J. Chil. Chem. Soc., 50, N 2 (2005), págs.: 501-504

CARBOHYDRATES FROM THE SEQUENTIAL EXTRACTION OF *Lessonia vadosa* (PHAEOPHYTA)

NANCY P. CHANDIA[1], BETTY MATSUHIRO*[1], JOHANNA S. ORTIZ[1] AND ANDRES MANSILLA[2]

[1]Departamento de Ciencias Químicas, Facultad de Química y Biología, Universidad de Santiago de Chile, Casilla 40, Correo 33, Santiago, Chile, bmatsuhi@lauca.usach.cl.
[2]Departamento de Ciencias y Recursos Naturales, Facultad de Ciencias, Universidad de Magallanes, Casilla 113-D; Punta Arenas, Chile.

ABSTRACT

Blades of *Lessonia vadosa* were sequentially extracted with 70% ethanol, followed by 2% $CaCl_2$ solution,, diluted HCl (pH 2), and finally with 2% Na_2CO_3 solution. D-mannitol was the only low molecular weight carbohydrate obtained from the ethanolic extract. By ion-exhange chromatography of the $CaCl_2$ extract a fraction contaning 36.7% of sulfate group was obtained. Chemical and spectroscopic analysis indicated the presence of a regular polymer of a-L-fucopyranose linked 1Æ3 and sulfated at positions *O*-2 and *O*-4. The main fraction isolated from the acidic extract by ion-exchange chromatography was shown to be composed of a complex a-L-fucan with 28.9% of sulfate. Alginic acid (17.0% of dry algae) was the major polysaccharide obtained in the sequential extraction.

Key words: *Lessonia vadosa*, alginic acid, mannitol, sulfated fucans.

INTRODUCTION

Members of Phaeophyceae class (brown seaweeds) produce a series of low and high molecular weight carbohydrates. D-mannitol is found universally in brown algae, in some species may account for a considerable proportion of the dried material. In addition, the presence of D-volemitol and of ß-metyl glycosides of alditols was reported[1]. The commercially important polysaccharide, alginic acid, is present in all the species so far studied. It is a copolymer of ß-D-mannuronic acid and a-L-guluronic acid linked 1 $\rightarrow$ 4. The ratio of the uronic acids can vary with the algal species, the habitat, the season, and the type of the tissue[2-4]. Laminaran, a neutral ß-D-glucan linked 1Æ3 is the food reserve material of the Phaeophyceae[2]. Many species of brown seweeds also produce sulfated fucans. Besides L-fucose, they frequently contain D-xylose, D-galactose, D-mannose and D-glucuronic acid[5,6]. The biological activities of sulfated fucans have been extensively examined but their structure are scarcely well established. These polysaccharides are heterogeneous and branched and they may contain acetyl groups[7,8]. Fucoidans are algal sulfated polymers containning substancial amounts of L-fucose and sulfate groups[6]. According to Patankar *et al*.[9], their structure corresponds to a branched α-1$\rightarrow$ 3-

linked L-fucose polymer mainly sulfated at *O*-4 position. Chevolot *et al*.[10,11] obtained by partial hydrolysis of the complex family of fucans from *Ascophyllum nodosum* and *Fucus vesiculosus* fucoidan fractions with a backbone of α-(1$\rightarrow$ 3) and α(1$\rightarrow$ 4) linkages with sulfate groups at *O*-2 and *O*-2,3 positions. Recently, Bilan *et al*.[12] informed that a fraction of the fucoidan from *Fucus distichus* is built up of alternating 3-linked-a-L-fucopyranose 2,4-disulfate and 4-linked-α-L-fucopyranose 2-sulfate residues.

Seaweeds of the genus *Lessonia* of the Laminariales grow abundantly in the chilean cost, four species have been described[13]. Percival *et al*.[14] by sequential extraction of *Lessonia nigrescens* obtained fucans with low content of sulfate groups (6-13%) and with considerable amounts of uronic acid (9-29%). On the other hand, *L. flavicans* afforded a fucan devoid of uronic acid with 35.5% of sulfate groups[15]. Extraction with acid of *L. trabeculata* gave a mixture of fucans which was fractionated by ion-exchange chromatography. It was found that as the ionic strength of the eluant increases, the amount of uronic acids decreases with increase in the content of sulfate groups[16].

