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Structural characterization of fucosylated chondroitin sulfates from sea cucumbers *Apostichopus japonicus* and *Actinopyga mauritiana*

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ABSTRACT

Two samples of fucosylated chondroitin sulfate (FCS), **AJ** and **AM**, were isolated from holothurian species *Apostichopus japonicus* and *Actinopyga mauritiana*, respectively. Purification of FCS was performed by ion exchange chromatography followed by gel filtration. Structure of the biopolymers was elucidated using chemical and NMR spectroscopic methods. Both polysaccharides were shown to contain a typical chondroitin core built up of repeating disaccharide units →3)-β-D-GalNAc-(1→4)-β-D-GlcA-(1→ and decorated by sulfate groups and α-L-Fuc branches. Two polysaccharides were different in pattern of sulfation of GalNAc and fucosyl branches connected to O-3 of GlcA. The ratio of GalNAc4*S*6*S*:GalNAc4*S* for **AJ** was about 2:1, whereas for **AM** this value was approximately 1:1. **AJ** contained Fuc*p*2*S*4*S* and Fuc*p*3*S*4*S* residues linked to O-3 of GlcA in a ratio of 3:1, while for **AM** this ratio was 1:4. Small portions of Fuc*p*4*S* units attached to O-3 of GlcA were also found in both polysaccharides. Moreover, in a structure of **AM** the presence of Fuc*p*3*S* residues linked to O-6 of GalNAc were determined using the data of NMR spectra.

1. Introduction

Fucosylated chondroitin sulfates (FCS) from sea cucumbers demonstrate a wide spectrum of biological activities, such as antithrombotic, anticoagulant, anti-inflammatory, anticancer, antiviral, etc. (Pomin, 2014). These biopolymers contain the typical chondroitin core built up of the repeating disaccharide units →3)-β-D-GalNAc-(1→4)-β-D-GlcA-(1→. At the same time, in contrast to linear mammalian glycosaminoglycans of the chondroitin family, the holothurian FCS are branched due to 3-*O*-fucosylation of the most part of glucuronic acid residues. FCS from different holothurian species vary in number of branches, as well as in degree and pattern of sulfation (Chen et al., 2011; Myron, Siddiquee, & Azad, 2014). These structural variations are responsible for differences in the types and levels of biological activity of FCS (Chen et al., 2013; Pomin & Mourão, 2014). In addition to structural features, the molecular mass and its distribution may influence the biological properties of FCS (Tsukamoto, Hattori, Sakabe, Haginaka, 2001; Wu, Xu, Zhao, Kang, Ding, 2010a,b). Several interesting observations on such relationships were made using products of partial depolymerization of native FCS (Suzuki, Kitazato, Takamatsu, Saito, 1991; Wu, Xu, Zhao, Kang, Ding, 2010c; Wu, Ye, Guo, Liao, Yin et al., 2013). Therefore, comparative structural analysis of FCS of different origin is very important for elucidation of structure-activity relationships within these biopolymers. They may be used in creation of new drugs (Mourão, 2015) or more complex hybrid biomedical systems (Ananikov et al., 2015) composed of carbohydrate part attached to proteins and synthetic polymer carriers, labels, oligodentate scaffolds for further applications as biotracers, artificial antigens, vectored drugs and other instruments for glycobiology investigations.

Structural characteristics of several FCS isolated from different holothurian species may be found in the literature. The sea cucumber *Apostichopus* (*Stichopus*) *japonicus* is the most popular source of FCS. Its main structural features were described for the first time in 1992 (Yoshida, Minami, Nemoto, Numata, & Yamanaka, 1992). According to the results of several hydrolysis conditions,

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the polysaccharide was shown to contain a typical chondroitin core with α-L-Fuc*p* residues as branches. Position of these branches and sulfation pattern were deduced from the carefully interpreted NMR spectra of the polymer. It was unambiguously shown that three type of fucose residues, namely, fucose 2,4-disulfate, fucose 3,4-disulfate, and fucose 4-sulfate, occur with a molar ratio 5:3:1 and are linked to O-3 of GlcA residues, whereas GalNAc residues are sulfated at both O-4 and O-6 positions.

