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Isolation and partial characterization of fucan sulfates from the body wall of sea cucumber *Stichopus japonicus* and their ability to inhibit osteoclastogenesis

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Abstract—Two types of fucan sulfate were isolated from chloroform/methanol extract of the body wall of the sea cucumber *Stichopus japonicus*. One type (type A) contained 3.41 mmol fucose/g and 2.35 mmol sulfate/g, and the molecular mass was determined to be 9 kDa by gel permeation chromatography (GPC). Structural analysis suggested that type A consists of a backbone of (1 → 3)-linked fucosyl residues that are substituted at C-4 with fucosyl residues, and that fucosyl residues are sulfated at C-2 and/or C-4. Another type (type B) contained 3.90 mmol fucose/g and 3.07 mmol sulfate/g, and the molecular mass was determined to be 32 kDa by GPC. Structural analysis showed that type B is largely composed of unbranched (1 → 3)-linked fucosyl residues, and that sulfate substitution(s) occur at C-2 and/or C-4. The potential of both types to inhibit osteoclastogenesis was examined by an in vitro assay system, showing that both types of fucan sulfate inhibit osteoclastogenesis more than 95% at 50 μg/mL concentration. These results suggest that types A and B fucan sulfate from sea cucumber are potent inhibitors of osteoclastogenesis.

Keywords: Fucan sulfate; Methylation analysis; NMR spectroscopy; Osteoclastogenesis; Sea cucumber body wall

1. Introduction

Fucan sulfates, which are commonly called fucoidans, have been isolated mainly from brown algae, including *Fucus vesiculosus*,[1–3] *Ascophyllum nodosum*,[2] *Sargassum stenophyllum*,[4] *Cladosiphon okamuranus*,[5] *Chorda filum*,[6] and *Ecklonia kurome*,[7] etc. They consist of (1 → 3)- and/or (1 → 4)-linked fucosyl linear backbones[1,2,5,6] that are partially substituted at C-2 and/or C-4 with fucosyl[1,6,7] and GlcA[5] residues. In some cases, seaweed fucan sulfates contain Gal, Glc, and Man residues as minor components.[8] Furthermore, a peculiar GlcN-containing fucan sulfate was isolated from *F. vesiculosus*.[8] It is noteworthy that these fucan sulfates are sulfated heterogeneously according to the sources from which they are prepared.[1–8] Recently, fucan sulfates were isolated from marine invertebrates and characterized.[9] These animal fucan sulfates consist of (1 → 3)- and/or (1 → 4)-linked fucosyl linear backbones that are partially substituted at C-2 and/or C-4 with sulfate groups.[9] In terms of biological effects on mammalian cells, fucan sulfates are involved in inhibition of sperm–egg interaction,[10,11] reduction of virus infection,[12] inhibition of cell–cell binding mediated by P- or L-selectin,[13] enhancement of anticoagulant activity,[9,14] and inhibition of fibroblast

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proliferation,[15] etc. However, the effect of fucan sulfate on osteoclastogenesis has not been reported. In the present study, we isolated two types of fucan sulfate from chloroform/methanol extract of the body wall of the sea cucumber *Stichopus japonicus*. Partial characterization of these two types was performed by methylation analysis, chemical composition analysis and NMR spectroscopy. In vitro assay of the present fucan sulfates revealed that both types strongly inhibited osteoclastogenesis. This is the first report on the inhibitory effect of fucan sulfate on osteoclastogenesis.

2. Results and discussion

2.1. Isolation and partial characterization of two types of fucan sulfate

We previously reported that chloroform/methanol extraction prior to purification of sea cucumber glycosaminoglycan can be used to isolate acidic polysaccharides such as fucan sulfate.[16] In the present study, we isolated two types of fucan sulfate from the chloroform/methanol extract of the body wall of the sea cucumber *S. japonicus*. In previous studies, fucan sulfates were isolated from water extracts of brown algae.[4–7] Similarly, a fucan sulfate from the body wall of the sea cucumber *Ludwigothurea grisea* was extracted in the aqueous phase.[17] It is therefore of interest to compare the organic solvent-extracted fucan sulfate with those which have been water extracted.

Figure 1 shows the elution profile of crude fucan sulfate on a Sepharose CL-6B column. Anthrone-positive fractions indicated by bold bar were collected, followed by further purification by a DEAE-cellulose column chromatography. By using a linear gradient of NaCl, two major anthrone-positive peaks were eluted, as shown in Figure 2. The faster eluting peak was designated type A fucan sulfate, and the slower was designated type B fucan sulfate. The chemical compositions of types A and B were determined (Table 1). One neutral sugar residue of type A contained 0.69 sulfate on average, while that of type B contained 0.79 sulfate on average. This difference in sulfate content explains the good separation between types A and B on DEAE-cellulose column chromatography (Fig. 2). The molecular masses of types A and B are 9 and 32 kDa, respectively, as determined by gel permeation chromatography (GPC) (Fig. 3).

