Carbohydrate Polymers 81 (2010) 448–460

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Carrageenan from *Solieria chordalis* (Gigartinales): Structural analysis and immunological activities of the low molecular weight fractions

Bondu Stephanie[a,b,*], Deslandes Eric[a,b,c], Fabre Marie Sophie[a,c], Berthou Christian[d], Guangli Yu[e]

[a] *Université Européene de Bretagne, Brest, France*
[b] *Université de Brest, LEBHAM EA3877, IUEM, place Nicolas Copernic, 29280 Plouzané, France*
[c] *Plate-forme d'extraction/purification BIODIMAR, Université de Brest, 6 Avenue V. Le Gorgeu, 29200 Brest, France*
[d] *Laboratoire de Thérapie Cellulaire et d'Immunobiologie du Cancer CHU Morvan, 5 avenue Foch, 29200 Brest, France*
[e] *Laboratory of Glycoscience and Glycoengineering, School of Medicine and Pharmacy, Ocean University of China, 266003 Qingdao, People's Republic of China*

ARTICLE INFO

Article history:
Received 5 January 2010
Received in revised form 19 February 2010
Accepted 25 February 2010
Available online 7 March 2010

Keywords:
Solieriaceae
Carrageenan characterization
Low molecular weight fractions
Antitumor activities
Immunomodulation

ABSTRACT

The investigations reported here are about a carrageenan extracted from the abundant red alga, *Solieria chordalis* (Gigartinales) settled along the coasts of Brittany. Its structural features were characterized by GC–MS, ^{13}C NMR and FTIR spectroscopies. The structural components of this polysaccharide are mainly a (DA2S-G4S)-type structure in association with methylated-ι-carrageenan, pyruvated α-carrageenan and the minor precursor, ν-carrageenen, in small amounts. The relative molecular weight of the native polysaccharide was estimated by LP-GPC as 913 kDa. The low molecular weight fractions (below 20 kDa) obtained by free-radical depolymerization and mild-acid hydrolysis presented substitution patterns similar to those of the native polysaccharide. These fractions proved to be devoid of direct cytotoxicity on Daudi (human Burkitt's lymphoma), Jurkat (human leukaemic T-cell lymphoblast) and K562 (human chronic myelogenous leukaemia) cells lines. On the other hand, they showed great immunostimulating properties: enhancement of neutrophil phagocytosis, cytotoxicity by natural killer cells, antibody-dependent cell cytotoxicity and stimulation of lymphocyte proliferation. Further to these investigations, it could be worth using the low molecular weight fractions of carrageenan from the red alga, *S. chordalis*, in immunotherapeutic approaches to cancer treatment.

1. Introduction

In recent decades, polysaccharides isolated from mushrooms, lichens, higher plants, bacteria have attracted a great deal of attention in the biomedical world because of their broad spectrum of therapeutic properties (Leung, Liu, Koon, & Fung, 2006). Marine algae are also great sources of polysaccharides. The sulfated polysaccharides produced by green, brown and red seaweeds are numerous and with a lot of various structures. They are acknowledged as endowed with a rather low toxicity and numerous biological activities, including antiviral (Gonzales, Alarcon, & Carrasco, 1987; Gonzales, Crance, Van Cuyck-Gandre, Renaudet, & Deloince, 1991; Mazumder et al., 2002; Pujol et al., 2006; Talarico et al., 2004; Talarico et al., 2005), anticoagulant (Carlucci et al., 1997; Opoku, Qiu, & Doctor, 2006), anti-tumoral (Alves de Sousa et al., 2007; Hoffman, Woodrow Burns, & Paper, 1995), antimetastatic (Xuelian, Jing, Xianliang, & Meiyu, 2006) and anti-inflammatory effects (Solimabi & Das, 1980), worthwhile for clinical uses. Among the sulfated polysaccharides, the carrageenans are isolated from the cell walls of some red algae; their economical value is high because of their gelling properties, which make them of current use in the food, cosmetic and medical/pharmaceutical industries. These polysaccharides are made up of a repeating disaccharide backbone of 3-linked β-D-galactopyranose (G units) and 4-linked α-D-galactopyranose (D units) or 3,6-anhydrogalactose (DA units) in alternation. Carrageenan is a collective term and covers several families denoted as κ-, λ-, β- or ω-carrageenan. Each family is sub-divided in different types identified from their 3,6-anhydrogalactose content and sulfate substitution pattern. The corresponding IUPAC nomenclature allotted names to Greek symbols such as κ-carrageenan (carrabiose 4′ sulfate, DA-G4S), ι-carrageenan (carrabiose 2,4′-disulfate, DA2S-G4S) or λ-carrageenan (carrabiose 2,6,2′-trisulfate, D2S6S-G2S), . . .

Several reports have dealt with animal models of chronic inflammation induced by carrageenans (Gábor, 2003; Moore, 2003; Morris, 2003) and enlightened the real pro-inflammatory potential of these molecules. Indeed, the systemic administration of carrageenans has proven to induce various effects, particularly on the immune system, e.g. induction of pro-inflammatory neutrophil migration, adhesion and expected action (Moreno et al.,

* Corresponding author at: Université de Brest, LEBHAM EA3877, IUEM, place Nicolas Copernic, 29280 Plouzané, France. Tel.: +33 298498867; fax: +33 298498772.
E-mail addresses: Stephanie.bondu@univ-brest.fr, stfbondu@yahoo.fr (B. Stephanie).

0144-8617/$ – see front matter.
doi:10.1016/j.carbpol.2010.02.046

2006). However, for further investigations, one should keep in mind that sulfated polysaccharides such as carrageenans interact with a variety of sulfated polysaccharides-binding proteins, and play two-edged roles, inhibitor and promoter, in immune response. Some sulfated polysaccharides act as immuno-suppressors: they block the transduction of an inflammatory signal induced by pro-inflammatory cytokines, suppress the activation of complement and inhibit the processes of leukocyte-adhesion and -passing through the endothelium. But, as an immune response can be initiated through interaction between immune cells and sulfated polysaccharides, the structure of the latter may play a critical role in their controversial effects (Chen, Wu, & Wen, 2008). Carrageenans are potential immunostimulatory agents liable to be used through local, or systemic, administration to enhance the natural immunity of patients with defenses altered because of cancer or infection, whereas oral administration of carrageenans seems to have strong adverse effects (Tobacman, 2001).

