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Inhibitory effect of sulfated galactans from the marine alga *Bostrychia montagnei* on herpes simplex virus replication in vitro

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Abstract

Sulfated polysaccharides exhibit many biological properties such as antiviral and anticoagulant activities. Herein, we report the antiviral activity of sulfated galactans extracted from the red seaweed *Bostrychia montagnei* against herpes simplex virus types 1 (strain F and the thymidine kinase-deficient strains Field and B2006) and 2 (strain G). Two crude extracts obtained with cold and hot water as well as some fractions obtained by anion exchange chromatography, inhibited significantly the replication of the different strains of herpesviruses as determined by plaque reduction assays. The inhibitory effect of the compounds studied here took place only when they were added during the adsorption period. They were found to be highly selective antiviral substances, causing no impairment of Vero cell viability. Furthermore, they had no direct inactivating effect on virions by incubation in a virucidal assay. The antiviral activity could be correlated with the molecular weight and sulfate content of the polysaccharides. Although sulfated polysaccharides are generally endowed with anticoagulant properties, the results of the activated partial thromboplastin time and the thrombine time assays indicated that the natural sulfated polysaccharides from *Bostrychia montagnei* have very low anticoagulant activity, confirming that there is no relation between the antiviral and anticoagulant properties.

Key words: polysaccharides, sulfated galactans, seaweed, herpes simplex virus, antiviral, anticoagulant

Introduction

The inhibitory effects of polyanionic substances on the replication of herpes simplex virus (HSV) and other viruses were reported almost four decades ago. In the last ten years, the activity spectrum of the sulfated polysaccharides has been shown to extent to various enveloped viruses, including viruses that emerge as opportunistic pathogens such as HSV and human cytomegalovirus in immunosuppressed (transplant) or immunocompromised (AIDS) patients (Witvrouw and De Clerq, 1997).

Several polysulfate compounds have the potential to inhibit the replication of herpesviruses by blocking the virus binding to the host cell (Witvrouw et al., 1994).

In previous studies, we reported that diverse types of polysaccharides isolated from algae such as carrageenans (Carlucci et al., 1997), sulfated mannans (Damonte et al., 1994; Pujol et al., 1998) and a sulfated galactan (Pujol et al., 1996), exerted a marked antiviral activity against herpesvirus in vitro. Red seaweeds biosynthesize a great variety of sulfated galactans that are the major components of the extracellular matrix, representing an interesting source for new antiviral agents (Craigie, 1990). The present study deals with the antiherpetic and anticoagulant activity and mechanism of action of the polysaccharides extracted with cold and hot water, as well as fractions obtained by

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anion-exchange chromatography, from the red seaweed *Bostrychia montagnei* (Noseda et al., 1999).

Materials and methods

Extraction and fractionation of the polysaccharides

Specimens of *Bostrychia montagnei* Harvey (Ceramiales, Rhodophyta) were collected in the Ilha do Mel (Paraná State, Brasil), sun-dried and ground in a mill to a fine powder. The extraction and fractionation procedures were described previously (Noseda et al., 1999). Briefly, the milled alga was treated with methanol (85%, 60 °C, 800 ml) until there was an absence of colour in the organic extract, and then dried in an oven at 40 °C. The pigment free powder was extracted with distilled water at 25 °C with mechanical stirring for 15 h (1 l, 4×). The residue was removed by centrifugation and the supernatant poured into ethanol (3 volumes), which precipitates the polysaccharides (BCW). The algal residue was re-extracted four times with hot water (1 l, 85 °C, 4 h) with mechanical stirring, centrifuged, and the supernatant also poured into ethanol (3 volumes), yielding the crude extract BHW. The ethanolic supernatants were concentrated, dialysed and freeze dried.

Anion-exchange chromatography

The extract BCW (3.51 g per 300 ml of water) was applied to a DEAE-Sephadex A-50 (Cl^-) column (6×51 cm) equilibrated with water. The polysaccharide fractions were eluted with water (fraction B1), 0.75 M (B2), 1 M (B3), 1.25 M (B4), 1.5 M (B5), and 4 M (B6) NaCl successively until the elutes were free from carbohydrates with phenolsulfuric reagents (Dubois et al., 1956). The fractions obtained were concentrated, dyalized against distilled water, and freeze dried.