New data about the structure of FCS from *A. japonicus* were published in 1997 (Kariya, Watabe, Kyogashima, Ishihara, & Ishii, 1997). Based mainly on the results of methylation analysis, the authors suggested that branches may be represented by differently sulfated 3-linked fucobiose residues, and these branches may be linked not only to O-3 of GlcA, but also to O-4 and O-6 of GalNAc residues. Recently the structure of similar FCS was carefully reinvestigated with the use of chemical, enzymatic and spectroscopic methods (Yang, Wang, Jiang, & Lv, 2015a). Presence of fucobiosyl branches was not confirmed, and some differences in position of sulfate and branches, as compared with the previous evidence, were explained by different regions of collection of sea cucumbers.

Due to the presence of some controversial data on FCS from *A. japonicus*, we performed reinvestigation of its chemical structure in comparison with FCS from sea cucumber *Actinopyga mauritiana*, which was not investigated earlier.

2. Experimental procedures

2.1. General methods

Quantitative determination of monosaccharides by gas-liquid chromatography of alditol acetates (for determination of hexosamines acid hydrolysis in 6 N HCl at 100 °C for 6 h was used) and turbidimetric determination of sulfate were carried out as described previously (Bilan et al., 2002; Bilan et al., 2007). Glucuronic acid was estimated colorimetrically with 3,5-dimethylphenol (Usov, Bilan, & Klochkova, 1995). The absolute configurations of fucose (L) and galactosamine (D) were established by GLC analysis of the corresponding acetylated (*S*)-(+)-*sec*-butyl glycosides (Gerwig, Kamerling, & Vliegenthart, 1978). The D-configuration of glucuronic acid was confirmed by comparison of the chemical shifts of the signals in ^{13}C NMR spectra of **AJ** and **AM** with the respective signals of FCS described previously (Yoshida et al., 1992). Relevance of such determination was proved earlier (Shaskov, Lipkind, Knirel, & Kochetkov, 1988).

2.2. Isolation of polysaccharides

The sea cucumber *Apostichopus japonicus* was collected from the Posyet Bay of the Sea of Japan, whereas *Actinopyga mauritiana* was collected from the coastal waters of the Gilbert Islands (the latter organisms were fixed with ethanol). According to the conventional procedure (Vieira, Mulloy, & Mourão, 1991), dried and minced body walls of *A. japonicus* (197.2 g) were suspended in 2 L of 0.1 M sodium acetate buffer (pH 6.0), containing papain (6.66 g), EDTA (2.92 g), and L-cysteine hydrochloride (1.56 g), and incubated at 45–50 °C for 24 h. An aqueous hexadecyltrimetylammonium bromide solution (10%, 200 mL) was added to the filtered extract, and the mixture allowed to stand overnight. The resulting precipitate was centrifuged and washed successively with water and ethanol. A portion (about one fourth) of precipitate was stirred with 20% ethanolic NaI solution (5 × 250 mL) for 2–3 days, washed with ethanol and dissolved in water. The solution was dialyzed, filtered and lyophilized to give the crude polysaccharide preparation **SP-1**, yield 3.28 g, composition: fucose, 14.9%, galactosamine, 8.0%, uronic acids, 4.9%, glucosamine, 2.3%, galactose, 2.6%, and sulfate, 33.0%.

Table 1
Composition[a] and pattern of sulfation[b] of fucosylated chondroitin sulfates **AJ** and **AM**.

Sample	GlcA	GalNAc	Fuc	SO_3Na	Pattern of sulfation	
					GalNA*c* **B**:**C**[c]	Fuc*p*(1 → 3) **D**:**E**
AJ	15	16	13	56	~2:1	3:1
AM[d]	17	18	13	52	~1:1	1:4

[a] mol%.
[b] Determined using NMR spectroscopy.
[c] Abbreviations as in Fig. 1 and Table 2.
[d] Unusual Fuc*p*3*S* branch (**G**) linked to O-6 of GalNA*c*p (**F**) was observed. Ratio of **D**:**E**:**G** was 1:4:1.

Similarly dehydrated by ethanol, dried and minced body walls of *A. mauritiana* (52.4 g) were suspended in 0.5 L of 0.1 M sodium acetate buffer containing papain, EDTA and L-cysteine hydrochloride and incubated as above. 100 mL of hexadecyltrimetylammonium bromide solution was added to the filtered extract, the resulting precipitate was treated as above to give the crude polysaccharide preparation **SP-2**, yield 2.57 g, composition: fucose, 25.1%, galactosamine, 6.8%, uronic acids, 6.1%, glucosamine, 1.5%, galactose, 3.2%, and sulfate, 27.8%.