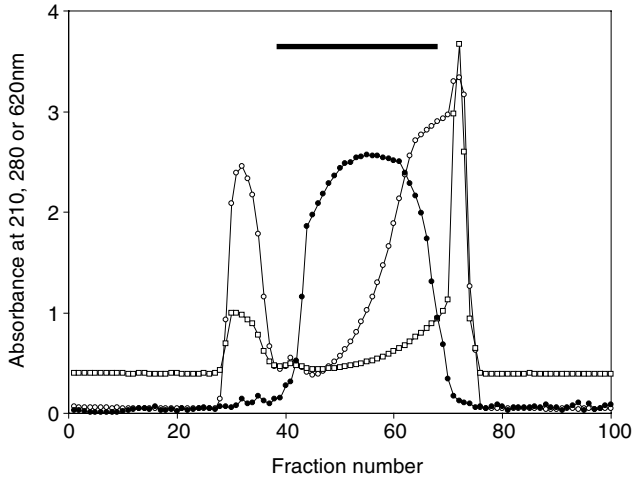

Figure 1. Elution profile of crude fucan sulfate on Sepharose CL-6B column chromatography. A portion (1 g) of the crude fucan sulfate was dissolved in 7 mL of 0.2 M NaCl and applied to the column. Elution was performed with 0.2 M NaCl and the fractions were monitored by anthrone method (●) together with spectrophotometry at 210 nm (○) or 280 nm (□).

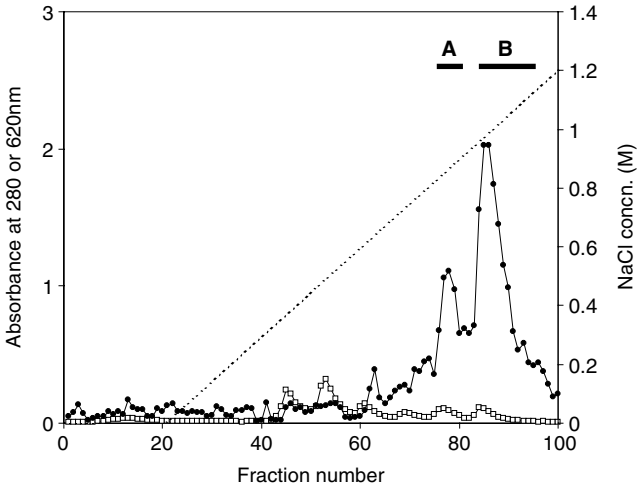

Figure 2. Elution profile of partially purified fucan sulfate on DEAE-cellulose column chromatography. A portion (100 mg) of gel filtration-purified fucan sulfate was dissolved in 50 mL of 100 mM sodium acetate (pH 5.0) and applied to the column. Elution was performed with linear gradient of NaCl up to 1.2 M and the fractions were monitored by anthrone method (●) together with spectrophotometry at 280 nm (□).

The glycosyl composition analysis of types A and B showed that both types consisted of L-fucose and a trace amount of D-Gal and D-Glc. These results show that types A and B are fucan sulfate. Although a small amount of uronic acid was detected in both types A and B, hexosamine was not found in either type A or B (Table 1).

Types A and B fucan sulfate were desulfated by the method of Nagasawa et al.[18,19] to give the desulfated types A and B with degrees of desulfation of 91% and 93%, respectively. Intact and desulfated types A and B were subjected to methylation analysis to determine the linkage positions of fucose branches and sulfate groups. In Table 2 are shown the molar ratios of various partially methylated alditol acetates (PMAAs) based on the peak area. Glycosyl linkage analysis of the desulfated type A fucan sulfate gave 2,3,4-tri-*O*-methyl-fucitol (T-Fuc), 2,4-di-*O*-methyl-fucitol (3-Fuc), and 2-*O*-methyl-

Table 1. Chemical compositions of two types of fucan sulfate (types A and B) from the body wall of the sea cucumber *Stichopus japonicus*

Fraction	Sulfate (mmol/g)	Hexosamine[a] (mmol/g)	Uronate[b] (mmol/g)	Neutral sugar[c] (mmol/g)
Type A	2.35	N.D.[e]	0.08	3.41
	(0.69)[d]	(0.00)	(0.02)	(1.00)
Type B	3.07	N.D.	0.12	3.90
	(0.79)	(0.00)	(0.03)	(1.00)

[a] D-Galactosamine was used as the standard.
[b] D-Glucuronolactone was used as the standard.
[c] L-Fucose was used as the standard.
[d] Numbers in parentheses represent molar ratios relative to neutral sugar.
[e] Not detected.

