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Structure of fucose branches in the glycosaminoglycan from the body wall of the sea cucumber *Stichopus japonicus*

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Abstract

Fucose-branched chondroitin sulfate E was prepared from the body wall of sea cucumber *Stichopus japonicus*. The purified glycosaminoglycan (GAG) was chemically desulfated, followed by carboxyl reduction. Intact, desulfated, and desulfated/carboxyl-reduced GAG fractions were subjected to per-*O*-methylation. GC–MS analyses of the resultant partially methylated alditol acetates demonstrated that the fucose branch is formed by two fucopyranosyl residues linked glycosidically through position (1 → 3), and that the fucose branch and glucuronic acid are almost equimolar. In addition, it was elucidated that about 20% of the branches stretch from O-3 position of a glucuronic acid moiety of the core chondroitin sulfate polymer, while remaining fucose branches are postulated to protrude from O-4 and/or O-6

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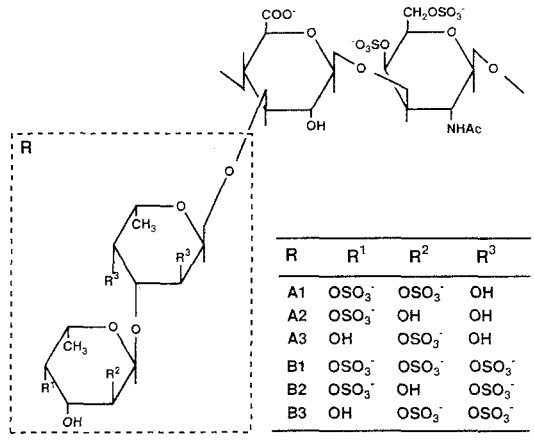
position(s) of a *N*-acetylgalactosamine moiety. This fucose branch was also confirmed to be highly sulfated according to six kinds of substitution pattern in methylation analysis.



R	R^1	R^2	R^3
A1	OSO_3^-	OSO_3^-	OH
A2	OSO_3^-	OH	OH
A3	OH	OSO_3^-	OH
B1	OSO_3^-	OSO_3^-	OSO_3^-
B2	OSO_3^-	OH	OSO_3^-
B3	OH	OSO_3^-	OSO_3^-

Keywords: Fucose branch; Glycosaminoglycan; Methylation analysis; Sea cucumber; Sulfate substitution

1. Introduction

The body wall of sea cucumber is easily and greatly hardened by heat, acid treatment, or even by handling stimulation under physiological conditions [1,2]. The last phenomenon is called "connective tissue catch" [3–10], which is specific not only to holothuroids but also to other classes of echinodermata. In self separation (autotomy), on the other hand, the body wall of sea cucumber is specifically softened and melted [7]. Chemical [8] and rheological [9,10] analyses of the body wall have not provided any clues about the mechanism involved. It has been argued that the glycosaminoglycan (GAG) of the sea cucumber (*Stichopus japonicus*) body wall is involved in the cation-dependent change of its connective tissue toughness [11,12]. In addition, it has been shown that the specific viscosity of the GAG solution was affected differently depending on the valency and species of cation. Interestingly, the mode of viscosity change of this GAG solution was quite different from those reported for known GAGs, such as chondroitin sulfate and dermatan sulfate [13,14]. Ultracentrifugal analysis of the sea cucumber GAG also demonstrated that its configuration is altered from an extended to a flexible form with the increased concentrations of cation, accompanying aggregation of the GAG molecule [15]. Furthermore, the sea cucumber GAG is not digested by both chondroitinase ABC (E.C. 4.2.2.4) and AC (E.C. 4.2.2.5), although in addition to neutral sugar (fucose) the GAG has a sugar backbone identical to chondroitin sulfate which contains glucuronic acid (GlcA) and *N*-acetylgalactosamine (GalNAc) [15].

