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Influence of sulphate on the composition and antibacterial and antiviral properties of the exopolysaccharide from *Porphyridium cruentum*

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ABSTRACT

Aims: The influence of two culture media and three different concentrations of sulphate in the medium on the growth of two strains of *Porphyridium cruentum* and on the production, composition and viscoelastic characteristics, and antimicrobial properties of the sulphated exopolysaccharide (EPS) were studied.
Main methods: A Bohlin C50 rheometer was used to evaluate the viscosity and elasticity of the EPS solutions. HSV virus, types 1 and 2, *Vaccinia* virus and *Vesicular stomatitis* virus were used along with two Gram-negative (*Escherichia coli* and *Salmonella enteritidis*) and one Gram-positive (*Staphylococcus aureus*) bacteria, for testing the antimicrobial activity of EPS.
Key-findings: The growth of microalgae was higher in NTIP medium and the production of EPS was enhanced by sulphate 21 mM. The protein content of the EPS was enhanced by the addition of sulphate 52 mM and 104 mM; this concentration also induced an increase in sulphate content of the EPS. However, neither the contents of EPS in carbohydrates and uronic acids were affected by the culture medium supplementation in sulphate. In general, the EPS from the Spanish strain presented a higher antiviral activity than the EPS from the Israeli strain. All EPS extracts revealed a strong activity against *V. stomatitis* virus, higher than the activity of all chemical compounds tested. The EPS from the Israeli strain also presented antibacterial activity against *S. enteritidis.*
Significance: Enrichment of the culture medium with sulphate improved protein and sulphate content of EPS. EPS extracts presented a relevant activity against *V. stomatitis* virus and *S. enteritidis* bacterium.

Introduction

The great potential of marine microalgae for diverse applications was recently reviewed (Raposo et al., 2013a,b). The polysaccharides and their derivatives have found applications in several fields, such as in food and cosmetics, as gelling agents, thickeners, emulsifiers, stabilizers, or even as biolubricants (Arad et al., 1985, 2006b). Marine red unicellular microalgae from the genus *Porphyridium* (Porphyridiales, Rhodophyta) produce an extracellular sulphated polysaccharide (EPS) (Arad and Levy-Ontman, 2010; Geresh et al., 2002) with acidic characteristics and with potential applications in cosmetics, as inhibitor of hyaluronidase, anti-allergic and anti-inflammatory agents (Matsui et al., 2003) and as a nutraceutical due to the antioxidant, hypolipidaemic and hypoglicaemic activities (Tannin-Spitz et al., 2005; Jiao et al., 2011), as well as a therapeutic agent (Geresh et al., 2002) due to activities as an anti-bacterial (Guzman-Murillo and Ascencio, 2000), antiviral, anti-inflammatory and anti-tumour (Huleihel et al., 2002; Matsui et al., 2003; Arad and Levy-Ontman, 2010).

The general mechanism of the antiviral activity of most polysulphates against enveloped viruses may be explained as follows. The viruses attach to cells by an interaction between the glycoproteic envelope and the cell surface heparan sulphate receptor (Witvrouw and De Clercq, 1997). The virus–cell complex is formed mainly by ionic interactions between the anionic (mainly sulphate) groups in this polysaccharide and basic amino acids of the glycoprotein

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(Damonte et al., 2004). Therefore, it seems that the antiviral activity is due to the shielding off the positively charged sites in the viral envelope glycoproteins. Therefore, the EPS could block the viral adsorption process. Buck et al. (2006) found that carrageenan, a sulphated polysaccharide similar to EPS, could directly bind to the Human Papillomavirus capsid, so as to inhibit not only the viral adsorption process but also the subsequent entrance and uncoating process of the virus.

Several authors studied the incorporation of sulphate in the EPS of *Porphyridium*. Ramus (1974) showed that *Porphyridium* can assimilate either sulphate or thiosulphate, both compounds supporting the growth of algae and the sulphate being transferred to the *Porphyridium* polysaccharide. Moreover, it is known that sulphate groups of the EPS can determine some of the characteristics of the polysaccharides, such as antiviral properties (Arad et al., 2006a). In fact, it was already found that higher sulphate content induced a higher antiviral activity (Arad and Levy-Ontman, 2010). In addition, Geresh et al. (2002) referred that polysaccharides with a higher sulphate content induced a decrease in viscosity but activity against FD cells (myeloid cell line) and 24-I and EL-4 T-lymphoma cell lines was enhanced.

In spite of all the previous studies on the effects of sulphate on the biological properties of the EPS, no information is available on the influence of the culture media and/or the incorporation of sulphate (from the media) neither on the composition and rheological characteristics of the EPS produced nor on the antiviral and antibacterial properties.