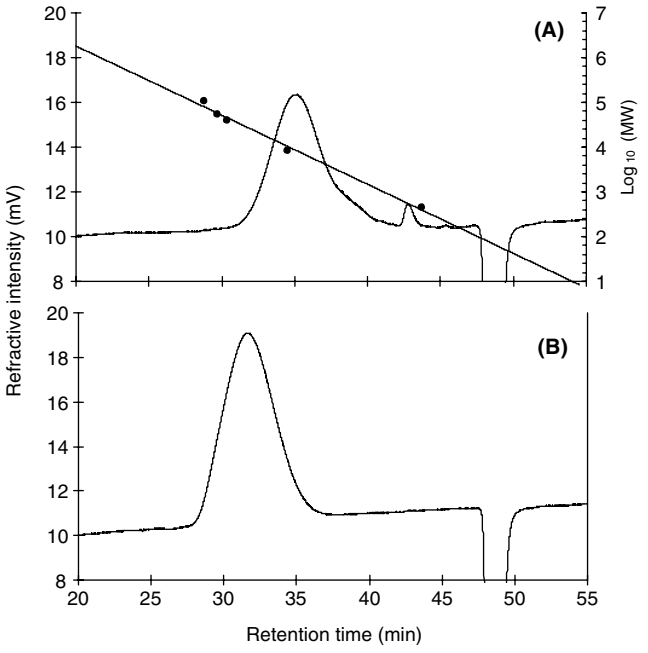

Figure 3. Elution profiles of types A and B fucan sulfate on gel permeation HPLC equipped with a series of TSK-gel G-4000, G-3000, and G-2500 PW_{XL} columns. Peak-maximum retention times of type A (A) and type B (B) were measured and compared with the molecular mass standard curve as shown in (A), in order to estimate their molecular masses.

fucitol (3,4-Fuc) residues, suggesting that the fucan sulfate has a $(1\rightarrow3)$-linked backbone together with fucose branches at C-4. The intact type A gave eight PMAAs, indicating that the type A fucan sulfate was highly sulfated. The presence of 3,4-di-*O*-methyl-fucitol (2-Fuc), 2,3-di-*O*-methyl-fucitol (4-Fuc), and 3-*O*-methyl-fucitol (2,4-Fuc), in addition to T-Fuc, indicated that terminal fucosyl residues are sulfated at C-2, or C-4, or C-2 and C-4. The presence of 4-*O*-methyl-fucitol (2,3-Fuc), 2-*O*-methyl-fucitol (3,4-Fuc), and fucitol (2,3,4-Fuc) indicated that $(1\rightarrow3)$-linked residues were sulfated at C-2, or C-4, or C-2 and C-4.

The desulfated type B gave T-Fuc (5%) and 3-Fuc (95%), showing that type B is largely linear. Glycosyl linkage analysis of the desulfated and intact type B fucan sulfates indicated that the sulfation occurred at C-2, or C-4, or C-2 and C-4 of a $(1\rightarrow3)$-linked fucan chain.

^{1}H NMR spectra of fucans A and B are shown in Figure 4A and B. They include resonances characteristic of sulfated α-fucans[20] such as narrow doublets from anomeric protons (H-1) between 5.0 and 5.6 ppm; signals from H-4 of 4-O-sulfated residues at 4.8–4.9 ppm; ring protons (H-2–H-5) between 3.6 and 4.7 ppm and prominent signals from the methyl protons H-6, one at about 1.37 ppm (minor) and a major envelope of signals at around 1.3 ppm. The signal from HOD at 4.42 ppm is reduced by presaturation; a sharp peak in the spectrum of fucan sulfate B at 1.9 ppm arises from a low molecular weight impurity. Broad signals between 1.5 and 3.5 ppm, and at about 0.8 ppm, can be attributed to a small proportion of peptide material in the samples These are particularly noticeable in fucan sulfate A (Fig. 4A).

Partial assignments of the spectrum were made by means of COSY and TOCSY spectra. It was apparent that the NMR spectra for fucans A and B did not resemble those of regular sulfated fucans identified in echinoderms, but were complex, like those of fucoidans from brown algae.[9] At least 13 separate spin systems attributable to α-fucose residues were distinguishable in the spectrum of fucan A, and these are listed in Table 3 and Figure 4C and D. The same spin systems were identifiable in the spectra of fucan B, with some of the minor contributors in lower proportions.

