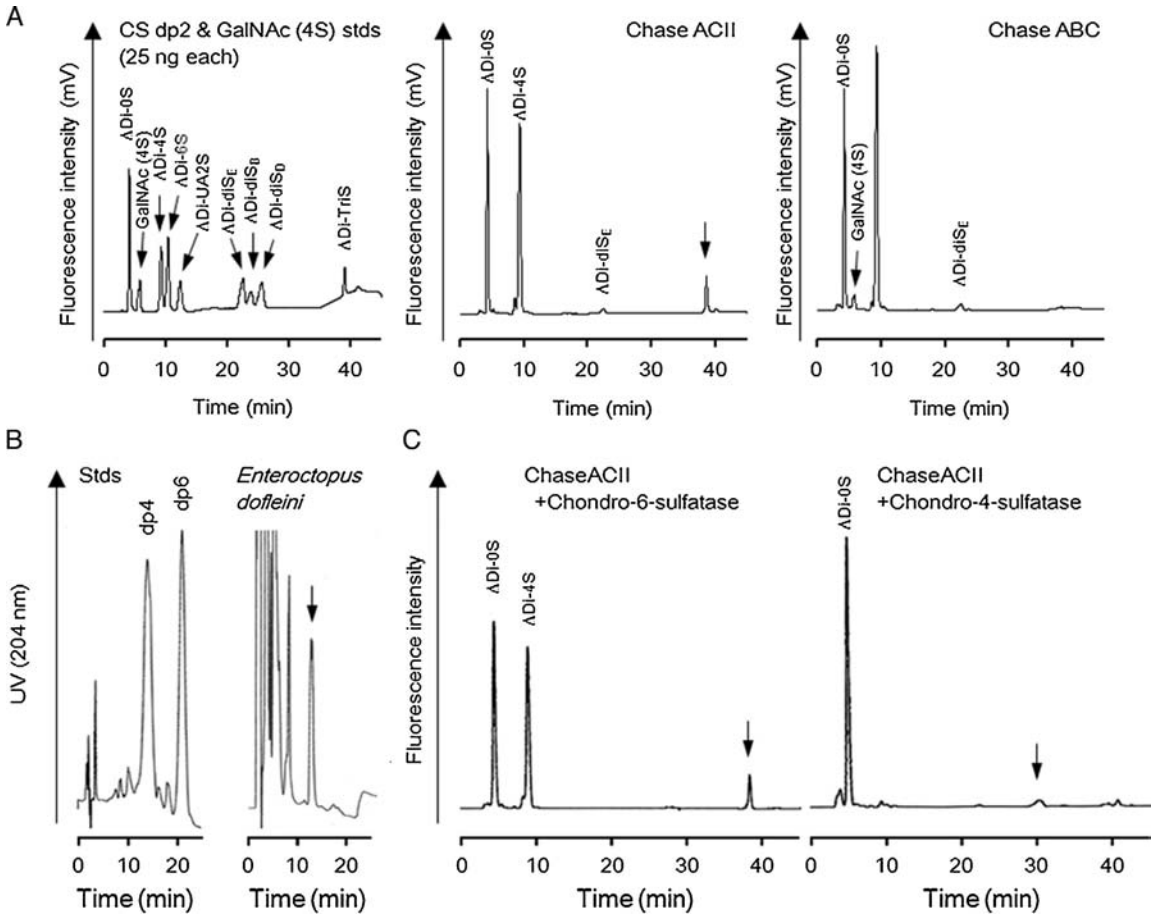

Fig. 1. Composition analysis of crude glycosaminoglycans from octopus cartilage. (A) Chromatogram of unsaturated disaccharides of CS obtained by digestion with Chase ACII or Chase ABC. Crude GAGs (5 μg) were incubated in reaction mixture (35 μL) which was contained 28.6 mM Tris acetate (pH 8.0), 50 mIU of Chase ABC or 50 mIU of Chase ACII. After incubation, depolymerized samples were submitted to gradient HPLC with fluorescence detection as described previously (Higashi et al., 2015). (B) Detection of tetrasaccharides of octopus CS obtained by Chase ACII treatment. HPLC analysis was performed as described under Section 2. (C) Effect of chondro-4-sulfatase or chondro-6-sulfatase on the sensitivity of tetrasaccharides obtained by Chase ACII treatment. Reaction mixture containing crude GAGs (5 μg), 28.6 mM Tris acetate (pH 8.0), 50 mIU of Chase ACII and 2.5 mIU of chondro-4-sulfatase (or chondro-6-sulfatase) was incubated at 37 °C for 16 h. Resulting oligosaccharides were subsequently subjected to gradient HPLC with fluorescence detection. Experiments were repeated twice with reproducible results.