Such unique physicochemical properties of the sea cucumber GAG seem to be related to its unusual structure. In this aspect, Vieira and Mourão [16] aimed to elucidate the precise structure of sea cucumber GAG. They succeeded in enzymatic digestion of GAG from the species of sea cucumber *Ludwigothurea grisea* with chondroitinases after mild acid treatment, finding a sulfated fucose branch stretching from O-3 position of a GlcA moiety of the core chondroitin sulfate polymer. Furthermore, it has recently been revealed that this fucose-branched chondroitin sulfate possesses an affinity for calcium ions five times greater than that of authentic chondroitin sulfate [17,18]. We also demonstrated the occurrence of a fucose-branched structure in the GAG from the body wall of the sea cucumber *S. japonicus*, using different procedures [19]. However, the linkage of the fucose branch of our GAG was not characterized.

In the present study, the purified sea cucumber GAG from *S. japonicus* was desulfated and carboxyl reduced. Each fraction was per-*O*-methylated and partially methylated alditol acetates were analyzed by

GC–MS. With the data obtained, the detailed structures of fucose branches were elucidated, which are different from those described by others [16,20].

2. Experimental

Preparation and purification of sea cucumber GAG.—Preparation and purification of GAG from the body wall of the sea cucumber *S. japonicus* were performed by a slight modification of the method described previously [19]. Briefly, sea cucumber body wall was minced and homogenized. After $CHCl_3$/MeOH (2:1, v/v) treatment, the mixture was filtered. The resulting residue was dried and autoclaved at 120 °C for 30 min. To the autoclaved residue was added 30 volumes of 50 mM phosphate buffer (pH 8.0) containing Actinase (E.C. 3.4.24.4) at a concentration of 50 mg/g protein, and the mixture allowed to stand at 55 °C for 8 h. The residue was treated with 0.4 M NaOH, followed by 10% trichloroacetic acid treatment. The resulting mixture was centrifuged and the supernatant was exhaustively dialyzed against running tap water. To the supernatant obtained by centrifugation of the dialyzate was added three volumes of ice-cold ethanol containing 2.5% sodium acetate. The formed precipitate of GAG was collected by centrifugation and washed with ten volumes of ice-cold ethanol. The resulting pellet was then dried under reduced pressure and pulverized (crude GAG). This preparation was purified by a combination of gel filtration with a Sephadex G-100 column and ion-exchange chromatography with a DEAE-cellulose column. The separated fractions were monitored for GlcA and neutral sugar by the carbazole method [21] and anthrone method [22], respectively. Finally, GAG fractions were combined, dialyzed exhaustively against distilled water, and lyophilized. The yield of sea cucumber GAG thus purified was about 500 mg from 1.5 g of crude GAG.

Desulfation of sea cucumber GAG.—Chemical desulfation was performed, essentially according to Nagasawa et al. [23,24]. Briefly, 55 mg of purified GAG was applied to a Dowex 50 W-X12 column equilibrated with distilled water and eluted isocratically with distilled water. Fractions obtained were monitored for cation-free material by pH measurement. Acidic pH fractions were combined and the pH of the solution was adjusted to 9.0 with pyridine, followed by lyophilization. Pyridinium salt of the GAG thus obtained (59.8 mg) was dissolved in 5 mL of DMSO/MeOH (9:1). This reaction mixture was kept at 80 °C for 5 h, followed by cooling at room temperature. After addition of 5 mL of distilled water, the pH of the reaction mixture was adjusted to 9.0 with 1 M NaOH. This solution was subjected to dialysis against running tap water for two days, and subsequently against distilled water for one day. Then the dialyzate was lyophilized. The yield of desulfated material was 24.2 mg. The desulfation degree was estimated according to our earlier report using ion chromatography [19].

Carboxyl reduction of desulfated sea cucumber GAG.—Carboxyl reduction was performed, essentially according to Taylor and Conrad [25] and Karamanos et al. [26]. Briefly, 10 mg of desulfated GAG was dissolved in 1 ml of distilled water, followed by addition of 10 mg of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). This solution was incubated at room temperature for 1 h under constant pH condition (about 4.8) by the continuous addition of 0.04 M HCl, resulting in the activation of carboxyl group by EDC. Then, to the reaction mixture was added freshly prepared 1 mL of 2 M sodium borohydride twice during the next 1.5 h at 50° C. The reaction was terminated by the addition of glacial acetic acid, and the reaction mixture was dialyzed against running tap water for two days, and subsequently against distilled water for one day. The dialyzate was then lyophilized and 7.1 mg of carboxyl-reduced material was obtained. The reduction degree was estimated by the difference of colour intensity developed by the carbazole method [21] before and after the reaction.