Expansions of the COSY spectrum in Figure 5A and B show cross-peaks between anomeric protons, H-1, and H-2 for all spin systems listed in Table 3. They can be divided into two groups; Figure 5A includes H-1–H-2 cross-peaks for 2-O-sulfated fucose residues, and Figure 5B shows H-1–H-2 cross-peaks for fucose residues not 2-O-sulfated. Positions of sulfation can be determined by the strong, down-field shift of protons at sulfated positions.[20] Using the TOCSY spectrum (data not shown) assignments can be extended for several of the spin systems, and where H-4 can be assigned it is possible to determine whether the corresponding residue is sulfated at C-4 or not. H-5 and H-6 are difficult to determine from COSY and TOCSY spectra due to the small value of $J_{H\text{-}4,H\text{-}5}$ for fucose, and assignments of H-6

Table 2. Methylation analysis of two types of fucan sulfate (types A and B) from the body wall of the sea cucumber *Stichopus japonicus* before and after desulfation reaction

PMAA	Type A		Type B	
	Intact (mol%)	Desulfated (mol%)	Intact (mol%)	Desulfated (mol%)
T-Fuc[a]	13.2	31.8	4.2	4.8
2-Fuc[b]	8.2	—	—	—
3-Fuc[c]	8.1	33.5	26.5	95.2
4-Fuc[d]	10.7	—	—	—
2,3-Fuc[e]	20.5	—	17.4	—
2,4-Fuc[f]	8.5	—	—	—
3,4-Fuc[g]	24.5	34.7	20.8	—
2,3,4-Fuc[h]	6.3	—	31.1	—

[a] 2,3,4-Tri-*O*-methyl-fucitol.
[b] 3,4-Di-*O*-methyl-fucitol.
[c] 2,4-Di-*O*-Methyl-fucitol.
[d] 2,3-Di-*O*-Methyl-fucitol.
[e] 4-*O*-Methyl-fucitol.
[f] 3-*O*-Methyl-fucitol.
[g] 2-*O*-Methyl-fucitol.
[h] Fucitol.

