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Structural and compositional characteristics of a sulfated galactan from the red alga *Gracilariopsis persica*

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ABSTRACT

An agar polysaccharide has been isolated from the red seaweed *Gracilariopsis persica* collected from the Persian Gulf (Iran). Characterization of the structure by chemical and spectroscopic methods showed a basic repeating structure of alternating 3-linked β-D-galactopyranosyl and 4-linked 3,6-anhydro-α-L-galactopyranosyl units. The main polysaccharide components were 3,6-anhydrogalactose (31.8%), galactose (52.1%) and 6-*O*-methyl-galactose (10.9%). In addition, minor components such as glucose (4.1%) and xylose (1.1%) were detected. The degree of substitution (DS) of sulfate, calculated from S%, was 0.18%. Data from ^{13}C NMR, FT-IR, methylation and desulfation–methylation provided evidence of sulfation at C-2 and C-6 of L-galactose and also C-4 of D-galactose. Gel permeation chromatography (GPC) indicated Mw = 2.22×10^5 g/mol for this polysaccharide.

1. Introduction

Red algal galactans are sulfated polysaccharides that found in the cell matrix of red algae (Rhodophyta). These polysaccharides usually have a linear backbone built on alternating 3-linked β-galactopyranose and 4-linked α-galactopyranose residues. The β-galactose residues are always belonging to the D-series, whereas the α-galactose residues are D in carrageenans and L in agars (Knutsen, Myslabodski, Larsen, & Usov, 1994; Painter, 1983). A substantial part or even all the α-galactose residues may exist in the form of 3,6-α-L-anhydro derivative (Usov, 1992). In general, some C-6 of the 4-linked α-L-galactose are substituted with sulfate groups resulting in α-L-galactose-6-sulfate, which is known as a biogenetic precursor of 3,6-α-L-anhydrogalactose (AG) (Rees, 1961). Various hydroxyl groups of this biopolymer may be substituted by sulfate ester, pyruvate acetal, methyl ether or single branching sugar residues such as 4-*O*-methyl-α-L-galactopyranose and β-D-xylopyranose (Usov, 1998). The location and amount of the substitutions, affect strongly the physical properties of agar gels and also their biological activity (Freile-Pelegrin & Murano, 2005; Shanmugan & Mody, 2000). These polysaccharides, due to their gel properties, are widely used as gelling agent in processed foods, cosmetics and pharmaceutical products, besides application in medicine and biotechnology (Marinho-Soriano & Bourret, 2005).

The genus *Gracilariopsis* of red seaweed is distributed throughout the tropical and warm-water regions of the world and the species *Gracilariopsis persica* was first described by Bellorin, Buriyo, Sohrabipour, Oliveira, and Oliveira (2008) based on cellular and molecular analysis. *G. persica* began to grow from late September to July and showing high growth rate from January to May. This is the first report on the chemical characterization of the sulfated galactan polysaccharide present in this seaweed.

2. Experimental

2.1. Collection of seaweed

G. persica was harvested after 45 days of cultivation in spring (April 2007) from the Persian Gulf (Bandarabbas city, Iran), cleaned of epiphytes, washed with distilled water, air dried and ground in a mill to a fine powder.

2.2. Isolation of sulfated polysaccharide

Prior to extraction of polysaccharides, dried grinded seaweeds were preliminary washed with acetone and hot 94% ethanol to eliminate pigments (Chinkichi, 1962). The depigmented algal powder obtained from *G. persica* (10 g) was autoclaved for 1 h at 120 °C in 500 ml of distilled water and successively filtered hot through celite bed and sintered glass filter CG = 2. The residue was re-extracted under the same conditions and added to the filtrate. Agar solution was cooled down to room temperature which changed to

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gel, frozen overnight and thawed; after removing the thawed liquid, the agar gel was lyophilized to obtain the dry polysaccharide.

2.3. Composition analysis

The monosaccharide composition was determined by GC and GC–MS as their alditol acetates. Derivatives were prepared by the reductive hydrolysis procedure of Stevenson and Furneaux (1991) that used *N*-methylmorpholine borane as reductant.

Configuration of 6-*O*-methyl-D-galactose and also D to L galactose ratio was determined by conversion of the monosaccharides to their diastereomeric 1-deoxy-1-(2-hydroxy-propylamino) alditol acetate by using chiral (S)-1-amino-2-propanol and analysis by GC and GC–MS in the previously described conditions (Cases, Cerezo, & Stortz, 1995).

