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COMPOSITION AND ANTIVIRAL ACTIVITIES OF A SULFATED POLYSACCHARIDE FROM *SCHIZYMENIA DUBYI* (RHODOPHYTA, GIGARTINALES)

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Abstract. *Schizymenia dubyi* (Gigartinales, Gymnophlaeaceae) contains an unusual sulfated heteropolysaccharide with uronic acids that is active against several viruses including human immunodeficiency virus type-1 (HIV-1), *Herpes simplex hominis* type 1 and type 2 (HSV-1 and HSV-2) and vesicular stomatitis virus (VSV).

Introduction.

Some polysaccharides have shown *in vitro* activity against animal viruses including adenovirus type 5 (Ad5), African swine fever virus (ASF), vesicular stomatitis virus (VSV), polio virus type 2, Semliki forest virus (SFV)[1, 2] and *Herpes simplex hominis* virus (HSV)[3]. In recent years, sulfated polysaccharides have also demonstrated *in vitro* inhibitory effects on human immunodeficiency virus (HIV)[4-6] infection. Red seaweeds contain large amounts of cell-wall polysaccharides, most of which are sulfated galactans[7-9]. These galactans are generally built on repeated alternating 1,3-linked α-galactopyranose and 1,4-linked β-D-galactopyranose units and differ in the level and pattern of sulfation, in the substitution of methoxyl and/or pyruvate groups and in other sugar residues (galactose, xylose). They also differ in 3,6-anhydrogalactose content and the configuration of the 1,3-linked α-galactopyranose residue. Among these galactans, carrageenans and agars are widely used as gelling or thickening additives by the food industry and in biotechnologies[10]. A sulfated polysaccharide isolated from the red alga *Schizymenia pacifica* has displayed an inhibitory effect on HIV reverse transcriptase and *in vitro* replication[11-13]. The chemical characteristics of this sulfated galactan suggest that it belongs to the carrageenan family.

The purpose of this work was to study the chemical composition of water-soluble polysaccharide from *Schizymenia dubyi* (Chauvin ex Duby) J. Agardh (Rhodophyta, Gigartinales, Gymnophlaeaceae) and its *in vitro* activity against human immunodeficiency virus (HIV), *Herpes simplex hominis* virus, vesicular stomatitis virus and polio virus.

Extraction techniques and chemical analysis methods.

Gametophytic *Schizymenia dubyi* samples were collected in May 1991 on the east coast of Sicily.

Water extraction: Sulfated polysaccharide from air-dried seaweed powder (5 x 20 g, < 5 mm particle size) was extracted in hot distilled water (5 x 1.5 L) at 80°C for 4 h with magnetic stirring. Separation of the soluble extract from insoluble debris was done by filtration on diatomaceous earth. The filtrate at 5°C was poured into 2 volumes of absolute ethanol, with stirring. The precipitate was recovered and washed with 95° ethanol, dehydrated with diethyl ether, dried overnight at 50°C, weighed and ground to a powder. The polysaccharide was redissolved in distilled water, dialysed against distilled water and freeze-dried (18-20% yield w/w on the initial algal weight basis).

Alkali extraction: Algal powder (100 g) was treated with 1.2 L containing NaOH (0.3 M) and KCl (1.6 M) in distilled water without sodium borohydride at 80°C for 4 h. Polysaccharide was recovered from the alkali solution as above.

Uronic acids in polysaccharide were reduced according to the method of Taylor and Conrad[14]. The acid form of the polysaccharide was obtained as follows: polysaccharide (50 mg) was dissolved in distilled water (5 mL), and the solution was eluted through a column of ion exchange resin (Amberlite IR-120 plus). The column was then washed with distilled water and the polysaccharide was recovered and freeze-dried. After sulfuric acid hydrolysis of the polysaccharide (2N, 100°C, 2 h), neutral sugars were derived into alditol acetate[15] and identified and quantified by gas liquid chromatography (GC) on a DB-225 (JW Scientific) fused silica capillary column eluted with hydrogen at 210°C. 3,6-anhydrogalactose content was determined by colorimetry according to the method of Yaphe and Arsenault[16]. Uronic acids of the acid hydrolysates resulting from the neutral sugar analysis by GC were measured by colorimetry according to the method of Ahmed and Labavitch[17]. Identification of the uronic acids in the trifluoracetic acid polysaccharide hydrolysate (2N, 120°C, 2 h) was attempted by high pressure liquid chromatography (HPLC) using an Aminex 87H column (Biorad) eluted with 10^{-3}M sulfuric acid and with differential refractive index detection.