Over the last decade, the depolymerization of carrageenans has become a subject of interest for several reasons: (i) the greater homogeneity of the chemical structure of the by-products issued from a native polysaccharide facilitates their chemical analysis, and (ii) the reduction of their anticoagulant activity makes them less toxic. Different methods have, thus, been developed to get some low molecular weight fractions (LMWFs), or oligosaccharides. Among them, mild-acid hydrolysis with HCl (Yu et al., 2002; Yuan & Song, 2005) and the enzymatic method by carrageenases (Haijin, Xiaolu, & Huashi, 2003; Knutsen et al., 2001) are the most common. More exotic techniques by microwave (Zhou et al., 2004), active oxygen with, or without, ultrasonication (Yamada et al., 1997; Yamada, Ogamo, Saito, Uchiyama, & Nakagawa, 2000) and free-radical depolymerization by H_2O_2 (Zuniga, Matsuhiro, & Mejias, 2006) or methanolysis (Knutsen & Grasdalen, 1992) have also been tested on carrageenans. Moreover, many biological activities, e.g. antiviral (Yamada et al., 1997), anti-angiogenic (Haimin, Yan, Lin, Wang, & Xu, 2007) or antitumor activities (Yuan, Song, Li, Li, & Dai, 2006), can be potentiated by depolymerizations. However, immunological tests carried on LMWFs or oligosaccharides from carrageenans are not yet fully documented and, to our knowledge, only λ- or κ-carrageenan have been investigated through *in vivo* (Haijin et al., 2003; Hu, Jiang, Aubree, Boulenguer, & Critchley, 2006; Yuan et al., 2006; Zhou, Sheng, Yao, & Wang, 2006; Zhou et al., 2004; Zhou et al., 2005) and *in vitro* studies (Haijin et al., 2003; Yuan et al., 2006). These compounds have demonstrated their ability to act on the immune system and upregulate the immune response. To our knowledge, to date no investigation has dealt with the immunological activities of ι-carrageenan.

Among the commercially exploited carrageenophytes, the most numerous ones belong to the Solieriaceae family, which is part of the Gigartinales order (Doty, 1988) known to contain the highest number of genera (Gabrielson & Hommersand, 1982; Womersley, 1994). Among them, the genus *Solieria* is acknowledged to contain ι-carrageenan as main component (Deslandes, Floc'h, Bodeau-Bellion, Brault, & Braud, 1985; Murano, Toffanin, Cecere, Rizzo, & Knutsen, 1997; Saito & De Oliveira, 1990). The aim of this study is to evaluate, *in vitro*, the antitumor and immunomodulatory activities of different LMWFs issued from the dominant ι-carrageenan extracted from Atlantic species of *Solieria* (*Solieria chordalis*).

2. Experimental part

2.1. Cell culture

2.1.1. Human cell lines

Daudi (Human Burkitt's lymphoma), Jurkat (Human leukaemic T-cell lymphoblast) and K562 (Human chronic myelogenous leukaemia) cells were obtained from ECACC (European Collection of Cell Cultures) and grown in the RPMI 1640 medium with L-glutamine (Lonza) supplemented with either 10% of heat-inactivated FBS (Cambrex), or 20% in the case of Daudi cells.

2.1.2. Human peripheric blood mononuclear cells (PBMCs)

Venous blood was collected from healthy donors, who had given their informed consent. For PBMC isolation, the cells were purified by sedimentation on Lymphocyte-Separation Medium (LSM 1077, PAA), a separation solution made with Ficoll™ 400. Then, after dilution with equal parts of culture medium, heparinized whole blood was carefully poured over the lymphocyte separation solution (LSM 1077). Centrifugation of the mixture at $400 \times g$ for 20 min led to the concentration of the lymphocytes (70–100% enrichment) in the interphase (white layer) between the plasma and the separation solution. These lymphocytes were subsequently extracted with a sterile Pasteur pipette and washed twice with the culture medium. Assessment of the cell viability was done with the use of 0.05% Trypan blue, which stains only the non-viable cells. The cell viability percentages were always above 95%. Then, after counting of the cell suspension, PBMC concentration was adjusted to the expected final one in EMEM (Lonza) with 10% FBS. Then, the PBMCs were split into two sets and incubated with the LMWFs of the carrageenans for either 48 h or 5 days prior to the bioactivity assays.

2.2. Algal material

Samples of the red algae, *S. chordalis* (C. Agardh) J. Agardh (Solieriaceae, Gigartinales), were collected in September 2006 in the Bay of Brest at Logonna Daoulas (Roz) France. After removal of the epiphytes, the thalli were rinsed in fresh seawater and then freeze-dried.

2.3. Polysaccharide extraction

The dried algal matter (200 g) was extracted with 4 L of hot water (85 °C) for 4 h. The pH of the solution was kept at 8.0–8.5 by addition of 1N NaOH. After centrifugation, the supernatant was collected and precipitated with twice its volume of ethanol. Then, the precipitate was freeze-dried and further dissolved in distilled water prior to treatment with amylase (EC 3.2.1.1) so as to remove the floridean starch. The solution was then dialyzed (MWCO 2 kDa) for 2 days and freeze-dried.

2.4. Polysaccharide treatment

The alkali treatment of polysaccharide was performed as described in Falshaw and Furneaux (1998). Briefly, 10 mg of the polysaccharide were dissolved in 2 mL of water before addition of 1 mg $NaBH_4$ and stirring overnight at room temperature. After addition of 1 mL of aqueous NaOH (3N) in solution with $NaBH_4$ (3 mg), the resulting mixture was heated at 80 °C for 24 h, and then cooled down for neutralization with acetic acid. Then, it was dialyzed against distilled water for 2 days (MWCO 2 kDa), and then freeze-dried.