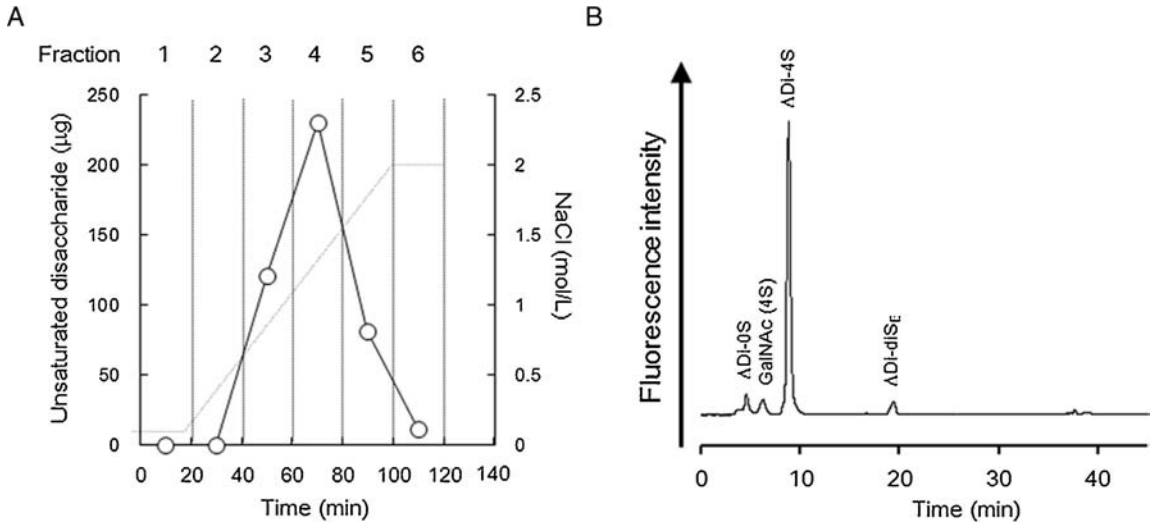

Fig. 2. Purification of CS using DEAE-cellulose column. (A) Purification of CS from crude GAGs. The crude GAGs (approximately 5 mg of dry powder) were purified on DEAE-cellulose column as described under Section 2. The dried powders obtained, after desalting and lyophilizing the 6 fraction samples, were treated with Chase ABC and then resulting unsaturated disaccharides were subjected to HPLC. Total amount of unsaturated disaccharides were exhibited as vertical axis. (B) Chromatograms of unsaturated disaccharides of CS derived from fraction 4. Experiments were repeated twice with reproducible results.