Gel filtration chromatography

The homogeneity of the fractions was tested by gel filtration chromatography on a Sepharose 4B column (0.8×50 cm). Solutions of fractions B1–B6 (2 mg per 0.5 ml of water) were applied to the column and the chromatography developed with water at 25 °C at a flow rate of 1 ml/min. Fractions (1 ml) were collected and assayed by the reaction of Dubois et al. (1956).

Chemical analysis

Sulfate content was determined by the turbidimetric method of Dodgson and Price (1962). Molecular weights were determined by the method of Park and Johnson (1949). The determination of monosaccharide composition was by gas chromatography, following the reductive hydrolysis procedure of Stevenson and Furneaux (1991) using N-methyl-morpholine-borane as the reductant.

Cells and viruses

Vero (African green monkey kidney) cells were grown in minimum essential medium (MEM) supplemented with 5% bovine serum. For maintenance medium (MM), serum concentration was reduced to 1.5%.

HSV-1 strain F and HSV-2 strain G were obtained from the American Type Culture Collection (Rockville, USA); B2006 and Field were HSV-1 TK^- strains received from Dr. E. De Clercq laboratory (Rega Institute, Leuven, Belgium).

Cytotoxicity test

Vero cell viability was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; Sigma-Aldrich) method. Confluent cultures in 96-well plates were exposed to different concentrations of the polysaccharides, with three wells for each concentration, using incubation conditions equivalent to those used in the antiviral assays. Then 10 µl of MM containing MTT (final concentration 5 µg/ml) was added to each well. After 2 hours of incubation at 37 °C, the supernatant was removed and 200 µl of ethanol was added to each well to solubilize the formazan crystals. After vigorous shaking, absorbance was measured in a microplate reader at 595 nm. The cytotoxic concentration 50% (CC_{50}) was calculated as the compound concentration required to reduce cell viability by 50%.

Antiviral assay

Antiviral activity was evaluated by reduction of virus plaque formation. Vero cell monolayers grown in 24-well plates were infected with about 50 plaque forming units (PFU) of virus/well in the absence or presence of various concentrations of the compounds. After 1 h of adsorption at 37 °C, residual inoculum was replaced by MM containing 0.7% methylcellulose and the corresponding dose of each compound. 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.

Virucidal assay

A virus suspension of HSV-1 containing 5×10^4 PFU was incubated with equal volume of MM with or without compound for 1 h at 37 °C. The samples were then diluted in cold MM to determine residual infectivity in a plaque formation assay using Vero cells.

Influence of various treatment periods on the anti HSV activity of the compounds

Vero cells grown in 24-well plates were infected with 50 PFU of HSV-1 following different treatment condi-

tions: 1) the cells were exposed to HSV-1 in the presence of the compound (50 µg/ml) and after 1 h of virus adsorption at 4 °C, both compound and unadsorbed virus were removed, the cells were washed three times with PBS and were further incubated with MM containing 0.7% of methylcellulose. 2) the cells were exposed to HSV-1 and after the virus adsorption period, unadsorbed virus was removed and the cells were further incubated with MM containing 0.7% of methylcellulose and 50 µg/ml of the compound. 3) the compound was present both during and after the adsorption period. After 2 days of incubation at 37 °C, virus plaques were counted.

Assay for anticoagulant activity

Anticoagulant activities of the sulfated polysaccharides were determined using the activated partial thromboplastin time (APTT) assay (Anderson et al., 1976) and thrombin time (TT) as described by Carlucci et al. (1997), using heparin (150 units/mg) as a standard and polysaccharides in various concentrations (50–200 µg/ml).

Results

Table 1 shows the chemical analysis of the extracts BCW and BHW from *Bostrychia montagnei*, obtained by aqueous extraction (25 and 85 °C, respectively) and polysaccharide fractions (B1–B6) purified by anion-exchange chromatography from BCW (Noseda et al., 1999). These fractions were homogeneous as shown by gel filtration chromatography on Sepharose 4B. Each fraction was included in the gel and eluted as a single symmetrical peak (data not shown). The extracts and all the fractions are constituted by galactose as the main sugar component, together with lesser amounts of 3,6-anhydrogalactose and its 2-*O*-methyl derivative. Other methylated sugars, such as 6-*O*- and 2-*O*-methylgalactose are also present, the latter in very low proportions. The sulfate content and molecular weight of the fractions B1–B3 (11.2–16.2% and 5,600–31,300) are comparatively lower than for B4–B6 (22.0–24.0% and 34,000–43,700, respectively).