The solvolytic desulfation was also performed on the native polysaccharide. Briefly, 100 mg of polysaccharide were dissolved in water (30 mL), passed through a cation-exchange column of AG® 50W (H^+) (Bio-Rad) and eluted with water. The eluent was collected, neutralized with pyridine and freeze-dried. The resulting pyridinium salt was desulfated with Me_2SO-MeOH-pyridine (89/10/1, v/v/v) at 100 °C for 4 h (Falshaw & Furneaux, 1994). After cooling and addition of water (10 mL), the pH was adjusted to 9.1 with 0.1N NaOH. The sample was then dialyzed against distilled water for 2 days (MWCO 2 kDa) and freeze-dried.

2.5. Preparation of low molecular weight fractions (LMWF)

For mild-acid hydrolysis, an aliquot of the native polysaccharide (500 mg, 10 mg/mL) was dissolved, at 60 °C, in 50 mL of 0.1N HCl under vigorous stirring. After 4 h, the hydrolysis was stopped by neutralization with 0.1N NaOH prior to centrifugation of the mixture. After collection of the supernatant, it was concentrated by rotary evaporation and freeze-dried.

The free-radical depolymerization reaction was performed on the native polysaccharide as follows: 500 mg were dissolved, in 47.5 mL of Na_2HPO_4 (10 mM) prior to the addition of 0.5 mL of 0.01 M $FeSO_4{\cdot}7H_2O$ and 2 mL of a 30% H_2O_2 solution. Then, after stirring of the mixture at 60 °C for 5 h and addition of 1 mL of the 30% H_2O_2 solution, the resulting mixture was stirred at 60 °C for 3 more hours. The reaction was stopped by addition of 0.5 mL of 0.075 mM $Na_2S_2SO_3{\cdot}5H_2O$. After centrifugation the supernatant was concentrated by rotary evaporation and freeze-dried.

Then, the acid and free-radical hydrolysates, respectively denoted as HA and F samples, were desalted by passage through a Sephadex G-10 column (100 cm × 1.5 cm) (Pharmacia) and use of MiliQ water as eluent at the flow rate of 0.2 mL/min (Biologic DuoFlow Chromatography System, Bio-Rad). Fractions of 4 mL, each, were collected and monitored through use of the phenol–H_2SO_4 acid reagent as described in Dubois, Gilles, Hamilton, Rebers, and Smith (1956). The sugar-containing fractions were pooled, concentrated by rotary evaporation and freeze-dried.

2.6. Fractionation

The HA and F samples were size-fractionated by low pressure gel permeation chromatography (LP-GPC) (Biologic DuoFlow Chromatography System, Bio-Rad). The hydrolysates (60 mg in 1 mL of 0.1 M NaCl) were loaded on a Bio-Gel P-10 column (100 cm × 1.5 cm) (Bio-Rad) marked out with glucose and a 11,300 Da dextran. Elution with 0.1 M NaCl at the flow rate of 0.2 mL/min was monitored through use of the phenol–H_2SO_4 acid reagent. The volume of each of the collected fractions was 1 mL/tube. Each hydrolysate (HA or F) was fractionated into six sub-fractions, respectively, denoted as HA1, HA2, HA3, HA4, HA5 and HA6 or F1, F2, F3, F4, F5 and F6. These fractions were desalted by passage on the Sephadex G-10 column as described above.

2.7. Chemical analysis

The total content in carbohydrates was determined by the phenol–sulfuric acid method, with use of galactose as standard, as described in Dubois et al. (1956). The sulfate content was analyzed by turbidimetry (Jackson & McCandless, 1978), and the amount of pyruvic acid was assessed by colorimetry (Sloneker & Orentas, 1962). The content of 3,6-anhydrogalactose was determined by the resorcinol method with 3,6-anhydro-D-galactose (Dextra Laboratories, United Kingdom) as standard (Yaphe & Arsenault, 1965), and the proteins were quantified by the Lowry procedure (Lowry, Rosebrough, Farr, & Randall, 1951). The ash content was determined after 4 h at 450 °C.

The monosaccharides were hydrolyzed through a two-steps procedure (Stevenson & Furneaux, 1991). Alditol acetates were generated from the polysaccharide (and from the LMWF) by reductive hydrolysis with 4-methylmorpholine-borane (MMB) and acetylation, as previously described in Jol, Neiss, Penninkhof, Rudolph, and De Ruiter (1999). Me_3Si-derivatives were also generated by methanolysis with anhydrous 1.5N MeOH–HCl at 80 °C for 16 h, and trimethylated as reported in Kamerling, Gerwig, Vliegenthart, and Higashi (1975). The alditol acetates and trimethylsilylated-derivatives were separated by High Resolution Gas Chromatography and Electron Impact Ionization Mass Spectrometry. The GC–MS apparatus (MSD 5973 from Agilent) was equipped with a Vf-5ms (60 m × 1.25 mm ID, DF 0.25 μm) (Varian) capillary column and the temperature conditions were programmed as follows: after heating of the oven at 50 °C and holding for 1 min, the temperature was raised to 90 °C at the rate of 40 °C/min, and then to 320 °C at the rate of 6 °C/min and held at the final temperature for 15 min. Identification was based on both the retention times relative to myo-inositol (Sigma, France) and the mass spectra. For quantification of alditols acetate, molar response factors were calculated relative to myo-inositol for D-galactose (Sigma, France) and 3,6-anhydro-D-galactose (Dextra Laboratories, United Kingdom) obtained from commercial sources. The unavailability of the 6′-*O*-methyl-D-galactose on the market drove us to assume that its response factor and that of galactose were alike.