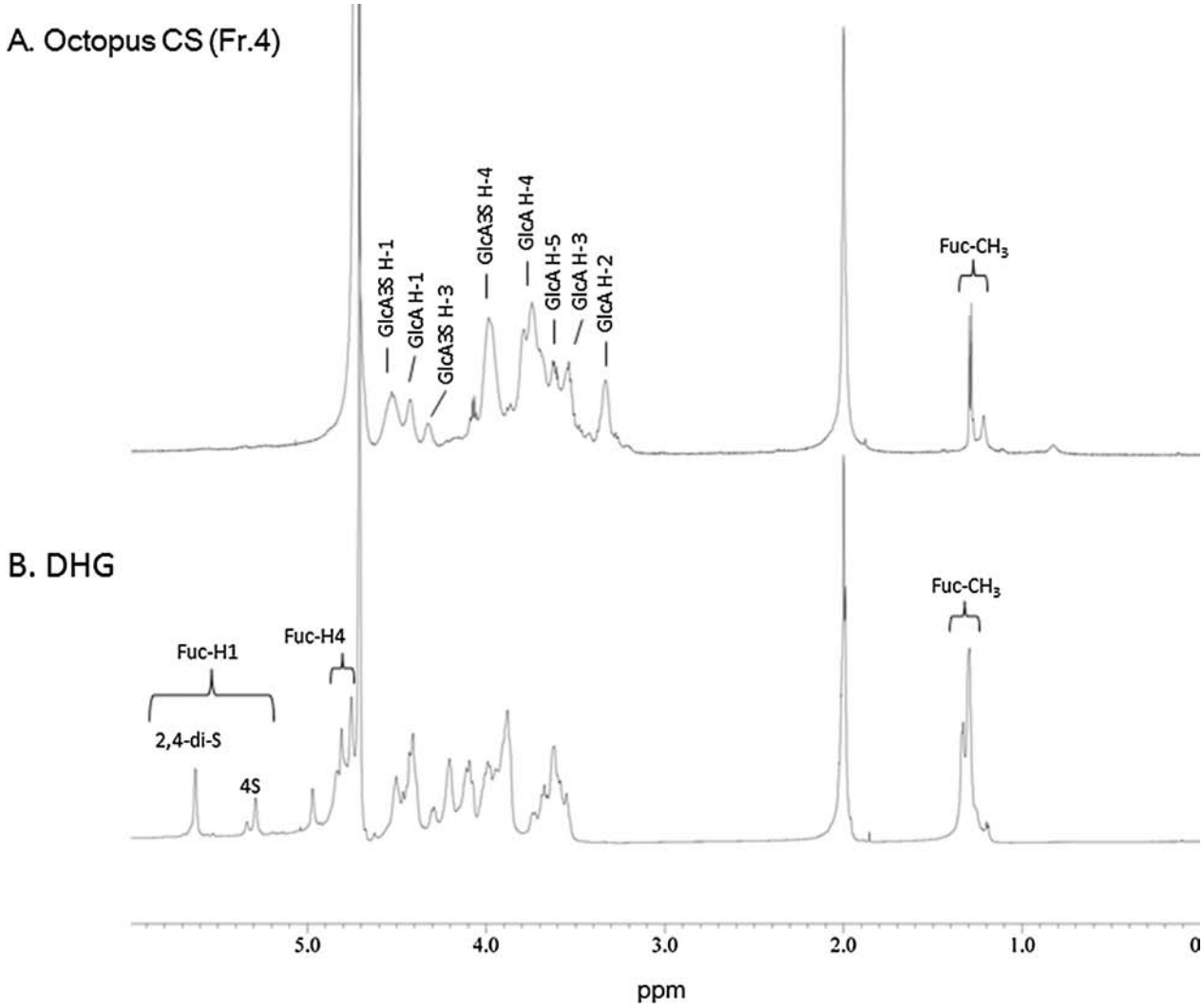

Fig. 3. One-dimensional ^{1}H NMR spectra of octopus CS (Fr. 4) (A) and partially depolymerized holothurian glycosaminoglycan (DHG), a fucosyl CS from sea cucumber (B). 600 MHz ^{1}H NMR spectra of octopus CS (Fr. 4) and DHG were recorded in D_2O at 25 °C.

2.5. NOP activity and affinity for growth factors of octopus CS-K

All animal experiments were approved by the Institutional Animal Care and Use Committee of Chiba University and carried out according to the guidelines for Animal Research of Chiba University. GAG pre-coating in 8-well chamber slide and evaluation of CS on neurite outgrowth were performed as described previously (Higashi et al., 2015). Briefly, 0.5 μg/well of the octopus CS, CS-E and CS-A were pre-coated with poly-DL-ornithine at 4 °C overnight. Subsequently, the cells were seeded on coverslips at a density of 16,000 cells/cm^2 and cultured for 18 h. Thereafter, the cells were fixed by 4% (w/v) paraformaldehyde for 30 min, the neurites were visualized by immunochemical staining using anti-microtubule-associated protein-2 (Lieco Technologies Inc., St. Louis, MO, USA) and anti-neurofilament (Sigma–Aldrich, St. Louis, MO, USA). Fifty cells with at least one neurite longer than the cell body were chosen at random to determine the length of the longest neurite. At least six independent experiments per condition were carried out.

Recombinant growth factors such as human pleiotrophin (PTN) and midkine (MK) were purchased from Peprotech and R&D systems, respectively. The binding of GAGs to growth factors was measured using a ProteOn XPR36 (Bio-Rad, Hercules, CA, USA). Briefly, recombinant growth factors were immobilized on a CM5 sensor chip (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. GAGs prepared specified concentration was applied to flow cells, and data was analyzed by ProteOn manager Ver3 using 1:1 Langmuir binding model.

3. Results and discussion

3.1. Composition of glycosaminoglycans derived from E. dofleini

Crude GAGs were extracted from octopus cartilage by actinase E digestion, and recovered by ethanol precipitation. The dried pellet (crude GAGs) was weighed after dialysis and freeze-drying. Finally, 1.66 mg GAG/g of dry tissue was recovered. The crude GAGs were analyzed by cellulose acetate membrane electrophoresis and visualized by alcian blue staining (Fig. S1). GAGs including two bands corresponding to the migration positions of hyaluronan (HA) and chondroitin sulfate (CS) standards were found. Based on this observation, we next investigated the composition of GAGs including CS, HA, heparan sulfate (HS), respectively. At first, crude GAGs were digested by Chase ACII to depolymerize HA and CS, and then the resulting unsaturated disaccharides of HA/CS were separated and detected using reversed phase ion-pair chromatography with fluorescence detection. As shown in Fig. 1A, an unidentified peak eluted at 38 min along with ΔDi-0S, ΔDi-4S and small amounts of ΔDi-diS_E. When crude GAGs were treated with Chase ABC, the