A solution of **SP-1** (248 mg) or **SP-2** (222 mg) in about 40–60 mL of water was placed on a column (3 × 10 cm) with DEAE-Sephacel in Cl^-–form and eluted with water, followed by NaCl solutions of increasing concentration (0.5, 0.75, 1.0 and 1.5 M), each time up to the absence of a positive reaction of eluate for carbohydrate (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). 1.0 M NaCl fractions contained FCS were chosen for further detail purification, while the investigation of other fractions would be described elsewhere. 1.0 M NaCl eluates obtained were desalted on Sephadex G-15 column. Polysaccharide fractions were concentrated to 1 mL and then subjected to gel filtration on Sephadex G-100 column. The main fractions were lyophilized giving the samples **AJ** and **AM** (32 and 29 mg, respectively). The composition of these fractions is given in Table 1.

2.3. Polyacrylamide gel electrophoresis (PAGE)

The polysaccharides **AJ**, **AM**, heparin (Sigma) and enoxaparin (Clexane®, Sanofi) (15 μg) were applied to a 0.75-mm-thick layer of 10% polyacrylamide (ICN Biochemicals), 100 mM Tris-borate, pH 8.3 gel in a buffer (10 mM Tris-borate, pH 8.3) with 10% (w/v) of glycerol. Electrophoresis was run at 400 V in a buffer (100 mM Tris-borate, pH 8.3) during 1 h. The gel was stained with 0.003% Stains-all (Merck, DE) in formamide (Sigma, EUA)- isopropanol-water (5:25:70) overnight in the dark and destained with water. The results are presented in Fig. 2.

2.4. Molecular weight estimation of polysaccharides

The polysaccharides **AJ**, **AM**, heparin (Sigma) and enoxaparin (Clexane®, Sanofi) were dissolved in aqueous 1 M NaCl to a concentration of 10 mg/mL. Gel chromatography of the samples was performed on analytical TSK2 column (Toyo Soda, Japan) 75 × 300 mm calibrated using pullulans (Fluka) at flow rate of 0.8 mL/min by elution with 1 M NaCl. The molecular weight of **AJ**, **AM**, heparin and enoxaparin were determined as 26980 Da, 26432 Da, 13649 Da and 2431 Da, respectively.

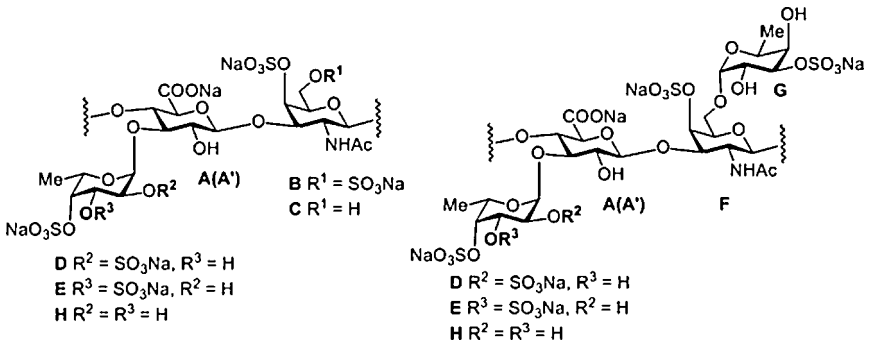

Fig. 1. Repeating blocks of fucosylated chondroitin sulfates **AJ** (constituent units **A**–**E,H**) and **AM** (constituent units **A**–**H**). Units **A** and **A'** bear Fuc2*S*4*S* (**D**) and Fuc3*S*4*S* (**E**)/Fuc4*S* (**H**) substituents, respectively, at O-3.

2.5. NMR spectroscopy

Samples (40 mg) were dissolved in 0.3 mL of imidazole-HCl buffer (90 mM, pH 7.2), freeze-dried, then dissolved in 99.9% D_2O and freeze-dried again followed by dissolution in 99.96% D_2O to a concentration about 120 mg/mL and putting into Shigemi tubes. ^1H and ^{13}C spectra were recorded using a Bruker AV-600 spectrometer at 303 K with HOD suppression by pre-saturation. COSY, HSQC, ROESY and HMBC spectra were recorded at 303 K using standard Bruker pulse sequences. Additional HMBC spectrum of **AM** was recorded at 333 K. Chemical shifts are relative to trimethylsilylpropionic acid at 0 ppm for ^1H and at −1.6 ppm for ^{13}C spectra.

