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Carrageenans from chilean samples of *Stenogramme interrupta* (Phyllophoraceae): structural analysis and biological activity

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Abstract

Carrageenans extracted from cystocarpic and tetrasporic *Stenogramme interrupta* were analysed by chemical and spectroscopic methods. The carrageenan from cystocarpic plants is composed predominantly of 0.5 M KCl-insoluble and 1 M KCl-soluble fractions. The insoluble fraction contained ι-carrageenan as the major component with α-carrageenan and pyruvated carrageenan as minor components. The soluble fraction is highly heterogeneous and did not contain the precursors μ- and ν-carrageenans. The polysaccharide from tetrasporic plants is composed of ξ- and λ-carrageenans, and low sulfated galactans. It is soluble in KCl and partly cyclized by alkaline treatment. The antiviral and anticoagulant properties of the insoluble polysaccharide fraction from cystocarpic *S. interrupta* and the polysaccharide from tetrasporic *S. interrupta* are reported the results of which suggest promising antiherpetic activity.

Keywords: *Stenogramme interrupta*; Carrageenans; Nuclear phases; Natural antiviral; Anticoagulant activity

1. Introduction

The soluble polysaccharides from different nuclear phases of the red seaweed *Stenogramme interrupta* (C.Ag.) Mont. (Phyllophoraceae, Gigartinales) were previously characterised in this laboratory by FTIR spectroscopy and double hydrolysis reduction (Cáceres, Faúndez, Matsuhiro & Vásquez, 1997). It was found that the polysaccharides from gametophytic and cystocarpic samples were of the κ-/ι-carrageenan type while the polysaccharide from tetrasporic samples were of λ-carrageenan type.

McCandless, West and Guiry (1982) found by IR spectroscopy and immunochemical reactivity that the gametophytes of *S. interrupta* from California yielded κ-/ι-hybrid carrageenans while tetrasporophytes yielded λ-carrageenan. According to Furneaux and Miller (1985), the polysaccharide extracted with hot water from carposporic samples of New Zealand specimens of *Stenogramme interrupta* was 75% floridean starch, and the remaining polysaccharide after enzymic digestion did not show a carrageenan structure by NMR spectroscopy. Recently, Miller (1998) reported that the polysaccharide extracted from gametophytic *S. interrupta* from New Zealand consists of 4-linked 3,6-anhydro-D-galactosyl-2-sulfate units alternating with either 3-linked D-galactosyl-4 sulfate or 3-linked D-galactosyl-4,6-pyruvate acetal units. The structure was solely determined by ^{13}C-NMR spectroscopy.

Sulfated polysaccharides have shown antiviral and anticoagulant properties (Witvrouw, Desmyter & De Clercq, 1994; Güven, Özsoy & Ulutin, 1991). In particular, polysaccharides isolated from red seaweed have been found to be selective inhibitors of several enveloped viruses, including such human pathogens as human immunodeficiency virus, herpes simplex virus (HSV), human cytomegalovirus and others

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Table 1
Assignments of the resonances (ppm) in the ^{13}C-NMR spectrum of the autohydrolysis product of F0.5 from polysaccharide C [a]

Sugar residue	Carbon atom							
	C-1	C-2	C-3	C-4	C-5	C-6	C=O	CH_3
3-Linked Gal 4-S	102.6	69.9	77.3	72.7	75.3	61.7		
4-Linked AnGal 2-S	92.7	75.2	78.4	78.8	77.3	70.3		
3-Linked Gal	102.6	69.9	81.9	66.9	75.3	61.7		
4-Linked AnGal 2-S	94.6	75.2	78.4	78.8	77.3	70.3		
3-Linked 4,6-PyGal	101.9	69.5	76.8	67.7	66.9	65.9	176.4	25.7
4-Linked AnGal 2-S	91.4	75.3	77.3	78.1	77.7	69.8		

[a] Gal : galactose; Gal 4-S : galactose 4-sulfate; AnGal 2-S : 3,6-anhydrogalactose 2-sulfate; 4,6-PyGal : 4,6-pyruvated galactose.

(Bourgougnon, Lahaye, Chermann & Kornprobst, 1993; Pujol, Errea, Matulewicz & Damonte 1996; Kolender, Pujol, Damonte, Matulewicz & Cerezo, 1997; Witvrouw et al., 1994). Recently, Carlucci et al. (1997) reported that the λ-carrageenan and partially cyclized μ-/ι-carrageenan from *Gigartina skottsbergii* showed potent antiviral effect against different strains of HSV type 1 and type 2.