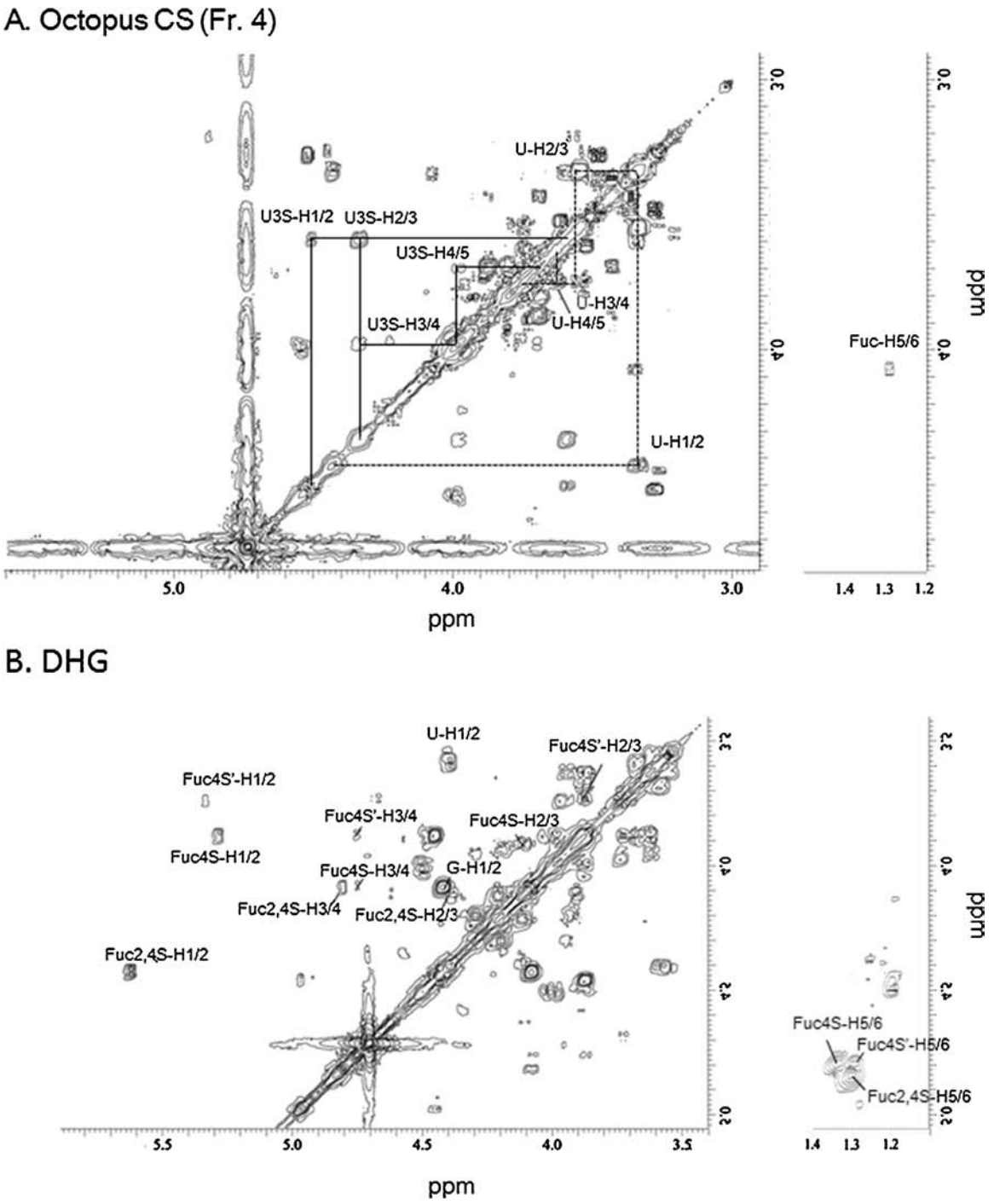

Fig. 4. Two-dimensional COSY spectra of octopus CS (Fr. 4) (A) and DHG (B) recorded in D_2O at 25 °C. Proton signals of GlcA, GlcA (3S) were assigned, respectively. The assignment pathway for the GlcA residue is drawn (- - -) and GlcA (3S) is drawn in (–), respectively. U corresponds to GlcA.

unidentified peak completely disappeared, whereas the intensity of ΔDi-4S peak increased, despite an unchanged intensity of the peak corresponding to Δdi-0S. Furthermore, small amount of new peak eluting at 4.68 min was observed. The retention time of the newly observed peak was nearly the same as the GalNAc (4S) standard. These results strongly suggested the possible presence of a 3-*O*-sulfo group-containing GlcA in octopus CS, based on a report by Fongmoon et al. The HA content was also analyzed on a graphitized carbon column, capable of separating ΔDi-0S from ΔDi-HA, and only ΔDi-0S was detected (data not shown) demonstrating that no HA was present.