GC analysis was performed using a Thermoquest-Finnigan Trace GC instrument equipped with a capillary DB-1 fused silica column (60 m × 0.25 mm i. d., film thickness 0.25 μm). The oven temperature was raised from 60 °C to 250 °C at a rate of 10 °C/min, then held at 250 °C for 25 min. Nitrogen was used as the carrier gas at a flow rate of 1.1 ml/min. The injector and detector (FID) temperatures were kept at 250 °C and 280 °C, respectively.

The GC–MS was carried out on a Thermoquest-Finnigan spectrometer equipped with a DB-1 column (60 m × 0.25 mm) using helium as a carrier gas. The temperature program was the same as GC analysis.

Sulfate and protein content were determined by elemental micro analysis (Vario III Elemental). DS sulfate was obtained by S% and C% according to Eq. (1), proposed by Melo, Feitosa, Freitas, and de Paula (2002). The protein content was calculated from N%, using the correction factor of 6.25 as proposed by Marks, Baum, and Swain (1985).

2.4. Desulfation

Desulfation of the sulfated galactan was performed as reported previously (Nagasawa, Inoue, & Kamaia, 1977), by treatment with 80:10:1 Me_2SO–MeOH–pyridine at 105 °C for 4 h.

2.5. Methylation analysis

Methylation analysis of native and desulfated polysaccharide was carried out two times by applying modified Hakomori (1964) procedure by Stevenson and Furneaux (1991) after converting the polysaccharide to triethylammonium salts. Methylated polysaccharide, after reductive hydrolysis and acetylation, was analyzed by GC and GC–MS.

2.6. Spectroscopic methods

Fourier transform infrared (FT-IR) spectrum was recorded from the polysaccharide powder in KBr pellets (0.5%, w/w) on a Bruker Tensor 27 FT-IR spectrometer (8 scans, at a resolution of 4 cm^{-1}) between 400 and 4000 cm^{-1}. For ^{13}C NMR spectroscopy, sample (60 mg/ml) was dissolved in D_2O by using ultrasonic radiation (30 min in 70 °C). Spectrum was acquired on a Bruker DRX 500 MHz Avance at 25 °C, using acquisition time of 1.05 and a relaxation delay of 3 S, for 30,720 scans. ^{13}C NMR chemical shifts were measured in ppm relative to internal acetone at 30.1.

2.7. Gel permeation chromatography (GPC)

Molecular weight distribution and weight-average molecular weight was analyzed by gel permeation chromatography (GPC) using an Agilent 1100 instrument equipped with a differential refractometer detector, an PL Aquagel-OH MIXED 8 μm column (300 mm × 7.5 mm) at room temperature, and flow rate of 0.5 ml/min. Concentration of polysaccharide was 0.5% in 0.1 M $NaNO_3$ as solvent. Pullulan samples were used as standard for calibration curve.

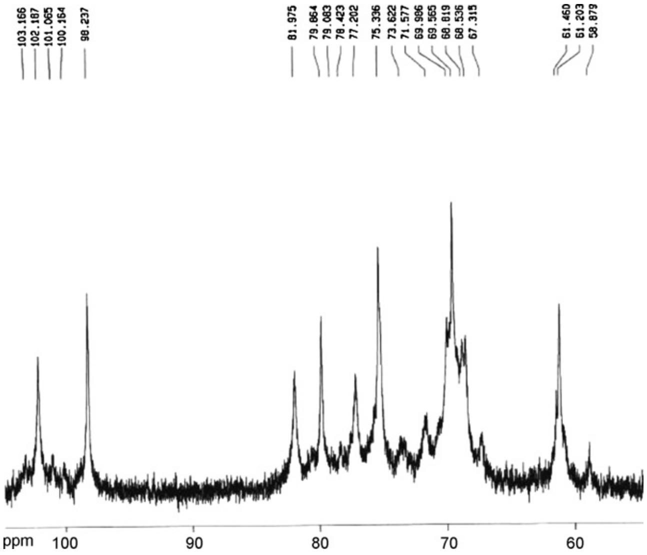

Fig. 1. ^{13}C NMR spectrum of *Gracilariopsis persica* agar polysaccharide.