2. Results and discussion

The polysaccharides extracted with hot water from cystocarpic and tetrasporic *Stenogramme interrupta*, hereafter known as polysaccharide C and polysaccharide T, were shown to be heterogenous by PAGE.

Polysaccharide C gave seven fractions by sequential treatment with 0.0625, 0.125, 0.25, 0.4, 0.5, 0.7 and 1.0 M KCl solutions. The major insoluble fraction F0.5 (41.6% yield), precipitating at 0.5 M KCl, presented in the FTIR spectrum (not shown) three characteristic bands at 930, 845 and 805 cm^{-1}, similar to the whole polysaccharide (Cáceres et al., 1997) indicating a κ-/ι-carrageenan type polysaccharide (Anderson et al., 1968). The second derivative spectrum (Matsuhiro, 1996) showed a weak band at 1740 cm^{-1} assigned to a C=O stretching vibration of a carboxyl group (Conley, 1966).

Alkaline treatment of F0.5 changed the galactose : 3,6- anhydrogalactose : sulfate ratio from 1.00 : 0.27 : 0.83 to 1.00 : 0.36 : 0.53, indicating the presence of a low but significant amount of μ- or ν-carrageenan type precursor units. Fraction F0.5 was submitted to autohydrolysis, with 70% of the material recovered. The autohydrolysis product (M_r 1650) was shown to be homogeneous by PAGE and was analysed by ^{13}C-NMR spectroscopy. In Table 1 the assignments of the signals are shown. The assignments of the major signals are in agreement with the values published for ι-carrageenan repeating unit (Usov, 1984; Stortz & Cerezo, 1992) and with the values published by Greer, Rochas and Yaphe (1985) for ι-carrageenan oligosaccharides obtained by enzymatic hydrolysis of commercial ι-carrageenan. The ^{13}C-NMR spectrum of the autohydrolyzate product did not present signals attributed to the anomeric carbon atoms of κ-carrageenan but contained minor signals which were assigned to a desulfated 3-linked D-galactopyranosyl residue linked to the 4-position of a 3,6-anhydro-D-galactopyranose residue by comparison with data published by Falshaw, Furneaux, Wong, Liao, Bacic and Chandrkrachang (1996), suggesting the presence of α-carrageenan repeating units (Zablackis & Santos, 1986). In addition, the spectrum showed signals attributed to the carbonyl carbon and methyl carbon of pyruvate residues (Usov, 1984; Lahaye, Yaphe, Viet & Rochas, 1989). These results are consistent with the finding in the second derivative FTIR spectrum of the band at 1741 cm^{-1} attributed to the C=O vibration of the carboxyl group (Conley, 1966). This signal was not observed in the normal spectrum indicating that only a small amount of pyruvic acid is present. At higher field, the ^{13}C-NMR spectrum showed three weak signals at 67.7, 66.9 and 65.9 ppm, assigned according to the literature to C4, C5 and C6 of the 3-linked pyruvated galactopyranosyl unit (Lahaye et al., 1989). As this work was being submitted to the journal, Miller (1998) reported that the predominant structure in the polysaccharide of cystocarpic *S. interrrupta* from New Zealand was pyruvated α-carrageenan. It can be pointed out that the fractionation in this case was done with lead nitrate. The presence of 3-linked 4,6-*O*-(1-carboxyethylidene)-D-galactopyranose residue is common in agar type polysaccharides but less common in carrageenans (DiNinno, McCandless & Bell, 1979; Stevenson & Furneaux, 1991). Recently, Chiovitti, Liao, Kraft, Munro, Craik and Bacic (1996) reported the presence of a significant proportion of pyruvate groups in the carrageenan from *Rhabdonia verticillata*. They also commented that several species of Australian red seaweeds produce sulfated galactans with significant proportions of pyruvated carrageenans.

From the ^{13}C-NMR spectroscopy and alkaline treatment results, it can be proposed that the homogenous fraction insoluble in 0.5 M KCl from cystocarpic *S. interrupta* is a hybrid polysaccharide composed predominantly of ι-carrageenan with contributions of α-carrageenan, pyruvated α-carrageenan and precursors structures.

