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Carbohydrate Polymers 66 (2006) 208–215
Carbohydrate Polymers
www.elsevier.com/locate/carbpol

Preparation of a low-molecular weight fraction by free radical depolymerization of the sulfated galactan from *Schizymenia binderi* (Gigartinales, Rhodophyta) and its anticoagulant activity

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Received 19 December 2005; received in revised form 1 March 2006; accepted 2 March 2006
Available online 2 May 2006

Abstract

Depolymerization of the sulfated galactan from *Schizymenia binderi* with H_2O_2 in the presence of copper (II) acetate afforded a homogeneous fraction (MW 8500) in 50% yield. Alkylation and NMR spectroscopy analysis of this fraction indicated the presence of an agaran–carrageenan hybrid polysaccharide composed of 3-linked β-D-galactopyranosyl residue partially sulfated at position O-2 and 4-linked α-D-galactopyranosyl and α-L-galactopyranosyl residues mainly sulfated at position O-3 and glycosylated at position O-2. Discreet anticoagulant activity, similar to that of the native polysaccharide was found in the derivative obtained by sulfation of this fraction.

Keywords: *Schizymenia binderi*; Rhodophyta; Agaran–carrageenan hybrid; Free radical depolymerization; Sulfation; Anticoagulant

1. Introduction

The water soluble polysaccharide from gametophytic *Schizymenia binderi* was previously studied in this laboratory. It was found by alkaline-treatment, alkylation analysis and spectroscopic techniques that it was an agaran–carrageenan hybrid with unusual sulfation pattern (Matsuhiro et al., 2005). Sulfated polysaccharides from red seaweeds have been found to exhibit various biological activities, such as antiviral, antitumor and anticoagulant (Bourgougnon, Chermann, Lahaye, & Kornprobst, 1996; Bourgougnon, Lahaye et al., 1996; Cáceres, Carlucci, Damonte, Matsuhiro, & Zúñiga, 2000; Carlucci et al., 1997; Duarte et al., 2001; Noda, Amano, Arashima, & Nisizawa, 1990). Recently, Pereira et al. (2005) reported the anticoagulant activity of a sulfated galactan from the red seaweed *Gelidium crinale*. It has been reported that seaweed polysaccharides can act as elicitors of plant defense (Bourarab, Potin, Correa, & Kloareg, 1999; Kupper, Kloareg, Guern, & Potin, 2001; Mercier et al., 2001).

It is of interest to obtain in good yield low molecular weight fractions with potential biological activity by chemical depolymerization of sulfated polysaccharides. Liu and Perlin (1994) prepared low molecular weight fractions by reaction of heparin with hydrogen peroxide in the presence of Cu^{2+} and ascorbate. The same reaction applied to dermatan sulfate, gave fractions with molecular weight from 25,000 to 2000 (Volpi, 1994). Fry (1998) reported that at pH 5.5, hydrogen peroxide caused the slow scission of xyloglucan, pectin, dextran, alginate, methyl cellulose and carboxymethylcellulose, but in the presence of ascorbate a very rapid scission took place. Nardella et al. (1996) degraded high molecular weight fucans from *Ascophyllum nodosum* to low molecular weight fucans on treatment with Cu^{2+} and hydrogen peroxide at neutral pH. The anticoagulant activity expressed as activated partial thromboplastin time activity of the fraction of low molecular weight (8300) was similar to that of the native fucan.

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doi:10.1016/j.carbpol.2006.03.007

The present work was undertaken to obtain a low molecular weight sulfated galactan and to study its anticoagulant activity.

2. Experimental

2.1. Material and general procedures

The origin and extraction of *Schizymenia binderi* have been reported previously (Matsuhiro et al., 2005). FT-IR spectra were obtained in KBr pellets according to the method described earlier (Matsuhiro, 1996). ^{1}H and ^{13}C NMR spectra were registered on a Bruker Avance DRTX 400 spectrometer operating at 400.13 MHz (^{1}H) and 100.62 MHz (^{13}C), at 80 °C after isotopic exchange with D_2O (3×0.75 mL) using D_2O as solvent with MeOH as internal reference ($\delta^{13}C$: 49.50 ppm, $\delta^{1}H$: 3.340 ppm). Two-dimensional spectra were registered using standard Bruker software. Human plasma was obtained by centrifugation (2500*g* for 5 min at 20 °C) of citrated blood (1/10 vol. of 3.8% sodium citrate) from a pool of healthy volunteer donors ($n = 12$) and stored at 4 °C. The ratio of D/L-galactose was determined through the formation of diastereomeric derivatives with (*S*)-1-amino-2-propanol and GC analysis of the corresponding 1-deoxy-1-1-(2-hydroxy-propylamino)alditol acetates according to Cases, Cerezo, and Stortz (1995).