3. Results and discussion

The mixture of water-soluble polysaccharides were isolated from the body walls of *Apostichopus japonicus* and *Actinopyga mauritiana* by conventional solubilization in the presence of papain added to destroy proteins (Vieira et al., 1991), followed by addition of cetyltrimethylammonium bromide to precipitate the sulfated components, which were then transformed into a water-soluble sodium salts by stirring with NaI in ethanol. Further the polysaccharide fractions were purified by anion-exchange chromatography on DEAE-Sephacel. 1.0 M NaCl eluates obtained were desalted and then subjected to gel filtration on Sephadex G-100 column. Monosaccharide content and degree of sulfation of the main fractions were consistent with the composition of putative FCS (Table 1). The main components of the polysaccharides were Fuc, GalNAc, GlcA and sulfate.

To a preliminary assessment of the molecular weight of FCS, gel electrophoresis of the samples **AJ** and **AM** was performed using sulfated polysaccharides heparin (Sigma) and enoxaparin (Clexane®, Sanofi) with defined MW as standards (Fig. 2). Based on mobility of the samples it was concluded that MW of FCS was higher than that of heparin. More accurate estimation of MW was performed by TSK gel chromatography using an appropriate analytical column calibrated with pullulans. Previously it was demonstrated that pullulans could be applied as standards for MW estimation of heparin using eluent with high ionic strength (Guo et al., 2003). As a result, the molecular weight of **AJ** and **AM** was assessed as ~27 kDa and ~26.5 kDa, respectively.

Characterization of the structure of polysaccharides **AJ** and **AM** in more details was performed using NMR spectroscopic methods. Application of two-dimensional techniques COSY, HSQC, ROESY, HMBC made it possible to assign all the signals of the major components in ^1H and ^{13}C NMR spectra of the polymers (Fig. 1, Table 2). Thus, the presence of fucose, galactosamine and uronic acid as the main monosaccharide residues was confirmed by the characteristic values of chemical shifts of C-6 for Fuc (δ 17.3 ppm) and GlcA (δ 176.3 ppm), as well as of C-2 for GalNAc (δ 52.7 ppm), in ^{13}C NMR

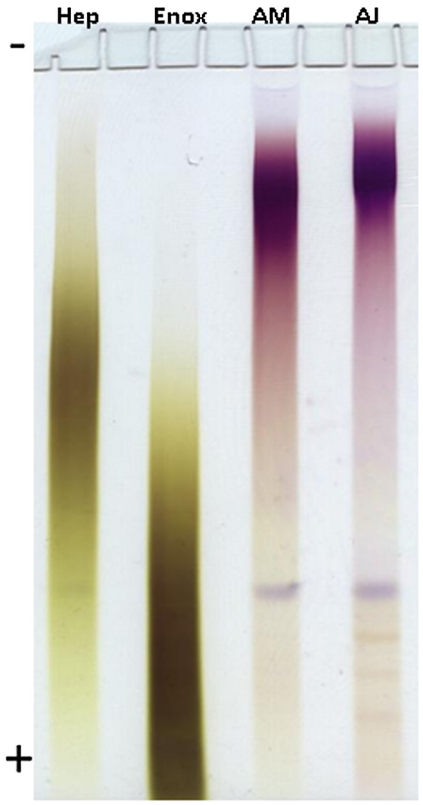

Fig. 2. Electrophoresis in polyacrylamide gel. Hep – heparin (Sigma), Enox – enoxaparin (Clexane®, Sanofi), **AM** and **AJ** – polysaccharide samples.

spectra (Fig. 3). The signals of GlcA and GalNAc were similar to those observed previously for other fucosylated chondroitin sulfates bearing →3)-β-D-GalNAc-(1→4)-β-D-GlcA-(1→ backbone (Panagos et al., 2014; Ustyuzhanina et al., 2016).

FCS from different species of sea cucumbers vary in pattern of sulfation of fucosyl residues (Panagos et al., 2014; Pomin, 2015). In the cases of **AJ** and **AM** two main types of fucosyl branches, Fuc*p*2*S*4*S* (**D**) and Fuc*p*3*S*4*S* (**E**), were observed (Fig. 4). According to the data of HSQC spectrum of **AJ**, positions of the H-1 signals responsible to units **D** and **E** were at 5.69 and 5.34 ppm, respectively (Fig. 5A). Further assessment of other signals of the resonance systems was easily performed using ^1H-^1H COSY spectrum (Fig. 5C, Table 2). Low-field position of H-2 (δ 4.48 ppm) and H-4 (δ 4.86 ppm) signals evidenced the presence of sulfate groups both at C-2 and C-4 in unit **D**. For unit **E** low-field shifts of H-3 (δ 4.53 ppm) and H-4 (δ 5.01 ppm) signals confirmed sulfation at O-3 and O-4. These data were consistent with those obtained previously

Table 2
The data of ^{1}H and ^{13}C NMR spectra of fucosylated chondroitin sulfates **AJ** (constituent units **A-E,H**) and **AM** (constituent units **A-H**) (the bold numerals indicate the positions of sulfate).