The antiviral activity of BCW, BHW and B1–B6 against HSV-1 strain F, HSV-2 strain G and the TK$^-$ mutant strains of HSV-1, Field and B2006, was evaluated in Vero cells by a virus plaque reduction assay. As shown in table 2, the extracts BSW and BHW were active against the standard strains of HSV-1 and HSV-2 at concentrations ranging from 11.2 to 20.7 µg/ml, being more active BCW than BHW for both serotypes of herpesviruses. Furthermore, both extracts elicited a strong antiviral activity against B2006 and Field with similar IC_{50} values. After the fractionation of BCW, no antiviral activity was observed in the fractions B1, B2 and B3 (IC_{50} > 50 µg/ml). Fractions B4 and B6 proved to be active against HSV-1 strain F and HSV-2 strain G at concentrations similars to those of BCW, while B5 was less active for these strains. Like BCW, B4 and B5 exerted a marked antiviral activity against the TK$^-$ strains of HSV-1, whereas the IC_{50} values of B6 were higher than those corresponding to BCW. In order to estimate the antiviral potency of the different compounds, the partially cyclized µ/ν carrageenan named

Table 1. Chemical analysis and monosaccharide composition of the polysaccharide fractions extracted from *Bostrychia montagnei*.

	BCW	B1	B2	B3	B4	B5	B6	BHW
Sulfate[a] (SO_3Na%)	23.0	15.0	11.2	16.2	22.0	24.0	22.0	17.0
Molecular weight[b]	27,500	31,300	5,600	11,900	43,700	42,500	34,000	45,700
Component monosaccharides[c] (mol%)[d]								
3,6-AG	10.9	8.9	3.9	10.4	11.6	13.0	11.6	13.6
2-Me-3,6-AG	4.0	8.7	2.0	5.3	9.2	4.2	3.7	6.2
Gal	71.5	65.5	56.2	60.0	67.2	70.5	70.8	63.0
6-Me-Gal	7.2	10.0	4.6	9.0	8.0	8.1	9.1	8.4
2-Me-Gal	0.2	1.0	0.2	2.5	0.9	1.0	–	2.4
Xyl	3.4	4.7	19.8	8.9	3.1	3.2	4.9	5.3
Glc	2.7	1.2	9.8	3.9	–	–	–	1.0
Man	tr.	–	3.5	–	–	–	–	–

[a,b] Determined by the method of: Dodgson & Price (1962) and Park & Johnson (1949), respectively.
[c] 3,6-AG = 3,6-anhydrogalactose, 2-Me-3,6-AG = 2-*O*-methyl-3,6-anhydrogalactose, 6-Me-Gal = 6-*O*-methylgalactose, etc.
[d] Means of 3 determinations (Stevenson and Furneaux, 1991).
– not detected.
tr. = traces (<0.2%).

Table 2. Antiviral activity and selectivity indices of the compounds isolated from *B. montagnei*.

	Inhibitory Concentration 50%, IC_{50} (µg/ml)				Selectivity Indices (CC_{50}/IC_{50})			
Compound	HSV-1 (F)	HSV-2 (G)	B2006	FIELD	HSV-1 (F)	HSV-2 (G)	B2006	FIELD
BCW	12.9 ± 0.5	11.2 ± 1.2	1.9 ± 0.2	1.2 ± 0.7	>77.5	>89.3	>526.3	>833.3
B1	>50	>50	>50	>50	–	–	–	–
B2	>50	>50	>50	>50	–	–	–	–
B3	>50	>50	>50	>50	–	–	–	–
B4	15.4 ± 4.9	12.4 ± 0.1	4.1 ± 0.1	5.8 ± 0.6	>64.9	>80.6	>243.9	>172.4
B5	25.7 ± 1.1	46.2 ± 5.3	3.9 ± 1.1	5.5 ± 1.2	>38.9	>21.9	>256.4	>181.8
B6	13.1 ± 2.1	19.4 ± 1.0	15.1 ± 1.8	12.7 ± 5.8	76.3	51.5	66.2	78.7
BHW	20.5 ± 6.8	20.7 ± 2.9	1.5 ± 0.1	3.3 ± 0.1	>48.8	>48.3	>666.6	>303.0
IC_3	0.7 ± 0.02	0.5 ± 0.01	0.5 ± 0.09	0.8 ± 0.1	>1428	>2000	>2000	>1250

