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Short communication

In vitro inhibition of the replication of haemorrhagic septicaemia virus (VHSV) and African swine fever virus (ASFV) by extracts from marine microalgae

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Abstract

We have screened for in vitro inhibition of viral replication with extracts from the following marine microalgae: *Porphyridium cruentum*, *Phaeodactylum tricornutum*, *Tetraselmis suecica*, *Chlorella autotrophica*, *Dunaliella tertiolecta*, *Dunaliella bardawil*, *Isochrysis galbana*, *Isochrysis galbana var Tiso*, *Ellipsoidon sp.* and *Tetraselmis tetrathele*. We have used as viral models two enveloped viruses of significant economic importance, the viral hemorrhagic septicemia virus (VHSV) of salmonid fish and the African swine fever virus (ASFV). The aqueous extracts from *P. cruentum*, *C. autotrophica* and *Ellipsoidon sp.*, produced a significant inhibition of the in vitro replication of both viruses in a dose-dependent manner. That this inhibition could be due to sulfated polysaccharides was suggested because the same pattern of viral inhibition was obtained by using exocellular extracts from microalgae enriched in these compounds and/or dextran sulfate of high molecular weight. However, the inhibition of viral replication did not correlate with the percentage of sulfatation of the exocellular polysaccharides. Extracts from marine microalgae may have prophylactic utility against fish and mammalian viral diseases.

Keywords: Disease; Marine microalgae; *Porphyridium cruentum*

Marine algae are a source of natural products with many pharmacological applications including substances inhibiting viral infection and/or replication (Nakashima et al., 1987; BoissonVidal et al., 1995; Hasui et al., 1995; Damonte et al., 1996; Saoo et al., 1996). However, only few reports have shown the existence of compounds inhibiting viral infection from marine microalgae (Borowitzka, 1995). In the few cases reported, the inhibition of viral infection was due to different

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mechanisms. Thus, 2% out of about 900 strains of cyanobacteria have been found positive for inhibition of the reverse transcriptase of human immunodeficiency virus (HIV) and avian myeloblastosis virus (Lau et al., 1993), and at least part of the activity could be attributed to a sulfolipid (Gustafson et al., 1989; Sudo et al., 1995) secreted into the exocellular medium (Minkova et al., 1996). On the other hand, sulfated polysaccharides from marine macroalgae are known to interfere with adsorption and penetration of some enveloped viruses. Sulfate residues are found in eukaryotic algal exopolysaccharides (Bourgougnon et al., 1993) and in some cyanobacterial polysaccharides (Sudo et al., 1995). We report here the screening of polysaccharide-rich fractions from 10 marine microalgae for inhibition of viral infection by using two enveloped virus as models, the rhabdovirus of viral hemorrhagic septicemia (VHSV) of salmonid fish and the Asfarvirus of African swine fever (ASF) of swine, both viruses of significant economic importance. Because some microalgae, including cyanobacteria are known to modulate production of their exopolysaccharide (Arad et al., 1985) in response to several environmental and culture conditions, we have also studied the possible correlation between the degree of sulfatation of this exopolysaccharide and the inhibition of viral infection.

Microalgal cultures were carried out using 80 ml glass tubes with an aeration of 100 ml/min. Aeration was supplemented with 10% CO_2 for 10 s every 10 min in order to maintain the pH below 7.6. Cultures were maintained at 22°C and submitted to 12 h light–12 h dark periodicity with a light intensity of 152.3 μmol m^{-2} s^{-1}. Tubes containing 60 ml of sterilized sea water (salinity 3.5%) were enriched with nutrients (Fabregas et al., 1984) to a final concentration of 2 mmol nitrogen per liter (Nl), except for *Porphyridium cruentum* (4 mmol/Nl). Cultures were inoculated at a cell density of $0.5–10 \times 10^6$ cells/ml depending on the microalgae.