3. Results and discussion

In order to study the chemical structure of polymer presented in G. persica, it was extracted from depigmented algal powder. The yield of extract was 29%. The protein content based on nitrogen content (0.37%) of polysaccharide was calculated to be 2.31%. The DS sulfate for *G. persica* was 0.18%. This value was calculated according to S% (1.4%) and C% (35.2%) of polysaccharide by using Eq. (1) (the number 12 in this equation corresponds to the number of carbons in disaccharide repeated units).

$$\mathrm{DS} = \frac{\mathrm{S\%/atomic\ mass\ of\ S}}{\mathrm{C\%/atomic\ mass\ of\ C} \times 12} = 4.5\frac{\mathrm{S\%}}{\mathrm{C\%}} \tag{1}$$

The GPC chromatogram of agar polysaccharide from *G. persica* by refraction index measurements showed one peak with $Mw = 2.2 \times 10^5$ g/mol.

3.1. Structure analysis

3.1.1. Gas chromatography

Reductive hydrolysis of the polysaccharide, followed by acetylation and subsequent GC and GC–MS analysis of the resulting acetylated alditols, indicated the presence of galactose (52.1%), 3,6-anhydro galactose (31.8%), 6-*O*-methyl-galactose (10.9%), glucose (4.1%) and xylose (1.1%) as the monosaccharide contents of this polysaccharide.

Analysis of absolute configuration showed that 23% of galactose units were in the form of L-galactose. The ratio of D-galactose plus 6-*O*-methyl-D-galactose to L-galactose plus 3,6-anhydro-L-galactose (its configuration obtained by ^{13}C NMR) was 1.16, showing an agaran backbone.

3.1.2. NMR spectroscopy

NMR spectroscopy is a convenient method to follow the composition and to give valuable structural information on the red algal galactans (Usov, 1998). The ^{13}C NMR spectrum of polysaccharide from *G. persica* is shown in Fig. 1. The observed resonances are consistent with an alternating structure of the agaran type, while the

Table 1
^{13}C NMR chemical shift assignments of *Gracilariopsis persica* agar polysaccharide.

Residue unit		^{13}C chemical shift						
		C1	C2	C3	C4	C5	C6	Me
(G-A)$_n$	G	102.2	70.0	82.0	68.5	75.3	61.2	
	A	98.2	69.6	79.7	77.2	75.3	69.6	
(G′-L6S)	G′	103.2	70.0	82.0	68.8	75.3	61.5	
	L6S	101.1	69.6	71.6	79.1	70.0	67.3	
(G6M-A)	G6M	102.2	70.0	82.0	68.8	73.6	71.6	59.0
FS	Glu	100.2	72.2	73.8	78.4	71.9	61.2	

(G-A): (1 → 3) β-D-galactose(1 → 4) 3,6-anhydro-α-L-galactose; (G′-L6S): (1 → 3) β-D-galactose(1 → 4) α-L-galactose-6-sulfate; (G6M-A): (1 → 3) 6-O-methyl-β-D-galactose(1 → 4) 3,6-anhydro-α-L-galactose; also FS signifying Floridean starch).

absence of any anomeric signals below 98 ppm indicated the lack of 4-linked D-galactose residues (Kolender & Matulewicz, 2002). Also the absence of signals of non-anomeric sugars carbons at a lower field than δ 82 in the ^{13}C NMR spectrum demonstrated the pyranoid form of all sugar residues (Bock & Pedersen, 1983). The signal assignments (Table 1) were made on the basis of comparison with spectra of model compounds, agarose and other related polysaccharides (Lahaye, Yaphe, Viet, & Rochas, 1989; Lai & Lii, 1998; Miller & Furneaux, 1997; Truus et al., 2006; Usov, Yarotsky, & Shashkov, 1980; Valiente, Fernandez, Perez, Marquina, & Velez, 1992).

The anomeric region (δ 90–110) shows two main signals as C-1 of β-D-galactose linked to 3,6-α-L-anhydro galactose (agarose unit) at 102.2 and C-1 of 3,6-α-L-anhydro galactopyranose at 98.2. The two minor but distinct peaks of anomeric carbons of →3)-β-D-galactose-(1 → 4)-6-*O*-SO$_3$-α-L-galactose-(1→ repeat unit (agarose biological precursor unit) were also assigned. The resonances due to anomeric carbon of β-D-galactose and anomeric carbon of 6-*O*-SO$_3$-α-L-galactose can be observed at 103.2 and 101.1, respectively.