The unidentified peak, obtained using Chase ACII, was subjected to normal phase chromatography and gel permeation chromatography to determine the oligosaccharide sequence in which the 3-*O*-sulfo group-containing GlcA resided (Fig. 1B). In this HPLC system, CS-derived tetrasaccharides and hexasaccharides are detectable within 25 min. As shown in Fig. 1B, tetrasaccharides derived from crude GAGs after digestion with Chase ACII were observed. It has been reported that chondro-6-sulfatase specifically acts on GalNAc (6S) at the reducing end of unsaturated CS oligosaccharides, while chondro-4-sulfatase preferentially removes a sulfo group from GalNAc (4S) at the reducing terminus

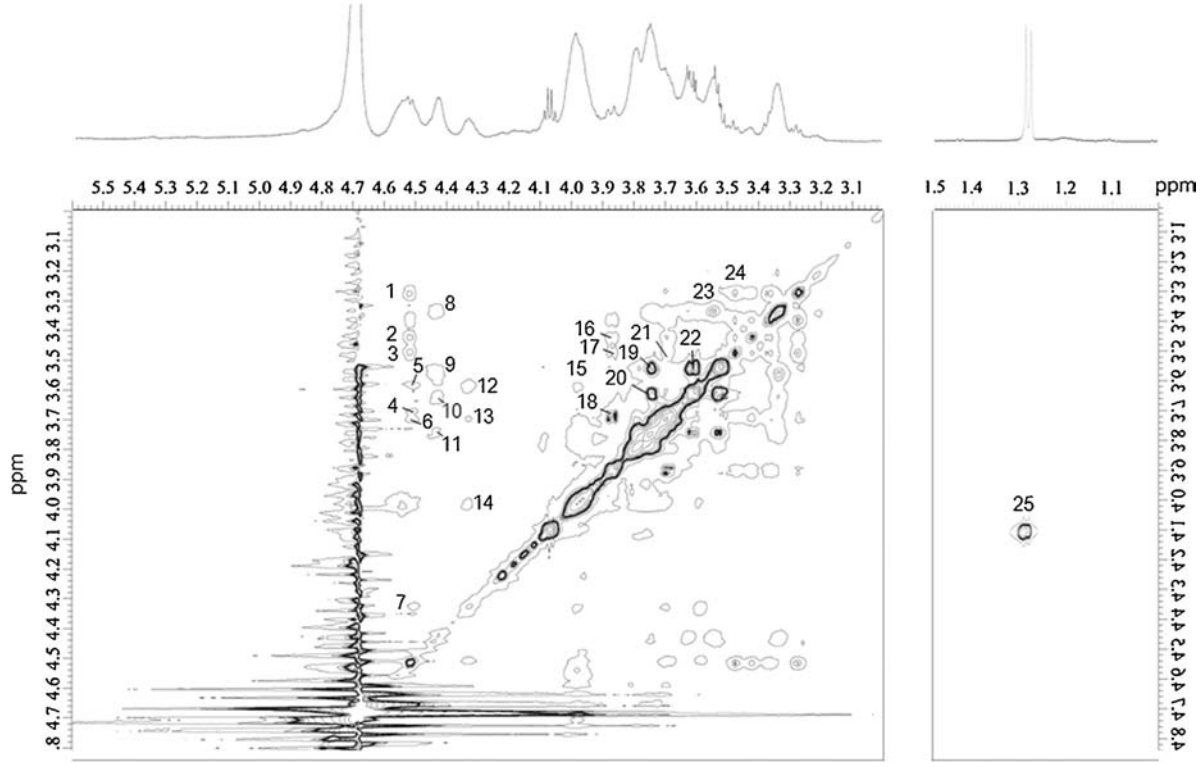

Fig. 5. Two-dimensional TOCSY spectrum of octopus CS (Fr. 4) recorded in D_2O at 25 °C. The cross-peaks are assigned as: (1) GalNAc H-1/H-2; (2) GalNAc H-1/H-6; (3) GalNAc H-1/H-3; (4) GalNAc H-1/H-5; (5) GlcA3S H-1/H-2; (6) GlcA3S H-1/H-5; (7) GlcA3S H-1/H-3; (8) GlcAH-1/H-2; (9) GlcA H-1/H-3; (10) GlcA H-1/H-5; (11) GlcA H-1/H-4; (12) GlcA3S H-2/H-3; (13) GlcA3S H-3/H-5; (14) GlcA3S H-3/H-4; (15) GlcA3S H-2/H-4; (16) GalNAc H-4/H-6; (17) GalNAc H-3/H-4; (18) GalNAc H-4/H-5; (19) GlcA H-3/H-4; (20) GlcA H-4/H-5; (21) GalNAc H-3/H-5; (22) GlcA H-3/H-5; (23) GlcA H-2/H-3; (24) GalNAc H-2/H-3; (25) Fuc H-5/H-6.

of CS oligosaccharides under the standard incubation conditions (Habuchi, Yamagata, & Suzuki, 1971; Seno, Akiyama, & Anno, 1974). As shown in Fig. 1C, ΔDi-4S was completely desulfated by chondro-4-sulfatase resulting in an increased intensity of the ΔDi-0S peak. Furthermore, an unidentified peak eluting at 30 min was observed following chondro-4-sulfatase treatment but not following chondro-6-sulfatase treatment. These results suggest that the unidentified peak was the tetrasaccharide $\Delta^{4,5}$HexA-GalNAc (4S)-GlcA (3S)-GalNAc (4S).

