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Vibrational spectroscopy characterization and anticoagulant activity of a sulfated polysaccharide from sea cucumber *Athyonidium chilensis*

Betty Matsuhiro[a,*], Igor O. Osorio-Román[b], Rodrigo Torres[a]

[a] Departamento de Ciencias del Ambiente, Facultad de Química y Biología, Universidad de Santiago de Chile, Av. B. O'Higgins 3363, Santiago, Chile
[b] Departamento de Química Inorgánica, Facultad de Química, Pontificia Universidad Católica de Chile, Vicuña Mackenna 4860, Santiago, Chile

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ABSTRACT

Extraction of *Athyonidium chilensis* body wall afforded a light brown powder in 4.2% yield which was fractionated by ion-exchange chromatography. The main fraction F1 contained glucuronic acid, galactosamine and fucose in a molar ratio of 1.0:1.1:1.1, and 32.9% of sulfate group. The second derivative FT-IR spectroscopy and Surface-enhanced Raman scattering (SERS) give more information than the normal IR and Raman spectroscopies. The broad band in the normal FT-IR spectrum of F1 at 850.6 cm^{-1} assigned to sulfate group was resolved in the second derivative spectrum into three bands at 856.4 cm^{-1}, 837.4 cm^{-1} and 818.6 cm^{-1} assigned to sulfate group attached to axial C4 and equatorial C2 and C6, respectively, in glycosidic residues. The most important bands in SERS of F1, but not observed in Raman spectrum are bands at 1648 cm^{-1} (νC=O), 1377 cm^{-1} (δC–H) assigned to methyl group of N-acetyl group, at 1173 cm^{-1} and at 956.2 cm^{-1} (νC–O–C) of glycosidic linkage; and bands at 925.5 cm^{-1} and 890.4 cm^{-1} (δC_1–H) assigned to α and β anomeric configurations of glycosidic residues, respectively. By NMR studies, glycosidic linkages and sulfation pattern of F1 was confirmed. Results obtained indicated that fraction F1 is chondroitin 4,6-disulfate substituted at position *O*-3 of glucuronic acid by partially 2,4-disulfated α-fucopyranosyl residues. Moreover, the anticogulant activity, measure in APTT of F1 showed at low concentration, a good correlation with heparin.

1. Introduction

The presence of sulfated polysaccharides in sea cucumbers (Echinodermata) is well known (Cássaro & Dietrich, 1977; Mourão & Bastos, 1987; Vieira & Mourão, 1988). The main polysaccharide isolated from the sea cucumbers *Stichopus japonicus* and *Ludwigotheura grisea* is a chondroitin sulfate containing substantial amount of fucose. Two residues of fucose, forming a 1–3 disaccharide branch were attached to the *O*-3 position of β-D-glucuronic acid residues, beside some residues of the uronic acid were sulfated at position *O*-3 (Kariya, Watabe, Hashimoto, & Yoshida, 1990; Kariya, Watabe, Kyogashima, Ishihara, & Ishii, 1997; Vieira, Mulloy, & Mourão, 1991). Ribeiro, Vieira, Mourão, and Mulloy (1994) reported that the sulfated polysaccharide from *L. grisea* was heterogeneous, comprising three fractions isolated by ion-exchange chromatography. The main fraction was the fucose branched chondroitin sulfate, a second fraction composed by fucose, galactose, and amino sugars was isolated in small amount, and a third fraction was a sulfated fucan. Fucosylated chondroitin sulfates have attracted attention due to their biological properties (Borsig et al., 2007; Fonseca & Mourão, 2006; Fonseca, Santos, & Mourão, 2009; Wu, Xu, Zhao, & Ding, 2010). Recently, Chen et al., 2011 characterized fucosylated chondroitin sulfates from four sea cucumbers. They found that the sulfation pattern of the fucose branch is important for the anticoagulant activity.

Sulfate groups in glycosaminoglycans were readily recognized by their infrared and Raman spectra (Bansil, Yannas, & Stanley, 1978; Cabassi, Casu, & Perlin, 1978; Neely, 1957). Longas and Breitweiser (1991) quantified the sulfate content in glycosaminoglycans by IR spectroscopy and informed that the results obtained were in good agreement with the values determined by chemical methods. Foot and Mulholland (2005) achieved the identification of different glycosaminoglycans by FT-IR spectroscopy and chemometric methods. Raman spectroscopy is a sensitive tool for the structural analysis of carbohydrates, especially Raman microspectrometry allowed the characterization of glycosaminoglycans and proteoglycans (Arboleda & Loppnow, 2000; Ellis, Green, & Winlove, 2009; Spencer et al., 2009). Recently, Mainreck et al. (2010) used both infrared and Raman microscopies for an effective differentiation of glycosaminoglycans. On the other hand, it was found that the second derivative spectra of Infrared spectra gave more information than normal IR spectra and allowed to differentiate sulfated galactans from red seaweeds and alginic acid block fractions (Cardenas-Jiron, Leal, Matsuhiro, & Osorio-Roman, 2011; Chandía,

* Corresponding author.
E-mail address: betty.matsuhiro@usach.cl (B. Matsuhiro).