Once the cultures reached the stationary phase, they were separated into pellet (endocellular) and supernatant (exocellular) extracts by centrifugation ($3000 \times g$, 20 min). The endocellular extracts were obtained by adding distilled water to the pellets, centrifugating again, sonicating and lyophilizing the new supernantants. The exocellular extracts were obtained from the first supernatants by double precipitation with ethanol (1:2 v/v), centrifugation and lyophilization of the new pellets. Lyophilized endo- and exocellular extracts were stored at −20°C until reconstituted at 2 mg/ml in distilled water and used.

Total soluble exopolysaccharide (TSEP) was quantified with the phenol–sulfuric method (Dubois et al., 1956). The sulfated soluble exopolysaccharide (SSEP) was quantified in the same samples with the Alcian Blue method, using the high viscosity alginic acid A7128 as reference (Sigma, St. Louis) (Ramus, 1977). Commercial dextran sulfate of average molecular weights 5000, 10 000 and 500 000 Da were obtained from Sigma.

To determine the inhibition of VHSV replication (Lorenzo et al., 1996), 10^3 $TCID_{50}$ per ml of VHSV 07.71 were incubated overnight at 4°C with serial dilutions of the microalgae extracts. Then cultures of epithelioma papulosum cyprini (EPC) cells in 96-well plates were infected with 100 μl of the different virus/extract mixtures, adsorbed during 1 h at 14°C, washed with cell culture medium and filled again with 100 μl/well with culture medium. After 16 h postinfection, the VHSV-infected EPC monolayers were fixed during 10 min in cold methanol and dried. To detect the infected foci, the Mab 2C9 specific for the N antigen of VHSV (Sanz and Coll, 1992), diluted 1000-fold in dilution buffer (130 mM NaCl, 2 mM KCl, 8 mM Na_2 HPO_4, 1.4 mM KH_2 PO_4, 0.24 mM merthiolate, 5 g of Tween 20/l, 50 mg of phenol red/l, pH 6.8) was added to the wells (100 μl/well) and incubated during 1 h. After washing with distilled water, 100 μl of peroxidase-labelled anti-mouse IgG (Nordic, Tilburg, The Netherlands) were added per well and incubation continued for 30 min. After 3 washings by immersion in distilled water, 50 μl of 1 mg/ml of diaminobenzidine (DAB) (Sigma) per well in the appropriate buffer was added, and the reaction allowed to proceed until brown foci were detected by inspection with an inverted microscope.