2.2. Chemical analyses

Total sugar content was determined by phenol–H_2SO_4 acid method (Chaplin, 1996). 3,6-anhydro-galactose content was determined by the resorcinol method of Yaphe and Arsenault (1965). Uronic acid was determined according to Filisetti-Cozzi and Carpita (1991). Molecular weight determination by the reducing end assay was performed as described earlier (Cáceres et al., 2000). Absorbance was registered with a Genesys 5 double beam spectrophotometer. Protein ($N \times 6.25$) content was determined by microanalysis in Facultad de Química, Universidad Católica de Chile. Sulfate content was analysed by the turbidimetric method of Dodgson and Price (1962). Reductive hydrolysis of the polysaccharide and GC analysis of the corresponding acetylated alditols were performed according to Stevenson and Furneaux (1991). Ethyl iodide was synthesized according to the literature (Vogel, 1961).

2.3. Free radical depolymerization

The native polysaccharide (250 mg) and 40 mg of copper (II) acetate monohydrate were dissolved in 15 mL of distilled water at 60 °C and 9% H_2O_2 solution was added at 12 mL/h during 4 h. The pH of the solution was maintained at 7.5 by addition of 2 M NaOH solution (Nardella et al., 1996). Aliquots were withdrawn at 15 and 30 min, and then every 30 min, treated with Chelex 100 resin to remove Cu^{2+} ions, dialysed extensively against distilled water using a 3500 cut-off membrane and freeze-dried.

2.4. Gel permeation chromatography

Each fraction (3 mg in 1 mL of water) was chromatographed on a Sephadex G-200 column (100×1.5 cm) using 0.4 M NaCl as eluant. The column was calibrated with 2 mL of 0.4% Blue dextran 2000 and D-glucose solutions. Fractions of 3 mL were collected and elution was monitored spectrophotometrically with the phenol–H_2SO_4 acid reagent (Chaplin, 1996). The column was calibrated, furthermore, with polysaccharides of narrow molecular weight distribution dextran sulfates (500 and 8 kDa) (Sigma), dextrans (482, 71.327, 41.272 and 10.200 kDa) (Sigma).

2.5. Solvolytic desulfation

Fraction obtained after 3 h of depolymerization in 2.3 (FRD-8) was converted into the pyridinium salts and the solvolytic desulfation was performed according to the method described by Falshaw and Furneaux (1998). Briefly, the sample (20 mg) was dissolved in 8 mL of a mixture of anhydrous DMSO–MeOH–pyridine (89:10:1 v/v/v) and heated at 100 °C for 4 h. After cooling, distilled water (5 mL) was added and the mixture was dialysed against distilled water and freeze-dried.

2.6. Methylation analysis

Fraction FRD-8 was converted into the triethylammonium salt as described by Stevenson and Furneaux (1991) and methylated with CH_3I–NaOH according to Ciucanu and Kerek (1984). Briefly, the sample (25 mg) in 2.5 mL of DMSO was stirred with 200 mg of finally powdered NaOH for 2 h at rt, then CH_3I (2.5 mL) was added and the mixture was stirred for 1 h. The addition was repeated twice. The reaction was stopped by addition of water (1 mL), neutralised with AcOH, dialysed against distilled water and freeze-dried. The solid obtained, was submitted again to the same methylation procedure and then, it was refluxed with CH_3I (4 mL) and 300 mg of dry Ag_2O for 8 h. After cooling, the mixture was filtered off, washed successively with hot water, 20% aqueous MeOH and MeOH. The filtrates were dialysed against distilled water and freeze-dried.

An aliquot of the methylated FRD-8 fraction was hydrolysed with TFA for 2 h at 120 °C, the partially methylated sugars were reduced with $NaBH_4$ and treated with acetic anhydride and anhydrous pyridine 1:1 (v/v). GC of partially methylated alditol acetates was carried out on a Shimadzu GC-17A gas-liquid chromatograph equipped with a SP-2330 column (0.25 mm i.d. × 30 m). Conversion of GC areas to molar basis was calculated for the partially methylated alditols acetates according to the effective carbon response theory of Sweet, Shapiro, and Albersheim (1975). The rest of methylated FRD-8 fraction was

desulfated as in 2.5 and methylated twice with CH_3I–NaOH, hydrolysed and analysed by GC and GC-MS as alditol acetates.