Per-O-methylation and GC–MS analysis of sea cucumber GAG fractions.—Per-*O*-methylation was performed by the modification by Sandford and Conrad [27] of the method of Hakomori [28], and per-*O*-methylated oligosaccharides and glycosyl alditols (partially methylated alditol acetates, PMAAs) were purified as described by Waeghe et al. [29]. Briefly, 1 mg each of sea cucumber GAG fractions was per-*O*-methylated, followed by purification with a SepPak C-18 cartridge. The methylated polysaccharides were hydrolyzed with 2.0 M TFA at 121 °C for 1 h, and reduced with sodium borodeuteride, whereas resulting alditols were acetylated with acetic anhydride. The resulting PMAAs were analyzed by GC–MS with a Shimadzu QP5000 type mass spectrometer equipped with a Supelco SP2330 capillary column (Ø 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.

^1H-1D NMR spectroscopy.—The spectrum was recorded with a JEOL JNM-EX 400 spectrometer at 70 °C, using 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid, sodium salt as internal standard.

3. Results and discussion

Intact, desulfated, and desulfated/carboxyl-reduced fractions were prepared and subjected to methylation analysis, in order to clarify the linkage manner and distribution of fucose branches together with substitution patterns of sulfate groups to the fucose branch. The degree of desulfation of the sea cucumber GAG and the carboxyl-reduced GAG fractions were about 80% and 85%, respectively. The degree of carboxyl-reduction of the desulfated/carboxyl-reduced sea cucumber GAG was about 70% with reference to the desulfated GAG fraction.

Fig. 1 shows the gas–liquid chromatograms of PMAAs derived from intact (Fig. 1a), desulfated (Fig. 1b) and desulfated/carboxyl-reduced (Fig. 1c) sea cucumber GAG fractions on GC–MS analysis. Peaks observed were identified with corresponding mass spectra and with reference to the data reported for another sea cucumber GAG [16]. In the case of intact GAG, five specimens of PMAA appeared; i.e., 2,4-di-*O*-methyl-fucitol (peak 2), 2,3-di-*O*-methyl-fucitol (peak 3), 3,4-di-*O*-methyl-fucitol (peak 4), 3-*O*-methyl-fucitol (peak 5) and fucitol (peak 6). On the other hand, the chromatogram of PMAAs from the desulfated GAG showed a quite different pattern

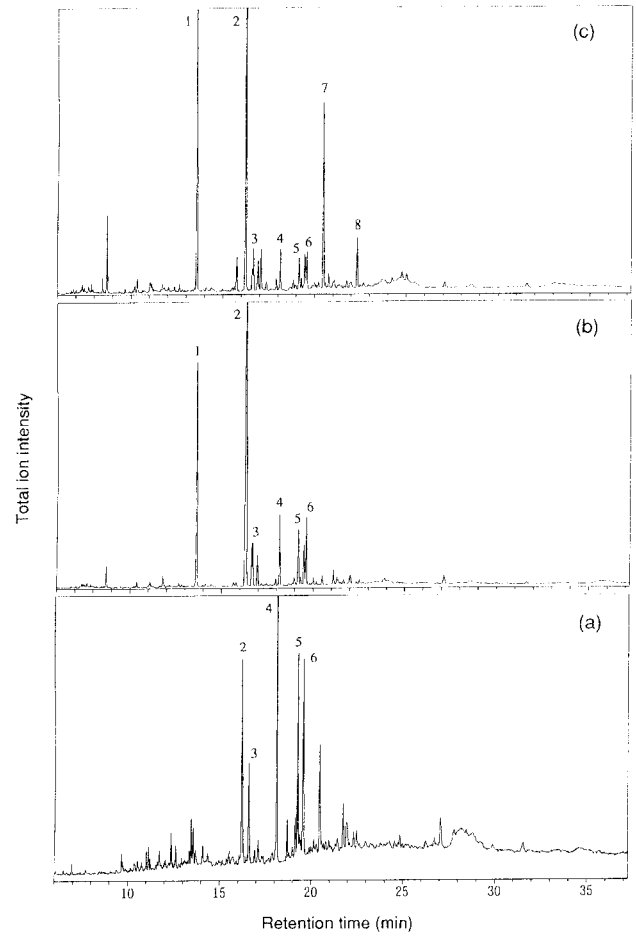


Fig. 1. Chromatograms of partially methylated alditol acetates derived from intact (a), desulfated (b), and desulfated/carboxyl-reduced (c) sea cucumber GAG on GC–MS equipped with a SP2330 capillary column.