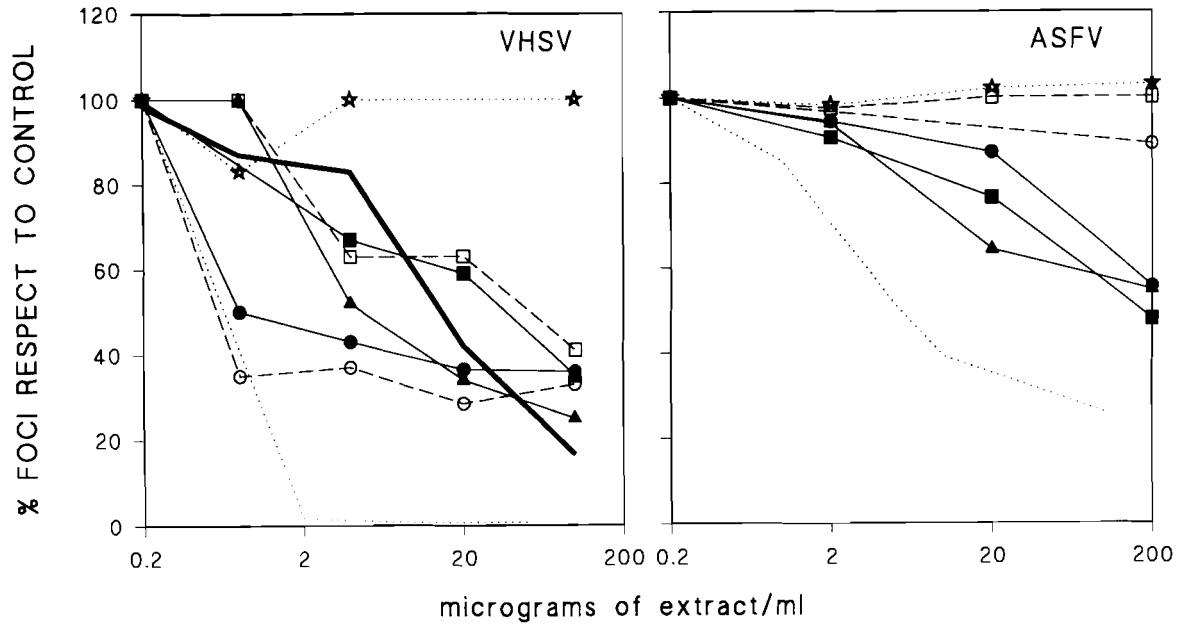

Fig. 1. Percentage of viral foci obtained in the presence of endocellular microalga extracts relative to viral controls in the VHSV and the ASFV assays. The results are expressed by the following formula, number of positive foci in the presence of extracts/number of DAB positive foci in the absence of extracts × 100. Positive controls of VHSV inhibition included the neutralizing MAb C10 obtained from Sanofi Diagnostic Pasteur (Marnes-La-Coquette, France), and of ASFV, the MAb 2A10 anti ASFV receptor (unpublished). As negative controls, virus in the absence of any compound was included in tetraplicate in every assay. The results of one of two experiments are shown in the Figure. Variation was between 10 and 30% of the presented values. ----, dextran sulfate of average molecular weight of 500 000 Da; ....., neutralizing anti-VHSV G MAb 1C10 (VHSV assay) and anti-ASFV receptor MAb 2A10 (ASFV assay); ●—●, *P. cruentum*; ○---○, *D. tertiolecta*; ■ ■, *Ellipsoidon* sp.; □...□, *I. galbana var Tiso*; ▲_____▲, *C. autotrophica*; *---*, *D. bardawil* (representative of the rest of microalgae of Table 1).

The inhibitory effect of microalgal extracts on African swine fever virus (ASFV) replication was estimated by using a recombinant ASFV expressing the β-galactosidase reporter gene introduced into the thymidine kinase locus of the viral genome (Gomez-Puertas et al., 1995). Vero cell monolayers were infected with 100 PFUs of the recombinant virus previously incubated overnight at 4°C with the microalgal extracts. The mixtures were incubated for 2 h at 37°C and then the Vero cells were covered with 0.8% agarose in DMEM 5% fetal bovine serum. After 6 days, the monolayers were washed, then stained with the chromogenic dye X-gal (300 μg/ml) and the blue plaques counted with an inverted microscope.

Fig. 1 shows that the endocellular extracts from *P. cruentum*, *Chlorella autotrophica*, *Isochrysis galbana var Tiso*, *Ellipsoidon* sp. and *Dunaliella tertiolecta* were capable of inhibiting VHSV replication and the extracts from *P. cruentum*, *C. autotrophica* and *Ellipsoidon sp.* also inhibited ASFV replication. Thus most of the endocellular extracts inhibiting VHSV replication, also inhibited ASFV replication, indicating a common activity against both enveloped viruses. The extent of inhibition of VHSV replication was higher than that of ASFV. However, the inhibition percentages obtained in ASFV were comparable to those obtained with the ASFV neutralizing MAb 2A10 or with neutralizing polyclonal antisera (Gomez-Puertas et al., 1995). Similar inhibition of VHSV was obtained with the commercial dextran sulfate (average molecular weight: 500 000 Da) (Fig. 1), in contrast with the lack of inhibition of dextran sulfates of lower average molecular weights (5000 and 100 000 Da, data not shown).

