Carbohydrate Polymers 101 (2014) 804–811

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

The system of fucoidans from the brown seaweed *Dictyota dichotoma*: Chemical analysis and antiviral activity

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ARTICLE INFO

Article history:
Received 17 July 2013
Received in revised form 3 October 2013
Accepted 7 October 2013
Available online xxx

Keywords:
Dictyota dichotoma
Brown seaweeds
Fucoidans
Galactofucans
Antiviral

ABSTRACT

Room-temperature acid (pH 2) extraction of *Dictyota dichotoma* thalli yielded 2.2% of sulfated polysaccharides. Further extraction with the same solvent at 70 °C was conducted sequentially for nine times, with a total yield of 7.2%. Fucose was the main monosaccharide only in the room-temperature extract (**EAR**) and in the first 70 °C extract (**EAH1**). The remaining fractions showed increasing amounts of mannose (the main neutral monosaccharide), xylose and uronic acids. Fractionation by means of cetrimide precipitation and redissolution in increasing sodium chloride solutions has allowed obtaining several subfractions from each extract. The fractions redissolved at lower NaCl concentrations have large amounts of uronic acids and lesser sulfate contents, whereas those redissolved at higher NaCl concentrations are heavily sulfated and have low uronic acid contents. For the fucose-rich extracts (**EAR** and **EAH1**), fractionation leads to uronoxylomannofucan-rich and galactofucan-rich fractions. The remaining extracts gave rise to complex mixtures, with mannose and uronic acid-rich polysaccharides. Moderate inhibitory effect against herpes virus (HSV-1) and Coxsackie virus (CVB3) were found for the galactofucan-rich fractions. Most of the other fractions were inactive against both viruses, although some xylomannan-rich fractions were also active against HSV-1.

1. Introduction

Brown seaweeds (Phaeophyta) produce different polysaccharides, namely alginates, laminarans and fucoidans (Painter, 1983). The latter polysaccharides, which contain mainly L-fucose and sulfate, but also minor amounts of other sugars (Bilan et al., 2010; Duarte, Cardoso, Noseda, & Cerezo, 2001; Ponce, Pujol, Damonte, Flores, & Stortz, 2003), have received preferential attention due to their different biological activities: anticoagulant (Athukorala, Jung, Vasanthan, & Jeon, 2006; Chandia & Matsuhiro, 2008; Chevolot et al., 1999; Chevolot, Mulloy, Rastiskol, Foucault, & Colliet-Jouault, 2001; Nardella et al., 1996; Nishino & Nagumo, 1992; Nishino, Kiyohara, Yamada, & Nagumo, 1991; Nishino, Nagumo, Kiyohara, & Yamada, 1991; Pereira, Mulloy, & Mourao, 1999; Wang, Zhang, Zhang, Song, & Li, 2010), antithrombotic (Mauray et al., 1995); antiinflamatory (Blondin, Fischer, Boisson-Vidal, Kazatchkine, & Jozenfovicz, 1994), antitumoral (Athukorala et al., 2009; Croci et al., 2011; Rocha de Souza et al., 2007; Yang et al., 2008; Zhuang, Itoh, Mizuno, & Ito, 1995), contraceptive (Mahony, Clark, Oehninger, Acosta, & Hodgen, 1993; Mahony, Oehninger, Clark, Acosta, & Hodgen, 1991), apoptotic (Foley, Mulloy, & Tuohy, 2011), antioxidant (Rocha de Souza et al., 2007; Ruperez, Ahrazem, & Leal, 2002; Wang, Zhang, Zhang, & Li, 2008; Wang et al., 2010) and antiviral (Bandyopadhyay, Navid, Ghosh, Schnitzler, & Ray, 2011; Feldman, Reynaldi, Stortz, Cerezo, & Damonte, 1999; Harden, Falshaw, Carnachan, Kern, & Prichard, 2009; Hayashi, Nakano, Hashimoto, Kanekiyo, & Hayashi, 2008; Hidari et al., 2008; McClure et al., 1991; Ponce et al., 2003; Trinchero et al., 2009; Venkateswaran, Millman, & Blumberg, 1989; Wang, Ooi, & Ang, 2008). They have particularly been described as inhibitors of the replication of several enveloped viruses, as human immunodeficiency virus, herpes simplex virus and human cytomegalovirus (Beress et al., 1993; McClure et al., 1991). Its action against HIV is of particular importance (Queiroz et al., 2008; Trinchero et al., 2009). Attempts to systematize the fine structure of the fucoidans as is already established for red seaweed galactans have been unsuccessful, as only few examples of regularity were found: linkages, branching, sulfate position, other sugars appear to be variable and thus the relationship between structure