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Matsuhiro, & Vásquez, 2001; Leal, Matsuhiro, Rossi, & Caruso, 2008; Matsuhiro, 1996). Second-derivative IR spectroscopy has been used for the characterization of other biomolecules such as proteins and nucleic acids (Byler, Wilson, Randall, & Sokoloski, 1995; Kong & Yu, 2007; Mehrotra, Tyagi, Jangir, Dawar, & Gupta, 2010; Sahu, Mordechai, & Manor, 2008; Susi & Byler, 1983; Whelan et al., 2011). Furthermore, bacteria were characterized and identified by second-derivative FT-IR spectroscopy (Al-Qadiri, Lin, Cavinato, & Rasco, 2006; Kuhn, Suter, Felleisen, & Ran, 2009; Li et al., 2004). However, the usefulness of vibrational spectroscopy may be limited by two factors; the high fluorescence of biological systems can mask the vibrational signals and low concentration of the samples result in poor spectra. In particular, there is one method that has attracted attention due its molecular specificity, high sensitivity and scanning time, the plasmon enhanced spectroscopy known as Surface-enhanced Raman scattering (SERS) with a wide range of practical applications (Aroca, 2006; Fleischmann, Hendra, & Mc Quillan, 1974; Moskovits, 1985). The technique is appealing for polysaccharides since water does not interfere with Raman measurements, thanks to its very low Raman cross section. Raman microscopy permits micrometer spatial resolution and allows to tap into the wealth of molecular fingerprint information available in vibrational spectroscopy. SERS is very useful in detecting conformational changes and structural differences regarding preferred orientations of molecules with respect to a metal surface (Campos-Vallette et al., 2010; Cardenas-Jiron et al., 2011; Osorio-Román et al., 2010).

Athyonidium chilensis is one of the most abundant species of sea cucumber of the coast of southern Pacific, it is distributed between Ancón (11°46′13″S, 77°10′23″W) in Perú, and Punta Gaviota (42°27′48″ S, 73°48′29″W) in Chile (Larraín, 1995; Ravest Presa, 2000). This sea cucumber has not been used as a source of food and drug like in the traditional Chinese medicine and others Asian countries; also it has not been widely exploited for commercial purposes. The present work shows the isolation and structural characterization by vibrational spectroscopy of a sulfated polysaccharide extracted from *A. chilensis.* Further, the anticoagulant activity of this biopolymer is evaluated.

2. Materials and methods

2.1. Materials

The sea cucumber *Athyonidium chilensis* was collected in the rocky intertidal area of Bahía de Quintay (33°10′59 77″S, 71°41′9 73″W), Chile. Papain, cystein, D_2O, and D_2O with 0.7% of sodium salt of 3-(trimethylsilyl)propionic 2,2,3,3-d_4 acid were purchased from Merck Chile (Santiago, Chile). The carbohydrate standards L-fucose, D-galactose, D-glucose, D-galacturonic acid, γ-D-glucuronolactone, D-galactosamine and D-glucosamine were from Sigma (St. Louis, MO, USA). For the anticoagulant activity the STA-Cephascreen and $CaCl_2$ 0.025 M solutions were purchased from Diagnostica Stago Inc. (New Jersey, USA), and the Heparine (25,000 IU/5 mL) were from Claris Lifescience (Ahmedabad, India).

2.2. FT-IR, Raman and SERS spectroscopy

FT-IR spectra in KBr pellets (10%, w/w) of polysaccharides were registered in the 4000–400 cm^{-1} region according to Leal et al. (2008) with a Bruker IFS 66v instrument. Derivation including the Savitzky-Golay algorithm with 23 smoothing points was performed using the OPUS/IRv.2.0 software incorporated into the hardware of the instrument.