IC_{50} (inhibitory concentration 50%): concentration required to reduce plaque number in Vero cells by 50%. Mean of two determinations ± SD.
CC_{50} (cytotoxic concentration 50%): concentration required to reduce 50% the number of viable Vero cells after 48 hours of incubation with the compounds. This concentration was >1000 µg/ml for all the compounds except for B6 that was 1000 µg/ml.

IC_3, isolated from the red seaweed *Gigartina skottsbergii*, was used as a reference substance (Carlucci et al., 1997). In comparison with IC_3, BCW, BHW and B1–B6 were less effective inhibitors of HSV-1 (F) and HSV-2 (G) although they were as active as the reference substance against the TK^- mutants with the exception of B6.

Cytotoxic effects of the polysaccharides were observed at a concentration much higher in comparison to the IC_{50}, with CC_{50} values above 1000 µg/ml for Vero cells. Thus, selectivity indices were estimated to be within the range of >21 to >833 for the different strains of herpesvirus assayed (Table 2).

One of the most active fractions of BCW, B4, and the crude extract BHW were further studied. In order to elucidate the possibility that these polysaccharides may act directly on the virus particle, a virucidal assay was carried out. Preincubation of the virus with B4 and BHW had no significant direct inactivating effect on HSV-1 virions (Fig. 1). Similar results were obtained with the other compounds when assayed in the same way (data not shown).

As a first approach to establish the stage of the virus replication cycle at which the compounds exert their antiviral activity, a virus plaque reduction assay for HSV-1 in Vero cells upon different treatment periods was employed. A high level of efficacy was attained if the compounds were present either only during HSV-1 adsorption or during the whole period of the plaque assay. When present only after adsorption, they were no longer effective, thus confirming that the compounds interfere with a very early stage of virus replication (Fig. 2).

The activated partial thromboplastin time (APTT) and thrombin time (TT) were measured to evaluate the anticoagulant activity of the sulfated galactans. The APTT and TT values of the blood treated with saline were 32.8 and 19.0 s, respectively. For the polysaccharides these values were only duplicate (or less) when they were tested at a concentration as high as 200 µg/ml. Heparin at concentrations of 4 and 5 µg/ml showed APTT and TT times higher than 120 and 100 s, respectively.

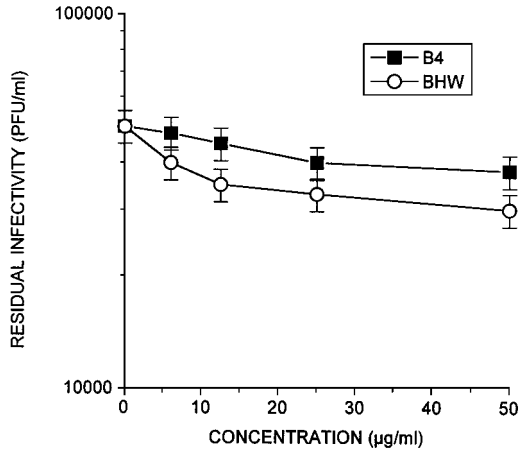

Fig. 1. Effect of B4 and BHW on HSV-1 inactivation. Samples of HSV-1 containing 5×10^4 PFU/ml were incubated at 37 °C for 1 h with different concentrations of the compounds, and remaining infectivity was determined by a plaque formation assay. Each value is the mean of two independent experiments.