Residue	H-1 (C-1)	H-2 (C-2)	H-3 (C-3)	H-4 (C-4)	H-5 (C-5)	H-6 (C-6)
A → 4)-β-D-Glc*p*A-(1 →	4.48 (105.0)	3.61 (75.0)	3.71 (78.1)	3.93 (76.6)	3.71 (78.1)	– (176.4)
A' → 4)-β-D-Glc*p*A-(1→	4.48 (105.0)	3.60 (75.0)	3.68 (80.7)	4.00 (76.6)	3.71 (78.1)	– (176.4)
B → 3)-β-D-Gal*p*NAc4*S*6*S*-(1 →	4.58 (100.9)	4.07 (52.7)	3.95 (77.9)	**4.81** **(77.2)**	4.00 (73.2)	**4.33, 4.20** **(68.5)**
C → 3)-β-D-Gal*p*NAc4*S*-(1 →	4.58 (100.9)	4.07 (52.7)	3.95 (77.9)	**4.81** **(77.2)**	4.02 (76.2)	3.81 (62.3)
D α-L-Fuc*p*2*S*4*S*-(1 →	5.69 (97.7)	**4.48** **(76.6)**	4.17 (67.8)	**4.86** **(82.5)**	4.90 (67.5)	1.37 (17.2)
E α-L-Fuc*p*3*S*4*S*-(1 →	5.34 (100.5)	3.95 (67.6)	**4.53** **(76.6)**	**5.01** **(80.6)**	4.80 (67.6)	1.37 (17.3)
F → 3)-β-D-Gal*p*NAc4*S*-(1 →	4.58 (100.9)	4.07 (52.7)	3.95 (77.9)	**4.81** **(77.2)**	4.00 (73.2)	4.29 (68.5)
G α-L-Fuc*p*3*S*-(1 →	5.02 (101.2)	3.80 (69.9)	**4.48** **(78.3)**	4.16 (71.1)	4.19 (67.6)	1.27 (16.7)
H α-L-Fuc*p*4*S*-(1 →	5.39 (ND)	3.80 (ND)	4.03 (ND)	**4.75** (ND)	ND[a] (ND)	ND (ND)

[a] Not determined.

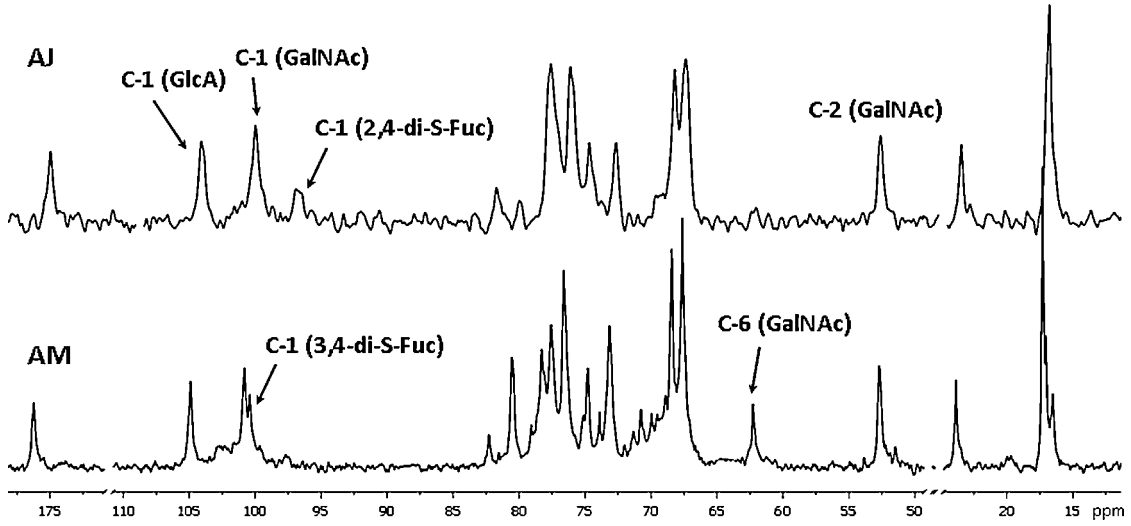

Fig. 3. ^{13}C NMR spectra of fucosylated chondroitin sulfates **AJ** and **AM**.