2.8. Linkage and substitution analysis

The native and desulfated polysaccharides were converted into Me_2SO-soluble pyridinium form and methylated with CH_3I or CD_3I as previously described in Stevenson and Furneaux (1991), except that a NaOH/Me_2SO suspension was used to generate the alkoxide (Ciucanu & Kerek, 1984). The permethylated alditol acetates were derived by reductive hydrolysis, acetylated, and separated by GC as described for the monosaccharide analysis. The eluted derivatives were detected and identified by mass spectra and the retention times relative to Meso-Erythritol (Sigma, France) hexa-acetate.

2.9. Determination of the relative Molecular weight

The weight-average molecular weight (Mw) of the crude polysaccharide was determined by Low Pressure Gel Permeation Chromatography (LP-GPC). The polysaccharide was dialyzed at different temperatures (20, 45, 60 and 80 °C) and then loaded on a Sepharose CL_4B (100 cm × 1.5 cm) (Pharmacia) with 0.5 M NaCl as eluent at a flow rate of 0.2 mL/min. The column was calibrated with glucose and dextrans of known weight (2000, 570 and 71 kDa). The elution of 4 mL fractions was monitored as previously with phenol–H_2SO_4 acid reagent.

The weight-average molecular weights (Mws) of the depolymerized fractions (from HA1 to HA6 and from F1 to F6) were determined by High Performance Gel Permeation Chromatography (HPGPC) on a TSK gel 3000SW × l column (7.8 mm × 300 mm, TOSOH, Japan) eluted with 0.2 M Na_2SO_4 at 0.5 mL/min in flow rate and 30 °C. The standards used for the calibration of the column were dextrans of known weights (11,700, 7100, 5700, 3650 and 2500 Da).

2.10. PAGE analysis

Gradient (12–22%) discontinuous Poly-Acrylamide Gel Electrophoresis (PAGE) analysis was performed on a vertical slab (0.1 cm × 16 cm × 20 cm) gel system (Mini Vertical Electrophoresis System, Hoefer). After loading of the gel with 20–50 μL of sample and electrophoresis at 400 V for 4 h, detection was achieved through use of Alcian blue (0.5% in 2% AcOH) staining.

2.11. FTIR and NMR studies

The Fourier Transform Infrared (FTIR) spectra of the crude polysaccharide prepared as KBr pellets were recorded with a Nicolet MS60 spectrophotometer.

All of the NMR studies were carried out on solutions of the polysaccharide (^{13}C NMR) or LMWFs (1D and 2D NMR) always prepared in 99.97% D_2O (700 μL). The apparatus was a Bruker Avance 500 spectrometer equipped with an indirect 5 mm triple resonance TBI $^1H/\{BB\}/^{13}C$ probehead, and the standard pulse sequences in

use were available in the Bruker software. The chemical shifts were always expressed in ppm relative to TMS as external reference.

The proton-decoupled ^{13}C NMR spectra of the polysaccharide were recorded under the following conditions: 85 °C, a 30° pulse with a 2 s delay as relaxation time and about 24,000 scans.

The 1D and 2D NMR spectra of the LMWFs were recorded at 25 °C. Double-quantum filtered ^{1}H–^{1}H correlated spectroscopy (DQF COSY), HMQC and HMBC experiments were performed with a delay of 60 ms according to standard pulse sequences in order to assign ^{1}H and ^{13}C resonances. For example, in an HMQC experiment, the raw data set consisted in a series of 1024 (F2) × 128 (F1) complex data points, zero-filled to 1 K in the F1 dimension prior to the Fourier transform with a spectral width of 2948.113 and 25,000 Hz in the F1 and F2 dimensions, respectively.

2.12. Biological activity analysis

2.12.1. Anti-tumoral assays

For the test, the cells were plated, in triplicate, at the concentration of 5×10^4 cells per well in 200 μL onto flat-bottomed 96-well ELISA plates and incubated in 5% CO_2 at 37 °C for 24 h prior to exposure to the drug. The LMWFs of carrageenans were added at the concentration of 10 mM. The stimulated cultures, the control ones (solvent alone) and the blank (cell-free medium) were incubated for an additional 48 h period. Their anti-tumoral activities were assessed by the cytotoxic assay after adaptation of the method described in Ishiyama et al. (1995). The mitochondrial function was assessed through the determination of the mitochondrial dehydrogenase (succinate-tetrazolium-reductase) activity by the WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) colorimetric assay (Roche Diagnostics); WST-1 is a tetrazolium dye, which contains an electron-coupling reagent cleaved by the mitochondrial dehydrogenase to a water-soluble formazan dye; the number of metabolically active cells is directly correlated with this reaction. After 24 h of incubation, 10 μL of the formazan dye were added to each well prior to 3 h incubation at 37 °C. Absorbance was measured at 450 nm with a multi-well spectrophotometer (TECAN Infinite). For each condition, the mean optical density (OD) of the 3 technical replicates per exposure condition was compared to the mean OD of the control (DMSO). For whole-blood cultures, heparin-anticoagulated venous blood was diluted 1/2 with EMEM. Then, 200 μL of the blood dilution were poured in wells of a flat-bottomed 96-well plate (Nunc) and incubated for 24 h at 37 °C in a chamber with 5% CO_2.

2.12.2. Phagocytosis assay

After dilution of human whole blood, aliquots were pre-incubated for 24 h with 10 mM of one of the LMWFs from carrageenans to investigate phagocytosis with an assay adapted from the commercially available Phagotest kit (Orpegen Pharma, Germany) (Hirt, Nebe, & Birr, 1994). This test relies on the measurement by flow cytometry (EPICS XL4, Beckman Coulter) of the uptake of fluorescence-labeled *E. coli* by the cells of interest. Briefly, heparinized whole blood is incubated for 10 more minutes with fluorescein-labeled *E. coli* bacteria at 37 °C while a negative control sample is kept on ice. The phagocytosis is stopped by placing the samples on ice. To exclude artifacts of bacteria, or cells, aggregation, a DNA staining solution is added just before the measurement, by flow cytometry, of the number of ingested bacteria.