This work is devoted to the isolation and chemical characterization of the soluble carbohydrates constituents of *Lessonia vadosa*.

EXPERIMENTAL

Materials and methods

Lessonia vadosa Searles was collected during winter in south Fuerte Bulnes (53° 37'55.6" S, 70° 55'17.9" W). Specimens were deposited in Sala de Colecciones, Departamento de Ciencias y Recursos Naturales, Universidad de Magallanes, Punta Arenas, Chile. FT-IR spectra in KBr pellets were registered in the 4000-400 cm^{-1} region using a Bruker IFS 66*v* instrument according to Cáceres et al.[17]. ^{1}H NMR (400 MHz) and ^{13}C (100 MHz) spectra of the polysaccharides, after isotopic exchange with D_2O (3 x 0.75 mL) were recorded in D_2O at 70°C on a Bruker Avance DRX400 spectrometer using internal methanol as reference. Two-dimensional spectra were registered using standard Bruker software.Optical rotations were measured with a Perkin Elmer 241 polarimeter. Microanalysis were performed at Facultad de Química, Universidad Católica de Chile. Total sugars were determined by the phenol-sulfuric acid method[18]. The content of uronic acid was determined following the method of Filisetti-Cozzi and Carpita[19] using D-galacturonic acid as standard. High performance liquid chromatography (HPLC) was carried out with a Whatman Partisil 10-Sax (4.6 mm x 0.25 m) column using KH_2PO_4 aqueous solution as eluant on a Merck-Hitachi L-6000 apparatus equipped with a L-4000A UV detector. Gas-liquid chromatography (GLC) was carried out on a Shimadzu GC-14B chromatograph equipped with a flame ionization detector using a SP 2330 column (0.25 mm x 30 m) and performed with an initial 5 min hold at 150 °C and then at 5 °C min^{-1} to 210 °C for 10 min. The helium flow was 20 mL min^{-1}.

Extraction

Blades of *Lessonia vadosa* were milled and treated at room temperature with formaldehyde in ethanol (1:4 v/v) for 72 h., filtered and air dried. One hundred grams of dried sample were sequentially extracted with 80% aqueous ethanol at 70°C, 2% aqueous $CaCl_2$, diluted HCl at pH 2.0 and finally with 3% aqueous Na_2CO_3 according to Percival et al.[14].

Examination of the extracts

The ethanolic extract was concentrated *in vacuo*, traces of water being removed by co-evaporation with several added portions of methanol and the resulting syrup crystallized from 98% ethanol. A portion of the crystals was acetylated with 1:1 acetic anhydride-dry pyridine.

The 2% $CaCl_2$ extract was dialysed against distilled water, concentrated *in vacuo* and freeze-dried. The resulting solid was dissolved in distilled water and fractionated on a DEAE Sephadex A-50 column by elution with water followed by increasing concentrations of potassium chloride solutions. Elution was monitored with the phenol-sulfuric acid reagent[18]. Fractions eluted at 0.5, 0.8, 1.0 and 1.2 M KCl were separately dialysed against distilled water (3500 Da cutt-off membrane), concentrated *in vacuo* and freeze-dried.

An aliquot of the fraction that eluted at 1.0 M KCl was heated with 2 M trifluoroacetic acid for 2 h at 120°C. The acid was removed *in vacuo* by co-evaporations with distilled water and the resulting syrup was treated with sodium borohydride in water. The reduced material was acetylated with acetic anhydride in dry pyridine and analysed by GLC. Acetates of L-fucitol, D-glucitol, D-mannitol, D-galactitol, and D-xylitol were used as standards.

The HCl extract was purified and fractionated as the 2% $CaCl_2$ extract.

The 3% Na_2CO_3 extract was dialysed against distilled water and purified as previously reported[20] . An aliquot of the purified material was hydrolysed with formic acid as described by Chandía *et al*. and analysed by HPLC[4].