The exocellular extracts (enriched in polysaccharides by the preparation method) from all the

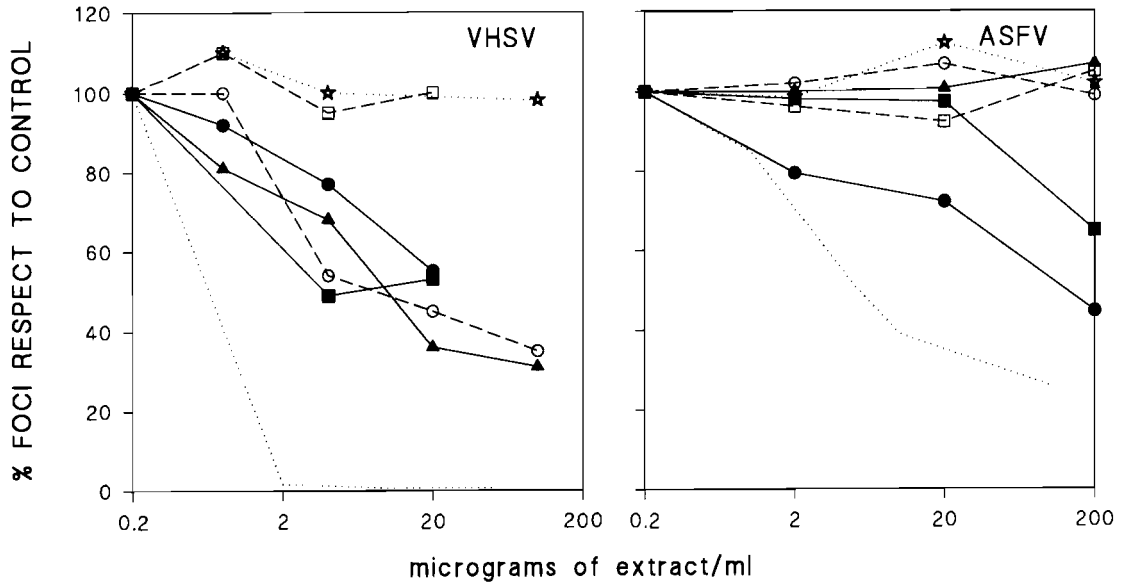

Fig. 2. Percentage of viral foci obtained in the presence of exocellular microalga extracts relative to viral controls in the VHSV and the ASFV assays. The results of one of two experiments are shown in the Figure. Variation was between 10 and 30% of the presented values. ....., neutralizing anti-VHSV G MAb 1C10 (VHSV assay) and anti-ASFV receptor MAb 2A10 (ASFV assay); ● ●, *P. cruentum*; ○--○, *D. tertiolecta*; ■_ _■, *Ellipsoidon* sp.; □_□, *I. galbana var Tiso*; ▲ ▲, *C. autotrophica*; *--*, *D. bardawil*.

10 microalgae tested were also assayed for inhibition of VHSV or ASFV replication (Fig. 2). The results obtained with the endocellular extracts were confirmed for most of the exocellular extracts, except for those from *Isochrysis galbana var Tiso* with both viruses and from *C. autotrophica* and *Dunaliella tertiolecta* with ASFV. Because the exocellular extracts are enriched in polysaccharides and because previous reported evidence links inhibition of viral infectivity by extracts from algae with their content in sulfated polysaccharides (Bourgougnon et al., 1993; Sudo et al., 1995), the content of sulfated soluble exopolysaccharides (SSEP) was measured for all the microalgae used (Table 1). However, no correlation between the SSEP of each microalga and the percentage of inhibition of viral replication could be shown. Thus the three microalgae which inhibited viral replication, *C. autotrophica* (15.3% SSEP), *Ellipsoidon sp.* (14.7% SSEP) and *P. cruentum* (6.1% SSEP) could be classified as average with respect to the SSEP content among all the microalgae tested (12.8% SSEP) (Table 1). The rest of microalgae which did not inhibit viral replication had either low, medium or high SSEP content (Table 1).