Methylation at the C-6 position of β-D-galactose was indicated by the characteristic signals due to 6-*O*-methyl-β-D-galactose (C-5; 73.6, C-6; 71.6 and C–Me; 59.0). Also a series of weak signals (C-1, 100.1; C-2, 72.2; C-3, 73.8; C-4, 78.4; C-5, 71.9; C-6, 61.2 ppm) were detected in ^{13}C NMR spectrum of polymer that shows the presence of fluridan starch in this polysaccharide (Dais & Perlin, 1982; Lahaye, Rochas, & Yaphe, 1986).

The structures of sulfated galactans isolated from two *Gracilariopsis* sp., i.e. *G. longissima* and *G. lemaneiformis*, have already been reported (Chirapart, Ohno, Ukeda, Sawamura, & Kusunose, 1995; Mollet, Rahaoui, & Lemoine, 1998). Similar to our finding, 6-*O*-methyl-galactose was reported from both species. However 4-*O*-methyl-galactose was reported from the former and 2-*O*-methyl-α-L-galactopyranose was reported from the later that were not detected in the agar from *G. persica*.

3.1.3. Methylation analysis

In order to confirm glycoside linkage position and identify sulfate group location on this sulfated galactan, methylation was performed before and after desulfation. Results of the methylation analysis of native and desulfated polysaccharide are given in Table 2. The results were in agreement with ^{13}C NMR chemical shift assignments and also composition analysis by gas chromatography. Major contents for 3-linked galactose units originated from D-galactose plus 6-*O*-methyl D-galactose (51.6%) and major content of 4-linked was related to L-anhydrogalactose (34.3%) and L-galactose-6-sulfate (6.4%).

Significant changes in 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methyl galactitol and 1,4,5,6-tetra-*O*-acetyl-2,3-di-*O*-methyl galactitol contents before and after desulfation were observed. Decrease in amount of second one related to desulfation of 4-linked L-galactose-6-sulfate (precursor of 4-linked L-anhydrogalactose). Furthermore, due to decrease in 1,2,4,5-tetra-*O*-acetyl-3,6-anhydro-galactitol and 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl galactitol contents in premethylated product after desulfation, minor amounts of 4-linked L-anhydrogalactose-2-sulfate and 3-linked D-galactose-4-sulfate residues, should have been present. Since there was no change in 1,3,5,6-tetra-*O*-acetyl-2,4-di-*O*-methyl galactitol content, xylose monosaccharide could be situated at C-6 position of 3-linked D-galactose as a single branch. The structure of the analyzed sulfated galactan is depicted in Fig. 2.

3.1.4. FT-IR analysis

The FT-IR spectrum of the agar from *G. persica* is shown in Fig. 3. The principal IR bands for *G. persica* were in good agreement for agars from other agarophyte species (Chopin & Whalen, 1993; Christian & Bodard, 1983; Lahaye & Yaphe, 1988; Lioyd, Dogson, Price, & Rose, 1961; Mollet et al., 1998; Prado-Fernandez, Rodriguez-Vazquez, Tojo, & Andrade, 2003; Rochas, Lahaye, & Yaphe, 1986; Sekkal et al., 1993; Stanley, 1963; Turvey & Williams, 1962). The most important bands were assigned at 1370 cm^{-1} corresponding to ester sulfates. The absorbance band at 1250 cm^{-1}

Table 2
Methylation analysis of native and desulfated *Gracilariopsis persica* agar polysaccharide.[a]

Derivative	Monosaccharide[b]	Proportion (mole%)	
		Native	Desulfated
1,5-Di-*O*-acetyl-2,3,4-tri-O-methyl xylitol	T-Xyl[c]	Tr[d]	1.4
1,4,5-Tri-*O*-acetyl-3,6-anhydro-2-*O*-methyl galactitol	4-AnGal	34.3	39.6
1,2,4,5-Tetra-*O*-acetyl-3,6-anhydro-galactitol	2,4-AnGal	1.9	Tr
1,5-Di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl galactitol	T-Gal	Tr	Tr
1,3,5-Tri-*O*-acetyl-2,4,6-tri-*O*-methyl galactitol	3-Gal	51.6	55.2
1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl galactitol	4-Gal	1.1	2.5
1,3,4,5-Tetra-*O*-acetyl-2,6-di-*O*-methyl galactitol	3,4-Gal	2.0	–
1,3,5,6-Tetra-*O*-acetyl-2,4-di-*O*-methyl galactitol	3,6-Gal	1.4	1.3
1,4,5,6-Tetra -*O*-acetyl-2,3-di-*O*-methyl galactitol	4,6-Gal	6.4	Tr
1,2,3,4,5,6-Hexa-*O*-acetyl galactitol	2,3,4,6-Gal	1.3	Tr