RESULTS AND DISCUSSION

Blades of *Lessonia vadosa* was sequentially extracted with ethanol, followed by aqueous solutions of $CaCl_2$, HCl and finally with 3% NaCO3. From the ethanolic extract, D-mannitol (0.5 % of dry algae), m.p. 165-166 C, $[a]^{23}$ -1.2 (c, 2.5, water), (lit.[21] mp. 166 C, $[\alpha]_D$-0.3) was obtained. Acetylation afforded hexa-O-acetyl-D-mannitol, mp. 125-126 C, $[\alpha]^{23}$+ 23.5 (c, 2.5, CHCl3), lit.[21], mp. 125 C, $[\alpha]_D$ +25.0. D-mannitol was the only low-molecular weight carbohydrate isolated from *L. vadosa.* In this respect, results are similar to those reported for L. nigrescens and L. trabeculata[14,16].

The 2% calcium chloride extract (2.9 % of dry algae) was fractionated by ion-exchange chromatography. No fraction was obtained by elution with water which indicates the absence of laminaran-type polysaccharide. Similarly, Percival et al.[14] did not obtain any laminaran by extraction of *L*. nigrescens, in the case of *L* trabeculata the presence of laminaran in less than 0.2% of dried algae was reported[16]. The main fraction (F-1) eluted at 1.0 M KCl. Acid hydrolysis of F-1 and GLC analysis of the corresponding alditol acetates showed that fucose is the major monosaccharide. Composition of F-1 is presented in Table 1.

Table 1. Composition of fraction F-1 obtained from the CaCl2 extract, and F-2 from the acidic extract.

	Chemical shifts (ppm)					
→3)-a-L-fucp-2,4-diSO3-(1→	H-1	H-2	H-3	H-4	H-5	H-6
	5.41	4.56	4.24	4.89	4.48	1.32
	C-1	C-2	C-3	C-4	C-5	C-6
	98.99	76.10	74.62	80.96	68.17	17.38

tr.: Traces
n. d.: No detected

The FT-IR spectrum of F-1 showed strong absorption bands at 1261.4 cm^{-1} assigned to the S=O stretching vibrations of sulfate groups and at 849.5 cm^{-1} assigned to the S-O stretching vibration of secondary axial sulfate group. The band at 582.1 cm^{-1} attributed to the asymmetric deformation of O-S-O group confirmed the presence of significant amounts of sulfate groups[22]. No signals assigned to the C=O vibrations of carboxyl groups were found, which is in agreement with the results obtained in the colorimetric determination of uronic acids. The ^{1}H NMR spectrum of F-1 (Fig. 1) contained an intense broad signal and several minor signals in the a-anomeric region and two intense signals in the high field region assigned to the protons of methyl groups. Thus, F-1 according to the these results and the high negative value of its optical rotation of $[\alpha]_D^{25}$ -152,5° (c, 1.16, water) is a sulfated a-L-fucan, with a molar ratio of fucose and sulfate of about 1:1.23. like other sulfated polysaccharides isolated from brown seaweeds[12].

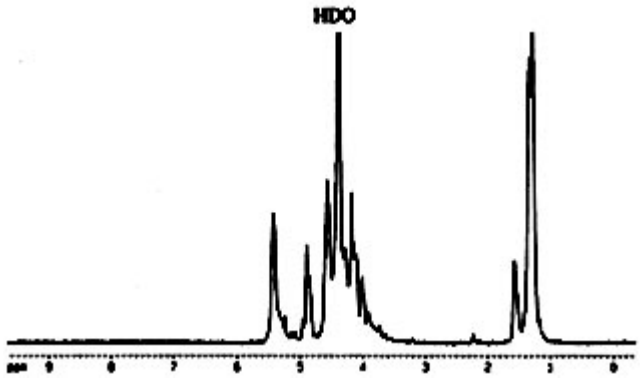

Fig. 1. ^{1}H NMR spectrum of fraction F-1 in D_2O recorded at 60° C

Assignments of the signals were deduced from the COSY spectrum (Fig. 2). Starting from anomeric protons connectivities from H-1 to H-4 could be established, confirming the assignements and indicating that the major proportion of fucose residues were sulfated at *O*-2 and *O*-4 positions. The methyl protons showed correlations with various H-5 resonances due to different substitution patterns on the fucosyl residue. The NOE spectrum confirmed the above results, additionally it showed only one inter-residue correlation between H-1 and H-3' which indicates that the fucose units are linked 1 → 3.