2.12.3. Natural killer cell cytotoxicity

The cytotoxicity by NK cells was quantified by a flow cytometric assay as reported in Kane, Ashton, Schmitz, and Folds (1996) and Ben and Quah (2007). Briefly, logarithmically growing K562 cells were washed and resuspended in 1 mL of PBS 5% FBS; then they were labeled with the green fluorescent dye, CFSE (Invitrogene) (final conc. 5 μM) at room temperature for 5 min, washed three times with PBS 5% FBS and resuspended in the culture medium that contained 10% FBS (Ben & Quah, 2007). PBMCs (stimulated or not with the LMWF of carrageenans) were diluted with 10% FBS-containing EMEM to get the desired effector-to-target ratio (E/C:10). CFSE-labeled K562 cells (50 μL, 5×10^4 cells) were mixed with the PBMCs (150 μL, 5×10^5 cells) in FACS tubes and vortexed. The cell mixtures were incubated at 37 °C for 4 h, mixed with ice-cold propidium iodide (PI) and transferred to ice. For flow cytometric analysis, the green (CFSE) and red (PI) fluorescences of the cells were read in an EpicsXL4 flow cytometer (Beckman Coulter). For each sample, 10,000 cells were analyzed as a dot plot of FL1 (CFSE fluorescence, total number of target cells) versus FL2 (PI fluorescence, number of dead target cells).

2.12.4. ADCC (antibody-dependent cellular cytotoxicity) assay

As reported in Papadopoulos et al. (1994), this flow cytometric assay is based on two fluorescent dyes and provides a measurement of NK cytotoxicity. Briefly, target Daudi cells were stained with 300 nM calcein-AM, a non-fluorescent substance which is converted by esterase to the green fluorescent calcein in viable cells. Effector PBMCs (stimulated or not with the LMWF of carrageenans) were washed, counted and adjusted to 1.5×10^6 cells/mL. The stained target cells (2×10^4 cells/mL), the effector one in a 15/1 ratio and an anti-CD20 antibody (rituximab [10 mg/mL]) were mixed in sterile Falcon polystyrene tubes and incubated at 37 °C in a humidified 5% CO_2 incubator for 4 h. Then, ethidium homodiner-1, a red DNA stain non-permeable to viable cells, was added at the concentration of 10 mM, and the reaction was let to develop for 15 min at room temperature prior to the acquisition of the Flow cytometric data. The dead target cells exhibit a green-red staining. Data analysis is performed by gating the regions of living and dead target and living effector cells from appropriate controls. Non-specific events are subtracted from the dead target region, and the ratio of specific dead target events to the total target events gives the percentage of cytotoxicity.

2.12.5. Lymphocyte proliferation assay

Purified PBMCs were resuspended in EMEM medium with 10% fetal bovine serum and cultured in triplicate, at the concentration of 5×10^4 cells per well, in flat-bottomed 96-well plates (Nunc). Mitogen-stimulated cultures (Phytohaemagglutinin, PHA, at 10 μg/mL) and antigen-stimulated cultures (10 mM of LMWFs of carrageenans) were incubated for 4 h (J0 control) or 5 days (J5). Lymphocyte cell proliferation was assessed with the WST-1 proliferation assay (Roche). Cell proliferation after antigen exposure was determined by colorimetric measurement of WST-1 cleavage. In viable cells, the tetrazolium salt, WST-1, is converted by mitochondrial dehydrogenases to a water-soluble formazan dye further quantified by spectrophotometry. WST-1 cleavage was quantified at 450 nm with a microplate reader (MRX® II, Thermo Labsystems). For each PBMC suspension, the relative proliferation was determined through comparison of the mean optical density (OD) of the 3 technical replicates per exposure condition against to the mean OD of the appropriate controls.

2.13. Statistical analysis

All of the data were analyzed with the Statistica Software. Data were expressed as the mean ± S.D. Comparisons of the measurement data between multiple groups and those of individual means were tested with one-way ANOVA test and Duncan tests, respectively. Prior to analysis, the resistant values were arcsin-transformed for normalization. Values of $p < 0.05$ or $p < 0.01$ were considered as statistically significant.

3. Results

3.1. Structure of the native polysaccharide from S. chordalis

The yield of extraction of the dried powder of *S. chordalis* with hot water and removal of the floridean starch by treatment of the native polysaccharide with amylase was 14.6 ± 1 wt.%. Table 1 gives the composition of the extracted polysaccharide. Low pressure gel permeation chromatography (LP-GPC), on a Sepharose CL_4B column, of the polysaccharide issued from the dialysis at 80 °C showed that was homogeneous, and its average molecular weight was estimated as 913 kDa.

The FTIR spectrum of the polysaccharide from *S. chordalis* (not shown) proved to be similar to those available in the literature (Deslandes, 1988; Prado-Fernández, Rodríguez-Vázquez, Tojo, & Andrade, 2003). Indeed, it exhibited an intense absorption at 1260 cm^{-1} indicative of sulfate ester substitutions (Stancioff & Stanley, 1969); this finding is in agreement with the high sulfate content (33.54 ± 0.25 wt.%) found by turbidity. The spectrum also displayed the characteristic absorption pattern of ι-carrageenan with peaks at 930 cm^{-1} (3,6-anhydrogalactose residue), 805 cm^{-1} (axial sulfate esters at *O*-2 of the 3,6-anhydrogalactose residue) and 840 cm^{-1} (axial sulfate ester at *O*-4 of the 3-linked galactose residue). The small shoulder at 900 cm^{-1} suggested the presence of an unsulfated, 3-linked galactose residues bearing a pyruvate acetal substitution (Chiovitti, Bacic, Craik, Kraft, et al., 1998; Chiovitti et al., 1997; Chiovitti, Bacic, Kraft, Craik, & Liao, 1999).