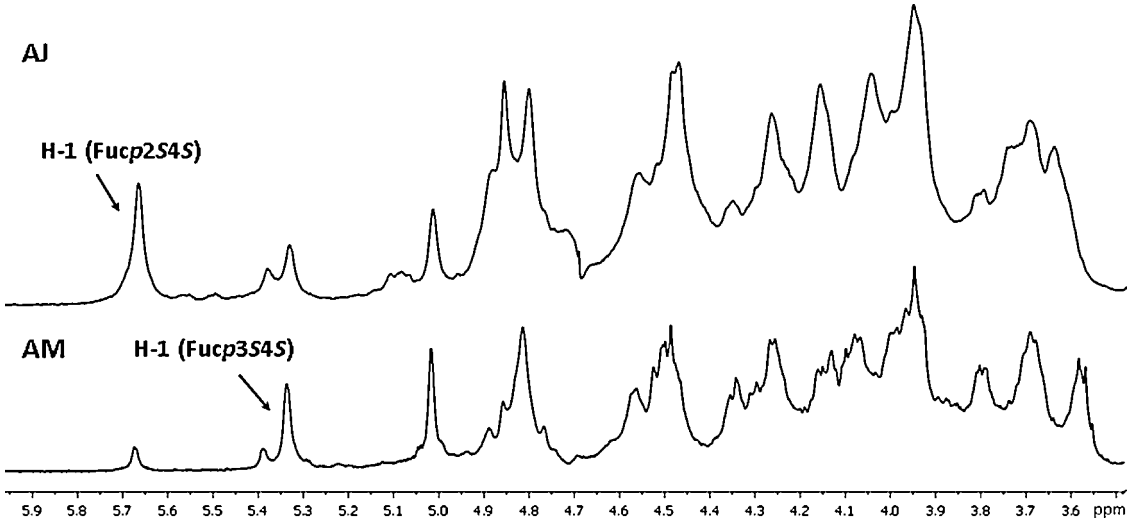

Fig. 4. Fragments of ^{1}H NMR spectra of fucosylated chondroitin sulfates **AJ** and **AM**.