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http://dx.doi.org/10.1016/j.carbpol.2013.10.019

and biological activity is not clearly established (Ale, Mikkelsen, & Meyer, 2011). However, about four different types of polysaccharides were found within fucoidan samples: one containing high proportions of fucose, galactose and sulfate groups (the "galactofucan" or "fucogalactan"), another one containing just fucose and sulfate (the "fucan") and another one containing different monosaccharide moieties, less sulfate, and important proportions of uronic acids, known first as "uronofucoidans" (Duarte et al., 2001; Ponce et al., 2003) but later subdivided into "fucoglucuronans" and "fucomannoglucuronans" (Bilan et al., 2010; Croci et al., 2011). It has been shown that only the formers have strong biological activities (Croci et al., 2011; Ponce et al., 2003). For the fucan moieties, in the last years two main types of fucoidans were differentiated: one carrying alternating 3- and 4-linked α-L-fucopyranosyl units, recognized earlier in *Ascophyllum nodosum* (Chevolot et al., 2001), but also present in some *Fucus* species as *F. vesiculosus* (Chevolot et al., 2001), *F. evanescens* (Bilan et al., 2002), *F. distichus* (Bilan et al., 2004) and *F. serratus* (Bilan, Grachev, Shashkov, Nifantiev, & Usov, 2006), and another one carrying mainly 3-linked α-L-fucopyranosyl units, as found in *Cladosiphon okamunarus* (Hidari et al., 2008), *Chordaria flagelliformis* (Bilan et al., 2008), *Lessonia vadosa* (Chandia & Matsuhiro, 2008) and *Saccharina latissima* (formerly known as *Laminaria saccharina*, Bilan et al., 2010). Other authors indicate both 3- and 4-linkages, but not an alternating fashion; usually, 3-linked fucose units tend to predominate (Adhikari et al., 2006; Lee, Takeshita, Hayashi, & Hayashi, 2011; Ponce et al., 2003). The presence of 2-linked fucose units has also been postulated (Karmakar et al., 2009) in *Padina tetrastromatica*. These backbones appear heavily sulfated, mainly on C-2 and/or C-4, and sometimes also branched. For the galactofucan, a core containing 6-linked β-D-galactopyranosyl units has been postulated (Bilan et al., 2010; Duarte et al., 2001), whereas for the glucuronic acid-containing polymers four different motifs were encountered: terminal GlcA units on C-2 of a 3-linked fucose backbone (Bilan et al., 2008; Hidari et al., 2008); a 3-linked β-D-GlcA*p* backbone carrying fucose units on C-4 (Bilan et al., 2010), a backbone containing both 4-linked β-D-GlcAp and 2-linked α-D-Man moieties, possibly alternating (Bilan et al., 2010; Li, Wei, Sun, & Xu, 2006), and the linear structure →4)-β-D-GlcA*p*-(1→2)-α-L-Fuc*p*-(1→3)-α-D-Man*p*-(1→, partially sulfated on O-3 of the fucose units, and on O-6 of the mannose units (Sakai et al., 2003). Xylose was found to appear mainly as branching points. Some terminal fucofuranose units have also been found (Bilan et al., 2008; Nishino, Nagumo, et al., 1991; Ponce et al., 2003).

Dictyota dichotoma is a cosmopolitan brown seaweed. Many reports, especially from Asian countries, have recently reported biological activities of its secondary metabolites (Ayrad et al., 2011; Ravikumar, Alí, & Beula, 2011; Tabarsa, Rezaei, Ramezanpour, Waaland, & Rabiei, 2012). However, this seaweed also appears in Europe, South Africa and Bermuda, according to molecular markers (Tronholm et al., 2010), and all around the world. In the late seventies, it has been determined that the polysaccharides from an Egyptian sample of *D. dichotoma* contained residues of D-glucuronic acid, D-galactose, D-mannose, D-xylose, and L-fucose in substantial amounts (Abdel-Fattah, Magdel-Din Hussein, & Fouad, 1978; Hussein, Fouad, & Abdel-Fattah, 1979). The antimicrobial activity of its non-characterized polysaccharides was reported (Kantachumpoo & Chirapart, 2010). In order to assess the potential of a cosmopolitan seaweed as a source of fucoidan, and to study its characteristics, we have studied the whole system of polysaccharides of *D. dichotoma* and screened their antiviral activity against HSV-1 and CVB3. Herein the extraction of the polysaccharides from this seaweed using diluted hydrochloric acid both at room temperature and at 70 °C is reported, together with their purification, analysis, fractionation and assessment of the antiviral activity of some selected fractions.

2. Materials and methods

2.1. Materials

The brown seaweed *Dictyota dichotoma* (Hudson) Lamouroux was collected in summer at the shores near Bahía Bustamante (Chubut Province, Argentina). The thalli were air-dried and milled to a fine powder. All the reagents indicated below were of analytical grade.

2.2. Analytical methods

Total carbohydrates were estimated by the phenol-H_2SO_4 method using fucose as standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acids were determined using the method of Filisetti-Cozzi and Carpita (1991) using glucuronolactone as standard. The percentages of sulfate were measured by turbidimetry (Dodgson & Price, 1962) after hydrolysis with 1 M HCl at 110 °C for 4.5 h, whereas the soluble proteins were determined by the procedure of Lowry, Rosebrough, Farr, and Randall (1951), using bovine serum albumin as standard. Average molecular weights were estimated as described by Park and Johnson (1949).