Protein content was measured by the Kjeldahl method (Nx 6.25). Sulfate content in polysaccharide (1g) was performed gravimetrically after complete hydrolysis (HCl 1N, 200 ml, 1h, 80°C followed by HCl 1N, H_2O_2 110 vol. (8: 1 v/v, 5 h, 80°C) followed by quantitative precipitation of the released sulfate by $BaCl_2$. Infrared spectra were recorded from a KBr pellet of the acid polysaccharide on a IRS 25-FT (Bruker Instrument). Proton broadband decoupling ^{13}C NMR of the acid-degraded water-soluble polysaccharide (HCl 1N, 90 min, 80°C) in D_2O was recorded on a Bruker AM 300 WB spectrometer at 75.5 MHz (room temperature). Chemical shifts in ppm were measured in relation to TMS as external reference.

Determination of antiviral activity of sulfated polysaccharide.

Monkey kidney cells (VERO cells Flow Lab. ref 03230) were cultured at 37°C in Eagle's minimal essential medium (MEM) with Earle's saline supplemented with 10 % inactive fetal calf serum[18]. HSV type 1, HSV type 2, polio-2 virus and VSV were isolated at Pontchaillou Hospital, Rennes, France. Virus stocks were prepared by inoculating VERO monolayers at low multiplicity of infection and incubation at 37°C. Two days after infection, cultures were frozen and thawed, and stocks were maintained at -70°C. Virus titration was performed by the Reed and Muench dilution method[19]. Viral titers were estimated from cytopathogenicity and expressed at 50 % of the tissue culture infective dose ($TCID_{50}$/ml).

Cytotoxicity test: The method used was essentially that described by Van Den Berghe *et al.*[20] and Hu *et al.*[21]. The cytotoxic concentration (CyD_{50}) value was defined as the maximum drug concentration causing cytotoxicity effects in 50% of the cultured cells after 96 h of incubation at 37°C. Values lower than those obtained in these tests were used in antiviral tests.

Antiviral activity was detected by measuring the inhibition of cytopathic effect (CPE), with estimation of a 50% antivirally effective concentration (ED_{50}) and therapeutic index (TI)[22]. Cell monolayers were infected with virus at low multiplicity [10^2 and 10^3 DI_{50} (50% infective doses) per microtiter plate] in the presence of polysaccharide at various concentrations. After several virus replication rounds (72 h for HSV, 32 h for polio and 28 h for VSV at 37°C), the cytopathic effects in infected and uninfected cells from 6 wells for each virus concentration of polysaccharide were examined under a phase-contrast microscope.

To assess the effect of polysacharide on uninfected MT4 cells, dilutions ranging from 1 mg/mL to 1µg/mL in the maintenance medium were added to MT4 cell line at 37°C with 5% CO_2 during 1h on 96-well microtiter plate containing $3x10^5$ MT4 cells for 100µL of compounds (in duplicate). The cells were then reajusted to $3x10^5$ MT4/mL on 24-well microtiter plate containing various concentrations of polysaccharide. After 4 days the cells were diluted to 1/3 with the corresponding dilution of compound.

Reverse Transcriptase (RT) assay: 1 mL sample of cell supernatants were concentrated 100 fold by ultracentrifugation at 95000 rpm at 4°C for 5 minutes in a TL100 rotor (Beckman). The pellet was resuspended in 10µL of NTE buffer containing 0.1% Triton X-100. The enzymatic reaction was performed in a 50µL of a reaction mixture containing: Tris 50mM, pH 7.8 ; $MgCl_2$ 20 mM ; KCl 20 mM ; dithiothreitol 2 mM ; Oligo(dT) 12-18 0.25 OD/mL ; poly(rA) 0.25 OD/mL and ^{3}HdTTP 2.5µCi. After 1h at 37°C, the reaction products were precipitated with 20% trichloracetic acid, filtered on Millipore 0.45µm and the β radioactivity was measured.