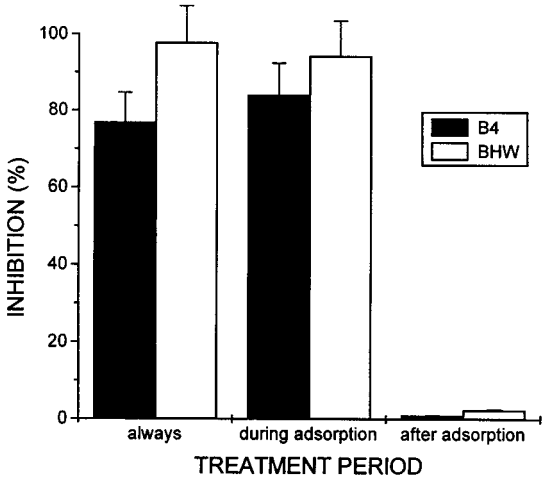

Fig. 2. Influence of various treatment periods on the anti HSV-1 activity of B4 and BHW. Vero cells were infected with 50 PFU of HSV-1. After 1 hr of virus adsorption at 37 °C in MEM with or without the compounds (50 µg/ml), unadsorbed virus and the compounds were removed. The cells were overlaid with medium with or without the compounds and further incubated at 37 °C for 2 days, whereafter the number of plaques was determined. Each value represents the mean of duplicate assays.

Discussion

The antiviral activity of acqueous extracts and the homogeneous polysaccharide fractions obtained from the red alga *B. montagnei* was demonstrated in Vero cells by a virus plaque reduction assay. Some polysaccharides proved to be active against various herpesviruses at concentrations ranging from 1.2 to 46.2 µg/ml, being the TK-strains the most susceptibles. The IC_{50} of these compounds was far from the cytotoxicity threshold and consequently these natural products possess good selectivity indices.

No virucidal effect was observed when the compounds were preincubated with the virions, confirming that the reduction observed in virus plaques was due to an interference with the virus replication cycle. Unlike other natural sulfated polysaccharides that inhibit not only the initial stages of viral infection, but also later replication steps after virus penetration (Hoshino et al., 1998; Lee et al., 1999), the inhibitory effect of the compounds studied here took place only when they were added during the adsorption period. A conclusion that may be drawn from this observation is that these polysulfates exert their anti HSV activity by shielding off the positively charged sites of the viral envelope glycoproteins, who are necessary for virus attachment to cell surface heparan sulfate, a primary binding site. This general mechanism also explains the broad antiviral activity of polysulfates against enveloped viruses (Witvrouw and De Clerq, 1997).

Among the fractionated sulfated galactans, only fractions B4–B6 showed antiviral activity (IC_{50} < 50 µg/ml). This activity could be correlated with the molecular weight and sulfate content of the polysaccharides, as the active fractions showed higher values for these characteristics than the inactive ones (B1–B3). Experiments carried out with dextran sulfate, sulfated cyclodextrins and fractions of chemically oversulphated galactosaminoglycans, have shown that antiviral activity increases with the molecular weight and the sulfation content (Marchetti et al., 1995; Witvrouw and De Clerq, 1997; Di Caro et al., 1999).

Sulfated galactans B1–B6 have an agaran type backbone, composed by alternating β-D-(1→4) and α-L-(1→3) linked galactosyl units (Noseda et al., 1999). This backbone is principally modified by sulfation on O-6, O-4 of the D- and O-3 of the L-galactosyl units (unpublished results). This type of backbone and pattern of sulfation is not strictly needed for the antiviral activity as the sulfated galactans B1–B3, do not show activity. Perhaps the differential activity between B1–B3 and B4–B6 may also be related with the distribution of the sulfate groups on the polysaccharide backbone and consequently with the effect that this distribution has on the conformation of the polysaccharide chains (Kolender et al., 1995; Carlucci et al., 1997).

The results of APTT and TT assays indicated that the natural sulfated polysaccharides from *B. montagnei* have very low anticoagulant activity, showing a negative correlation between antiviral properties and intrinsic coagulation pathways or antithrombin activity. In conclusion, the high selectivity indices and the lack of significant anticoagulant activity of the galactans obtained from *B. montagnei* warrant a further characterization of their antiherpetic properties. It may be of interest to assay the action of these polysaccharides in combination with other compounds in clinical use against herpesviruses infections.

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