The micro-Raman and SERS spectra were recorded with a Renishaw Raman RM1000 instrument equipped with the 632.8 nm laser line, an electrically refrigerated CCD camera, and a notch filter to eliminate the elastic scattering. Typical spectra were obtained using a 20× microscope objective. The output laser power at the samples was about 0.2 mW and the spectral resolution was 4 cm^{-1}. The spectral scanning conditions were chosen to avoid sample degradation. To measure the SERS spectra was necessary to prepare a solution of the polysaccharide in nanopure water at a final concentration of 1 mg/mL. Colloidal silver nanoparticles were obtained by the reduction of silver nitrate with trisodium citrate in aqueous medium using the method of Lee and Meisel (1982). SERS samples were prepared by adding 10 μL of the polysaccharide solution to 1 mL of the silver colloid to give a final solution of 10 μg/mL. The solutions were incubated at 4 °C for 12 h to improve the interaction of macromolecules with nanoparticles. The colloid was also activated by addition of 0.3 M aqueous NaCl. The silver colloid containing the target polysaccharide was cast onto quartz slides. The quartz slide containing the dried activated Ag nanoparticles, was placed as a lid onto a glass slide with a shallow groove containing aliquot of silver colloids. Therefore, SERS is measured from a liquid–solid interface through the quartz slide. The arrangement minimizes the burning of the organic material that is seen when the laser is used directly on the dried colloidal sample (Campos-Vallette et al., 2010; Cardenas-Jiron et al., 2011; Osorio-Román et al., 2010).

2.3. NMR spectroscopy

^1H NMR (400.13 MHz) and ^{13}C (100.62 MHz) spectra of the polysaccharide were recorded in D_2O at 60 °C on a Bruker Avance DRX400 spectrometer using the sodium salt of 3-(trimethylsilyl)propionic 2,2,3,3-d_4 acid as standard. All the two-dimensional experiments were acquired using a pulse field gradient incorporated into NMR pulse sequences. The two dimensional homonuclear $^1H/^1H$ correlation spectroscopy (NOESY) was acquired with 128 × 2048 data points having a spectral width of 2800 Hz and processed in a 1024 × 1024 matrix to give a final resolution close to 2.3 Hz/point in the two dimensions. The two dimensional heteronuclear-single quantum coherence correlation (2D $^1H/^{13}C$ HSQC) spectra were acquired with 128 × 1024 data point and processed in a 1024 × 1024 matrix to give a final resolution close to 2.3 Hz/point in ^1H and close to 2.4 Hz/point in ^{13}C. The number of scans (ns) in each experiment was dependent on the sample concentrations.

2.4. Determination of chemical composition

Sulfate content was determined by the turbidimetric method of Dodgson and Price (1962). The content of uronic acid was determined following the method of Filizzetti-Cozzi and Carpita (1991) using D-galacturonic acid as standard. Total sugars were determined by phenol–H_2SO_4 acid method (Chaplin, 1996) using L-fucose as standard. The total hexosamine content was carried out using an adapted Elson–Morgan method for quantification in glycosaminoglycans with glucosamine as standard (Carney, 1996).

2.5. Extraction of sulfated polysaccharides from Athyonidium chilensis

Extraction of sea cucumber polysaccharide was performed using the method reported by Vieira and Mourão (1988). Briefly, the body wall of sea cucumber was defatted using acetone and dried. The tissue was cut in small pieces and incubated with papain in a 0.1 M sodium acetate buffer solution (pH 6) containing 5 mM EDTA and 5 mM cysteine at 60 °C for 24 h. The digestion product was centrifuged and the supernatant was precipitated with 3% cetrimide solution for 12 h at 40 °C. The precipitate was dialyzed against distilled water at 40 °C in Spectra/Por membranes (MWCO 3500) for

two days and it was dissolved in a 2 M NaCl:EtOH (100:15, v/v) solution and precipitated in ethanol for 18 h at 4 °C. The mixture was centrifuged at 8000 g, and the pellet was dissolved in water, frozen and lyophilized.

2.6. Fractionation of the extract from Athyonidium chilensis

The crude extract was chromatographed on DEAE-Sephadex A-50 column (20 cm × 1 cm). Elution was carried out using a gradient increasing concentrations of NaCl (0.2, 0.4, 0.8, 1.2, 1.6, and 2.5 M). All fractions were monitored spectrophotometrically by using the phenol–sulfuric acid reagent (Chaplin, 1996).

2.7. Reduction of uronic acid residues in polysaccharide and total hydrolysis

The polysaccharide was reduced by the method of Taylor, Shively, and Conrad (1976). Briefly, a sample of the polysaccharide (5 mg) was dissolved in a minimum volume of distilled water, and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (15 mg) was added with stirring. After 2 h, $NaBH_4$ (10 mg) was added and the mixture was stirred for 2 h, dialyzed against distilled water and freeze-dried. The procedure was repeated once more and the reduced polysaccharides were hydrolyzed using 2 M TFA (1 mL) during 2 h at 120 °C. The acid was removed *in vacuo* by repeated addition of distilled water, and the resulting syrup was reduced with $NaBH_4$, acetylated with Ac_2O in dry pyridine and analyzed by gas-liquid chromatography (GLC). GLC was carried out in a Shimadzu GC-14B gas–liquid chromatograph equipped with a flame ionization detector using a SP 2330 column (0.25mm × 30 m) and performed with an initial 3 min hold at 100 °C and then to 160 °C at 3 °C/min to 160 °C and at 12 °C/min to 220 °C for 10 min. Injector and detector temperature was 220 °C and helium flow was 20 mL/min.