We had previously reported that the IdoA and B-type units as well as A- and C-type units were found in clusters in shark CS/DS (Higashi et al., 2015). Based on this experience, we examined whether or not oligosaccharides containing multiple consecutive K-type units were observed after Chase ACII treatment. The depolymerized crude GAG fraction was subjected to polyacrylamide gel electrophoresis (PAGE) and then, alcian blue staining was performed. Unlike the case of the repeating DS units found in shark CS/DS (Higashi et al., 2015), no oligosaccharides containing consecutive repeating K-type units were observed in octopus CS (data not shown). These results suggest that the K-type units occur randomly dispersed through octopus CS.

The crude GAGs (approximately 5 mg) from octopus cartilage were further fractionated by anion-exchange chromatography, and each fraction was collected, desalted, lyophilized and total amounts of unsaturated disaccharides obtained by Chase ABC treatment are shown in Fig. 2A. The chromatogram of unsaturated disaccharides of Fr. 4 in Fig. 2A is shown in Fig. 2B. The disaccharide composition of octopus CS was 4.9% of ΔDi-0S, 76.9% of ΔDi-4S, 4.2% of ΔDi-diS_E and 13.9% of GalNAc (4S) (K-type units). The molecular weight was also determined by gel permeation chromatography, and octopus CS (Fr. 4) had an average molecular weight of 67.2 kDa (data not shown).

The HS content of the crude GAGs was also determined and the unsaturated disaccharide composition of HS was ΔUA-GlcNAc (9.9%), ΔUA-GlcNS (12.2%), ΔUA-GlcNS6S (42.3%), ΔUA2S-GlcNS (16.2%), and ΔUA2S-GlcNS6S (19.4%). Taken together, these data show that the crude GAGs from octopus cartilage contained CS (19.2%) and HS (0.5%).

3.2. NMR analysis of chondroitin sulfate (Fr. 4)

The structure of octopus CS (Fr. 4) was investigated by 600 MHz ^{1}H NMR spectroscopy (Fig. 3A). The signals at 2.0 ppm and 3.34 ppm can be readily assigned to the *N*-acetyl methyl signal of GalNAc and H-2 of GlcA, respectively. The H-3 of GlcA(3S) was also assigned to a signal at 4.33 ppm, as the *O*-sulfo group results in a downfield shift of proton signals on the adjacent carbon atom by 0.4–0.8 ppm (Kitagawa et al., 1997).

Most of the chemical shifts of protons of the octopus CS could be assigned using correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY) (Figs. 4A and 5). Since cross-peaks of H-1/H-2, H-2/H-3, H-3/H-4, H-4/H-5 signals corresponding to GlcA(3S) residues were reliably assigned, confirming the presence of K-type units in octopus CS.

Kitagawa et al. reported that CS-K from king crab cartilage was fucosylated (Kitagawa et al., 1997) and the H-6 methyl proton signal was clearly observed around 1.2–1.3 ppm. Additionally, the one-dimensional ^{1}H NMR spectrum of a partially depolymerized holothurian glycosaminoglycan (DHG), a fucosyl chondroitin sulfate from sea cucumber (Imanari, Washio, Huang, Toyoda, Suzuki, & Toida, 1999), showing fucose-dependent signals, served as positive controls (Fig. 3B). Significant fucose signals, corresponding to the anomeric H-1 (5.64 and 5.29 ppm), H-4 (4.86 and 4.76 ppm) and H-6 methyl protons (1.3 ppm) signals, are clearly observed in