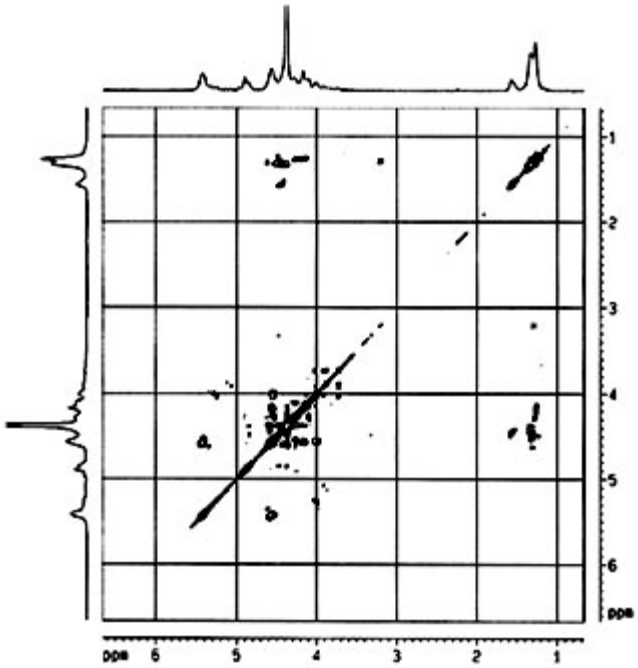

Fig. 2. Homonuclear ^{1}H COSY spectrum of F-1 recorded at 60 °C.

The ^{13}C NMR spectrum (figure not shown) presented considerable complexity. It presented three signals in the α-anomeric region. Signals between 81-74 ppm can be assigned to sulfated or glycosylated carbons in the fucopyranose ring while the group of signals at 71-66 ppm might arise from unsubstituted carbons. The methyl resonances appeared at 17.38 and 16.85 ppm. According to the literature, on sulfate group substitution, the resonance of C-6 is expected to shift from about 16.5 up to 19 ppm[11]. Resonances of the major signals in the ^{13}C NMR spectrum were assigned with the aid of heteronuclear ^{13}C-^{1}H HSQC and HMBC experiments and data reported in the literature[11,12,23]. The HSQC sprectrum showed that H-1 at 5.41 ppm was correlated to carbons at 98.99 and 94.91 ppm and the one at 5.38 ppm with carbon at 101.45 ppm. In the HMBC spectrum H-1/C-3 correlation was observed. All together, the spectroscopic data suggest that F-1 is mainly composed of a-L-fucopyranosyl-2,4-disulfate residues linked 1→ 3. Assignments of the ^{1}H and 13C NMR resonances are presented in Table 2. The values are very consistent with those reported by Mulloy et al.[24] for fucans isolated from echinoderms. The fucoidans from sea cucumber and sea urchin have a linear backbone of 1→ 3 linked a-L-fucopyranose residues with some sulfate substitution at the O-2 and O-4 positions. The F-1 fraction differs from the fucoidan with alternate 1→ 3 and 1→ 4 linkages isolated from Fucus distichus by Bilan et al.[12]. Several signals of minor intensity in the spectra could be due to unsulfated and monosulfated fucose residues. Correlation in the HSQC spectrum of the proton at 4.77 ppm with carbon at 81.43 ppm may indicate the presence of fucosyl residues monosulfated at O-4[10].

Table 2. NMR data for fraction F-1

	Chemical shifts (ppm)					
→3)-a-L-fucp-2,4-diSO3-(1→	H-1	H-2	H-3	H-4	H-5	H-6
	5.41	4.56	4.24	4.89	4.48	1.32
	C-1	C-2	C-3	C-4	C-5	C-6
	98.99	76.10	74.62	80.96	68.17	17.38

The fractions eluted with 0.3, 0.5, 0.8, 1.2, and 2.0 M KCl aqueous solutions were obtained in minor amounts, they presented lower negatives values of optical activities ($[\alpha]_D$ 26.0 to 48.0) which indicates the presence of heterogeneous fractions and were not further studied.