2.7. Ethylation analysis

Ethylation of FRD-8 was conducted as 2.6 using C_2H_5I instead of CH_3I, and a third ethylation cycle was performed using C_2H_5I–Ag_2O as in 2.6. A portion of the ethylated polysaccharide was hydrolysed with 2 M TFA at 120 °C during 2 h and the partially ethylated sugars were reduced with $NaBH_4$, and acetylated with acetic anhydride in anhydrous pyridine. The alditol acetates produced were analysed by GC as the methylated derivatives. The partially ethylated FRD-8 was desulfated as in 2.5 and submitted to two cycles of ethylation with C_2H_5I–NaOH, hydrolysed with 2 M TFA and the resulting partially ethylated sugars were analysed as alditol acetates by GC as in 2.6.

2.8. Sulfation

Sulfation was carried out with chlorosulfonic–pyridine complex according to Mähner, Lechner, and Nordmeier (2001). Briefly, fraction FRD-8 (200 mg) was dried over phosphorous pentoxide in vacuum for 24 h, and then dissolved in 5 mL of formamide. The solution was added drop by drop to a solution of 0.8 mL of chlorosulfonic acid in 4 mL of anhydrous pyridine at −15 °C. The mixture was stirred for 4 h at 65 °C, and was poured into 20 g of ice water and precipitated with methanol. The solid was dissolved in water, neutralised with 15% NaOH solution, dialysed for 120 h against distilled water, concentrated and finally desalted by gel permeation on Sephadex G-10 (100 × 1.5 cm). The yield was 41.5%.

2.9. Anticoagulant activity

Thrombin time (TT), expressed as the clot formation time in relation to the control time was carried out as previously described (Matsuhiro, Zúñiga, Jashes, & Guacucano, 1996). Normal human plasma (0.10 mL) containing various concentrations of heparin (100 UI mg^{-1} mL^{-1}, Laboratorio Sanderson S.A., Chile), native polysaccharide, fraction FRD-8, sulfated and desulfated FRD-8 were incubated at 37 °C for 2 min. Then, 0.05 mL of 10 U mL^{-1} thrombin (Pacific Hemostasis, Fisher Diagnostic, USA) was added, and the clotting time was measured in quadruplicate using a Thrombotimer 1 coagulometer (Behnk Elektronik, Germany) and repeated on three different days. The Coagulation Control Normal (Pacific Hemostasis, Fisher Diagnostic, USA) was used as control of thrombin time (TT) of human plasma.

3. Results and discussion

Depolymerization of the native polysaccharide from *S. binderi* with H_2O_2 in the presence of Cu^{2+} was followed by withdrawing samples at different times and analyzing by gel-permeation chromatography (GPC). Results are shown in Fig. 1 and the compositions of the fractions are presented in Table 1. Fraction obtained after 3 h of treatment (50.0% yield) showed to be homogeneous, the composition of this fraction is presented in Table 2. Its molecular weight by GPC was 8500 which is in good agreement with that (8300) determined by the reducing-end method. It can be observed that protein disappeared after 1 h of treatment, which is in accordance with the finding reported by Rice-Evans, Diplock, and Symons (1991) that OH radicals promote the degradation of proteins.

Fraction FRD-8 was solvolytically desulfated and analysed by NMR spectroscopy. The ^{13}C NMR spectrum (Fig. 2) was very similar to that reported for the desulfated alkali-treated native polysaccharide from *S. binderi* (Matsuhiro et al., 2005) and the ^{13}C resonances showed 0.0–0.2 ppm differences with those reported by Lahaye, Yaphe, and Rochas (1985) for agar type polysaccharides and by Usov, Yarotsky, and Shaskov (1980) for carrageenans (Table 3). Signals of ^{1}H NMR spectrum were assigned with the aid of HSQC (Fig. 3) and COSY 2D spectra (figure not shown) (Table 4) and the assignments were very similar to those reported by Farías, Valente, Pereira, and Mourao (2000). Results indicate a backbone

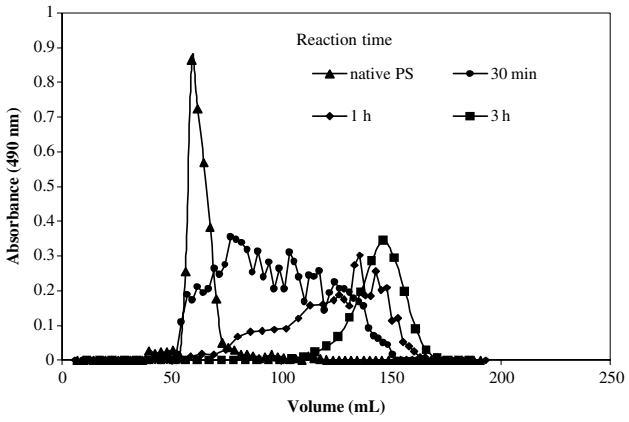

Fig. 1. Representative elution profiles on Sephadex G-200 of fractions obtained by free radical depolymerization of the native polysaccharide from *Schizymenia binderi*.