Fig. 1 shows the ^{13}C NMR spectrum of the native polysaccharide, and Table 2 sums up the resonance assignments. Though the complexity of the spectrum reflected the polysaccharide heterogeneity, comparison of most of the major signals with literature data (Deslandes et al., 1985; Van de Velde, Knutsen, Usov, Rollema, & Cerezo, 2002) allowed us to assign them to the repeating carrabiose 2,4′-disulfate of ι-carrageenan. However, the difference of 2 ppm noticed in the shifts (δ) through comparison with literature data was attributed to variations in pH and calibration methods. The ^{13}C NMR spectrum also displayed signals assigned to 6′-*O*-methylated carrabiose 2,4′-disulfate (i.e. 6′-*O*-methylated ι-carrageenan) (Table 2) through comparison with the literature available about the highly methylated carrageenans from the genera *Rhabdonia* (Chiovitti et al., 1996) and *Claviclonium* (Chiovitti, Bacic, Craik, Kraft, & Liao, 2004). Concerning the 6′-*O*-methylated ι-carrageenan, the NMR assignments exhibited, for the C-4 and C-5 of the 3-linked residues, chemical shifts induced by the methyl substitution in α- and β on the C-6. Moreover, the signal at 61.26 ppm was identified as corresponding to the methylene group on the C-6 of the 3-linked galactopyranosyl-4-sulfate residues. In addition, from the literature (Chiovitti, Bacic, Craik, Kraft, et al., 1998; Chiovitti et al., 1997; Chiovitti et al., 1999) the small signals at 27.77 and 177.98 ppm were, respectively, assigned to the methyl and carboxyl carbons of the pyruvated galactopyranosyl residues. According to the study reported in Garegg, Lindberg, and Kvarnström (1979), the finding of a chemical shift at 27.77 ppm for the methyl signal is in favor of an R configuration for the acetal carbon of the pyruvate substituent. Besides, a set of minor resonances at 69.75, 69.19 and 67.87 ppm was assigned from Chiovitti et al. (1997), Chiovitti, Bacic, Craik, Kraft, et al. (1998), Chiovitti et al. (1999) to the C-4, C-5 and C-6 of the 3-linked, 4′,6′-pyruvated galactopyranosyl residue.

The GLC and GLC–MS data about the alditol acetates of the polysaccharide showed that galactose (determined as 1,2,3,4,5,6-hexa-*O*-acetyl-D-galactitol) and 3,6-anhydrogalactose (determined as 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol) were both the major monosaccharides (Table 1). In addition to these sugars, 6′-*O*-methyl-galactose was also identified from the MS spectrum of 1,2,3,4,5-penta-*O*-acetyl-6-*O*-methyl-D-galactitol in agreement

Table 1
Yield and composition of the native polysaccharide from *Solieria chordalis*.

Yield (% w/w)[a]	Ash (% w/w)[b]	Proteins (% w/w)[b]	Sugar (% w/w)[b]	AnGal (% w/w)[b]	Pr[c] (% w/w)[b]	MW[d] (kDa)	Sulfate (% w/w)[b]	Pyruvate (% w/w)[b]	Monosaccharides[e] (mol%)			
									Gal	AnGal	6-MeGal	Glu
14.6 ± 1	24.55 ± 3	7.10 ± 0.5	51.58 ± 1.3	40.03 ± 2.8	9.93 ± 1.1	913	33.54 ± 0.3	6.17 ± 0.4	52	37	11	–

[a] Based on the dry weight of the seaweed.
[b] Calculated on the carrageenan fraction.
[c] Precursor amount estimated after the alkali treatment of carrageenan.
[d] Molecular weight estimated by LP-GPC.
[e] Monosaccharides: Gal = galactose, AnGal = 3,6-anhydrogalactose, 6-MeGal = 6′-*O*-methylgalactose, Gluc = Glucose.

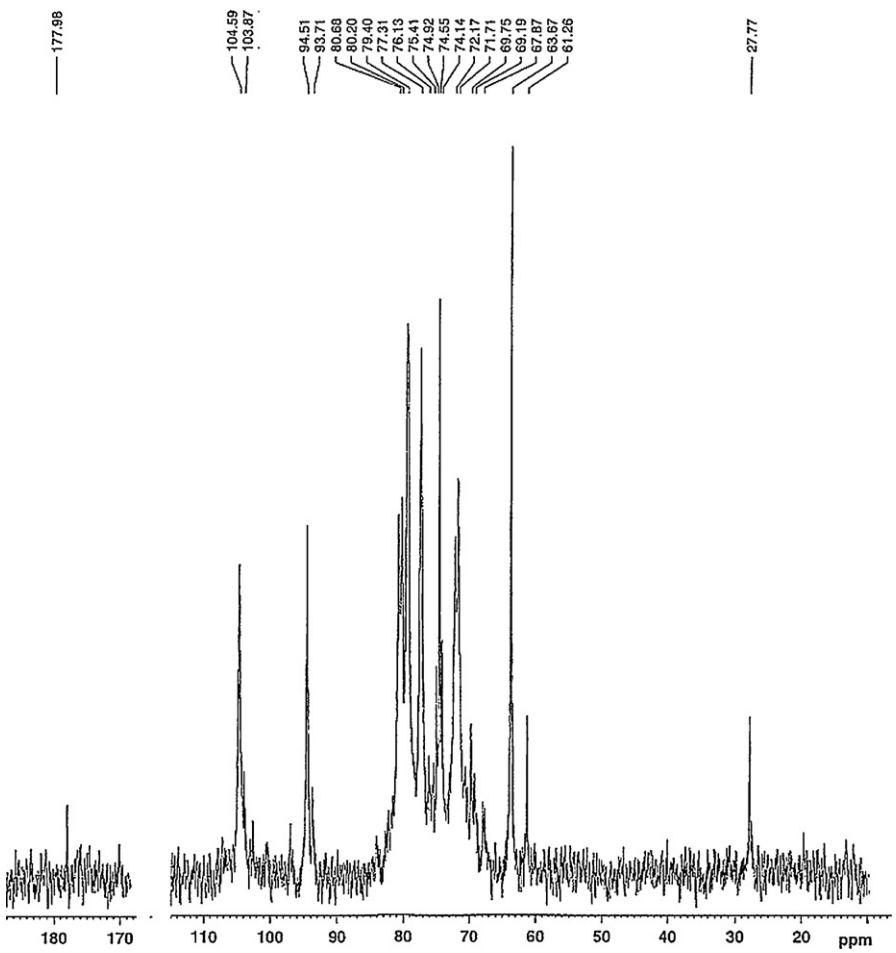

Fig. 1. ^{13}C NMR spectrum of the native polysaccharide from *Solieria chordalis*.