Hydrolysis of the polysaccharides was carried out with 2 M CF_3COOH (90 min, 120 °C), *i.e.* the best conditions reported by Albersheim, Nevins, English, and Karr (1967). Hydrolyzates were derivatized to the alditol acetates and analyzed by GLC using a capillary column (30 m × 0.25 mm) coated with SP-2330 (0.20 μm) on a HP-5890 Gas Chromatograph equipped with a flame ionization detector (FID). Nitrogen was used as the carrier gas, with a head pressure of 15 psi and a split ratio of 100:1. Chromatography runs were isothermal at 220 °C, while the injector and detector were set at 240 °C. When a confirmation of identity was needed, the GLC–MS analyses was carried out on a Shimadzu QP 5050 A GC/MS apparatus working at 70 eV using similar conditions to those described above, but using He as gas carrier with a split ratio of 60:1.

2.3. Extraction

The milled seaweed (500 g) was extracted with 80% aqueous EtOH (2.5 L) under mechanical stirring at room temperature and then at 70 °C (each for 24 h), yielding, after evaporation and freeze-drying fractions **EOH_1** and **EOH_2**, respectively. The final residue was recovered by centrifugation and then extracted with aqueous HCl (diluted to pH 2), for 7 h at room temperature. The extract (**EAR**) was neutralized, concentrated at reduced pressure, dialyzed (mol.wt cutoff 6000–8000) and recovered by freeze-drying. The residue was reextracted exhaustively with the same solvent, but working at 70 °C, until negligible amounts of carbohydrates appeared in the supernatant. Nine different extracts were obtained (**EAH1**–**EAH9**).

2.4. Fractionation

A 10% (w/v) aqueous solution of hexadecyltrimethylammonium bromide (cetrimide, Sigma) was added slowly to **EAR** (500 mg) in water (100 mL) with stirring, until no further formation of complex occurred (usually after adding 6–8 mL). The mixture was kept stirring overnight, and then the precipitates were centrifuged off, suspended in 0.5 M NaCl (60 mL), and stirring was continued overnight. The precipitate was centrifuged off, and the supernatant was extracted with 1-pentanol (3 × 30 mL), dialyzed, concentrated and freeze-dried. The remaining precipitate was submitted to similar consecutive procedures with NaCl concentrations increased to 1, 1.5, 2, 3, 4 and 5 M. The same procedure was carried out with each of the **EAH** fractions. The **EAR** fractions were submitted to gel permeation chromatography on a Biogel P-30 column (1.2 cm × 33 cm) eluted with a NaCl 0.1 M aqueous solution. Fractions of 0.4 mL were

Table 1
Yields and analyses of the products extracted from *Dictyota dichotoma*.

Fraction	Yield (%)	Carbohydrate (% anh.)	Protein (%)	Mol.wt. (kD)	Uronic acid (%)	Sulfate (% SO_3Na)
EAR	2.2	48	6	6.5	21	15
EAH1	2.0	52	3	6.0	32	11
EAH2	2.0	54	3	6.1	38	9
EAH3	1.4	55	4	7.3	45	7
EAH4	0.7	55	9	5.6	50	5
EAH5	0.3	60	9	3.7	50	8
EAH6	0.2	53	8	3.2	49	7
EAH7	0.2	56	12	3.7	47	8
EAH8	0.2	48	12	2.6	37	8
EAH9	0.2	28	17	1.2	36	7

isolated, and aliquots were assayed by the phenol-sulfuric acid method.

2.5. Antiviral assays

Vero (African green monkey kidney) cells were grown in minimum essential medium (MEM) supplemented with 5% bovine serum. Both Herpes simplex virus 1 (HSV-1) strain F and Coxsackievirus B3 (CVB3) strain Nancy were obtained from the American Type Culture Collection (Rockville, USA).

Vero cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) method (Denizot & Lang, 1986). The CC_{50} (cytotoxic concentration 50%) was calculated as the compound concentration required to reduce cell viability by 50%.

Antiviral activity was evaluated by reduction of virus plaque formation. Vero cell monolayers grown in 6-well plates were infected with about 100 plaque-forming units (PFU) of virus/well in the absence or presence of various concentrations of the compounds. Plaques were counted after 2 days of incubation at 37 °C. The inhibitory concentration 50% (IC_{50}) was calculated as the compound concentration required to reduce virus plaque by 50%. All determinations were performed twice and each in duplicate. Ribavirin was used as a positive control.