Table 1
Chemical composition of the fractions obtained by free radical depolymerization of the native polysaccharide from *Schizymenia binderi*

Fraction (FRD)	Reaction time	% Proteins	% Sulfate (as $-SO_3Na$)	% Uronic acids by weight
1	0 min	9.5	22.3	4.9
2	15 min	3.8	23.5	4.1
3	30 min	1.6	23.2	4.4
4	1.0 h	0.0	24.8	4.5
5	1.5 h	0.0	23.2	4.3
6	2.0 h	0.0	25.6	4.4
7	2.5 h	0.0	25.5	3.9
8	3.0 h	0.0	24.7	4.0
9	3.5 h	0.0	26.5	4.1

Table 2
Chemical composition (%) of fraction FRD-8 obtained by free radical depolymerization of the native polysaccharide from *Schizymenia binderi*

Components	FRD-8 (%)
Total sugar	55
Neutral sugars[a]	
Galactose	49.8
D-galactose[b]	39.8
L-galactose[b]	10.0
Glucose	0.5
Xylose	1.8
3-*O*-Methylgalactose[c]	1.4
3,6-Anhydrogalactose	1.5
Uronic acids	4.0
Proteins	0.0
Sulfate (as $NaSO_3$)	24.7

[a] By GC analysis.
[b] By GC analysis of diastereomeric derivatives produced by reductive amination with (*S*)-1-amino-2-propanol.
[c] By GC-MS analysis.

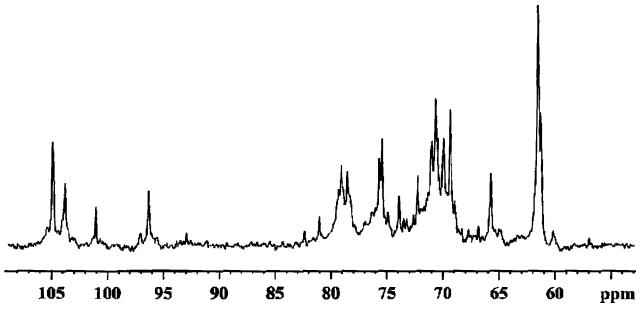

Fig. 2. ^{13}C NMR spectrum (100.62 MHz) in D_2O of desulfated fraction FRD-8 obtained by free radical depolymerization of the native polysaccharide from *Schizymenia binderi*.

Table 3
^{13}C NMR chemical shifts (ppm) of desulfated FRD-8 from *Schizymenia binderi*

Unit	Desulfated FRD-8	C-1	C-2	C-3	C-4	C-5	C-6
A	→3-*O*-β-D-Galp-1→	103.80	69.94	81.06	68.95	75.70	61.55
B	→4-*O*-α-L-Galp-1→	101.05	69.38	70.99	79.10	72.28	61.28
C	→3-*O*-β-D-Galp-1→	104.90	70.67	79.10	65.73	75.47	61.28
D	→4-*O*-α-D-Galp-1→	96.31	69.38	70.99	78.56	70.67	61.55

of alternating 3-linked β-D-galactopyranosyl residue and 4-linked α-galactopyranosyl units that are predominantly of D-configuration and partly of L-configuration. The D:L galactose ratio (4.7:1.0) found by total hydrolysis of FRD-8 and GC analysis of the derived diastereomeric amines sustained this assumption.