with Bellion, Brigand, Prome, Welti, and Bociek (1983) and found to be present in small amount in the polysaccharide. The composition analysis of the trimethylsilylated methyl glycoside derivatives issued from the methanolysis of the polysaccharide highlighted the presence of 4′,6′-*O*-(1-carboxyethylidene)-D-galactose (detected on the MS spectrum of 2,3-di-*O*-trimethylsilyl-4,6-*O*-(1-methoxycarbonyl-ethylidene)-D-galactoside in agreement with Cérantola, Marty, & Montrozier, 1996). A similar analysis performed on the depyruvated polysaccharide issued from a treatment with 2% AcOH at 100 °C for 2 h showed that the peak corresponding to the 4′,6′-*O*-(1-carboxyethylidene)-D-galactose was missing on the GC spectrum of the depyruvated product. The content in pyruvate in the polysaccharide was estimated at 6.17 ± 0.40 wt.% (Table 1). It is worth noting that these GC–MS data corroborated those obtained by NMR and FTIR analyses.

The linkage analysis performed on the native polysaccharide from *S. chordalis* cell wall after methylation with CH_3I ((SC-CH_3I) Table 3) led to the finding, for every sugar, of linkage between *O*-1 and *O*-5. This was attributed to the presence of pyranosyl residues glycosidically linked through *O*-1. The major components were, thus, identified as 3,4-linked-galactopyranosyl (40 mol%) and 2,4-linked anhydrogalactopyranosyl residues (26.5 mol%), issued from 3-linked galactopyranosyl 4-sulfate and 4-linked anhydrogalactopyranosyl 2-sulfate residues that are both characteristic of ι-carrageenan. Moreover, the Gal/AnGal ratios deduced from the composition analysis about sugars and linkages were not 1/1 (see

Table 2
Assignments of resonances[a] observed in the ^{13}C NMR spectum[b] of the native polysaccharide *from S. chordalis*.

Repeating units	Sugar	Carbon atom									
		C-1	C-2	C-3	C-4	C-5	C-6	*O*-Me	Pyruvate acetal		
									Methyl	Acetal	Carboxyl
G4S-DA2S	3-Linked	104.59	71.71	79.40	74.55	77.31	63.67				
	4-Linked	94.51	77.31	80.20	80.68	79.40	72.17				
G4S,6Me-DA2S	3-Linked	104.59	71.71	79.40	75.41	74.90	74.14	61.26			
	4-Linked	94.51	77.31	80.20	80.68	nd	72.17				
GP-DA2S	3-Linked	104.30	nd	nd	69.75	69.19	67.87		27.77	103.87	177.98
	4-Linked	93.71	77.31	80.20	80.68	79.40	72.17				

Repeating units: G4S-DA2S = carrabiose 2,4′-disulfate of ι-carrageenan, G4S6Me-DA2S = 6′-*O*-methylcarrabiose 2,4′-disulfate, GP-DA2S = 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate. nd: coincident resonances.

[a] Chemical shifts were expressed in ppm relative to TMS.

[b] Spectrum was recorded in D_2O at 85 °C.

Table 3
Linkage analysis of constituent sugars (mol%) of native polysaccharide (SC) and desulfated polysaccharide (SCDS) from *S. chordalis*, methylated with CH_3I or CD_3I.

Constituent monosaccharide[a]			
Deduced linkage[b]	SC-CH3I	SC-CD3I	SCDS-CH3I
AnGal*p*			
4-			17
2,4-	26.5	31	
Gal*p*			
None[c]	2	1	tr[d]
3-	4	4.5	21.5
4-	1.5	2	23.5
3,4-	40	31[e]	6.5
4,6-	1	1	13
2,3,4-	0.5	0.5	0
3,6-	1	2	5.5
3,4,6-	9.5	11	10.5
2,4,6-	6.5	3	0
x	3.5	4	0
2,3,4,6-	4	1	2.5
6-MeGal*p*			
3,4-		8[e]	

x: monosaccharide not determined.

[a] AnGal*p* = 3,6-anhydrogalactopyrannose, Gal*p* = galactopyrannose, 6-MeGal*p* = 6′-*O*-methylgalactopyrannose.

[b] 4-Linked AnGal*p* deduced from 1,4,5-tri-*O*-acetyl-2-*O*-methyl/deuterio-3,6-anhydrogalactitol; 2,4-linked AnGal*p* deduced from 1,2,4,5-tetra-*O*-acetyl-3,6-anhydrogalactitol; 3-linked Gal*p* deduced from 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl/deuterio-galactitol; 3,4-linked 6-*O*-MeGal*p* deduced from 1,3,4,5-O-acetyl-2-*O*-deuterio-6-*O*-methylgalactitol.

[c] None Gal*p* deduced from 1,5-di-*O*-acetyl-2,3,4,6-hexa-*O*-methyl/deuterio-galactitol.

[d] tr = trace (<0.5 mol%).

[e] Co-eluting derivatives were distinguished by permethylation with CD_3I and quantified by mass spectrometry from specific primary fragment ions.