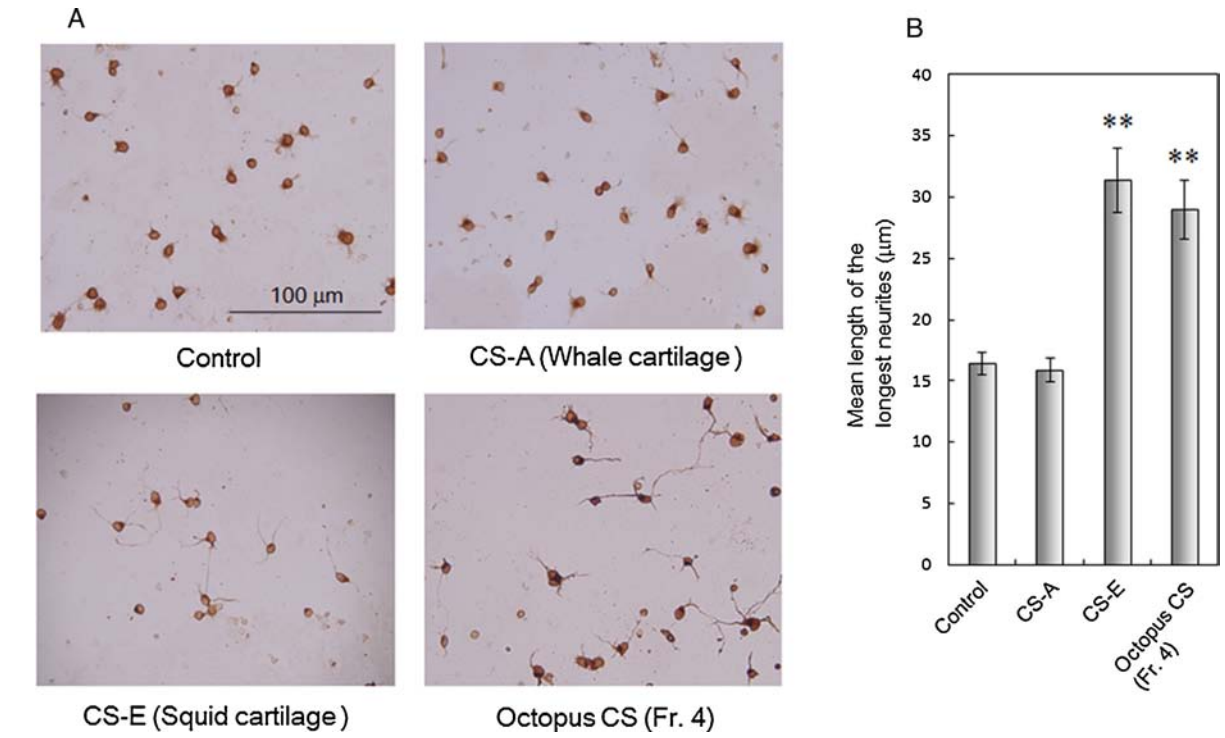

Fig. 6. Effect of octopus CS (Fr. 4) on neurite outgrowth of hippocampal neurons. (A) Representative morphological features of E16 hippocampal neurons cultured with octopus CS. E16 hippocampal neuronal cells (16,000 cells/cm^2) were cultured for 18 h on various substrates coated on poly-DL-ornithine, fixed and immunostained as described under Section 2. (B) The mean length of the longest neurite was measured for more than 50 randomly selected neurons cultured on various substrates (see Section 2). The values obtained from six independent experiments are expressed as the mean ± SE. Mann–Whitney's *U* test was used to evaluate the significance of differences between means ($^{**}p < 0.01$).