The FTIR spectrum of F1.0, the fraction soluble at 1.0 M (45.6% yield), presented bands at 930 and 805 cm^{-1}, and a broad one centered at 830 cm^{-1}, which in the second derivative mode is resolved into two bands at 845 and 830 cm^{-1} (Matsuhiro & Rivas, 1993). The latter was assigned to sulfate linked to equatorial sec-

ondary alcoholic group on D-galactopyranosyl residues which may indicate the presence of a range carrageenan structures including types not observed in F0.5. This fraction was shown to be heterogeneous by PAGE. A very small increase in the 3,6-anhydrogalactose content from 11.01 to 13.92% and a concomitant decrease in sulfate content were found by alkaline treatment. These results indicate that the precursors μ- and ν-carrageenans are not present in significant proportions.

In the case of polysaccharide T, no fraction was obtained by treatment with KCl up to 1.0 M. The FTIR spectrum of the recovered polysaccharide presented a shoulder around 930 cm^{-1} and a broad band at 819 cm^{-1}. The latter in the second derivative spectrum was resolved into three signals at 837, 821 and 806 cm^{-1} assigned to sulfate residues attached to secondary equatorial, primary and secondary axial alcoholic groups, respectively. Alkaline treatment of polysaccharide T afforded a modified polysaccharide with an increase in the 3,6-anhydrogalactose content (2.56–8.63%) and a decrease in the sulfate content (23.6–18.31%). Its FTIR spectrum showed the presence of a peak of medium intensity at 934 cm^{-1}. The lack of the signal at 819 cm^{-1} due to the primary sulfate (i.e. attached to C-6) in the second-derivative FTIR spectrum of alkali-modified polysaccharide T may indicate the presence of alkali-modified λ-carrageenan (i.e. θ-carrageenan) and of ξ-carrageenan. Data from the ^{13}C-NMR spectrum of the autohydrolysis product (M_r 1567) from polysaccharide T provided support for this assumption. The spectrum was complex, reflecting the heterogeneity of the sample, but it showed signals at 104.2, 79.3, 78.4, 66.3 and 61.7 ppm assigned according to Stortz and Cerezo (1992) to carbons 1, 2, 3, 4 and 6, respectively of 3-linked 2-sulfated galactopyranose residue (unit A of ξ-carrageenan). The signals at 94.0, 79.1 and 76.8 were assigned to carbons 1, 4, and 2 respectively, of 4-linked 2-sulfated galactopyranose residues (unit B of ξ-carrageenan). The signal at 70.9 ppm was assigned to C5 of the same residue. Furthermore, the spectrum showed two characteristic signals at 68.6 and 68.1 ppm, due to C6 and C5 of the residue when a sulfate group is attached on C6 (λ-carrageenan repeating unit). The anomeric resonances are close to those found by Noseda and Cerezo (1993) for a room temperature ^{13}C-NMR spectral analysis of partially hydrolyzed λ-carrageenan but differ from the values reported for native λ-carrageenans isolated from *Gigartina decipiens* (Falshaw & Furneaux, 1994) and *Iridaea undulosa* (Stortz, Bacon, Cherniak & Cerezo, 1994). According to Stortz et al. (1994) the lower than expected chemical shift value (92.0 ppm) for the anomeric 4-linked galactopyranosyl residue in the native polysaccharide is attributed to the shielding effect of the sulfate on carbon 2 of the 3-linked galactopyranosyl unit caused by modification of the preferred torsional angle around the glycosidic bond.

Table 2
Antiherpetic activity of the fraction F0.5 from polysaccharide C and of polysaccharide T [a]

Virus strain	IC_{50} (μg/ml)	
	F0.5	PT
HSV-1 strain F	9.33 ± 1.19	2.88 ± 0.29
HSV-2 strain G	4.32 ± 0.08	0.92 ± 0.02
HSV-1 strain 1213 LCR/94	4.75 ± 0.92	1.48 ± 0.53
HSV-2 strain 244 BE/94	1.90 ± 0.20	0.65 ± 0.27
HSV-1 TK^- strain B2006	6.23 ± 1.55	1.48 ± 0.18
HSV-1 TK^- strain field	3.55 ± 0.17	0.85 ± 0.20

[a] Each value is the mean of duplicate assays ± standard deviation.