2.8. Identification of uronic acids

The polysaccharide (4 mg) was dissolved in 2 M TFA (1 mL) and heated during 2 h at 120 °C. The acid was removed *in vacuo* by repeated co-evaporations with distilled H_2O, and the resulting syrup was dissolved in a minimum amount of distilled H_2O and applied in a column (15 cm × 1 cm) of DEAE Sephadex A-50 (Cl^-). The column was eluted with distilled H_2O, until the eluant monitored by phenol–sulfuric acid reagent became free from carbohydrates, and then with 10% formic acid. The acidic fraction was concentrated *in vacuo*, the acid removed as previously described and the residue was examined by HPLC for uronic acids (Gacesa, Squire, & Winterburn, 1983). The HPLC was carried out on a Waters 600 (Waters, Milford, Massachusetts, USA) instrument equipped with a Waters 2996 photodiode array detector using a Phenomenex Sax 250 mm × 4.6 mm column (Phenomex, Torrance, California, USA). The sample was eluted with a 0.002 M KH_2PO_4, pH 4 solution using a 1 mL/min flow. D-Galacturonic acid and γ-D-glucuronolactone were used as standards.

2.9. Anticoagulant studies

The anticoagulant activity was measured as activated partial thromboplastin time (APTT). The APTT was determined with a STA Compact (Diagnostica Stago, Inc., New Jersey, USA) at 37 °C. Healthy samples of human plasma containing different concentrations of polysaccharide (0.2–20.0 μg/mL) and heparine (0.2–6.0 μg/mL) were added a STA-cephascreen solution and incubated for 4 min. Then, a 0.025 M $CaCl_2$ solution was added and the clotting time was counted by triplicate in three different days. The clotting time results were expressed as sample APTT/negative control APTT ($APTT_s/APTT_{NC}$).

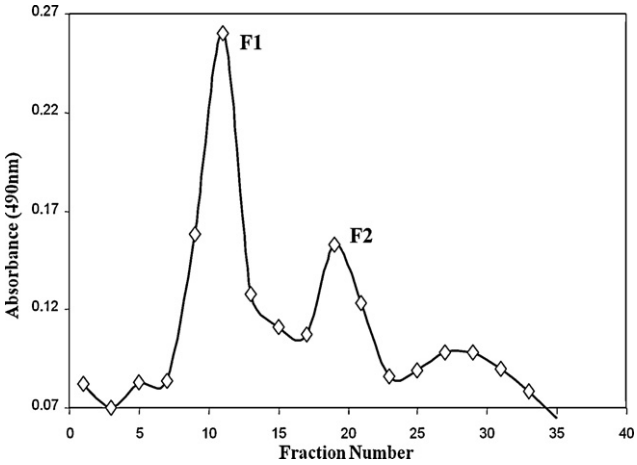

Fig. 1. Elution profile on Sephadex A-50 of the extract from *A. chilensis*.

3. Results and discussion

The digestion process followed by cetrimide fractionation of the extract from *Athyonidium chilensis* afforded a light brown powder in 4.2% yield, which was fractionated by ion exchange chromatography. At a concentration of 1.2 M of NaCl (Fig. 1) gave fraction F1 (21.4% yield) and fraction F2 (12.2%). The IR-FT spectrum of F1 in the region 4000–1800 cm^{-1} showed the characteristic O–H and C–H stretching vibrations at 3482 and 2987 cm^{-1}, respectively. In the region 1800–400 cm^{-1} (Fig. 2A) it showed a band a 1739.4 cm^{-1} assigned to stretching vibration of C=O group and characteristic bands of glycosaminoglycans at 1650.9 cm^{-1} assigned to the amide I band, at 1558.7 cm^{-1} corresponding to an amide group II vibration and at 1419.0 cm^{-1} due to C–N vibration of N-acetyl group (Conley, 1970; Foot & Mulholland, 2005). Moreover, three signals assigned to sulfate groups appeared at 1256.9 cm^{-1} corresponding to S=O asymmetric stretching vibration, at 850.6 cm^{-1} assigned to the symmetric C–O–S stretching vibration and at 583.4 cm^{-1} due to S–O stretching vibration (Matsuhiro, 1996). The second derivative spectrum of F1 presents more signals (Fig. 2B). The broad band centered at 1650.9 cm^{-1} in the normal spectrum is resolved in two bands, the latter and a new band at 1625.8 cm^{-1} which may be assigned to asymmetric stretching vibration of