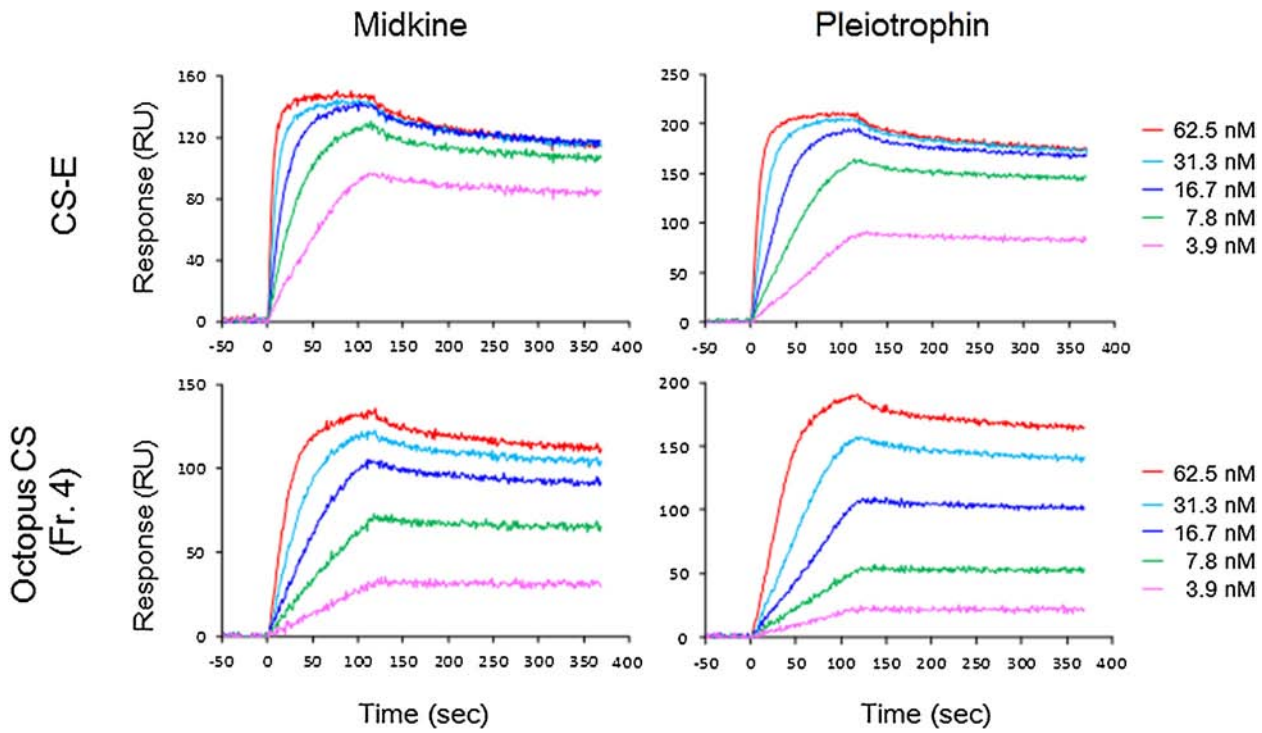

Fig. 7. Binding of octopus CS (Fr. 4) to immobilize to growth factors. Various concentrations of octopus CS and squid CS-E (Seikagaku Corp., Tokyo, Japan) were injected onto the surface of a pleiotrophin- or midkine-immobilized sensor tip. Sensorgrams obtained with various concentrations of octopus CS were evaluated by ProteOn manager ver3 using 1:1 Langmuir binding model. RU, resonance units.

Table 1
Kinetic parameters for the interaction of octopus CS-K with immobilized pleiotrophin and midkine.

Ligand	Analyte	Kinetic parameter		
		K_a (1/Ms)	K_d (1/s)	K_D (nM)
Midkine	CS-E (squid cartilage)	3.97×10^6	6.93×10^{-4}	0.17
	CS-K (*E. dofleini* Fr. 4)	1.34×10^6	6.12×10^{-4}	0.46
Pleiotrophin	CS-E (squid cartilage)	4.09×10^6	6.19×10^{-4}	0.15
	CS-K (*E. dofleini* Fr. 4)	2.39×10^6	7.35×10^{-4}	0.31

sea cucumber CS. Furthermore, cross-peaks between H-1/H-2, H-2/H-3, H-3/H-4, H-4/H-5 signals of fucose residues in DHG are also observed (Fig. 4B). Since the signals around 1.2–1.3 ppm that are observed might correspond to the H-6 methyl proton of fucose residues (Fig. 3A), we tried to assign the cross-peaks corresponding to fucose residues using COSY and TOCSY experiments for octopus CS (Figs. 4A and 5). The signal intensity of cross-peaks between H-6 methyl protons and H-5 signal are very low and none of the other expected cross-peaks are observed (Fig. 4A). While, TOCSY experiments showed clear cross-peaks between H-6 methyl protons and H-5 signal of fucose residues, our inability to observe any other fucose ring protons suggests that proportion of fucosylated disaccharide units in octopus CS was extremely low (Fig. 5).

3.3. Effect of CS-K derived from octopus cartilage on neurite outgrowth

It is known that CS-K from king crab cartilage exhibits neurite outgrowth-promoting (NOP) activity toward E16 embryonic mouse hippocampal neurons (Fongmoon et al., 2007). Based on this observation, we examined the effect of octopus CS (Fr. 4) on neurite outgrowth of E16 mouse hippocampal neurons (Fig. 6). Hippocampal neurons were cultured with octopus CS-K, CS-E (a positive control) and CS-A (a negative control) that were each immobilized onto coverslips pre-coated with the poly-DL-ornithine. Both CS-E and octopus CS-K stimulated neurite outgrowth when compared with the negative control, CS-A.

Additionally, to confirm the effects of CS on neurite outgrowth, we subsequently analyzed the interaction between octopus CS and growth/neurotrophic factors expressed in the brain during embryonic development using surface plasmon resonance (SPR) (Fig. 7). Various concentrations of octopus CS-K and squid cartilage CS-E were next evaluated by SPR as analytes on the surface of a sensor chip coated with pleiotrophin or midkine to determine the association and dissociation rate constants (k_a and k_d) as well as the dissociation equilibrium constants (K_d). The K_d values for midkine and pleiotrophin of octopus CS-K are 0.46 and 0.31 nM, respectively (Table 1). These results demonstrate that affinities of octopus CS-K toward these growth factors are comparable to those of squid CS-E.

4. Conclusions

There are significant levels of K-type units in CS isolated from octopus cartilage (*E. dofleini*). The disaccharide composition of octopus CS was 4.9% of ΔDi-0S, 76.9% of ΔDi-4S, 4.22% of ΔDi-diS_E and 13.9% of GalNAc (4S) (K-type units). One difference between octopus CS-K and previously reported king crab CS-K is that the composition of the octopus CS-K disaccharide units substituted with fucose residues is very low. Additionally, the K_d values, of the octopus CS-K binding to several growth factors, were quite similar to those of squid CS-E as demonstrated by SPR experiments. These results demonstrate that octopus cartilage represents a rich source of CS-K useful for future investigations of growth factor-mediated biological activities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2015.07.082.

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