Table 1
Methylation analyses of intact, desulfated, and desulfated/carboxyl-reduced fractions prepared from the sea cucumber *S. japonicus* GAG

Peak	PMAA [a]	t_R [b]	Molar ratio [c]		
			Intact	Desulfated	Desulfated/carboxyl-reduced
1	2,3,4-Fuc	1.00	N.D. [d]	0.21 (21)	0.21 (21)
2	2,4-Fuc	1.19	0.16 (16) [e]	0.45 (45)	0.25 (25)
3	2,3-Fuc	1.22	0.08 (8)	0.03 (3)	0.04 (4)
4	3,4-Fuc	1.33	0.23 (23)	0.07 (7)	0.03 (3)
5	3-Fuc	1.41	0.17 (17)	0.05 (5)	0.03 (3)
6	Fuc	1.43	0.18 (18)	0.04 (4)	0.04 (4)
7	2,3,6-Glc	1.50	N.D.	N.D.	0.15 (15)
8	2,6-Glc	1.64	N.D.	N.D.	0.04 (4)

[a] Partially methylated alditol acetate with methoxy groups at the positions shown.
[b] Retention time on a SP2330 capillary column relative to 2,3,4-tri-*O*-methyl-fucitol.
[c] The molar ratios were based on the peak area.
[d] Not detected.
[e] Numbers in parentheses represent percentage distribution of PMAAs.

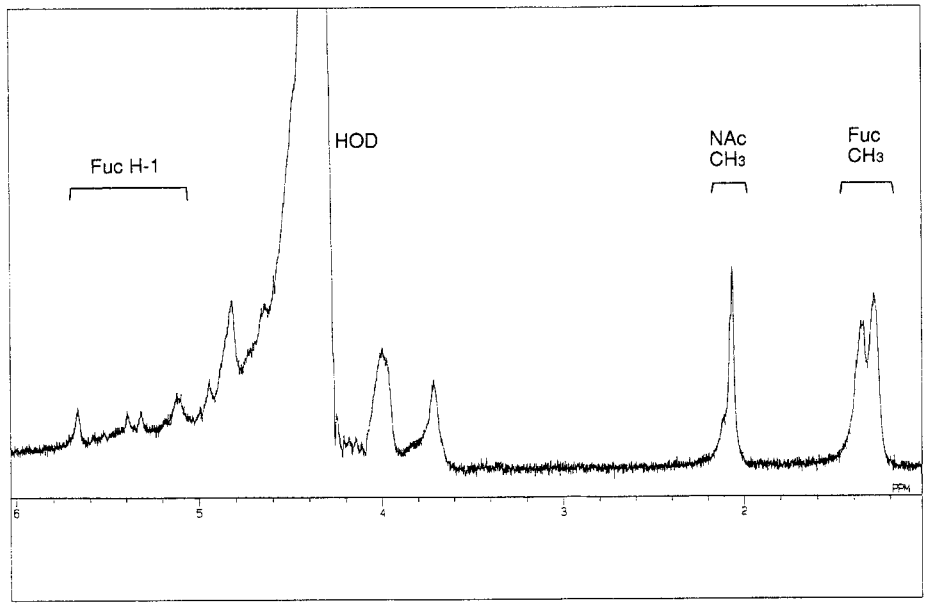


Fig. 2. A 400-MHz ^{1}H-1D NMR spectrum of intact sea cucumber GAG in D_2O at 70 °C.

from that of intact GAG-derived PMAAs. Namely, 2,3,4-tri-*O*-methyl-fucitol (peak 1), which was not observed for intact GAG, appeared and the quantities of 2,4-di-*O*-methyl-fucitol (peak 2) slightly increased, while those of peaks 3, 4, 5 and 6 markedly decreased. When desulfated/carboxyl-reduced GAG-derived PMAAs were analyzed, 2,3,6-tri-*O*-methyl-glucitol (peak 7) and 2,6-di-*O*-methyl-glucitol (peak 8) newly appeared, while the composition of peaks 1–6 was very similar to that of desulfated GAG-derived PMAAs.