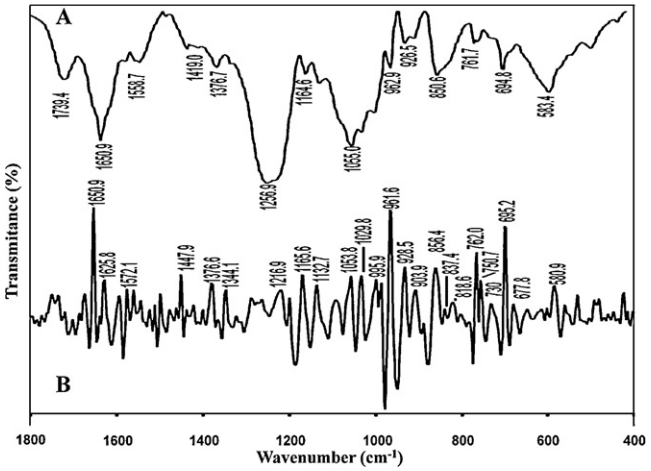

Fig. 2. FT-IR (A) and second derivative FT-IR (B) spectra of fraction F1 of the extract from of *A. chilensis*.

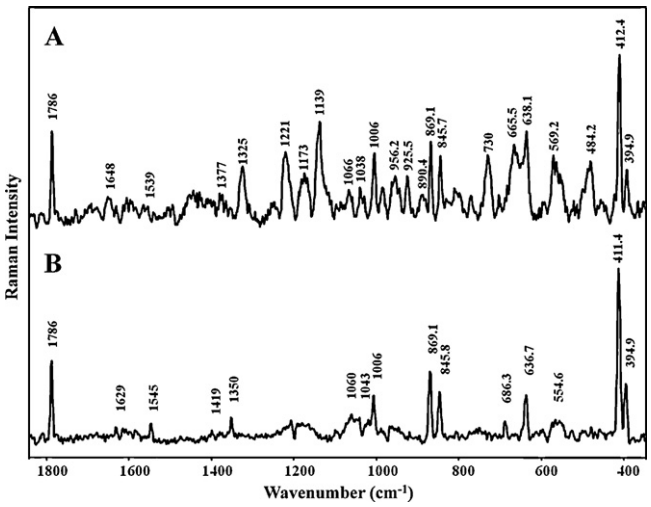

Fig. 3. SERS (A) and Raman (B) spectra of fraction F1 of the extract from of *A. chilensis*.

carboxylate group. Carbohydrates present in the 1500–1200 cm^{-1} region deformation vibrations of H–C–H, C–O–H, C–H and C–O–C groups. FT-IR spectrum of F1 presents bands in this region at 1447.9 and at 1376.6 cm^{-1} that may be assigned to asymmetric and symmetric deformation of CH_3, these bands were also shown in the second-derivative spectrum of fucoidan, and may indicate the presence of L-fucopyranosyl residue in F1 (Chandía & Matsuhiro, 2008; Chandía, 2005; Conley, 1970; Mathlouthi & Koenig, 1986); the new band at 1216.9 cm^{-1} was assigned to deformation of C–H group. The 1200–950 cm^{-1} region is the C–O stretching region with contributions of C–C vibrations; the second-derivative spectrum of F1 presents bands at 1165.6 cm^{-1} assigned to C–O symmetric stretching vibration of glycosidic linkage, at 1132 cm^{-1} which may be assigned to stretching of C–O and C–O–C groups, and C–C–C deformation. Furthermore, in this region signals at 1053 and 1029.8 cm^{-1} assigned to C–O–C, C–O–C and C–C stretching vibrations are present. The strong band at 961.6 cm^{-1} may be attributed to C–C stretching vibration with contribution of C–C–H deformation. In the finger print or anomeric region, bands at 928.5 cm^{-1} assigned to asymmetrical ring vibration, and at 903 cm^{-1} due to β-anomeric C–H deformation are shown; the broad band at 850.6 cm^{-1} is resolved in three bands in the second derivative spectrum, at 856.4 cm^{-1} which may be assigned to C–O–S vibration of sulfate at C-4 axial with contributions of α-anomeric C–H-deformation, C–C and C–H vibrations, and at 837.4 cm^{-1} and 818.6 cm^{-1}, due to sulfation in C-2 equatorial and C-6 equatorial positions, respectively (Amasekara, Opoku, Qiu, & Doctor, 2007; Matsuhiro, 1996). Moreover, it was possible to see that the second-derivative spectrum showed a better resolution of the weak signal at 761.7 cm^{-1} in the normal spectrum, the former showed two bands at 762.0 and 750.7 cm^{-1} which may be assigned to "ring breathing" vibrations; finally, the band at 695.2 cm^{-1} may be attributed to deformation of O–H group (Cardenas-Jiron et al., 2011).