3. Results and discussion

3.1. Extraction using room-temperature acid solution

The milled seaweed was extracted with ethanol first in order to remove low-molecular weight material. About 40% of the original dry weight of the seaweed was extracted with ethanol; mannitol was an important constituent of that extract, although it also contained reducing carbohydrates, proteins, uronic acids and sulfate. The residue was extracted with aqueous hydrochloric acid (pH 2) for 7 h. This solvent usually maximizes the extraction of fucoidans while it minimizes the coextraction of proteins, with no evident sign of degradation (Ponce et al., 2003). The yield of the extracted polysaccharide (**EAR**) was 2.2%. Its analytical characteristics (Table 1) show the presence of important proportions of neutral carbohydrates (48%), uronic acids (21%) and sulfate ester groups (15%), and less protein (6%). Fucose was the main monosaccharide, but important proportions of galactose, xylose and mannose also appear (Table 2). These results are similar with those reported previously for this seaweed (Abdel-Fattah et al., 1978). In most brown seaweed "fucoidans", fucose is by far the most predominant monosaccharide, usually in proportions larger than 70% (Bilan et al., 2002, 2004, 2010; Ponce et al., 2003), or less often in proportions between 50 and 70%, accompanied mainly by xylose and galactose (Bandyopadhyay et al., 2011; Duarte et al., 2001; Wang, Zhang, et al., 2008) or by glucose, in laminaran-rich crude extracts (Bilan et al., 2006; Foley et al., 2011). However, it is even less common to find fucoidans with proportions of fucose lower than 50%, and containing important amounts not only of xylose and galactose, but also of mannose and uronic acids. Most of the examples of such heterogeneous polysaccharides are found within the family *Dictyotaceae* (order Dictyotales) to which the current seaweed belongs: these "heterofucans" were found besides in *Dictyota metensii* (Queiroz et al., 2008), *Dictyota menstrualis* (Albuquerque et al., 2004), *Padina tetrastromatica* (Karmakar et al., 2009) and *Spatoglossum schröederi* (Leite et al., 1998). An old work showed that the *D.dichotoma* polysaccharides also share similar characteristics (Abdel-Fattah et al., 1978; Magdel-Din Hussein, Fouad, & Abdel-Fattah, 1979). To the best of our knowledge, the only two extracts originated in brown seaweeds outside this order where large heterogeneity were found become from *Hizikia fusiforme*, from the Sargassaceae (Li et al., 2006), and from *Kjellmaniella crassifolia*, from the Laminariaceae (Sakai et al., 2003).

3.2. Extraction using acid solutions at 70 °C

When the residue of the room temperature extraction was re-extracted with diluted acid at 70 °C, a similar amount of extract (**EAH1**) was obtained, with analytical characteristics indicating a larger amount of uronic acids, and less sulfate and proteins (Table 1). Fucose was still the main monosaccharide, but the

Table 2
Monosaccharide composition (mols/100 mols) of the products extracted from *Dictyota dichotoma*.[a]

Monosaccharide	Rha	Fuc	Ara	Xyl	Man	Gal	Glc
EAR	2	36	1	18	14	23	6
EAH1	2	42	2	29	16	8	1
EAH2	2	28	2	30	29	7	2
EAH3	2	18	4	33	35	5	3
EAH4	2	12	2	25	51	5	3
EAH5	2	9	1	23	55	5	5
EAH6	2	20	–	16	50	8	4
EAH7	2	15	2	15	49	9	8
EAH8	2	20	2	17	41	11	7
EAH9	1	19	2	11	44	9	14

[a] Fractions **EOH1** and **EOH2** only contain mannitol.

Table 3
Analyses of the fractions obtained by cetrimide precipitation and redissolution[a] of the fraction **EAR** from *Dictyota dichotoma* and of the subfractions of **EAR-0**.[b]

	Yield (%)	Carbohyd. (% anh.)	Uronate (%)	Sulfate (% SO_3Na)	Mol.wt. (kD)	Neutral sugars (mol/100 mols)						
						Rha	Fuc	Ara	Xyl	Man	Gal	Glc
EAR-0	4.1	40	5	11	1.5	10	16	5	13	13	5	38
EAR-0.5	10.6	63	40	13	8.4	2	40	2	30	16	6	4
EAR-1	5.5	67	24	24	11.5	1	41	–	27	16	13	2
EAR-1.5	7.5	59	16	30	16.3	1	46	–	23	18	10	2
EAR-2	8.8	59	14	33	23.6	1	43	–	16	10	28	2
EAR-4	8.5	59	4	34	26.3	1	37	1	8	1	51	1
EAR-5	0.8	n.d	n.d	n.d	n.d	3	38	2	7	4	43	3
EAR-0A	15	47	4	8	2.4	7	9	10	30	11	15	19
EAR-0B	19	57	5	9	1.0	3	5	5	13	11	3	62
EAR-0C	13	16	2	15	1.1	4	17	4	22	18	8	27

[a] The acronym of the original fraction incorporates a number indicating the concentration of NaCl necessary to redissolve the fraction, in tenths of molarity (*e.g.* **EAR-1** is the fraction of the **EAR** product redissolved with 1 M NaCl).
[b] Peaks obtained after gel filtration.

amount of xylose increased at expenses of galactose (Table 2). The residue was sequentially re-extracted with the same solvent until negligible amounts of carbohydrates appeared in the supernatant. This extraction procedure led to the isolation of eight more fractions (**EAH2**–**EAH9**), with decreasing yields (Table 1). The proportion of carbohydrates was similar in all of the fractions (48–60%) with the exception of **EAH9**, which showed lower amounts. The proportion of uronic acids increased first as the extraction proceeded, rising from 32% in **EAH1** to 50% in **EAH4**/**EAH5** and then falling back to 36% in **EAH9**. Only small variations in the proportion of sulfate were observed for most fractions (Table 1). Few proteins (≈3%) were extracted in the first extracts (**EAH1** and **EAH2**), but more were extracted as the extraction progressed, reaching a proportion of 17% in **EAH9**. Regarding the sugar composition, **EAH1** was the only fraction actually fucose-rich and mannose-poor. Galactose was always a minor component (Fig. 1), whereas rhamnose and arabinose were even less important (Table 2), and glucose started to be an important component in the last fractions (**EAH7**–**EAH9**). In **EAH2**, the proportion of mannose increased and that of fucose decreased in such a way that equals proportions of Man, Fuc and Xyl appeared (*ca.* 30% each). In the main following fractions (**EAH3**–**EAH7**), Man gained preponderance reaching proportions of up to 55% (Table 2), whereas Xyl decreased progressively from 33 to 15%, and Fuc varied between 9 and 20%. These results suggest that most of the galactofucan/fucan residues present in *Dictyota dichotoma* were extracted at room temperature, and an important amount was also removed by the first hot-acid extraction. The remaining hot-acid extractions removed components rich in uronic acids, mannose and xylose, certainly with a different structure. This is also shown by the diverse molar ratios observable in Fig. 1. The analysis of the ratios between uronic acid and Man, Xyl and Man, Gal and Fuc, and Fuc and sulfate suggests that Xyl, Man and uronic acid are probably part of the same molecules equivalent to those observed by Li et al. (2006) and Bilan et al. (2010), and that a sulfated galactofucan is also constituting the polysaccharides of this seaweed. The analysis of the Xyl/Fuc and the Xyl/Man ratios suggests that xylose appears probably substituting both the mannoglucuronan and the galactofucan moieties (Fig. 1).