Previous work has demonstrated the importance of the use of semicontinuous cultures for the production of marine microalgae because of the simplicity of operation and the added possibility of manipulating the biochemical composition of the biomass by changing some culture parameters like renewal rate or nutrient concentration. Thus, the unicellular red alga *P. cruentum*, encapsulated by a polysaccharide envelope composed of sulfated polysaccharides (Otero et al., 1997), changes its content of sulfated polysaccharides under different field conditions (Arad et al., 1985) or culture parameters (Fabregas et al., 1999). Therefore, the microalgae *C. autotrophica*, *P. cruentum* and *Ellipsoidon sp.* were grown in semicontinuous cultures in order to study the effect of the renewal rate on the percentage of SSEP and whether or not it would affect its inhibitory effect on viral replication. Whereas the renewal rate had little effect on the percentage of SSEP of *C. Au-*

totrophica (variation of SSEP from 3.89 to 5.78%), *P. cruentum* showed an increment on the percentage of SSEP from 2.9 to 10.56% with increasing renewal rates and *Ellipsoidon sp.* showed a decrease in the percentage of SSEP from 53.4 to 9.7% with increasing renewal rates (Table 2).

Comparison of the virus-inhibitory values obtained with the above mentioned samples (Fig. 3), showed that *C. autotrophica* had about the same inhibition index (47.4–67.4%) independently of the renewal rate, *P. cruentum* had a decreasing inhibition index (46.8–31%) with the increasing renewal rate despite their increment in percentage of SSEP, and *Ellipsoidon sp.* had a decreasing inhibition index (44–0%) concomitantly with a decrease in SSEP and increasing renewal rate. Therefore, no correlation was found between the inhibition of VHSV replication and the content of sulfated polysaccharides in the three microalgae selected. It seems that the different polysaccharide molecular species that constitute these different samples (and differing in sulfate content, carbohydrate composition, etc) may be responsible for the observed effects. The different inhibitory effects obtained with sulfated polysacharides of different molecular weight suggest that the differences observed in inhibition activity might also be due to differences in molecular size. Further studies should address the identification of these molecular species.

In the antiviral assays, the test compounds were present before and during the viral adsorption period to increase the probability of detection of inhibitory activity of the microalgae in this preliminary screening. The fact that the commercial dextran sulfate showed inhibitory activities at similar concentrations as the extracts from marine microalgae suggests that the observed activities must be attributed to the inhibition of viral adsorption (Bourgougnon et al., 1993; Sudo et al., 1995). However, a direct action of the test compounds on the virus cannot be completely ruled out because of the low purification of the extracts. Further purification of the test compounds will be necessary to correctly interpret the antiviral mechanism(s) involved.

Table 1
Percentage of sulfated soluble exopolysaccharides (SSEP) of microalgae in batch cultures[a]

Microalgae	% SSEP
P. tricornutum	29.8
T. tetrathele	19.9
Tetraselmis suecica	17.3
C. autotrophica	15.3
Isochrysis galbana	15.1
Ellipsoidon sp.	14.7
Dunaliella tertiolecta	6.7
P. cruentum	6.1
Dunaliella bardawill	1.6
Isochrysis Tiso	1.5

[a] The microalga strains were obtained from the Culture Center of Algae and Protozoa (Cambridge, England), Provasoli-Guillard Center for the Culture of Marine Phytoplankton (CCMP) Bigelow, Maine, USA. 'The University Texas Collection' (Austin, TX). Collection Sammlung von Algen Kulturen, Planzen Physiologisches Institut 'Universitt Gottingen Deutschland'. Ellipsoidon sp. was isolated by Dr Ralph A. Lewin (Lewin and Cheng, 1989). *T. suecica* and *P. tricornutum* were isolated from NW Spain.