Comparison of methylation and ethylation data of FRD-8 (Table 5) showed good correlations, but ethylation allowed the separation of 2,6-di-*O*-galactitol acetate from 4,6-di-*O*-galactitol acetate. Values presented in Table 5 are very similar to those obtained in the alkylation analysis of the alkali-treated native polysaccharide (Matsuhiro et al., 2005). 4,6-Di-*O*-ethyl-galactitol acetate originated from 3-linked D-galactopyranosyl residues sulfated at position O-2 of FRD-8 (19.4%). The presence of 2,4,6-tri-*O*-alkylgalactitol acetate indicated that part of the 3-linked galactopyranosyl units are not sulfated (20%). After desulfation, the content of 2,4,6-tri-*O*-alkylgalactitol acetate increased considerably, with the concomitant decrease of 4,6-di-*O*-ethyl-galactitol content, indicating the presence of 3-linked galactopyranosyl residues with sulfate groups at position O-2. The presence of 2,6-di-*O*-ethyl-galactitol acetate could be ascribed either to 4-substituted, 3-linked galactopyranosyl units or 3-substituted, 4-linked galactopyranosyl units. Taking into consideration the total amount of 2,4,6-tri-*O*-ethyl-galactitol acetate after desulfation (38.6 %) and the contributions of the different sulfated units to its formation, it can be deduced that the major amount of 2,6-di-*O*-ethyl-galactitol acetate could be originated from 4-linked galactopyranosyl units sulfated at position O-3. The presence of 2,6-di-*O*-ethyl derivative after desulfation may be due to incomplete desulfation (5–6% of remaining sulfate) and/or under alkylation, although glycosylation at position O-4 in 3-linked D-galactopyranosyl units and/or at position O-3 of 4-linked galactopyranosyl units could not be excluded (Takano, Nose, Hayashi, Hara, & Hirase, 1994). The presence of 3,6-di-*O*-ethyl-galactitol acetate (10.4%) after desulfation indicated glycosylation at O-2 of 4-linked galactopyranosyl units. Since only 2% of 3-*O*-methyl-2,4,6-tri-*O*-ethyl-galactitol acetate was detected, branching could originated also from uronic acids and xylose residues. It is known that 2,3,4-tri-*O*-methyl-xylitol acetate is quite volatile and could had

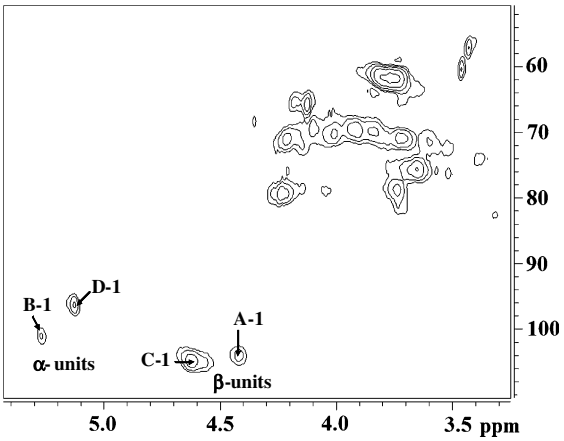

Fig. 3. $^{13}C/^{1}H$ HSQC NMR spectrum in D_2O of desulfated fraction FRD-8 obtained by free radical depolymerization of the native polysaccharide from *Schizymenia binderi*.

Table 4
^{1}H NMR chemical shifts (ppm) of desulfated FRD-8 from *Schizymenia binderi*

Unit	Desulfated FRD-8	H-1	H-2	H-3	H-4	H-5	H-6
A	→3-*O*-β-D-Galp-1→	4.42	3.83	–	–	3.67	3.78
B	→4-*O*-α-L-Galp-1→	5.26	3.98	4.03	–	–	3.78
C	→3-*O*-β-D-Galp-1→	4.61	3.77	3.75	4.01	3.67	3.78
D	→4-*O*-α-D-Galp-1→	5.13	3.93	3.73	4.10	4.21	3.78

Table 5
Linkage analysis of the constituent sugars of fraction FRD-8 obtained by free radical depolymerization of the native polysaccharide from *Schizymenia binderi*

Monosaccharide	Deduced unit and substitution pattern	Polysaccharide[a]			
		Methylation		Ethylation	
		FRD-8	DS-FRD-8[b]	FRD-8	DS-FRD-8
2,3,4,6-Gal	T[c]	5.4	4.3	4.1	8.2
2,4,6-Gal	→3-Gal	20.9	40.5	20.0	38.6
2,3,6-Gal	→4-Gal	2.2	28.4	4.9	28.8
2,6-Gal	→3-Gal 4S[d] + →4-Gal 3S	–	–	27.5	10.4
2,4-Gal	→3-Gal 6S + →3-Gal 6R[e]	2.4	1.5	3.3	1.8
3,6-Gal	→4-Gal 2S + →4-Gal 2R	nd[f]	11.1	1.3	10.4
6-Gal	→3-Gal 2,4S + →4-Gal 2,3S + →-4-Gal 2R, 3S	19.1	1.1	11.4	2.5
2-Gal	→3-Gal 4,6S + →4-Gal 3,6S	4.6	1.6	5.5	1.6
3-Gal	→4-Gal 2,6S	nd	nd	1.7	1.8
4,6-Gal	→3-Gal 2S	–	–	19.4	nd
2,6 + 4,6-Gal[g]		48.6	8.8	–	–
3-*O*-Me-2,4,6-tri-*O*-ethyl-Gal	T	–	–	2.0	2.1