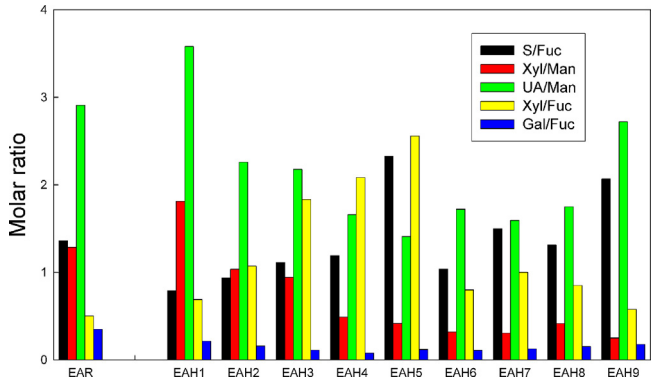

Fig. 1. Diverse molar ratios calculated for the fractions extracted from *Dictyota dichotoma*.

3.3. Fractionation

EAR and the nine EAH fractions were subfractionated with the aid of cetrimide precipitation. The insoluble salts were stepwise redissolved with the aid of increasing concentrations of sodium chloride: yields and analyses are shown in Tables 3–5. For **EAR**, as expected, only small amounts of products (identified by the suffix **-0**) remained soluble in presence of the cationic detergent, but surprisingly they carried significant amounts of anionic groups (especially sulfate). Glucose was the main monosaccharide, but other six monosaccharides appear in significant amounts. Fucose is the main monosaccharide in all fractions, but the monosaccharide compositions also show a straightforward behavior regarding the concentration of redissolution: the galactose content tends to increase with the NaCl concentration, while those of xylose and mannose tend to decrease, as occurred with *Adenocystis utricularis* (Ponce et al., 2003). A similar extract from *A. utricularis* fractionated in the same fashion gave rise to two main fractions with dissimilar characteristics: one redissolved at 0.5 M NaCl represented a low-sulfate high-uronate fucoidan with significant heterogeneity of monosaccharides (Rha, Xyl, Man, Gal), while the other, redissolved at 2 M NaCl was a heavily sulfated galactofucan. For *Dictyota dichotoma* five fractions were obtained in similar proportions. The first one (**EAR-0.5**) shares the general characteristics of the uronofucoidans previously found: significant heterogeneity of monosaccharides, high uronic acid and low sulfate contents (Ponce et al., 2003). Its analytical characteristics assimilate this polysaccharide with the xylofucoglucuronan from *Spatoglossum schröederi* (Leite et al., 1998). Fractions **EAR-1.5**, **EAR-2** and **EAR-4**, appear to be more compatible with a fucoxylogalactan structure, as uronic acids and mannose are less important constituents. However, among the three fractions, the proportion of galactose increases sharply as the concentration of NaCl necessary to redissolve the fraction increases, at the same time that the proportion of fucose and xylose diminishes. Simultaneously, the sulfate content and the average molecular weight increase (Table 1). Mannose can also be part of these molecules. On the other hand, fraction **EAR-1** shows characteristics intermediate between those observed for **EAR-0.5** and **EAR 1.5**, suggesting a continuous structural change, rather than

Table 4
Analyses of the fractions obtained by cetrimide precipitation and redissolution[a] of the products **EAH1**, **EAH2**, **EAH3** and **EAH4** extracted from *Dictyota dichotoma*.