The Raman spectrum (Fig. 3B) shows characteristic bands of carbonyl, carboxylate and amide II and III bands. Assignments of the signal were performed with the aid of literature data and are shown in Table 1 (Bansil et al., 1978; Cardenas-Jiron et al., 2011; Ellis et al., 2009). The SERS spectrum (Fig. 3A) afforded more information. The more important bands in SERS (Table 1), but not observed in Raman spectrum are bands at 1648 cm^{-1} (νC=O), 1377 cm^{-1} (δC–H) assigned to methyl groups, at 1173 cm^{-1} and 956.2 cm^{-1} (νC–O–C) corresponding to glycosidic linkage, at 925.5 cm^{-1} and 890.4 cm^{-1} (δC_1–H) indicating the α and β, anomeric configurations of the monosaccharide residues, respectively (Bansil et al., 1978; Ellis et al., 2009; Grant, Long, Moffat, & Williamson, 1989; Mainreck et al., 2010). Furthermore, an important band at 730.0 cm^{-1} was observed, associated to ring breathing with contribution of dihedral deformations of N–H, due to the interaction between the N-acetyl group and the nanoparticles surface (Cardenas-Jiron et al., 2011; Osorio-Román et al., 2010). Vibrations below 700 cm^{-1} were assigned to skeletal vibrations, and also to nonplanar bending absorption of hydroxyl group (Bansil et al., 1978; Cardenas-Jiron et al., 2011; Mathlouthi & Koenig, 1986).

Table 1
Wavenumber assignments (cm^{-1}) for Raman and SERS bands of fraction F1 of the extract from *A. chilensis*.

Wavenumber cm^{-1}		Assignments[a]
Raman	SERS	
1786	1786	νC=O
	1648	νC=O
1629		$\nu_s COO^-$
1545	1539	Amide II vibration
1419		νCOO^-
	1377	δCH_3 (N-acetyl group)
1350		Amide III vibration
	1325	δC–H, δO–H
	1221	δC–H, δC–O–H
	1173	νC–O–C (glycosidic linkage)
	1139	νC–C–C, νC–O
1060	1066	ν_sS=O, νC–O (glycosidic, and ring)
1043	1038	νC–O–C (glycosidic linkage)
1006	1006	ν_sC–O–C, νC–O–S (equatorial)
	956.2	νC–C, νC–O–C, δC–C–C
	925.5	δC–H (α anomeric)
	890.4	δC–O, δC–H (β anomeric)
869.1	869.1	νC–C, δ_ωO–H
845.8	845.7	νC–C, δ_ωO–H, νC–O–S vibration (axial)
	730	Ring breathing, δ_ωN–H
	665.5	Ring deformation
636.7	638.1	Ring deformation, δ_ωO–H
	569.2	Ring deformation, δ_ωO–H
554.6		Ring breathing
	484.2	
411.4	412.4	Ring δC–C–C, δC–C–O
394.9	394.9	Ring δC–O–C

[a] ν: stretching vibration; ν_s: symmetric stretching vibration; δ: deformation; δ_ω: wagging.

The chemical analysis showed that the F1 was composed for 32.9% of sulfate, 34.1% of total hexosamine, and 15.8% uronic acids. HPLC analysis of the acidic fraction of the total acid hydrolysis of F1 allowed the identification of glucuronic acid. Furthermore, the presence of glucuronic acid was analyzed by reduction of fraction F1 followed by total acid hydrolysis and GLC analysis of alditol acetates. The chromatogram showed three peaks corresponding to peracetates of glucitol, fucitol and galactosaminitol in a molar ratio of 1.0:1.1:1.1, respectively.

Results obtained by vibrational spectroscopy were corroborated by NMR studies. The ^{1}H NMR spectrum of F1 presented some complexity, but two signals at high field (2.07 and 1.37 ppm) can be readily assigned to the methyl protons of N-acetylgalactopyranosylamine (CH_3CO) and fucopyranosyl residues (CH_3), respectively (Fig. 4A). The ^{13}C spectrum (Fig. 4B) showed three signals in the anomeric region at 106.00, 102.45 and 102.21 ppm corresponding to the anomeric carbons of β-GlcA, β-GalNAc and α-Fuc, respectively (Vieira et al., 1991). Further, it was possible to assign signals corresponding to carbonyl carbon of acidic group (177.75 ppm), carbonyl carbon of N-acetyl group (177.86 ppm), carbons of methyl of N-acetyl group (25.51 ppm), and methyl group of deoxysugar (19.03 ppm). Moreover, a signal at 54.44 ppm corresponding to C2 of pyranosyl ring bonded to N-acetyl group was found. The complete assignment of the ^{1}H and ^{13}C NMR was achieved using the ^{1}H/^{13}C HSQC spectrum (Fig. 5). This