No signals assigned to pyruvated residues were found in the ^{13}C-NMR spectrum of polysaccharide T, but hydrolysis of the acetal group under the acidic conditions used in the autohydrolysis may have taken place. Besides, from the low sulfate content of polysaccharide T, with galactose : 3,6-anhydrogalactose : sulfate molar ratio of 1.00 : 0.16 : 0.60, the presence of unsulphated galactose units could not be excluded.

2.1. Antiviral activity

No cytotoxicity was observed with F0.5 from polysaccharide C or with polysaccharide T when cell viability was evaluated in Vero cells in the presence of concentrations up to 1000 μg/ml. In Table 2, data on the antiviral activity against diverse strains of HSV virus are presented. Polysaccharide T was the most active compound with IC_{50} values in the range 0.65–2.88 μg/ml, whereas the fraction F0.5 of polysaccharide C was slightly less effective than polysaccharide T. However, both polysaccharides showed a wide spectrum of action against the two serotypes of HSV, including TK^- acyclovir resistant variants and clinical isolates. For reference strains and clinical isolates, the strains of HSV-2 were more sensitive to the antiviral effect of polysaccharide T and F0.5 than was HSV-1. The antiherpetic activity of these polysaccharides was comparable to that previously reported for the λ- and κ-/ι-carrageenans obtained from *Gigartina skottsbergii* (Carlucci et al., 1997). To determine whether polysaccharide T and fraction F0.5 had virion-inactivating properties, a suspension of HSV was directly mixed with each compound diluted in medium to provide final concentrations ranging from 1 to 100 μg/ml. After 60 min of incubation at 37°C the samples were titrated in Vero cells. No differences in remaining infectivity titers between polysaccharide-treated and untreated virus suspensions were detected (data not shown), even when the carrageenans were used at con-

centrations higher than the IC_{50}. Thus, polysaccharide T and fraction F0.5 did not exert a direct virucidal effect but are true antiviral agents interfering the replication cycle of HSV.

As an approach to determine the mode of action of the carrageenans on HSV replication, the IC_{50} against the most susceptible virus strain, HSV-2 strain 244 BE/94, was determined by a plaque reduction assay under different treatment conditions: the compounds were present either during the virus adsorption period only, after virus adsorption only, or both during and after virus adsorption. The IC_{50} values obtained for polysaccharide T were 2.56 μg/ml (polysaccharide only at adsorption) and 0.65 μg/ml (polysaccharide at adsorption and post-adsorption), whereas for F0.5 the corresponding values of IC_{50} were 6.81 (F0.5 only at adsorption) and 1.90 μg/ml (F0.5 at adsorption and post-adsorption). When the carrageenans were added only after adsorption, they were no longer effective even at a concentration of 50 μg/ml. These results strongly suggest that, like other sulfated polysaccharides (Pujol et al., 1996; Baba, Pauwels, Balzarini, Arnout, Desmyter & DeClercq, 1988; Mitsuya, Looney, Kuno, Ueno, Wong-Staal & Broder, 1988; Callahan, Phelan, Mallinson & Norcross, 1991), the main target for polysaccharide T and F0.5 action in the replicative cycle is the virus attachment to the host cell, interfering with the initial binding of the viral glycoprotein gC to the cellular heparan sulfate proteoglycans (Lycke, Johansson, Svennerholm & Lindahl, 1991; Herold, WuDunn, Soltys & Spear, 1991).

2.2. Anticoagulant activity

Fraction F0.5 of the polysaccharide from cystocarpic *S. interrupta* and the polysaccharide extracted from tetrasporic plants at various concentrations were examined for blood-anticoagulant activity. Thrombin time (TT) was determined for human plasma with bovine thrombin and the time to clot formation was registered. At the concentrations assayed for antiviral activity polysaccharide T did not exhibit anticoagulant activity. A thrombin time (TT) value of 2.29 was determined at a polysaccharide T concentration of 60 μg/ml and the TT value increased to 6.29 at 120 μg/ml. Fraction F0.5 from polysaccharide C exhibited very slight activity, a thrombin time (TT) of 3.00 was obtained at 1200 μg/ml, twenty times the concentration of polysaccharide T needed to give similar values. These results are indicative of a lack of correlation between antiviral and anticoagulant properties of the polysaccharides studied in this work, which consequently become very promising antiherpetic compounds.