	Yield (%)	Carbohyd. (% anh.)	Uronate (%)	Sulfate (% SO_3Na)	Mol.wt. (kD)	Neutral sugars (mol/100 mols)						
						Rha	Fuc	Ara	Xyl	Man	Gal	Glc
EAH1-0	1.0	62	21	11	2.6	5	22	1	17	6	13	36
EAH1-0.5	17.7	50	51	6	5.1	2	22	1	45	27	2	1
EAH1-1	14.6	63	36	14	14.8	1	47	–	23	22	6	1
EAH1-1.5	17.1	64	30	19	13.7	2	41	–	26	25	5	1
EAH1-2	3.9	44	12	21	18.2	3	30	1	15	9	40	2
EAH1-4	2.1	35	3	21	18.3	4	27	1	8	4	49	7
EAH1-5	0.4	n.d	n.d	n.d	n.d	4	16	3	26	18	25	8
EAH2-0	6.4	58	26	9	3.5	5	18	2	20	23	12	20
EAH2-0.5	31.9	80	42	10	9.0	1	26	–	36	33	4	–
EAH2-1	12.3	59	25	18	8.5	1	22	3	36	29	5	4
EAH2-1.5	4.3	64	24	23	16.2	2	37	–	26	24	8	3
EAH2-2	0.9	22	4	32	16.7	5	32	–	21	11	27	4
EAH2-4	0.5	57	2	19	16.8	4	9	–	12	6	2	67
EAH2-5	0.8	66	2	24	n.d	2	9	4	39	23	15	8
EAH3-0	6.7	57	43	5	3.7	6	11	2	21	33	15	12
EAH3-0.5	24.9	62	48	7	6.4	1	12	–	41	41	2	3
EAH3-1	10.0	66	38	12	8.2	1	24	–	31	35	6	3
EAH3-1.5	0.5	52	3	15	9.3	3	18	–	16	31	10	22
EAH3-2	0.6	40	3	22	7.6	4	8	3	23	29	24	9
EAH3-4	1.0	30	2	22	17.6	2	3	4	47	18	8	18
EAH3-5	0.5	n.d	n.d	27	n.d	5	2	–	40	28	4	21
EAH4-0	2.6	53	18	4	2.1	6	9	1	8	27	18	31
EAH4-0.5	47.2	68	48	5	8.0	1	10	–	30	51	5	3
EAH4-1	0.8	n.d	n.d	n.d	3.5	8	11	–	8	46	7	20
EAH4-1.5	0.4	n.d.	n.d	n.d	n.d	1	1	2	55	33	3	5
EAH4-2	0.7	n.d.	n.d	n.d	n.d	7	2	11	23	35	5	17
EAH4-4	1.6	30	2	17	6.2	2	32	4	19	26	13	16

[a] The acronym of the original fraction incorporates a number indicating the concentration of NaCl necessary to redissolve the fraction, in tenths of molarity (*e.g.* **EAH2-1** is the fraction of the **EAH2** product redissolved with 1 M NaCl).

a sharp one. Abdel-Fattah et al. (1978) also encountered a fraction rich in Fuc, Xyl and Gal by ethanol precipitation.

In order to search for possible mixtures of polysaccharides in each of those fractions, they were submitted to gel permeation chromatography on a BioGel P-30 column. Results showed that only fraction **EAR-0** could be subdivided further, whereas the fractions redissolved in NaCl gave only one peak, thus suggesting that, if two components are present, they have similar molecular weights. The three subfractions of fraction **EAR-0** were isolated and further analyzed (Table 3). The heterogeneity persists for the three fractions, although one (**EAR-0C**) shows more characteristics of a fucan sulfate, and another one (**EAR-0B**) laminaran-like characteristics. In any case, the presence of almost every monosaccharide, sulfate ester and uronic acids in every fraction shows that they are still mixtures of polysaccharides. The presence of ionic groups like sulfate and uronic acid in a fraction not precipitated by a cationic detergent

Table 5
Analyses of the fractions obtained by cetrimide precipitation and redissolution[a] of the products **EAH5**–**EAH9** extracted from *Dictyota dichotoma*.

	Yield (%)	Carbohyd. (% anh.)	Uronate (%)	Sulfate (% SO_3Na)	Mol.wt. (kD)	Neutral sugars (mol/100 mols)						
						Rha	Fuc	Ara	Xyl	Man	Gal	Glc
EAH5-0	5.8	58	42	3	3.5	3	7	2	12	42	12	22
EAH5-0.5	30.4	73	50	5	8.4	2	7	1	15	64	5	6
EAH5-1	0.5	n.d	n.d	n.d	n.d	7	6	–	21	30	11	25
EAH5-2	0.5	n.d	n.d	n.d	n.d	2	5	–	17	31	13	32
EAH5-4	0.9	n.d	n.d	n.d	n.d	8	5	–	27	21	9	30
EAH6-0	5.8	26	24	20	2.9	4	25	1	18	30	14	8
EAH6-0.5	25.3	58	42	6	7.5	1	6	–	21	62	5	5
EAH6-1	4.1	n.d	n.d	n.d	n.d	7	5	3	15	29	10	31
EAH6-1,5	3.8	n.d	n.d	n.d	n.d	9	14	8	19	21	8	22
EAH6-2	2.4	n.d	n.d	n.d	n.d	1	3	3	20	38	8	27
EAH6-4	0.8	26	2	n.d	15.1	–	2	1	10	17	4	66
EAH7-0	19.6	37	17	13	2.1	3	13	11	15	27	8	23
EAH7-0.5	39.8	61	35	9	3.5	–	11	–	19	57	7	6
EAH7-1	3.0	55	4	14	8.5	4	6	20	34	15	7	14
EAH7-1.5	1.6	49	1	15	15.1	4	4	19	16	22	5	30
EAH7-2	2.2	36	1	16	18.8	4	3	16	19	33	8	17
EAH7-4	13.3	9	1	20	n.d	–	4	9	59	14	4	10
EAH8-0	13.1	57	44	9	2.6	3	9	2	18	53	7	8
EAH8-0.5	15.7	64	42	9	4.9	–	6	–	17	64	6	7
EAH8-1	2.0	n.d	n.d	n.d	n.d	1	9	2	26	48	5	9
EAH8-4	3.1	n.d.	n.d	n.d	n.d	2	19	5	19	26	17	12
EAH9-0	38.3	23	10	30	2.1	1	21	5	17	29	10	17
EAH9-0.5	15.8	43	20	12	3.2	1	7	4	21	51	6	10
EAH9-1	2.4	48	2	12	n.d	2	5	13	44	9	5	22

[a] The acronym of the original fraction incorporates a number indicating the concentration of NaCl necessary to redissolve the fraction, in tenths of molarity (*e.g.* **EAH6-1** is the fraction of the **EAH6** product redissolved with 1 M NaCl).