Determination of antiviral activity with HIV-1 was based on measuring the protective effect of the sulfated polysaccharide against this virus-induced cytopathogenicity in MT4 cells. Four to six days after infection, multinucleated giant cell (syncitium) formation was observed, preceding cell death. MT4 cells were incubated in RPMI 1640 culture medium with 10% fetal calf serum (FCS) and 1% PSN antibiotics, 1% glutamine and 2µg/mL polybrene at 37°C during 1 h on 96-well microtiter plate containing various concentrations of sulfated polysaccharide ($3x10^5$ cells for 100 µL of compound). 100 µL of HIV-1 (dilution 10^{-3}) suspension was added in wells. One hour after incubation, infected cells were washed three times with RPMI and then cocultured at $3x10^5$ cells/mL on 24-well microtiter plate containing various concentrations of the sulfated polysaccharide. Every three or four days, cells were diluted three times and the cell concentration was adjusted at $3x10^5$ cells/mL RPMI, 10% FCS, 1% glutamine and 2µg/mL polybrene in the presence of sulfated polysaccharide. Three days after cultures, infected MT4-HIV-1 cells with and without sulfated polysaccharide and uninfected MT4 cells were observed for syncitial formation every one or two days[23].

Results.

The chemical composition of the water-soluble polysaccharide is shown in Table 1.

Table 1

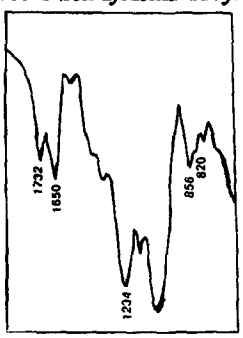
Chemical composition of the sulfated polysaccharide from *Schizymenia dubyi*[a]

	Water-extracted polysaccharide	Reduced polysaccharide[b]
Neutral sugars		
galactose	29.9	25.2
xylose	1.6	1.6
mannose	1.3	-
glucose	3.3	6.7
3,6-anhydrogalactose	0.9	0.9
Uronic acids	33.7	2.8
Sulfate	15.5	
Proteins	0.5	
Ash	19.0	

[a] % dry weight ; [b] polysaccharide reduced twice.

Fig. 1

IR spectra of phycocolloid from *Schizymenia dubyi*



Neither galacturonic, mannuronic nor glucuronic acid was detected by HPLC analysis of an acid hydrolysate of the polysaccharide. Two successive reductions of the polysaccharide decreased the uronic acid content from 33.7 to 2.8%. Neither marked increase of a particular sugar (except a small increase in glucose) nor the appearance of a new sugar was detected by gas chromatography after reduction. The presence of uronides was confirmed by the characteristic ^{13}C NMR signal at 177 ppm for carboxylic carbons and by typical absorbance at 1732 cm^{-1} on the infrared spectrum of the polysaccharide in acid form. The latter spectrum also confirmed the presence of sulfate (1234 cm^{-1}), and the absorbance at 856 and 820 cm^{-1} indicated that sulfate groups might be located on carbon 4 and 6 of the galactose units[24] (Fig. 1). Sulfate content (14.0%) and infrared spectrum of the alkali-extracted polysaccharide were similar to those of the water-extracted polymer. No methyl from methoxyl or pyruvate groups was detected by ^{13}C NMR spectroscopy.

The *in vitro* antiviral activities of the water-extracted polysaccharide from *Schizymenia dubyi* are shown in Table 2. The maximal non-toxic concentration of the polysaccharide for the VERO cell line was 93.7 µg/mL, giving a cytotoxic concentration of CyD_{50} at 187.5 µg/mL. The polysaccharide was most effective against HSV-1 and least effective against polio-2 virus. The compound was toxic on the MT4 cell lines within the concentration range of 1mg to 50µg/mL while toxicity was much less at 25µg/mL, 10µg/mL and 1µg/mL doses showed no apparent cytotoxicity.

Table 2

In vitro antiviral activity of water-soluble polysaccharide from *Schizymenia dubyi* against HSV-1, HSV-2, VSV and Polio-2 viruses

Viruses	ED_{50} (µg/mL)[a]		TI[b]	
	10^2DI_{50} /50µL[c]	10^3DI_{50} /50µL[c]	10^2DI_{50} /50µL[c]	10^3DI_{50} /50µL[c]
HSV-1	1.5	5.0	125.0	37.5
HSV-2	25.0	6.0	75.0	31.2
VSV	10.0	15.0	18.7	12.5
Polio-2	44.0	30.0	4.0	2.0

[a] antivirally effective concentration; [b] Therapeutic index: C_yD_{50}/ED_{50}.

Evaluation of the anti-HIV effect of the polysaccharide (Table 3) indicated that syncitial formation was completely suppressed at 20 µg/mL and delayed at 10µg/ml. No toxicity was found at these concentrations. Furthermore, the sulfated polysaccharide is inhibitory to the HIV-associated Reverse Transcriptase.