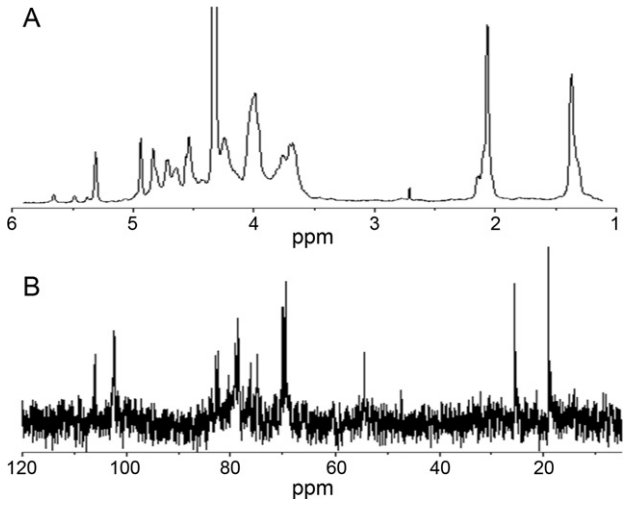

Fig. 4. ^{1}H (400.13 MHz) (A) and ^{13}C (100.62 MHz) (B) NMR spectra in D_2O of fraction F1 of the extract from *A. chilensis*.

information is summarized in Table 2. The three anomeric protons at 4.54, 4.64, and 5.31 ppm corresponding to β-glucuronic acid (β-GlcA), β-N-acetyl-galactosamine (β-GalNAc) and α-fucopyranose (α-Fuc) residues, respectively were found by correlations with the anomeric carbons signals. The chemical shifts to lower fields of C4 and C6 of β-GalNAc suggested sulfations at the 4-*O* and 6-*O* positions. Further, the down-field shifts of H2 and H4 of α-Fuc residue showed sulfations at 2-*O* and 4-*O* positions. The $^{1}H/^{1}H$ NOESY spectrum showed the correlation between H1 of α-Fuc and H3 of β-GlcA, H1 of β-GalNAc and H4 of β-GlcA, and H1 of β-GlcA and H3 of β-GalNAc indicating that glycosidic linkage of these residues are $1 \rightarrow 3$, $1 \rightarrow 4$ and $1 \rightarrow 3$, respectively. Altogether the results found by chemical analyses and spectroscopic methods indicate that the F1 is mainly composed of 4,6-sulfated chondroitin-4,6-disulfated substituted at position *O*-3 of glucuronic acid by partially 2,4-disulfated α-fucopyranosyl residues, and the proposed structure is shown in Fig. 6. In the other hand, the ^{1}H NMR spectrum of F2 (figure not shown) was very similar to that of F1 fraction and it was possible to see four additional signals in the anomeric region (5.5, 5.3, 5.25 and 5.1 ppm) in relation to F1 spectrum, corresponding to α glycosidic residues. Moreover, three signals at up field, between 1.45 and 1.2 ppm, were observed, which may be assigned to methyl groups protons of fucopyranosyl residues. Therefore, F2 fraction may be a mixture of sulfated polysaccharides where is possible to find mainly a fucose-branched chondroitin sulfate and in minor proportions sulfated fucans. F2 was obtained in very low yield (0.5% dry weight) and was not further studied.

Table 2
Chemical shifts assignments in the ^{1}H and ^{13}C NMR spectra of fraction F1 of the extract from *A. chilensis*.

Nucleus	δ (ppm)		
	β-GlcA	β-NAcGal	α-Fuc
H1/C1	4.54/106.00	4.64/102.45	5.31/102.21
H2/C2	4.24/69.42	–/54.44	–/78.95
H3/C3	3.66/76.26	–/78.41	3.99/69.48
H4/C4	3.70/82.80	4.83/79.10	4.94/82.32
H5/C5	–/79.10	3.99/74.87	4.71/69.48
H6/C6	177.75	4.32/69.99	1.37/19.03
CH_3		2.07/25.51	
C=O		177.86	

β-GlcA: β-glucuronic acid; β-NAcGal: N-acetyl-β-galactosamine; α-Fuc: α-L-fucose.