Table 6
Antiviral and cytotoxic activities of some subfractions isolated from *Dictyota dichotoma*.[a]

Fraction	IC_{50} (μg/mL)		CC_{50} (μg/mL)	SI	
	HSV-1	CVB3		HSV-1	CVB3
EAR-0.5	12.5	125	1250	100	10
EAR-1	31.2	15.6	312.5	10	20
EAR-1.5	62.5	15.6	>1000	>16	>64
EAR-2	7.5	15.6	312.5	42	20
EAR-4	15.5	15.6	>1000	>65	>64
EAH1-0.5	375	100	250	1	3
EAH1-1	50	25	500	10	20
EAH1-1.5	125	>100	187	2	>2
EAH1-4	93.7	12.5	>1000	>11	>80
EAH2-0.5	375	25	750	2	30
EAH2-1	50	>100	>1000	>20	>10
EAH2-2	93.7	>100	>1000	>11	>10
EAH3-1	25	>100	500	20	>5
EAH3-2	93.7	>100	500	5	>5
EAH4-0.5	12.5	>100	500	40	>5
EAH5-0.5	100	50	500	5	10
EAH6-0.5	100	50	>1000	>10	>20
EAH8-0.5	15.6	>100	250	16	>3
EAH8-4	375	>100	500	1	>5
EAH9-0	25	>100	>1000	>40	>10
Ribavirin[a]	12	16	82	6.8	5.1

[a] Ribavirin was used as a positive control.

might seem odd, but it has already been shown to occur (Ponce et al., 2003). In those cases, it has been considered that some interaction of the sulfate groups (probably through calcium bridges) precludes that which should occur with the cationic detergent.

Cetrimide fractionation of **EAH1** (Table 4) shows three main fractions redissolved at NaCl concentrations of 0.5, 1 and 1.5 M (**EAH1-0.5**, **EAH1-1**, and **EAH1-1.5**). They are characterized by the presence of Fuc, Xyl and Man: coarsely speaking **EAH1-0.5** exhibits a Fuc:Xyl:Man ratio of 1:2:1, whereas the other two fractions show a 2:1:1 ratio. The minor fractions precipitating at higher NaCl concentrations show an increased proportion of Gal, which becomes the main monosaccharide. The cetrimide fractionation of **EAH2** and **EAH3** produces large proportions of a fraction redissolved in 0.5 M NaCl (25–32%) rich in uronic acid (42–48%). Xyl is the main monosaccharide for the **EAH2** fractions, closely followed by Fuc and Man. For **EAH3**, on the other hand, Xyl and Man share importance at expenses of Fuc. Fractionation of **EAH4**, **EAH5** and **EAH6** (Tables 4 and 5) give rise to just one important fraction, redissolved at 0.5 M NaCl, and scarcely sulfated (5–6%). Its analysis suggests the presence of xylomannans (or xylomannoglucuronans). It is noteworthy to find, at the minor fractions which are heavily sulfated, the presence of significant proportions of glucose, possibly constituting polysaccharides different from laminaran or floridean starch. In the fractionation of **EAH7**, **EAH8**, and **EAH9**, the cetrimide-soluble fraction starts to gain weight with the sequential extraction (Table 5). The subfractions redissolved in 0.5 M NaCl (indicated by **-0.5**), also abundant in these fractions, show the same characteristics as those found previously: high amounts of uronic acid-containing polysaccharides mixed or interspersed with xylomannans. The soluble fractions (indicated by **-0**), on the other hand, exhibit a large heterogeneity: although laminarans are possibly present, other polysaccharides containing the remaining monosaccharides (especially Xyl and Man) are also at hand. They carry significant amounts of anionic groups like uronic acids or sulfate, suggesting polysaccharides similar to those found in the **-0.5** fractions, but unable to interact with the cationic detergent. Arabinose, a monosaccharide unusual in brown seaweeds appears significantly in some fractions. The presence of products with a composition compatible with that of xylomannans is not common in brown seaweeds. However, Magdel-Din Hussein et al. (1979) found oligosaccharides after partial hydrolysis of a purified fraction from *D. dichotoma*, carrying both Xyl and Man as main constituents.