[a] Normalised mol % of monosaccharide having methyl or ethyl groups at the positions indicated.
[b] DSFRD-8, desulfated FRD-8.
[c] T, terminal.
[d] S, $-SO_3^-$.
[e] R, glycosylated unit.
[f] nd, not detected.
[g] According to GC-MS analysis.

been lost during the analysis (Stevenson & Furneaux, 1991). Then, it can be deduced that ~10% of 6-*O*-ethyl-galactitol acetate comes from 4-linked galactopyranosyl units sulfated at position O-3 and branched at position O-2. The small amount of 2-*O*-methyl-galactitol acetate observed could be originated from 4-linked and/or 3-linked galactopyranosyl units disubstituted by sulfate or glycosyl groups at positions O-3 and O-6, and at positions O-4 and O-6, respectively. Minor amount (~2%) of 2,4-di-*O*-galactitol acetate may indicate glycosylation at position O-6 of 3-linked galactopyranosyl units. As in the sulfated galactan from *S. binderi* it is noteworthy that the alkylation pattern is different from those expected for λ- and/or ξ-carrageenans.

The DEPT 135 NMR spectrum (figure not shown) of FRD-8 showed only one inverted signal centered at 61.75 ppm indicating that most of the primary alcoholic groups in the galactopyranosyl residues were not substituted by sulfate or glycosyl groups. In the ^{13}C NMR spectrum (Fig. 4) the signals in the anomeric region, at 104.07 ppm was assigned to C-1 of 3-linked β-D-galactopyranosyl residue (unit A) linked to →4-α-L-galactopyranosyl unit sulfated at position O-3 (unit B), at 103.38 ppm to C_1 of β-D-galactopyranosyl residue (unit C) linked to →4-α-D-galactopyrnosyl unit unsulfated or sulfated at O-3 (unit D) and at 101.59 ppm to C_1 of 3-linked β-D-galactopyranosyl unit sulfated at O-2 (unit E) linked to →4-α-D-galactopyranosyl residue sulfated (or glycosylated) at O-2 and at O-3 positions (Farías et al., 2000; Usov et al., 1980; Van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002). Two-dimensional HSQC spectrum (Fig. 5) allowed the identification of the anomeric ^{13}C and ^{1}H spin systems of the galactopyranosyl residues and the ^{1}H–^{1}H COSY spectrum (figure not shown) allowed, starting from H-1 the assignments of H-2 and some H-3 signals of the galactopyranosyl units (Table 6). In the anomeric region of the HSQC spectrum connectivities among six anomeric ^{13}C–^{1}H systems were observed. The 104.76/4.72 ppm correlation was assigned to C-1/H-1 of unit A (C-2–C-6: 70.07, 81.29, 69.19, 75.86 and 61.5 ppm) which is linked to unit B. In unit B (C-2–C-6: 68.42, 76.63, 78.01, 72.17 and 61.02 ppm), the sulfation at position O-3 was deduced from the downfield shifts of H-3 compared with the chemical shifts of H-3 in 4-linked α-L-galactopyranosyl unit (4.05 ppm) in the desulfated FRD-8 and it is in good agreement with the value reported in the literature (Farías et al., 2000). Similarly, sulfation at position O-2 in unit E (C-2–C-6: 78.23, 75.86, 73.96, 73.96 and 61.50 ppm) was deduced from the downfield chemical shift of H-2 in relation to the H-2 shifts in unsulfated units A and C, and in desulfated FRD-8 (Zibetti, Noseda, Cerezo, & Duarte, 2005). Unit C (C-2: 70.32 ppm); C-4–C-6: 65.83, 75.68 and 61.50 is linked to unit D. The ^{13}C chemicals shifts for unit D (C-2–C-6: 67.41, 78.58, 75.86, 71.46 and 61.50 ppm) were