The acid extract (3.1 % of the dried algae) was fractionated by ion-exchange chromatography.The fraction eluted at 0.8 M KCl had $[a]^{23}$ -126.1 (c, 1.25, water). Composition is presented in Table 1. It contained fucose and sulfate in a molar ratio 1:0.74. Its FT-IR spectrum showed bands at 1261.1, 849.9 and 582.1 cm^{-1} assigned to sulfate groups. The second-derivative spectrum presented a small signal at 1736.5 cm^{-1} assigned to the C-O stretching vibration of uronic acids. Its ^{1}H NMR spectrum (not shown) presented in the a anomeric region a broad band which probably is the envelope of more than one resonance due to H-1 of α-L-fucans, and two signals at 1.34 and 1.30 ppm assigned to the methyl protons. The rest of the main signals could be assigned to the protons H-2, H-3, H-4 and H-5 of fucopyranosyl residues linked 1 → 3, sulfated at O-2 and O-4 positions. Several minor signals also appeared in this zone which may indicate the presence of desulfated residues. The ^{13}C NMR spectrum showed considerable complexity, displaying more than 5 lines in the a anomeric zone From these results it can be concluded that F-2 is not a regular fucoidan, probably with some ramification. Its structure will be further studied.

The purified 3% sodium carbonate extract (17.0 % of dry algae) showed by FT-IR analysis the characteristics bands at 948, 893, 818 and 781 cm^{-1} assigned to alginic acid[4]. Total hydrolysis and HPLC analysis showed a mannuronic acid (M) to guluronic acid (G) ratio of 0.73, the value is very similar to that (0.79) determined for the alginic acid obtained by direct extraction of the same seaweed sample with aqueous Na_2CO_3 [25].

The results found in this work showed that *Lessonia vadosa* biosynthesizes D-mannitol, a family of fucans and alginic acid like other members of the Lessoniaceae. The fucans extracted from *Lessonia vadosa* resemble the sulfated fucan of *L. flavicans* and differ from those extracted from *L .trabeculata* and *L. nigrescens* which contained considerable amounts of uronic acids. Others members of the family Lessoniaceae such as *Macrocystis pyrifera* and *M. integrifolia* also produced fucoidans[7,26]. The one from *M. pyrifera* is composed of fucose, galactose and xylose in the ratio of 36:2:1. Cruz-Suárez *et al*.[27] in a study of the composition of *Macrocystis pyrifera* found similar yields in alginate and laminaran as compared with *L. vadosa*. Besides, it contained 0.5-2% of fucoidan, but no information about its chemical structure was reported. Studies on the fucoidans from *Laminaria brasiliensis, Ecklonia kurome and Chorda filum* of the order Laminariales indicate that they present an average structure based on a backbone of L-fucose linked α 1→ 3, mainly sulfated at position *O*-4 and *O*-2[24, 28-29]. In this respect, a re-investigation of the structure of the fucoidan from *Lessonia flavicans* studied by Villarroel and Zanlungo[15] should be accomplished.

The highly regular a 1→ 3 fucoidan obtained from the $CaCl_2$ extract of *Lessonia vadosa* constitutes an important polysaccharide for the study of the relation of chemical structure with biological properties.

ACKNOWLEDGEMENTS

The financial support of FONDECYT (Grant 1010594), MINEDUC-Acuicultura, MECESUP MAG0002, and of Dirección de Investigaciones Científicas y Tecnológicas of Universidad de Santiago de Chile is gratefully acknowledged. The authors thank Dr. Juan Guerrero and Grant MECESUP USA-007 for the NMR spectra. N. P. Chandía thanks CONICYT for a doctoral fellowship.

REFERENCES

1. Percival, E., McDowell, R.H. Chemistry and Enzymology of Marine Algal Polysaccharides, Academic Press, New York, 1965, pp. 4-5.

2. Painter, T. J. Algal Polysaccharides. In: The Polysaccharides, Vol. 2, Aspinall, G. O. (Ed.) Academic Press, New York, 1983, pp. 196- 285.

3. Panikkar, R., Brasch D. Carbohydr. Res., 300, 229-238 (1997).

4. Chandía, N.P., Matsuhiro, B., Vásquez, A. Carbohydr. Polym. 46, 81-87 (2001).

5. Percival, E., McDowell, R.H. Chemistry and Enzymology of Marine Algal Polysaccharides, Academic Press, New York, 1965, pp. 157- 164.