Table 3

Evaluation of antiviral effect for polysaccharide with HIV-1

Cells	polysaccharide concentration (µg/mL)	D4[a]	D5	D6	D7	RT (cpm/mL) (in duplicate)
MT4-HIV-1	500	-/Tox	-/Tox	Tox	Tox	104/80
	100	- -	- -	- -	- -	178/156
	50	- -	- -	- -	- -	140/512
	20	- -	- -	- -	- -	482/134
	10	- -	- -	++ +	++/T ++/T	700/1666
	2	++ ++	++ ++	++/T ++	++/T ++/T	39874/98632
	0.2	++ ++	++ ++	++/T ++	++/T ++/T	
	0.0	+ (+)	++ ++	++/T ++	++/T ++	20470/10984
MT4	0.0	- -	- -	- -	- -	136/184

[a] Day ; - : absence of syncytia ; + : presence of syncytia ; ++ : syncytia ; T: cell death ; Tox: Toxicity.

Discussion.

The chemical composition of *Schizymenia dubyi* water-soluble polysaccharide has never been described in the literature, although that of *S. pacifica* has been reported by Whyte *et al.* [25] and Nakashima *et al.* [12]. Whyte *et al.* reported galactose, 3,6-anhydrogalactose and sulfate in a molar ratio of 1, 0.04, and 0.75 respectively, with the presence of 0.5% methyl and the absence of uronic acids. Nakashima *et al.* reported galactose, 3,6-anhydrogalactose and sulfate in a molar ratio of 1, 0.01 and 0.51 respectively and attributed this sulfate galactan to a λ-carrageenan. The water-soluble polysaccharide from *S. dubyi,* another sulfated galactan encountered in many red algae[8,9], differed because of its unusually high content of

uronic acids. Neither the methoxyl nor the pyruvate group was associated with these polymers. The presence of uronic acids was confirmed by ^{13}C NMR and IR spectroscopy and chemical reduction, but the sugar-bearing carboxylic group remained unidentified. Uronic acids have been rarely reported in polysaccharides from marine red algae, whereas D-glucuronic acid is present in the mucilage of several unicellular red algae. Craigie *et al.*[8] and Okazaki *et al.*[26] reported guluronic-rich alginate-like polysaccharides in *Serraticardia maxima* and other coralline red algae. The galactan of *Dilsea edulis* contains 9.5 to 11% uronic acids[7], although it has recently been reported that the Atlantic and Pacific gametophytes and tetrasporophytes from *Dilsea, Halymenia, Cryptonemia* and *Schizymenia* species are very anatonomically similar[27].

The similarity in sulfate content and between IR spectra of the water- and alkali-extracted polysaccharide suggests that the sulfate groups are alkali-stable and thus not located on C-6 or C-3 of 4-linked galactose residues[28]. Research is in progress in our laboratory to determine the identity of the uronic acid and the position of the sulfate groups on the water-soluble sulfated galactan from *S. dubyi.*

Antiviral activity of sulfated polysaccharides has been recognized for some years. As early as 1947, polysaccharides from diverse sources were found to inhibit viral growth[29,30]. The polysaccharide-rich fractions from two marine red algae, *Cryptosiphonia woodii* and *Farlowia mollis* (Dumontiaceae), were found to exhibit *in vitro* activity against HSV-1 and HSV-2, vaccinia virus and VSV[31,32]. In recent years, sulfated polysaccharides have been shown to inhibit *in vitro* replication of many enveloped viruses, including HIV[33,34]. A sulfated polysaccharide isolated from the sea alga *Schizymenia pacifica* may also suppress *in vitro* HIV infection by interfering with virus adsorption as well as inhibiting reverse transcriptase[11, 12]. Multinucleated giant cell formation induced by interaction between the glycoprotein expressed on the surface of cells infected with HIV-1 and the receptor of uninfected cells may play an important role in the depletion of T4 lymphocytes in acquired immune deficiency syndrome (AIDS) patients[35]. Pentosan polysulfate, fucoidan, mannan sulfate and λ-carrageenan are inhibitors of giant cell formation and hence protect target cells against destruction by killer HIV-1-infected cells. Their mode of action have been attributed to an inhibition of virus adsorption to the cell membrane[36].

In the present study, the water-soluble polysaccharide of *Schizymenia dubyi* showed activity against HSV-1, HSV-2 and VSV, but the relation between the chemical structure and antiviral activity remains to be determined. The antiviral mode of action of this polysaccharide is now under investigation. As the nature of anionic groups are a decisive factor in anti-HIV process[37,38], the fine chemical structure of this polysaccharide is currently being studied in our laboratory.

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