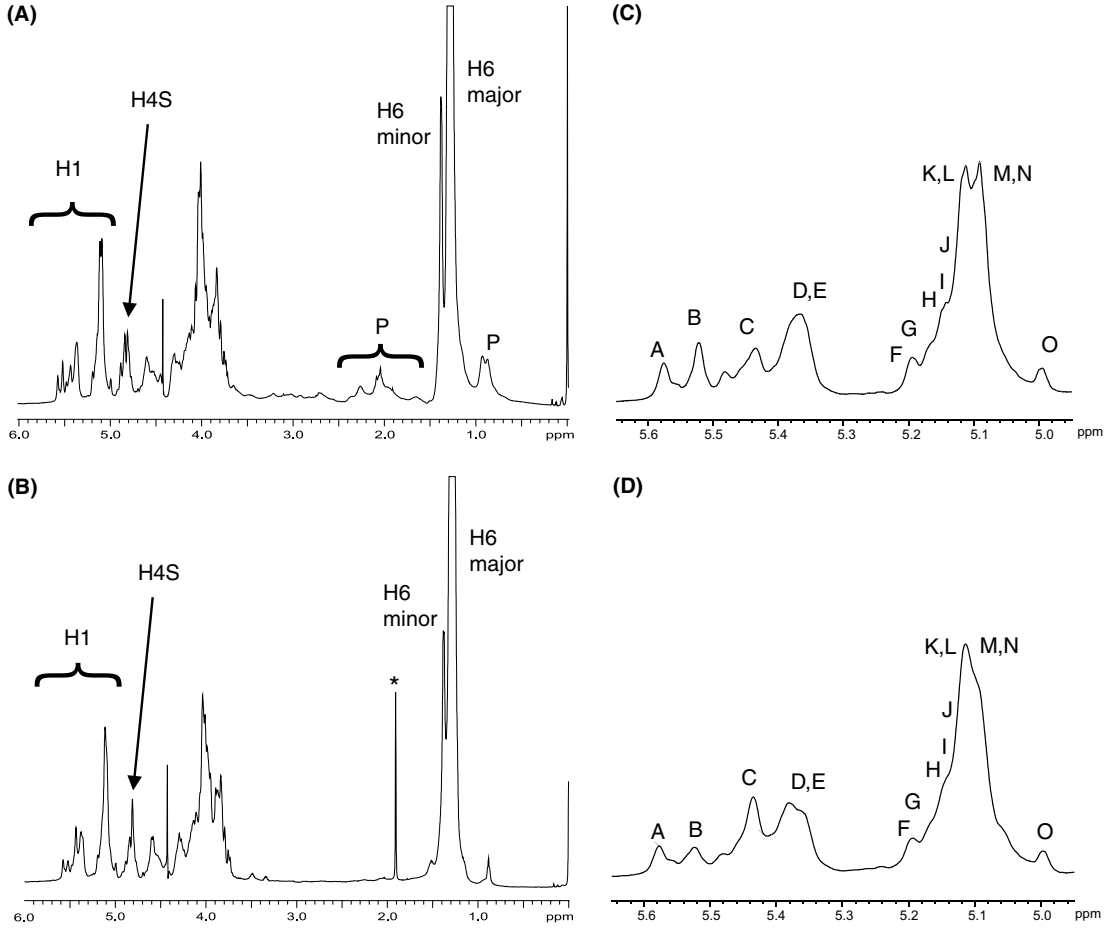

Figure 4. One-dimensional ^{1}H NMR spectra at 500 MHz of (A) fucan A and (B) fucan B. Well-resolved signals from anomeric protons (H-1), protons at sulfated C4 (H-4S), methyl protons at C6 (H-6 major and minor), and signals from peptides (P) are marked. Expansions of the spectra (C: fucan A and D: fucan B) showing anomeric (H-1) signals are labeled in accord with Table 3.

are based on H-4–H-6 cross-peaks in the NOESY spectrum (data not shown). Assignments of H-5 may be made by COSY cross-peaks from H-6, but in this case only the residues with H-6 at 1.37 ppm can be assigned.

Table 3. ^{1}H NMR of fucan sulfate A

Spin system	H-1	H-2	H-3	H-4	H-5	H-6
A	5.58	**4.54**	4.20	**4.81**	N.D.[a]	1.29
B	5.53	**4.65**	4.30	**4.90**	4.49	1.37
C	5.45	**4.60**	4.28	**4.85**	N.D.	N.D.
D	5.39	**4.58**	4.16	N.D.	N.D.	N.D.
E	5.38	**4.54**	4.34	N.D.	N.D.	N.D.
F	5.20	4.02	3.80	N.D.	N.D.	N.D.
G	5.18	3.99	4.11	**4.85**	N.D.	N.D.
H	5.16	3.85	4.05	N.D.	N.D.	N.D.
I	5.15	3.75	3.85	3.81	N.D.	1.29
J	5.14	3.91	N.D.	N.D.	N.D.	N.D.
K	5.12	4.01	4.18	**4.85**	N.D.	N.D.
L	5.10	3.99	4.22	**4.87**	N.D.	1.29
M	5.10	3.93	4.02	N.D.	N.D.	N.D.
N	5.10	3.83	4.01	N.D.	N.D.	N.D.
O	5.01	3.86	4.03	N.D.	4.36	1.37[b]

Sulfated positions are indicated in bold type.
[a]Not determined.
[b]Tentative assignment based on NOESY.

NMR spectroscopy gives results consistent with both fucans A and B being largely 3-linked, and sulfated at either or both of the 2- and 4-positions. This agrees well with the results of methylation analysis. The residues with H-6 signals at about 1.37 may be 4-linked,[20] and a prominent cross-peak in the NOESY spectrum (data not shown) links H-1 of D with H-1 of H, indicating a 2-linkage between these two residues. These signals are found in both fucans A and B, indicating that fucan B is not a wholly linear, 3-linked polysaccharide. This result is not completely in accord with the results of methylation analysis of desulfated fucan B; it may be that under the conditions of desulfation, 4- and 2-linked branches were lost from a 3-linked backbone. Fucan B has a similar spectrum to fucan A, but some minor residues are less prominent, and three residues, all account for a high proportion of the NMR intensity: residue C, both 2- and 4-sulfated, corresponds with 2,3,4-Fuc (Table 2); residue L, 4-sulfated, corresponds to 3,4-Fuc, and residue M, not 2-sulfated (possibly 3-Fuc in Table 2), are also prominent in the spectrum of fucan B.

All the above experimental evidence is consistent with the presence of a 3-linked fucan backbone with 2- and 4-linked branches, for both fucans A and B, and this is the most likely structure (Fig. 6) though conclusive proof that 2- and 4-linkages are not part of the main chain is lacking. Neither fucan has a regular repeating structure, as found for fucans extracted from other echinoderm species.[9] None of the regular fucans described so far have had a branched structure, or have had variable linkage positions within the main chain. Though the heterogeneity and complexity of these fucans from *S. japonicus* is comparable with that of fucoidans from brown algae, methylation analysis and NMR spectroscopy show that the fine structure is different from any algal fucoidan so far described.[1,4–7]