6. Nishino, T., Nishioka, C., Ura, H., Nagumo, T. Carbohydr. Res., 255, 213-224 (1994).

7. Nagumo, T., Nishino. T. Fucan sulfates and their anticoagulant activities. In:Polysaccharides in Medicinal Applications, Dumitriu, S. (Ed.), Marcel Dekker, New York, 1996, pp. 545-574.

8. Tako, M., Yoza, E., Tohma, S. Bot. Mar. 43, 393-398 (2000).

9. Patankar, M.S., Oehninger, S., Barnett, T., Williams R.L., Clark, G. F. J. Biol. Chem., 268, 21770-21776 (1993).
[Medline]

10. Chevolot, L., Foucault, A., Chaubet, F., Kervarec, N., Sinquin, C., Fisher, A.-M., Boisson-Vidal, C. Carbohydr. Res., 319, 154-165 (1999).

11. Chevolot, L., Mulloy, B., Ratiskol, J., Foucault, A., Colliec-Jouault, S. Carbohydr. Res. 330, 529-535 (2001).

12. Bilan, M. I., Grachev, A. A., Ustuzhanina N. E., Shaskov, A. S., Nifantiev, N. E., Usov, A. I. Carbohydr. Res., 339, 511-517 (2004).

13. Santelices, B. Algas Marinas de Chile, Ediciones Universidad Católica de Chile, Santiago, 1989, pp. 80-91.

14. Percival E., Venegas Jara, M.F., Weigel, H. Phytochemistry, 22, 1429-1432 (1983).

15. Villarroel L., Zanlungo A.B. Rev. Latinoamer.Quím. 6, 127-130 (1975).

16. Matsuhiro, B., Zambrano D.M.J. Appl. Phycol. 2, 183-185 (1990).

17. Cáceres P. J., Faúndez, C.A.., Matsuhiro, B., Vásquez, J. A. J. Appl. Phycol. 8, 523-527 (1997).

18. Chaplin, MF. Monosaccharides. In: Carbohydrate Analysis, a Practical Approach, Chaplin, M.F., Kennedy, J.F. (Eds.). IRL Press, Oxford, 1986, pp. 1-36.

19. Filisetti-Cozzi, T.M.C., Carpita N.C. Analyt. Biochem. 197, 157- 162 (1991).
[Medline]

20. Venegas, M., Matsuhiro, B., Edding, M.E. Bot. Mar., 36, 47-59 (1993).

21. Hough L., Richardson A.C. Penta-, hexa- and higher Polyhydric Alcohols. In: Rodd's Chemistry of Carbon Compounds, Vol. I, Coffey, S. (Ed.), Elsevier, New York, 1967, pp. 18-24.

22. Matsuhiro, B. Hydrobiologia, 326-327, 481-489 (1996).

23. Bilan, M.I., Grachev A.A., Ustuzhanina N.E., Shashkov A.S., Nifantiev N.E., Usov, A.I. Carbohydr. Res. 337, 719-730 (2002).

24. Mulloy, B., Ribeiro, A.-C., Alves, A.-P., Vieira, R.P., Mourão P.A.S. J. Biol. Chem. 269, 22113-22123 (1994).
[Medline]

25. Chandía N.P., Matsuhiro B., Mejías E., Moenne A. J. Appl. Phycol., 16, 127-133, (2004).

26. Berteau O., Mulloy B. Glycobiology, 13, 29-40, 2003.

27. Cruz-Suárez E., Ricque-Marie D., Tapia-Salazar M., Guajardo- Barbosa C. Memoria V Simposium Internacional de Nutrición Acuícola, Mérida, 2000, pp. 227-266.

28. Nishino T., Nagumo, T. Carbohydr. Res., 211, 77-90 (1991).

29. Chizov A.O., Dell A., Morris H.R., Haslam, S.M., McDowell, R.A., Shaskov, A.S., Nifantiev N.E., Khatuntseva E.A., Usov A.I. Carbohydr. Res., 320, 108-119 (1999).