Sulfated xylomannans have been commonly found within the order Nemaliales of red seaweeds, usually carrying a α-(1→3)-D-mannan backbone with β-D-xylopyranosyl substitution at C-2 (Kolender, Matulewicz, & Cerezo, 1995; Matulewicz & Cerezo, 1987; Recalde, Noseda, Pujol, Carlucci, & Matulewicz, 2009; Usov, Adamyants, Yarotskii, & Anoshina, 1975), or with xylose at other positions (Mandal et al., 2008; Usov & Dobkina, 1988). These polysaccharides had antiherpetic and anticoagulant properties (Kolender, Pujol, Damonte, Matulewicz, & Cerezo, 1997; Mandal et al., 2008). A different sulfated xylomannan with a β-(1→4)-D-mannan backbone and xylose stubs at C-6 was reported for two red seaweeds of the order Ceramiales (Cardoso, Noseda, Fujii, Zibetti, & Duarte, 2007).

3.4. Antiviral activity

The antiviral and cytotoxic activities of the main fractions against HSV-1 and CVB3 are shown on Table 6. Regarding HSV-1, fractions **EAR-0.5, EAR-2** and **EAR-4** showed the higher activities and selectivities; **EAH4-0.5**, **EAH9-0** and **EAH2-1** gave acceptable values, whereas the remaining fractions showed less antiviral activities or higher cytotoxicities, thus leading to low selectivities (<20). These results are quite different to those encountered for *A. utricularis* (Ponce et al., 2003) for which the uronofucoidan fractions redissolved at low ionic strengths exhibited no activity up to 100 μg/mL, and the galactofucan fractions exhibited maximal activities. Several fucoidans with IC_{50} against HSV-1 and HSV-2 in the order of 1 μg/mL (or even less) were reported (Bandyopadhyay et al., 2011; Ponce et al., 2003; Wijesekara, Pangestuti, & Kim, 2011). However, other authors have reported fucoidans with an IC_{50} of 18 μg/mL and considered them as antiviral agents if the selectivity indexes were above 10 (Lee et al., 2011). In any case, fractions **EAR-0.5**, **EAR-2**, and **EAR-4** with IC_{50} of 12.5, 7.5 and 15.5 μg/mL and SI of 100, 42, and >65, respectively, should be considered with moderate antiherpetic activity. A survey of crude extracts from Hong Kong seaweeds which included *Dictyota dichotoma* gave a IC_{50} value of *ca.* 25 μg/mL (Wang, Ooi, et al., 2008), not far from the average of the current fractions. On the other hand, the inhibitory effect against CVB3 shows a different pattern. The

room-temperature extracted fractions **EAR-1**, **EAR-1.5**, **EAR-2**, and **EAR-4**, as well as the early-appearing **EAH1–4** exhibit the best activity (IC_{50} = 12.5–15.6 μg/mL), but most of the remaining fractions are inactive (Table 6). The best selectivity indexes for this virus are observed for **EAR-1.5**, **EAR-4** and **EAH1–4** (>64). Thus, **EAR-4** appears as the best antiviral fraction for both viruses. This fraction, as well as **EAH1–4**, are clearly sulfated fucogalactans (Table 2). In other fractions, the large amounts of xylose present, as well as the relatively low molecular weights encountered for the current highly sulfated fractions might be responsible for their decreased inhibitory activity.

The antiviral activity has been related with the sulfate content and the molecular weight (Chevolot et al., 1999; Nishino, Kiyohara, et al., 1991; Nishino, Nagumo, et al., 1991; Ponce et al., 2003). This relationship does not hold with the current results, where the sulfated galactofucans/fucogalactans seem to be responsible of the inhibitory effect of both viruses, as previously reported (Ponce et al., 2003; Zhuang et al., 1995). The hydrophobicity of the methyl group on C-6 of fucose might be responsible for this effect (Damonte, Matulewicz, & Cerezo, 2004; Feldman et al., 1999; Venkateswaran et al., 1989). However, in the current work, some fractions carrying larger amounts of Xyl, Man and uronic acids seem to have a moderate inhibitory action against HSV-1 (although none against CVB3). The anti-HSV effect of red seaweed xylomannans has been already reported (Kolender et al., 1997).

4. Conclusion

The brown seaweed *Dictyota dichotoma* produces large amounts of heterogeneous polysaccharides when submitted to the extraction procedures used to obtain fucoidans. By cetrimide fractionation of the two first extracts, *i.e.* that extracted with room-temperature acid (**EAR**), and that extracted in the first place by hot acid (**EAH-1**), typical heavily sulfated galactofucans/fucogalactans are obtained, with no major signs of heterogeneity. However, the proportion of subfractions from these two extracts are minor when compared to those also carrying fucose but with large proportions of xylose, mannose and uronic acids, redissolved at lower NaCl concentrations from the same extracts. Fucose is not the most important monosaccharide in most of the subfractions of the subsequent extracts. The proportions of mannose, xylose and uronic acids increase in such a way as to make typical fucans minor components of the whole polysaccharide system of this seaweed.

Moderate antiviral activity (against HSV-1 and CVB3) was determined for the galactofucan fractions; the remaining fractions were inactive against CVB3, but some of them, rich in xylose, mannose and uronic acid were also active against HSV-1.

Acknowledgements

This work was supported by grants from University of Buenos Aires and CONICET. M.R. is a recipient of a Fellowship from CONICET, whereas N.M.A.P., D.A.N., R.M.G. and C.A.S. are Research Members of the same Institution. The authors are indebted to Dr. L. Cavallaro (UBA) for the gift of a sample of ribavirin.

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