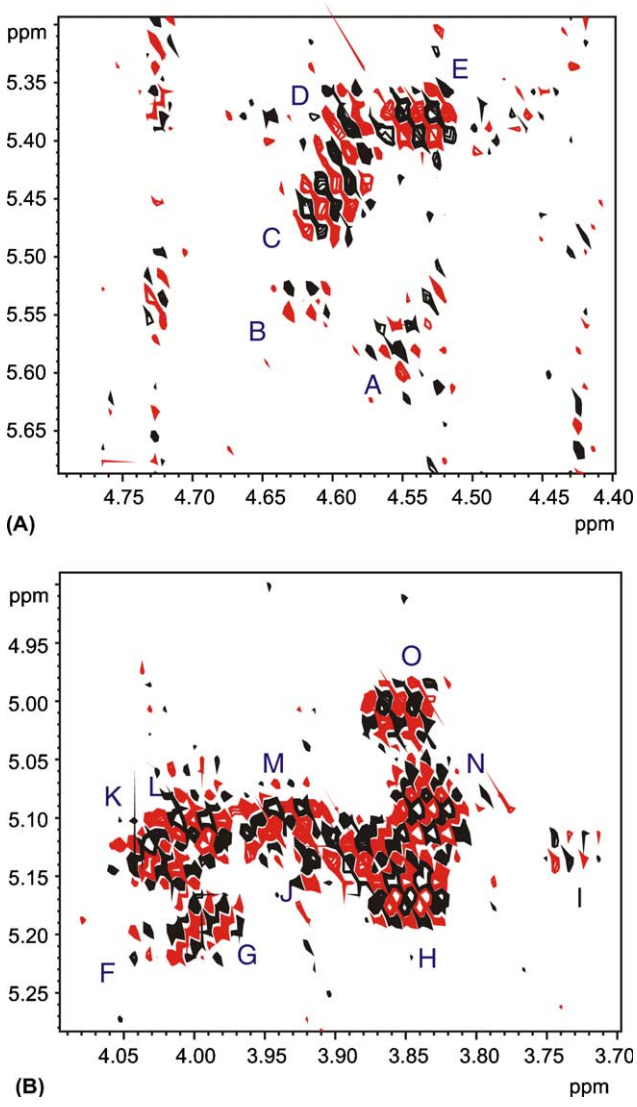

Figure 5. Expansions of two-dimensional COSY spectra of fucan A showing H-1–H-2 cross-peaks from 2-sulfated residues (A) and from nonsulfated residues (B) by which the separate spin systems listed in Table 3 are differentiated.

2.2. Activities of two types of fucan sulfate to inhibit osteoclastogenesis

Figure 7 shows the inhibitory effects of types A and B fucan sulfate on an in vitro osteoclast-formation assay. In the absence of fucan sulfate samples (control), the average number of osteoclast-like cells generated was 171.8. In contrast, the average numbers of osteoclast-like cells generated in the presence of types A and B decreased to 0.3 and 6.3, respectively. Both specimens of fucan sulfate used were shown to exhibit no cytotoxicity (data not shown). These results indicate that the presence of fucan sulfates strongly hindered the formation of osteoclast-like cells in the culture medium. From this viewpoint, types A and B inhibited 99.8% and 96.3% of osteoclast-like cell formation, respectively, as taking the

A 3-linked backbone of α-L-fucose:

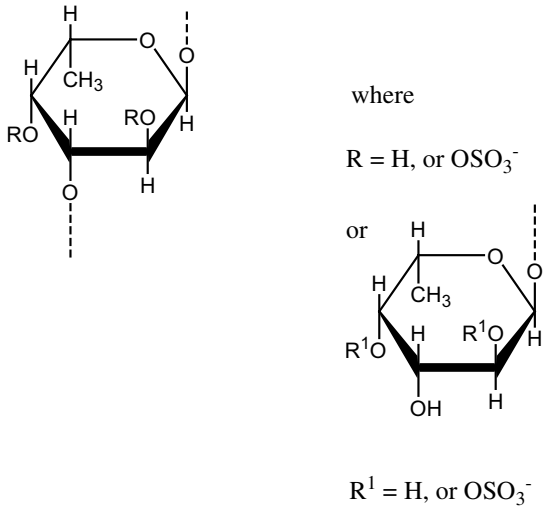

Figure 6. Hypothetical structures of type A and type B. Both fucans A and B probably consist of a backbone of (1 → 3)-linked fucose residues substituted with fucosyl residues at C-2 and C-4. Sulfate substitution(s) occur at C-2 and/or C-4 position(s).