Table 2
Influence of the percentage of renewal rate of semicontinuous cultures in the percentage of SSEP of microalgae

%[a]	*P. cruentum*	*C. autotrophica*	Ellipsoidon sp.
Batch culture	–	5.12	53.4
1	2.90	–	–
2	2.29	5.78	
5	2.75	4.15	–
10	4.23	4.59	21.7
20	3.52	4.72	22.1
30	5.28	5.41	20.8
40	6.27	3.89	18.7
50	10.56	4.78	9.7

[a] %, percentage of renewal rate. Tubes containing 60 ml of sterilized sea water with nutrients (Fabregas et al., 1984) to a final concentration of 4 mmol/Nl except for 8 mmol/Nl in *C. autotrophica*, were inoculated at a cell density of $1–10 \times 10^6$ cell/ml depending on the microalgae with the same conditions of Table 1. Once cultures had reached early stationary phase, the semicontinuous regime was initiated with a daily renewal rate of 1, 2, 5, 10, 20, 30, 40 and 50% of the culture volume. Renewal of the cultures with sterilized seawater enriched with nutrients at their initial concentration 4 or 8 mmol/Nl, was carried out during the first hour of the light period (Otero et al., 1997). Cultures were kept in the semicontinuous regime for 15–20 days with no significant change in steady-state density. Biomass was harvested by centrifugation.

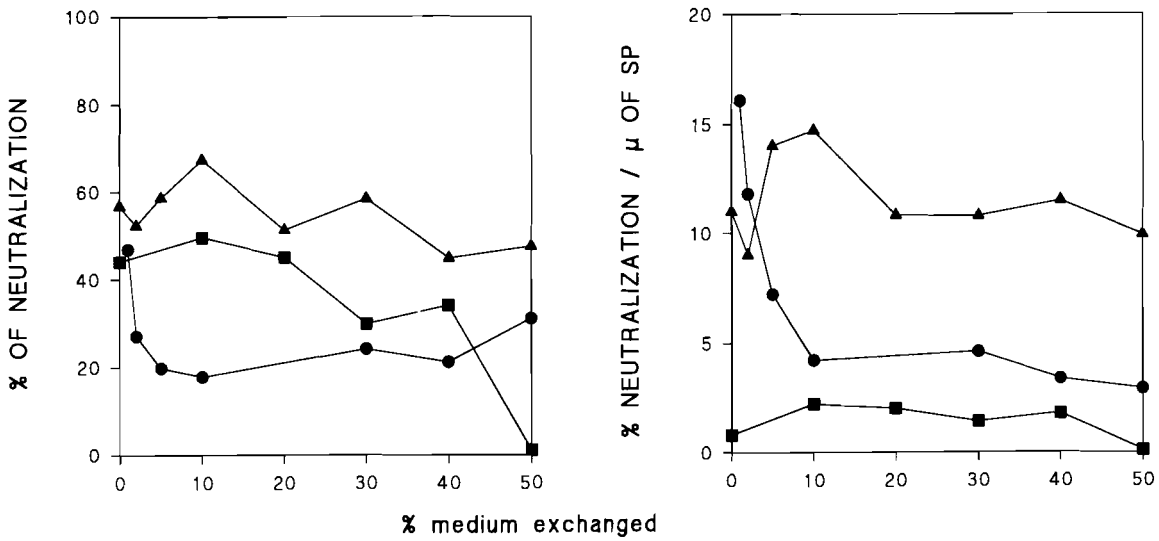

Fig. 3. Variation in the percentage of VHSV inhibition (right) and percentage of inhibition per µg of SSEP (left) with the microalga culture conditions. Supernatants from *P. cruentum* (●___●), *C. autotrophica* (▲ ▲) and Ellipsoidon sp. (■ ■) obtained by culture with different percentage of exchanged medium (0% is a discontinuous batch culture) were assayed at four different dilutions (from 100 to 0.8 µg/ml). Results at 100 µg/ml and Table 2 were used for the calculations. Percentage of neutralization was obtained by the following formula: 100-percentage of foci relative to control.

This study has shown the tremendous opportunity that microalgae-related molecules offer for a further development of compounds with viral infectivity inhibitory-properties. More precisely since marine microalgae are used in aquaculture, conditions could be manipulated so that their viral inhibitory effects could be optimized to protect fish in hatcheries against VHSV and perhaps also against other fish enveloped viruses. In this respect, substances inhibiting viral infectivity are being searched in peptides of VHSV (Estepa et al., 1999) as well as other enveloped viruses (Wild et al., 1995; Judice et al., 1997).

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