3. Materials and methods

The origin and extraction of *Stenogramme interrupta* have been reported previously (Cáceres et al., 1997). Polyacrylamide gel electrophoresis (PAGE) was conducted after Usov and Arkhipova (1975), staining with Toluidine Blue and destaining with 5% HOAc aq. solution.

3.1. Chemical analyses

Sulfate content was determined by microanalysis in Facultad de Ciencias Químicas, Universidad Católica de Chile. Total sugar content was determined by the phenol-sulfuric acid method (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). 3,6-anhydrogalactose was determined according to Yaphe and Arsenault (1965), using D-fructose as standard. Absorbance was registered with a Shimadzu UV-150-02 double beam spectrophotometer.

3.2. Spectroscopic analyses

^{13}C-NMR spectra at 50.32 MHz of D_2O : H_2O (1 : 1 v/v) solutions were recorded with a Bruker 200 spectrometer operating at 32°C according to Stortz and Cerezo (1991). Chemical shifts were measured relative to internal acetone (29.7 ppm). The number of acquisitions varied from 30,000 to 70,000. FTIR spectra of polysaccharides were obtained in KBr pellets according to the method described earlier (Matsuhiro & Rivas, 1993).

3.3. Fractionation of extracts with KCl

Solutions of each extracts in H_2O were fractionated by addition of solid KCl as described (Matulewicz & Cerezo, 1980).

3.4. Autohydrolysis

Fraction F0.5 from polysaccharide C and polysaccharide T were treated with Amberlite IR-120 H^+ resin and hydrolyzed for 7 h according to Ref. (Stortz & Cerezo, 1987). The molecular weight of the hydrolysate was determined by adapting the reducing end assay of Park and Johnson (1949). Briefly, aliquots of 0.5 ml of the hydrolysate with an initial concentration of 3 mg/ml, were mixed with 0.5 ml of aqueous solutions of KCN (0.65 g/l), Na_2CO_3 (5.3 g/l) and of $KFe(CN)_6$ (0.5 g/l). The mixture was heated for 15 min at 100°C, cooled at room temperature and treated with 2.5 ml of ammoniun ferrisulfate (1.5 g/l) and SDS. After 15 min absorbance was determined at 690 nm. Solutions of D-galactose (1–70 μM) were used as standards.

3.5. Alkaline treatment of polysaccharides

Fraction F1.0 from polysaccharide C and polysaccharide T were treated with $NaBH_4$–NaOH according to Ciancia, Matulewicz and Cerezo (1995).

3.6. Cells and viruses

HSV-1 strain F and HSV-2 strain G were obtained from the American Type Culture Collection and were used as reference strains. B2006 and Field were HSV-1 TK^- strains obtained from Dr. E. De Clerck (Rega Institute, Belgium). The 1213 LCR/94 strain of HSV-1 and the 244 BE/94 strain of HSV-2 were clinical isolates obtained from infected patients and provided by Instituto Nacional de Microbiología Carlos Malbrán (Buenos Aires, Argentina). Vero cells were grown in Eagle minimum essential medium (MEM) (GIBCO, USA) supplemented with 5% inactivated calf serum.

3.7. Antiviral activity and cytotoxicity assays

Antiviral activity was evaluated by a plaque reduction assay. Vero cells monolayers grown in 24-well plates were infected with 50 PFU of virus/well in the absence or presence of various concentrations of the polysaccharides. After 1 h adsorption, residual inoculum was replaced by medium containing 0.7% methylcellulose and the corresponding dose of compound. Plaques were counted after 2 days of incubation at 37°C. The 50% inhibitory concentration (IC_{50}) was calculated as the compound concentration required to reduce virus plaques by 50%. The cytotoxicity of the polysaccharides was evaluated in parallel with the antiviral activity by the MTT [3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide] method (Denizot & Lang, 1986) and the 50% cytotoxic concentration (CC_{50}) was calculated as the compound concentration required to reduce the MTT signal by 50% compared to controls.

3.8. Anticoagulant activity of polysaccharides

Thrombin Time (TT), expressed as the clot formation time in relation to the control time, was determined according to the method described earlier (Matsuhiro, Zúñiga, Jashes & Guacucano, 1996). As control the polysaccharide sample was replaced by phosphate buffer (pH 7.4).

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