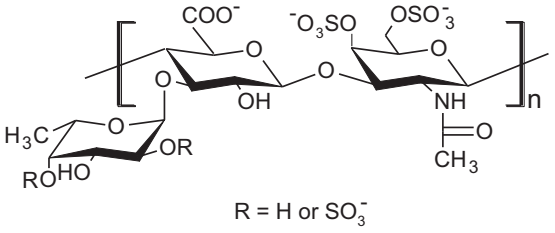

Fig. 6. Proposed structure for fraction F1 of the extract from *A. chilensis*.

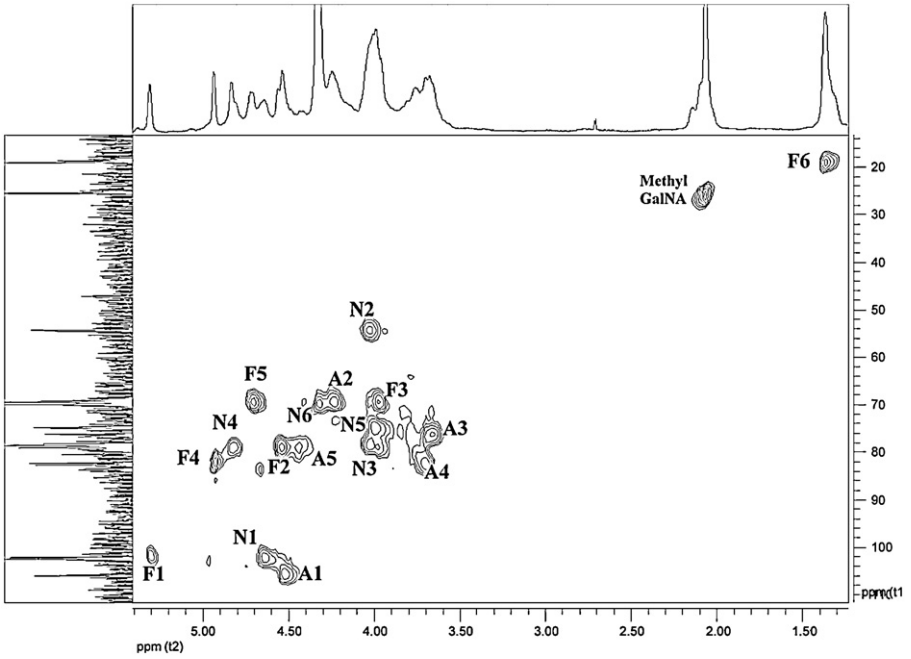

Fig. 5. $^{1}H/^{13}C$ HSQC NMR spectrum of fraction F1 of the extract from *A. chilensis*, where F, N and A correspond to α-Fucopyranosyl, N-acetyl-2-amino-2-deoxy-β-Galactopyranosyl and β-Glucuronopyranosyl residues, respectively.

Table 3
Anticoagulant activity of fraction F1 of the extract from of *A. chilensis* and heparin.

F1	Concentration (μg/mL)	20.0	10.0	5.0	2.5	1.0	0.5	0.2
	APTT*,**	4.15±1.04	2.83±0.61	1.92±0.07	1.52±0.14	1.19±0.03	1.10±0.07	1.05±0.06
Heparin	Concentration (μg/mL)	6.0	4.9	2.1	0.7	0.4	0.2	
	APTT*,**	4.12±0.66	4.00±1.09	1.92±0.22	1.13±0.10	1.11±0.11	1.02±0.02	

* APTT is expressed as the quotient between sample APTT and negative control APTT.
** Error value is expressed using a $p = 0.05$.

The anticoagulant activity for various concentrations of F1 fraction of the extract from *A. chilensis* was determined as APTT (activated partial thromboplastin time) (Table 3). The measure of clotting time was related to the clotting time of heparin. It is interesting to find that F1 at a concentration between 0.2 to 1 μg/mL behaved similarly to heparin. Chen et al. (2011) in a study of fucosylated chondroitin sulfates from four sea cucumbers found that sulfation pattern in fucosyl residue is important for the anticoagulant activity. They reported that the sulfated polysaccharide with 2,4-*O*-disulfation in fucosyl residues showed the strongest anticoagulant activity, higher than heparin. However, F1 is not so effective as heparine at higher concentration, probably the former adopts a different conformation due to the presence of fucopyarnosyl branch.

4. Conclusions

The body wall of the *Athyonidium chilensis*, like other species of sea cucumbers synthesized a fucosylated chondroitin sulfate and minor amounts of fucans. The second derivative FT-IR and SERS give more information than normal FT-IR and Raman spectroscopies for the characterization of polysaccharides. These techniques may be useful for the differentiation of glycosaminoglycans. The results of anticoagulant activity showed that the Chilean sea cucumber might have applications in food and medicinal industries.

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