[a] Data were normalized to exclude glucose.
[b] 2,4-AnGal means a 2,4-disubstituted and/or linked galactopyranosyl unit, etc.
[c] T- means a terminal sugar unit.
[d] Tr means trace (<1.0%).

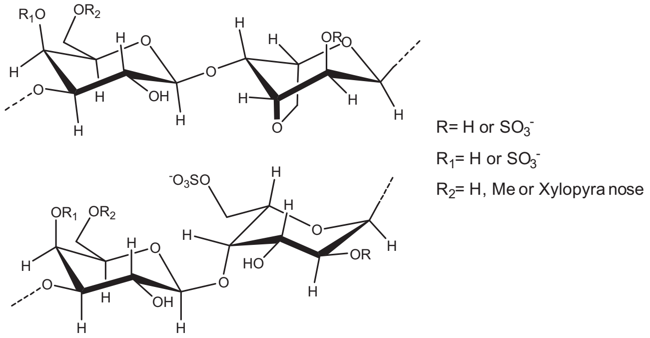

Fig. 2. Structure of sulfated galactan polysaccharide extracted from *Gracilariopsis persica*.

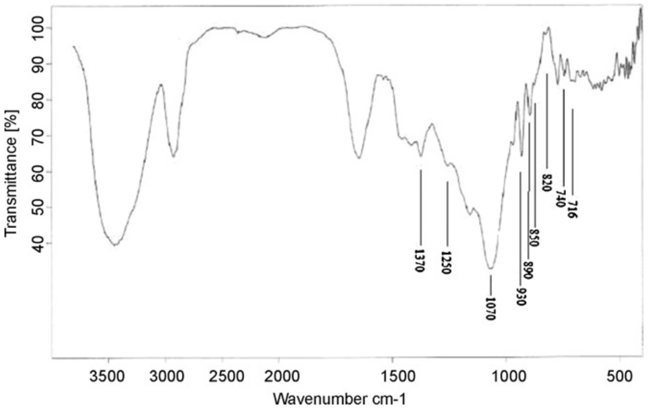

Fig. 3. FT-IR spectrum of *Gracilariopsis persica* agar polysaccharide.

belongs to asymmetric stretching of sulfate ester group (Lioyd et al., 1961; Turvey & Williams, 1962), 1070 cm^{-1} showing the skeletal made of the galactan (Sekkal et al., 1993), 930 cm^{-1} due to the vibration of the C–O–C bridge of 3,6-anhydro galactose (Stanley, 1963), and 890 cm^{-1} is an agar specific band (Christian & Bodard, 1983). Also the two bands at 740 cm^{-1} and 716 cm^{-1} in the spectrum were attributed to the C–O–C bending mode in glycosidic linkages (Sekkal et al., 1993).

The region around 800–850 cm^{-1} is used in order to identify the position of the sulfate group in agar biopolymer (Chopin & Whalen, 1993; Lahaye & Yaphe, 1988; Mollet et al., 1998; Prado-Fernandez et al., 2003; Rochas et al., 1986). The weak band close to 820 cm^{-1} indicated the presence of sulfate group on C-6 of the α-L-galactose and the presence of a shoulder at 850 cm^{-1} indicated a small degree of substitution on C-4 of β-D-galactose sugar units.

4. Conclusion

A sulfated galactan polysaccharide was extracted from the red seaweed *G. persica* and its structure was investigated by different chemical and spectroscopic techniques for the first time. Analysis showed basic repeating structure of alternating 3-linked β-D-galactopyranosyl and 4-linked 3,6-anhydro-α-L-galactopyranosyl units as an agaran backbone with minor precursor [→3)-β-D-galactose-(1 → 4)-6-*O*-SO_3-α-L-galactose-(1→] units. Methyl group was located on C-6 of β-D-galactopyranosyl and there was evidence of substitution of some sulfate group on C-4 of β-D-galactopyranosyl, also C-2 and C-6 of α-L-galactopyranosyl.

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