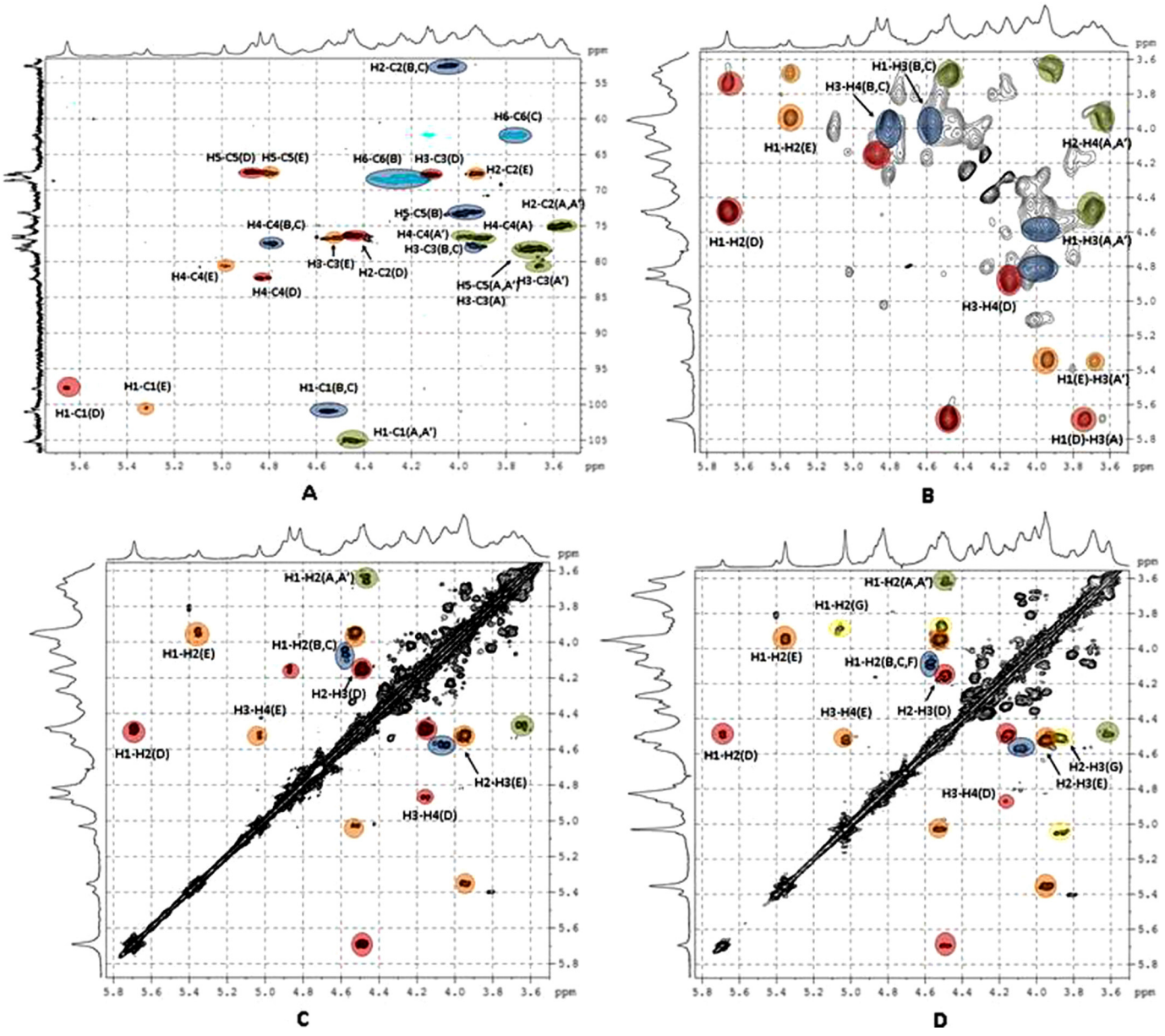

Fig. 5. NMR spectra of polysaccharides **AJ** and **AM**: (A) HSQC spectrum of **AJ**, (B) ROESY spectrum of **AJ**, (C) COSY spectrum of **AJ**, (D) COSY spectrum of **AM**. Correlation peaks are marked on the spectrum: GlcA (A,A') – green, GalNAc (B,C,F) – blue, Fuc2*S*4*S* (D) – red, Fuc3*S*4*S*(1 → 3) (E) – orange, Fuc3*S* (1 → 6) (G) – yellow (abbreviations as in Fig. 1 and Table 2) (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

for many other fucosylated chondroitin sulfates bearing sulfated fucosyl branches (Chen et al., 2011; Gao et al., 2015; Panagos et al., 2014; Wu et al., 2012; Wu et al., 2013; Yang et al., 2015a; Yang et al., 2015b; Yoshida et al., 1992). The same fucosyl branches were found in a structure of **AM** (Figs. 5D and S1, Table 2). The ratio of units **D**:**E** was about 3:1 for **AJ** and 1:4 for **AM**. These values were determined using integral intensities of the respective H-1 signals. Small portions of Fuc*p*4*S* units (**H**) attached to O-3 of GlcA were also found in both polysaccharides, which was confirmed by the data of ^{1}H NMR spectra (Table 2). Unfortunately, the content of units **H** in both polymers was insufficient to detect all signals of these units in ^{1}H and ^{13}C NMR spectra.

Determination of the position of branches **D** and **E** was performed using the data of ROESY spectra of the polysaccharides. The cross peaks respective to H-1 (Fuc2*S*4*S*, **D**) – H-3 (GlcA, **A**) and H-1 (Fuc3*S*4*S*, **E**) – H-3 (GlcA, **A'**) interactions unambiguously evidenced the substitution of GlcA at O-3 by fucosyl residues (Fig. 5B for **AJ**, Fig. S2 for **AM**).

An unusual branch Fuc*p*3*S* (**G**) linked to O-6 of GalNAc*p* (**F**) was observed in a structure of **AM**. The values of chemical shifts of H-1 (5.02 ppm) and C-1 (101.2 ppm) of **G** were significantly different from those for the fucosyl residues linked to O-3 of GlcA (Fig. S1). The position of sulfate group in unit **G** was confirmed by low-field shift of H-3 signal (δ 4.48 ppm) in ^{1}H NMR spectrum (Fig. 5D, Table 2). The site of attachment of the branch **G** to the backbone was determined using HMBC experiment. As the H-1 signal of **G** overlaps with H-4 (**E**) signal, the sample was heated to 333 K to separate the signals. The correlation between C-1(**G**) and H-6 (**F**) (Fig. 6) confirmed the presence of Fuc(1 → 6)GalNAc fragment in a structure of **AM**.