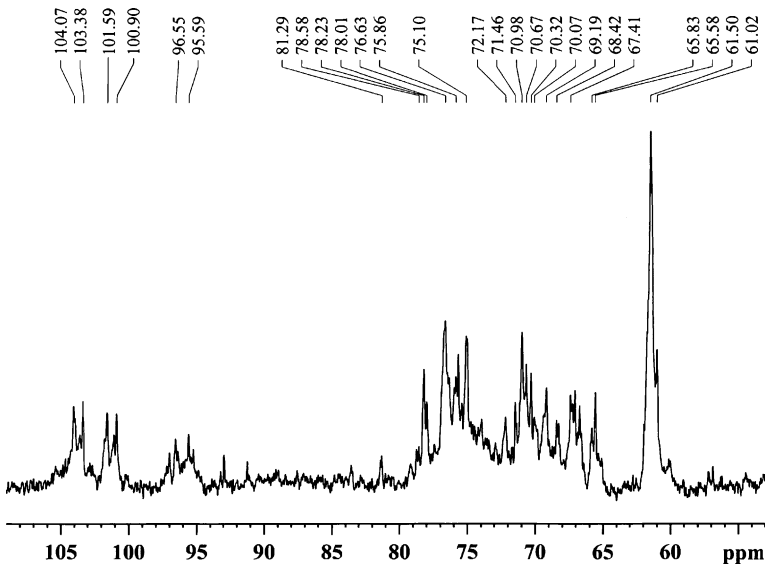

Fig. 4. ^{13}C NMR spectrum (100.62 MHz) in D_2O of fraction FRD-8 obtained by free radical depolymerization of the native polysaccharide from *Schizymenia binderi*.

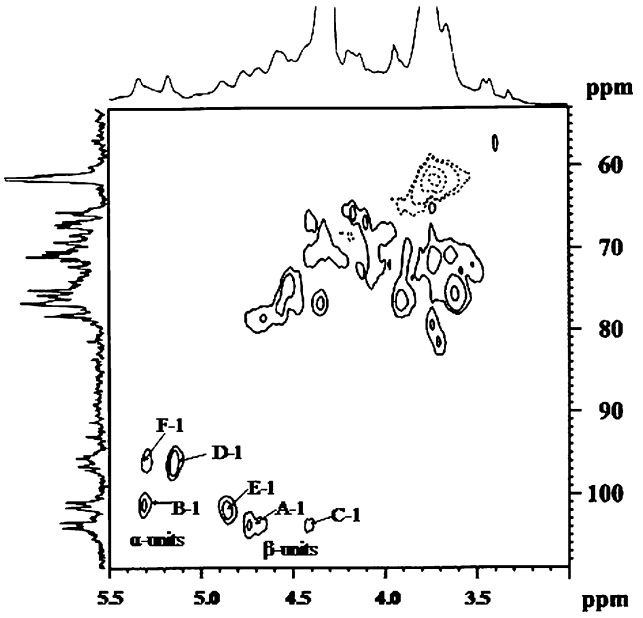

Fig. 5. $^{13}C/^{1}H$ HSQC NMR spectrum in D_2O of fraction FRD-8 obtained by free radical depolymerization of the native polysaccharide from *Schizymenia binderi*.

assigned according to the ^{13}C chemical shifts reported for methyl α-D-galactopyranoside 3-SO_3, considering a ~8 ppm glycosylation shift on C-4 (Duarte, Noseda, Cardoso, Tulio, & Cerezo, 2002; Farías et al., 2000). The HSQC spectrum allowed the identification of C-1 and H-1 of unit F. The COSY spectrum, starting from H-1 identified H-2 and H-3 of unit F (Table 6). The signals of H-2 and H-3 at lower field than in the unsubstituted 4-linked α-D-galactopyranosyl residue are indicative of the presence of disulfated derivative or most probably, in accordance to the results obtained in the alkali-treated

Table 6
Assignments of ^{13}C and ^{1}H chemical shifts (ppm) present in the $^{13}C/^{1}H$ HSQC and COSY $^{1}H/^{1}H$ NMR spectra of FRD-8

Unit		δ (ppm)						
		C_1	H_1	H_2	H_3	H_4	H_5	H_6
A	→3-β-D-Gal	104.07	4.72	3.55	3.72		3.60	3.72
B	→4-α-L-Gal-3S[a]	101.09	5.31	4.02	4.71			
C	→3-β-D-Gal	103.38	4.42	3.75				
D	→4-α-D-Gal-3S	96.55	5.13	3.89				
E	→3-β-D-Gal-2S	101.59	4.85	4.59				
F	→4-α-D-Gal-2R[b],3S	95.59	5.28	4.06	4.63			

[a] S, $-SO_3^-$.
[b] R, glycosylated unit.

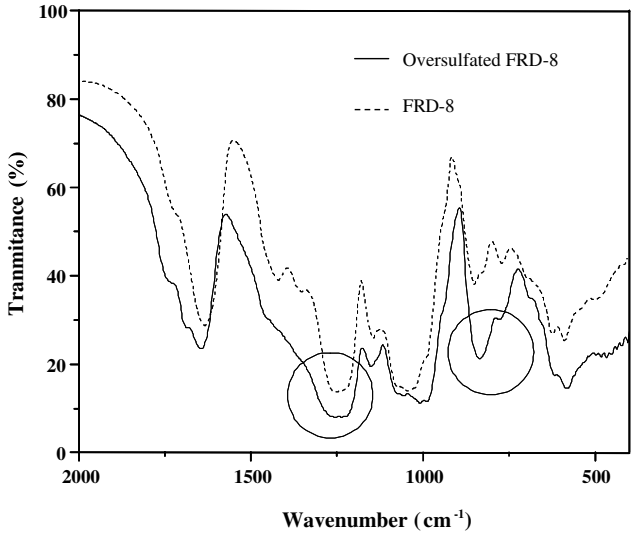

Fig. 6. IR spectrum of oversulfated FDR-8 fraction.