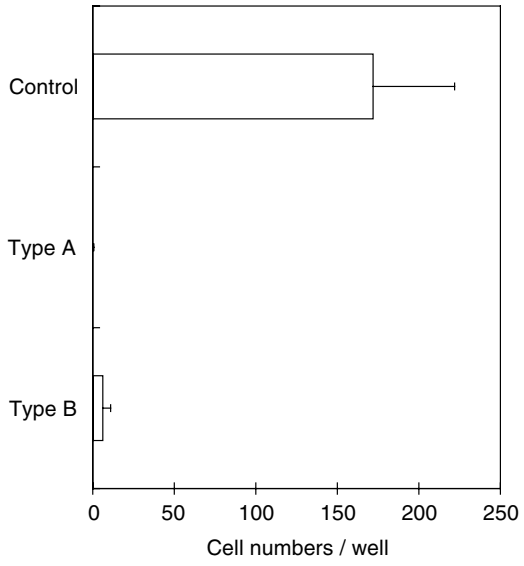

Figure 7. Inhibitory effects of types A and B fucan sulfate on an in vitro osteoclast-formation assay system. Data were expressed as mean ± SD ($n = 3$). Types A and B inhibited 99.8% and 96.3% of osteoclast-like cell formation, respectively, as taking the control as 0%.

control as 0%. When the inhibition potential of types A and B were compared, that of type A was slightly higher than that of type B, although the difference was not significant. However, it is possible that this difference in the inhibition potential of types A and B would reflect the difference in the structures of types A and B.

Homeostasis of mammalian bone tissue is maintained upon the equilibrium of two dynamic processes of resorption by osteoclast[21,22] and formation by osteoblast.[23] Once this equilibrium is disturbed, various bone disorders such as rheumatoid arthritis (RA)[24] and osteoporosis[25] are induced. In the case of RA and osteoporosis, resorption of bone tissue by osteoclast is more accelerated than formation of bone tissue by osteoblast. Accordingly, potent inhibitors of bone resorption by osteoclast might possess the potential to alleviate some symptoms of RA and osteoporosis, and the potential may lead to the production of new drugs for the treatments of such diseases.

3. Experimental

3.1. Materials

Live specimens of a sea cucumber *S. japonicus* (about 400 g in body weight) were purchased at the Tokyo Central Wholesale Market and immediately transported to the Central Research Laboratories of Seikagaku Corporation. The dorsal portion of the body wall was dissected, desalted by running tap water, and used for preparation of fucan sulfates in the following experiments.

3.2. Isolation of fucan sulfate from the body wall of the sea cucumber

Desalted sea cucumber body wall (ca. 10 kg) was minced and homogenized with a Masco MKZ B-10-20 grinder. To the homogenate was added two volumes of chloroform/methanol (2:1, v/v), and the mixture was blended using a Nihonseiki AM-1 blender and filtered. After repeating this procedure one more time, both filtrates were combined and evaporated to dryness. Distilled water (300 mL) was added, and the suspension was allowed to stand at 4 °C for 16 h. The suspension was filtered to remove suspended lipid components. Three volumes of ice-cold ethanol was added to the filtrate, followed by centrifugation at 5000*g* for 15 min. The resulting pellet was dried under reduced pressure and pulverized. The resulting material (ca. 2.5 g) was used as 'crude fucan sulfate' in the following experiment.

A portion (1 g) of the crude fucan sulfate was dissolved in 7 mL of 0.2 M NaCl and applied to a Sepharose CL-6B column (∅ 3.4 × 110 cm) equilibrated with 0.2 M NaCl. Elution was performed with the same solvent at a flow rate of 20 mL/h. The eluent was monitored by spectrophotometry at 210 or 280 nm, and neutral sugar was quantitated by the anthrone method.[26] Fucan sulfate fractions (shown by bold bar in Fig. 1) were pooled, dialyzed exhaustively against distilled water, and lyophilized (135.4 mg). A portion (100 mg) of this powder was dissolved in 50 mL of 100 mM sodium acetate (pH 5.0), and applied to a DEAE-cellulose column (∅ 2.3 × 18 cm) equilibrated with 100 mM sodium acetate (pH 5.0). After washing the column with 100 mL

of 100 mM sodium acetate (pH 5.0), elution was performed by a linear gradient using 200 mL each of 100 mM sodium acetate (pH 5.0) with and without 1.2 M NaCl. Fractions (5 mL) were collected and neutral sugars were determined by the anthrone method. Two peaks, peak A (Fraction number 75–80; type A) and peak B (Fraction number 82–89; type B), were pooled (Fig. 2), dialyzed exhaustively against distilled water and lyophilized, respectively. The yields of types A and B fucan sulfate were 14.2 and 33.6 mg, respectively.

3.3. Chemical composition analysis of two types of fucan sulfate

Neutral sugar and uronic acid contents were determined by the anthrone method[26] using authentic L-fucose as the standard and by the carbazole method[27] using D-glucuronolactone as the standard, respectively. Hexosamine content was determined essentially according to the previous method,[28] using authentic D-galactosamine as the standard. Sulfate content was determined as previously reported.[29]

3.4. Glycosyl composition analysis of two types of fucan sulfate

Glycosyl composition analysis was performed according to the method of Yasuno et al.[30] using authentic Gal, Man, Glc, Ara, Rib, ManNAc, Xyl, GlcNAc, Fuc, Rha, and GalNAc as the standards.