An intensive cross peak in the ROESY spectra of **AJ** and **AM** respective to H-3–H-4 (GalNAc) interaction indicated the down-field shift of H-4 (δ 4.81 ppm) (Figs. 4B, S2), which means that all

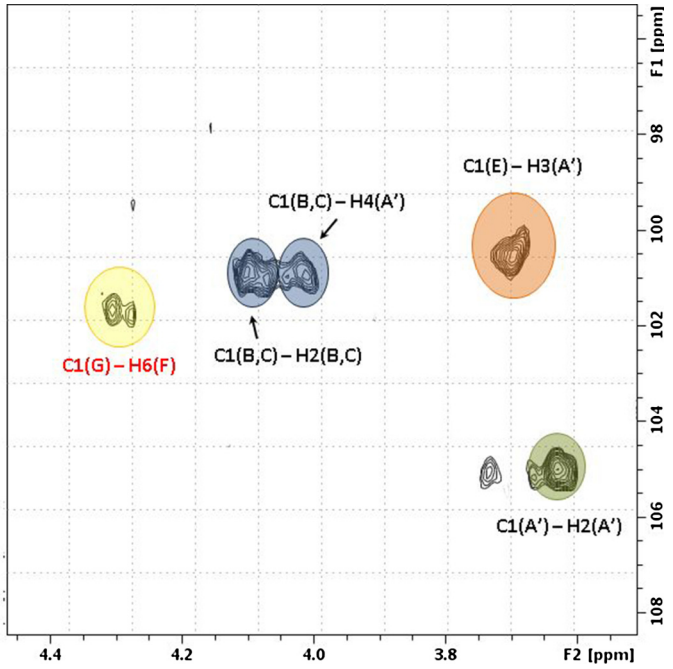

Fig. 6. A fragment of HMBC spectrum of **AM**.

GalNAc residues were sulfated at C-4. According to the presence of two peaks of C-6 related to sulfated (δ 68.5 ppm) and non-sulfated (δ 62.3 ppm) GalNAc in ^{13}C NMR spectra, it was concluded that GalNAc residues of the core are partially sulfated at C-6. Integration of the intensity of the cross-peaks related to H-6–C-6 interaction of GalNAc4*S*6*S* (**B**) and of GalNAc4*S* (**C**) in HSQC spectrum led to determine the approximate ratio between these units. The ratio of GalNAc4*S*6*S*:GalNAc4*S* for **AJ** was about 2:1, whereas for **AM** this value was approximately 1:1.

Structural differences between **AJ** and **AM** illustrate the variations in FCS depending on the taxonomic position of sea cucumbers. Geographical area of the habitat is often also regarded as the factor determining the fine structure of FCS (Chen et al., 2011; Yang et al., 2015a). It may be supposed that several additional factors, such as season, ecological conditions, age and physiological status of the organisms, may probably have some influence on the chemical structures of FCS. Our structure **AJ**, coinciding qualitatively with the formula suggested by Yoshida et al. (1992), differs considerably in several minor features from the structures of FCS of the same species found by subsequent investigators (Kariya et al., 1997; Yang et al., 2015a). It should be noted that conclusions about fucosylation of GalNAc residues at O-4 and O-6 in these previous papers were based on several indirect calculations, which were not confirmed by methylation analysis of galactosamine derivatives. In contrast, our data about the structure of **AM** represent the first direct spectral evidence on the fucosylation of GalNAc at position 6.

4. Conclusion

Structural characterization of two fucosylated chondroitin sulfates, **AJ** and **AM**, isolated from two species of sea cucumbers *Apostichopus japonicus* and *Actinopyga mauritiana*, respectively, was performed. Both polysaccharides were shown to contain a typical chondroitin core built up of repeating disaccharide units →3)-β-D-GalNAc-(1→4)-β-D-GlcA-(1→. The polysaccharides were different in pattern of sulfation of GalNAc and fucosyl branches linked to O-3 of GlcA. **AJ** contained Fuc*p*2*S*4*S* and Fuc*p*3*S*4*S* residues in a ratio of 3:1, while for **AM** the ratio of such units was 1:4. Small amounts of Fuc*p*4*S* units attached to O-3 of GlcA were observed in both polysaccharides. Moreover, in a structure of **AM** a small portion of Fuc*p*3*S* residues linked to O-6 of GalNAc was determined using the data of NMR spectra. The ratio of GalNAc4*S*6*S*:GalNAc4*S* for **AJ** was about 2:1, whereas for **AM** this value was approximately 1:1.

Conflict of interest

There are no conflicts of interest to report.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carbpol.2016.07.076.

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