In Table 1 are shown the molar ratios of various PMAAs based on the peak area. In desulfated/carboxyl-reduced GAG-derived PMAAs, 2,3,4-tri-*O*-methyl-fucitol and 2,4-di-*O*-methyl-fucitol accounted for 21% and 25%, respectively. These data indicate that the fucose branch of intact sea cucumber GAG contains two fucopyranosyl residues linked glycosidically through position (1 → 3). The presence of 2,6-di-*O*-methyl-glucitol indicates that some fucose branches are linked glycosidically through the O-3 position of a GlcA moiety in the intact sea cucumber GAG. Since the sum of 2,6-di-*O*-methyl-glucitol and 2,3,6-tri-*O*-methyl-glucitol accounted for 19% of total area, the true value of the sum would be calculated to be 27% taking the reduction degree (70%) into consideration. From the sum of derivatives of fucitol (peaks 1–6) (60%) and that of two derivatives of glucitol (27%), the molar ratio of fucose to GlcA is estimated to be 2.2 in desulfated/carboxyl-reduced sea cucumber GAG. This also supports a branching structure composed of two fucopyranosyl residues

Table 2
Percentage distribution of core disaccharide units (GlcA β1 → 3GalNAc derivatives) and possible branching positions [a]

	Disaccharide unit [b]				Total
	Zero-sulfated	4-*O*-Sulfated	6-*O*-Sulfated	4,6-Di-*O*-sulfated	
Percentage	11.2	10.4	56.0	22.4	100.0
GalNAc	O-4,6 [c]	O-6	O-4	–	88.8 [d]
GlcA	O-3 [c]	O-3	O-3	O-3	21.1 [e]

[a] Data were partially quoted from [19].
[b] Disaccharide units were classified according to sulfation patterns of GalNAc moiety.
[c] Possible fucose-branching positions in GalNAc moiety or GlcA moiety.
[d] Percentage of maximum substitution of fucose to GalNAc moiety, calculated as in the following equation:
$11.2 \times 2 + 10.4 + 56.0 = 88.8$.
[e] Percentage of 2,6-di-*O*-Me-Glcol compared with the sum of 2,6-di-*O*-Me-Glcol and 2,3,6-tri-*O*-Me-Glcol (see Table 1).

and that the fucose branch and GlcA are almost equimolar (1.1:1).

In order to compare the fucose contents before and after chemical modifications, a ^{1}H-1D NMR spectrum of intact sea cucumber GAG was taken (Fig. 2). The signal intensity of the methyl protons of fucose moiety observed between 1.2 and 1.5 ppm was 2.2 times greater than that of the methyl protons of GalNAc observed between 2.0 and 2.2 ppm, although both signals were rather broad possibly due to the heterogeniety of sulfate substitution. This indicates that the molar ratios of fucose to GalNAc and GlcA are both 2.2 in the intact sea cucumber GAG, since GlcA and GalNAc are equimolar in the core chondroitin sulfate. As mentioned above, the molar ratio of fucose to GlcA was estimated to be 2.2 from gas–liquid chromatogram of PMAAs derived from desulfated/carboxyl-reduced sea cucumber GAG. Accordingly, it was confirmed that removal of fucose hardly occurred during desulfation, carboxyl reduction and methylation.

Table 2 shows the percentage distribution of core disaccharide units of chondroitin sulfate and possible branching positions through core polymers. The data were partially quoted from reference [19]. Since 21.1% of the disaccharide units are fucosylated through the O-3 position of a GlcA moiety from the molar ratios of 2,6-di-*O*-methyl-glucitol (4%) and 2,3,6-tri-*O*-methyl-glucitol (15%) (Table 1), the remaining fucose branches (up to 88.8%) could be linked through O-4 and/or O-6 position(s) of a GalNAc moiety (Table 2). The sum of 21.1% and 88.8% is about 110%, corresponding to the molar ratio of fucose branch to GlcA (1.1). Fucosylation through either GlcA or GalNAc occurs at almost every disaccharide unit. If fucosylation through the O-3 position of a GlcA moiety does not occur in the disaccharide units, a GalNAc moiety is fucosylated through O-4 and/or O-6 position(s). Thus, it is probable that almost every disaccharide unit of the core GAG polymer contains the fucose branch linked either through GlcA moiety (about 20%) or through GalNAc moiety (about 80%). It has been demonstrated that about 22% of disaccharide units of the core GAG polymer is of the E-type structure of chondroitin sulfate ([19], Table 2). The core disaccharide unit bearing 4,6-di-*O*-sulfate in a GalNAc moiety (E-type) possibly contains the fucose branch linked glycosidically through O-3 position of a GlcA moiety.