3.5. Measurement of molecular masses of two types of fucan sulfate

For calibration of molecular masses, five kinds of molecular mass markers were used: hyaluronic acid [HA] of 104 kDa: chondroitin sulfate Cs [CS-Cs] of 52.2 kDa, 39.1 kDa, 8.05 kDa; and, ΔDiHS-(U, 6)S [ΔUA(2S)(1→4)GalNAc(6S) of 458 Da]. These were applied to a Tosoh CCPM HPLC equipped with a series of TSK-gel G-4000, G-3000, and G-2500 PW_{XL} columns (∅ 7.5×300 mm each) using 0.2 M NaCl at 40 °C and a flow rate of 0.6 mL/min, and the eluent was monitored by refractometry. The molecular masses of the standard HA and CS-Cs were determined by light scattering.[31] Peak-maximum retention times of types A and B fucan sulfate were measured and compared with the molecular mass standard curve to estimate their molecular masses.

3.6. Desulfation of two types of fucan sulfate

Chemical desulfation of two types of fucan sulfate was performed according to the method of Nagasawa et al.[18,19] The degree of desulfation was estimated by ion chromatography.[29]

3.7. Estimation of glycosyl linkage position(s) and sulfate substitution pattern of two types of fucan sulfate by methylation analysis

To evaluate the glycosyl linkage position(s) and sulfate substitution pattern of two types of fucan sulfate, intact and desulfated fucan sulfates were subjected to per-O-methylation by the modification of Sandfold and Conrad[32] of the method of Hakomori,[33] and per-O-methylated polysaccharides were purified as described by Waeghe et al.[34] The methylated polysaccharides were hydrolyzed with 2 M TFA at 121 °C for 1 h, and reduced with sodium borodeuteride, and then acetylated with Ac_2O. The resulting PMAAs were analyzed by GC–MS with a JEOL JMS-DX303HF mass spectrometer (Tokyo, Japan) equipped with a Supelco SP2330 capillary column (0.25 mm×30 m) in the splitless mode. The column was programmed to run at 50 °C for 2 min and then to 170 °C at 30 °C/min. Column temperature was further raised to 235 °C at 4 °C/min and held for 15 min.

3.8. NMR spectroscopy

NMR spectra were recorded using a Varian 500 MHz INOVA spectrometer. Samples of fucan sulfates A and B (about 10 mg each) were lyophilized twice from 98% D_2O for deuterium exchange, then taken up in 0.7 mL of 99.9% D_2O and transferred to a 5 mm NMR tube. All spectra were recorded at 60 °C; chemical shifts are quoted relative to trimethylsilylpropionic acid-d_4 (TSP) at 0 ppm. Two-dimensional spectra (double quantum filtered COSY, TOCSY, HSQC, and NOESY), were recorded using pulse sequences supplied by the spectrometer manufacturer.

3.9. Activities of two types of fucan sulfate to inhibit osteoclastogenesis

Tibia and femur were aseptically excised from 6-weeks old ddY mice, being free of adhering tissues. After removing the terminal portions of the both bones, the marrow cavity was flushed with alpha minimal essential medium (α-MEM) (Gibco Co., Grand Island, NY) by injecting it into one end of the bone using a sterile needle. Bone marrow cell suspension thus obtained was subjected to centrifugation at 250*g* for 5 min. The cell pellets were dispersed in adequate amount of α-MEM containing 10% heat-inactivated fetal calf serum (HIFCS), attaining the concentration of 5×10^7 cells/mL. The cell suspension was shaken gently and then uniformly divided into 100 μL portions. Each portion was put into each well of a 24-well microtiter plate, followed by the addition of 350 μL of α-MEM containing 10% HIFCS and 50 μL of 500 μg/mL fucan sulfate to be tested. Thus, final concentration of cells and fucan sulfates were maintained at 5×10^6 cells/well and 50 μg/mL,

respectively. After addition of 1 μM prostaglandin E_2 (PGE_2), culture was initiated at 37 °C in 5% CO_2. During the culture, 350 μL of medium in each well was replaced by 350 μL of new medium (300 μL of α-MEM containing 10% HIFCS and 1.67 μM PGE_2 together with 50 μL of 500 μg/mL fucan sulfate) at least two times. After 7 days culture, tartrate-resistant acid phosphatase (TRAP), which is indicative of osteoclast-like cells generated, was stained with a commercial kit (Sigma, St. Louis, MO). By means of light microscopy at 100-fold magnification, the number of osteoclast-like cells in each well was measured. Osteoclast-like cells were defined as TRAP-positive multinucleated cell containing more than three nuclei.

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