Table 7
Blood anticoagulant activity of FRD-8, oversulfated FRD-8, desulfated FRD-8 and native polysaccharide from *Schizymenia binderi*

Polysaccharide	Thrombin time (TT)[a]				
FRD-8	0 μg/mL	50 μg/mL	150 μg/mL	250 μg/mL	500 μg/mL
	1.0	1.7	2.5	2.9	6.2
Oversulfated FRD-8	0 μg/mL	50 μg/mL	150 μg/mL	250 μg/mL	500 μg/mL
	1.0	2.6	5.3	8.9	>10.0
Desulfated FRD-8			>1000 μg/mL		
			1.0		
Native PS	0 μg/mL	50 μg/mL	150 μg/mL	250 μg/mL	500 μg/mL
	1.0	2.3	6.5	10	>10.0
Heparin	0 μg/mL	1.0 μg/mL	1.5 μg/mL	2.0 μg/mL	5.0 μg/mL
	1.0	1.4	2.5	4.4	>10.0

[a] TT, thrombin time expressed as the clot formation time in relation to the negative control clot time.

native polysaccharide from *S. binderi* (Matsuhiro et al., 2005) of 4-linked α-D-galactopyranosyl unit glycosylated at position O-2 and sulfated at position O-3.

It can be proposed that the main fraction (FRD-8) obtained by free radical depolymerization of the sulfated galactan from *S. binderi* is a hybrid polysaccharide composed of 3-linked β-D-galactopyranosyl residue partially sulfated at position O-2 and 4-linked α-D-galactopyranosyl and α-L-galactopyranosyl residues mainly sulfated at position O-3 and glycosylated at position O-2. The structure of fraction FRD-8 seems more regular than that of the native polysaccharide. The obtainment in good yield of a homogeneous fraction by free-radical depolymerization might be due to the coordination of Cu^{2+} with sulfate group at positions O-2 and O-3 of galactopyranosyl residues which protected the glycosidic linkages (Rej, Holme, & Perlin, 1990).

Fraction FRD-8 was sulfated with chlorosulfonic acid. The FT-IR spectrum (Fig. 6) of sulfated FRD-8 showed a very strong band at 1263 cm^{-1}, characteristic of S=O stretching vibration of sulfate groups and a broad band centered at 837 cm^{-1}, which indicated the presence of primary and secondary sulfate groups. After sulfation reaction the content of sulfate increased to 46.9%.

3.1. Anticoagulant activity

The anticoagulant activity of native polysaccharide, FRD-8, sulfated FRD-8 and desulfated FRD-8 fractions was assayed in terms of the thrombin time, results are shown in Table 7. The native polysaccharide presented a discreet inhibition of coagulation in relation to heparin activity. The lower activity of FRD-8 fraction compared to that of native polysaccharide is not only due to the decrease in molecular weight (Shanmugam & Mody, 2000; Toida, Chaidedgumjorn, & Linhardt, 2003), since its sulfation increased the activity to values similar to those of the native polysaccharide. As expected, after desulfation, FRD-8 fraction lost completely the anticoagulant activity. Among carrageenans, λ-carrageenan showed the highest activity, probably due to its higher sulfate content, but its activity was one-fifteenth the activity of heparin (Shanmugam & Mody, 2000). A sulfated galactan with anticoagulant activity similar to that of heparin was isolated from the red algae *Botryocladia occidentalis* by Farías et al. (2000). The activity was attributed to the sulfation at O-2 and O-3 positions of the α-D-galactopyranosyl residues but required molecular sizes between 16 and 46 kDa (Melo, Pereira, Foguel, & Mourao, 2004).

The homogeneous low molecular weight fraction obtained by free-radical depolymerization of the sulfated galactan from *S. binderi* showed low anticoagulant activity compared to heparin, yet it may have other biological properties and it constitutes a model compound with potential biological applications.

Acknowledgements

The financial support of FONDECYT, Chile (Grant 1010594) and CONICYT, Chile (Grant AT-4040212) is gratefully acknowledged. E.A. Zúñiga is grateful for a doctoral fellowship (CONICYT). The authors are indebted to Dr. María C. Matulewicz (Universidad de Buenos Aires, Argentina) for enantiomeric analysis.

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