In Fig. 3a are shown hypothetical structures of the fucose branch in the GAG from the body wall of sea cucumber *S. japonicus* through a GlcA moiety. The branch structures (R) are depicted in two types, A and B, depending on the sulfation of an inner fucopyranose (R^3). Because the two fucopyranosyl residues are linked glycosidically through position (1 → 3) in the branch structure, an inner fucopyranose will only produce the two types of PMAA, 2,4-di-*O*-methyl fucitol and fucitol. Accordingly, an outer fucopyranose will have three types of PMAA, 3-*O*-methyl-fucitol, 2,3-di-*O*-methyl-fucitol and 3,4-di-*O*-methyl-fucitol. Sulfate substitution patterns were actually classified into six types as shown in Fig. 3a.

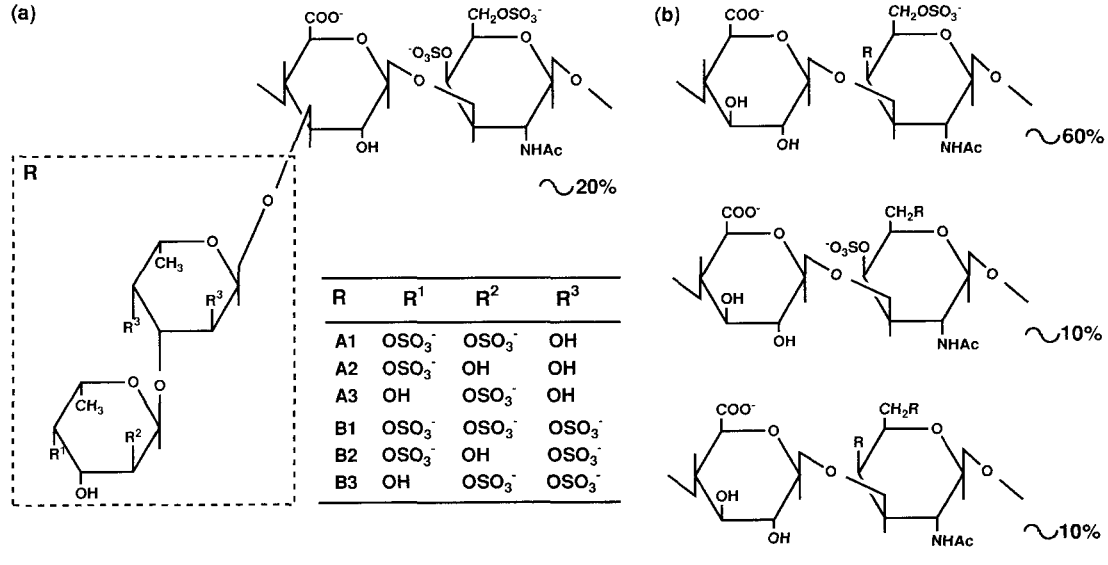


Fig. 3. Hypothetical structures for fucose branches stretching from a core chondroitin sulfate unit of E-type (a) and from other core disaccharide units (b) of the sea cucumber GAG.

The sulfate content of this fucose branch structure was estimated to be about 2.5 for one fucose branch on an average from the percentage distribution of intact GAG-derived PMAAs (Table 1). Fig. 3b shows the structures and percentage distribution of mono- and zero-sulfated core disaccharide units containing fucose branch(es) through the GalNAc moiety.