In this study, two different marine strains of *Porphyridium cruentum* were grown in two different culture media and three concentrations of sulphate, and the influence of these conditions on the production, composition and viscoelastic characteristics of the exopolysaccharide produced was studied. The antiviral and bactericidal properties of the EPS produced were also evaluated.

Materials and methods

Strains

Strain SP of *P. cruentum* was gently supplied by the Department of Microbiology of the Faculty of Pharmacy, University of Santiago, Spain. Strain ISR was kindly provided by the Laboratory of Microalgae Biotechnology of the Department of Biotechnology Engineering, University of Ben-Gurion, Negev, Israel.

Culture media

Study of the culture medium

In the first experiment, two different media were tested: Brody's medium (Brody and Emerson, 1959) and NTIP medium (Table 1).

Study of the of sulphate concentration in the culture medium

In the second experiment, sulphate concentration of NTIP was adjusted up to that of Brody's medium (21 mM), as sulphate in NTIP is only at very low concentration, as being part of the micronutrients. Two other concentrations in sulphate (52 and 104 mM) were also tested in order to verify the improvement of the biomass growth and EPS production and also to study the characteristics and quality of the polysaccharide obtained. In this experiment, $MgCl_2$ was used as the control for $MgSO_4$, in order to assess whether modifications in algal biomass and EPS production and characteristics were due to sulphate. Simple NTIP was used as the control.

Growth conditions of P. cruentum

The axenic cultures of the two *P. cruentum* strains were grown in a walk-in chamber, under a continuous light (30 μE m^{-2} s^{-1}) and constant temperature (25 °C); compressed air was gently bubbled into the culture flasks, through sterilised 0.2 μm filters. Every experiment was carried out twice. There were three replicates of 2 L-flasks, with 1.750 mL culture medium, for every condition (Brody/NTIP; $MgSO_4/MgCl_2$; different concentrations of sulphate). Three samples were taken from each replicate every other day. Growth was monitored by determination of biomass dry weight.

Determination of algal biomass dry weight

For the determination of the biomass dry weight, filters (GF/C Whatman, 47 μm diameter, 22 μm pore size) were first washed twice with distilled water and dried in an oven overnight, at 105 °C. After taking filters out, they were stored in a desiccator and weighed before using (first weight). A 5 mL sample of well-stirred microalgal culture was filtered through previous treated filters, and dried overnight at 105 °C. Filters with the dried biomass were taken out and stored in the same desiccator until weighed. Dry weight biomass was then calculated by subtracting the last weight from the first weight. Biomass dry weight is expressed in g/L culture.

EPS extraction and purification

Before extraction of the EPS, cultures were first centrifuged at 10,000 rpm, for 20–30 min, at 4 °C. The pellet was rejected and the supernatant, with the EPS, was put under a ventilated hotte to concentrate the EPS containing suspension. After 3–4 days, samples were placed in a water bath at 80 °C, for 1 h. Suspensions with the EPS were filtered (simple filter paper) and extraction was accomplished by precipitation with two volumes of cold ethanol. EPS thus obtained was lyophilized under 0.100 mbar in a freeze dryer CHRIST Alpha 1–4 (B. Braun — Biotech International, Melsungen, Germany) before and after a dialysis procedure. Dialysis was performed by resuspending dried EPS in deionised-bidistilled water and dialysing with a cellulose membrane (MWCO = 12000, size 33 mm × 21 mm, Sigma-Aldrich) against deionised-bidistilled water, several times. Dialysed EPS was used in all the analyses performed.

Analyses of the EPS

Carbohydrates (total sugars) and uronic acids

Carbohydrates (as neutral oses) and uronic acids were determined colorimetrically (Shimadzu UV-1601) by the phenol-sulphuric acid (Dubois et al., 1956) and *m*-hydroxydiphenyl-NaOH (Blumenkrantz and Asboe-Hansen, 1973), respectively, using galactose and galacturonic acid as standards.

Sulphate

Sulphate content was obtained turbidimetrically, as barium sulphate (Dodgson and Price, 1962).

Protein

Protein content of the EPS was determined according to the biuret method of Gornall (Zaia et al., 1998).

Sugar composition

Determination of the sugar composition was performed by gas chromatography–mass spectrometry (GC–MS) after enzymatic hydrolysis and methylation, in collaboration with the University of Aveiro, Portugal.

Viscosity and elasticity

A Bohlin C50 rheometer was used to evaluate the viscosity and elasticity of the aqueous solutions with 2% (w/v) of EPS from the Israeli and Spanish strains. Viscosity was evaluated as a function of the shear rate at a constant temperature (frequency sweep = 0.01–30; steady shear rate = 3.14 s^{-1}; strain rate = 0.8%). The slope of temperatures 20–80–20 °C was carried out at a controlled frequency (1Hz),