Vieira and Mourão [16] demonstrated the presence of two fucopyranosyl residues linked glycosidically through position (1 → 4) and/or (1 → 2) for the fucose branch in the GAG from the sea cucumber *L. grisea*. The two fucopyranosyl residues of the fucose branch in the present study were linked (1 → 3) only. In addition, about 20% of total fucose branches were linked glycosidically through the O-3 position of a GlcA moiety (the remaining 80–90% were assumed to be linked through GalNAc).

References

[1] J. Uexküll, *Pflüger Arch.*, 212 (1962) 1–14.
[2] S.E. Buddenbrock, *Vie Milieu*, 14 (1963) 55–70.
[3] G.N. Smith and M.J. Greenberg, *Biol. Bull.*, 144 (1973) 421–436.
[4] D.L. Meyer, *Mar. Biol.*, 9 (1974) 235–241.
[5] I.C. Wilkie, *J. Zool.*, 186 (1978) 311–330.
[6] R.H. Emson and I.C. Wilkie, *Mar. Biol.*, 18 (1980) 155–250.
[7] L.C.U. Junqueira, G.S. Montes, P.A.S. Mourão, J. Corneiro, L.M.M. Salles, and S.S. Bonetti, *Rev. Can. Biol.*, 39 (1980) 157–164.
[8] T. Motokawa, *Comp. Biochem. Physiol.*, 70C (1981) 41–48.
[9] T. Motokawa, *Comp. Biochem. Physiol.*, 73C (1982) 223–229.
[10] T. Motokawa, *Proc. Jpn. Soc. Biorheol. (Tokyo)*, 5 (1982) 85–88.
[11] Y. Kariya, S. Watabe, Y. Ochiai, S. Srikantha, K. Hashimoto, and K. Murata, *Connect. Tissue (Tokyo)*, 18 (1986) 312–313.
[12] Y. Kariya, S. Watabe, Y. Ochiai, K. Murata, and K. Hashimoto, *Connect. Tissue Res.*, 25 (1990) 149–159.
[13] M.B. Mathews, *Arch. Biochem. Biophys.*, 42 (1953) 41–53.
[14] M.B. Mathews and A. Dorfman, *Arch. Biochem. Biophys.*, 43 (1953) 181–193.
[15] Y. Kariya, S. Watabe, Y. Ochiai, K. Hashimoto, and K. Murata, *Comp. Biochem. Physiol.*, 95B (1990) 387–392.
[16] R.P. Vieira and P.A.S. Mourão, *J. Biol. Chem.*, 263 (1988) 18176–18183.
[17] J. Ruggiero, R.P. Vieira, and P.A.S. Mourao, *Carbohydr. Res.*, 256 (1994) 275–287.
[18] P.A.S. Mourão and R.P. Vieira, *Trends Glycosci. Glycotechnol.*, 7 (1995) 235–246.
[19] Y. Kariya, S. Watabe, K. Hashimoto, and K. Yoshida, *J. Biol. Chem.*, 265 (1990) 5081–5085.
[20] K. Yoshida, Y. Minami, H. Nemoto, K. Numata, and E. Yamanaka, *Tetrahedron Lett.*, 33 (1992) 4959–4962.
[21] T. Bitter and H. Muir, *Anal. Biochem.*, 4 (1962) 330–334.
[22] R.L. Dimler, W.C. Schaefer, C.S. Wise, and C.E. Rist, *Anal. Chem.*, 24 (1952) 1411–1414.
[23] K. Nagasawa, Y. Inoue, and T. Tokuyasu, *J. Biochem. (Tokyo)*, 86 (1979) 1323–1329.
[24] K. Nagasawa, H. Uchiyama, and N. Wajima, *Carbohydr. Res.*, 158 (1986) 183–190.
[25] R.L. Taylor and H.E. Conrad, *Biochemistry*, 11 (1972) 1383–1388.
[26] N.K. Karamanos, A. Hjerpe, T. Tsegenidid, B. Engfeldt, and C.A. Antonopoulos, *Anal. Biochem.*, 172 (1988) 410–419.
[27] P.A. Sandford and H.E. Conrad, *Biochemistry*, 5 (1966) 1508–1516.
[28] S. Hakomori, *J. Biochem. (Tokyo)*, 55 (1964) 205–208.
[29] T.J. Waeghe, A.G. Darvil, M. Mcneil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.