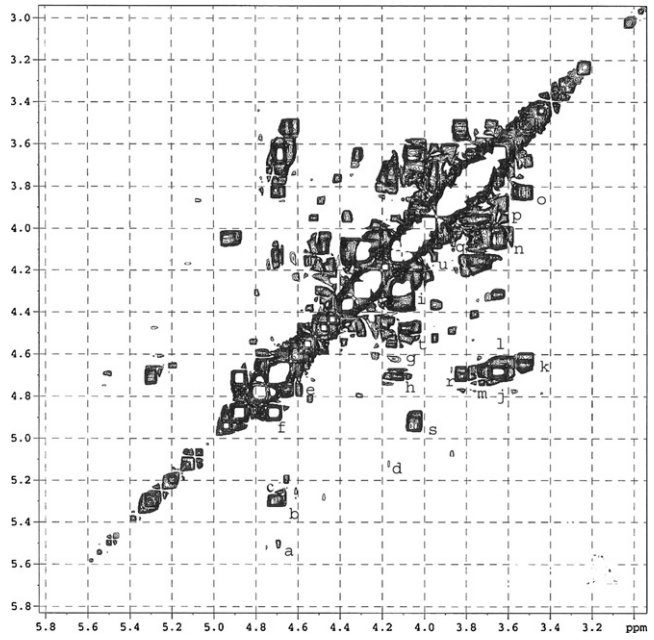

Fig. 3. NMR characterization of the HA4 fraction using two-dimensional spectroscopy. $^1H/^1H$ homonuclear correlation spectroscopy (COSY) spectrum: (a) AnGal*p*2S adj. Gal*p*4′,6′pyr H1/H2, (b) AnGal*p*2S adj. to Gal*p*4S H1/H2, (c) AnGal*p*2S adj. to Gal*p* H1/H2, (d) AnGal*p* H1/H2, (e) AnGal*p*2S adj. to Gal*p* H2/H3, (f) AnGal*p*2S adj. to Gal*p*4S H2/H3 et H3/H4, (g) AnGal*p* H2/H3, (h) AnGal*p*2S H5/H6a, (i) AnGal*p*2S H6a/H6b, (j) Gal*p*4S adj. to AnGal*p*2S H1/H2, (k) Gal*p*4S adj. to AnGal*p* H1/H2, (l) Gal*p* adj. to AnGal*p*2S H1/H2, (m) Gal*p*4′,6′pyr adj. to AnGal*p*2S H1/H2, (n) Gal*p*4S adj. to AnGal*p*2S H2/H3, (o) Gal*p*4S adj. to AnGal*p* H2/H3, (p) Gal*p* adj. to AnGal*p*2S H2/H3, (q) Gal*p*4′,6′pyr adj. to AnGal*p*2S H2/H3, (r) Gal*p*4S adj. to AnGal*p* H3/H4, (s) Gal*p*4S adj. to AnGal*p*2S H3/H4, (t) Gal*p*4′,6′pyr adj. to AnGal*p*2S H3/H4.

Tables 1 and 3) as expected for ideal galactans. This unexpected result likely comes from an incomplete recovery of AnGal over the reductive hydrolysis step; a similar observation was reported in the literature for AnGal2S of ι-carrageenan (Falshaw et al., 1996; Stevenson & Furneaux, 1991). However, in the native polysaccharide, the content of AnGal estimated by a colorimetric method was not 50%, but 40.03 ± 2.76 wt.% (Table 1). So, the polysaccharide may also contain some 4-linked Gal*p* 2,6-disulfate (precursor residues of 4-linked anhydrogalactopyranosyl-2-sulfate). This hypothesis is supported by the occurrence of 2,4,6-linked-galactopyranosyl

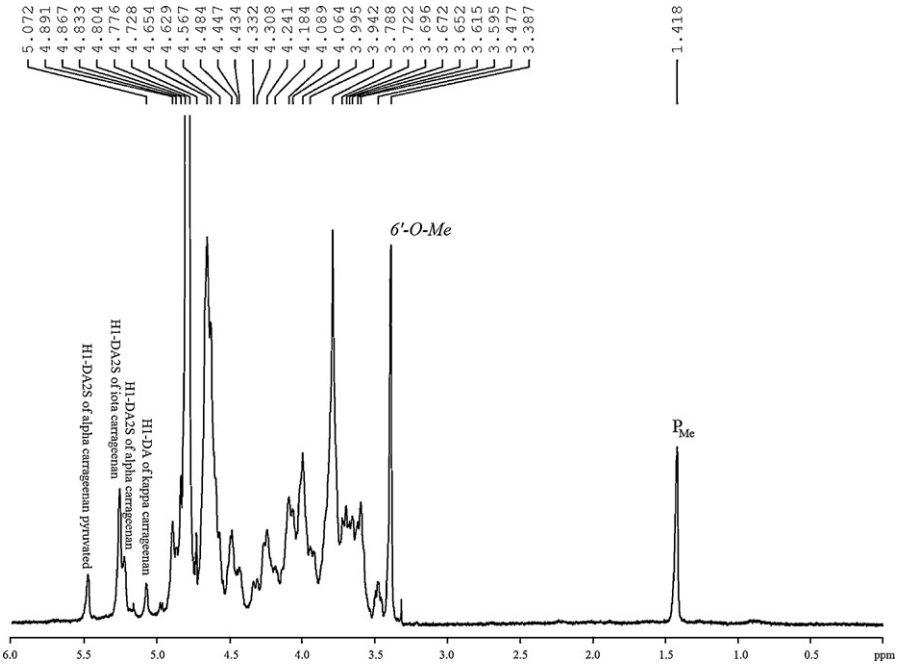

Fig. 2. ^1H NMR spectrum of the low molecular weight fraction (HA4) issued from the mild-acid hydrolysis of the polysaccharide of *S. chordalis* (about 4 kDa) (in D_2O, 500 MHz). DA2S: anhydroglactose2S, DA: anhydrogalactose, 6′-*O*-Me: CH_3 of the Gal*p*4S6Me, PMe: methyl carbon of pyruvate acetal group of Gal*p*4,6pyr

residues in the linkage analysis data (6.5 mol%, Table 3). Moreover, after the treatment with alkali, assessment by colorimetry of the amount of built AnGal led to 9.93 ± 1.09 wt.% (Table 1). The 3,4,6-linked-galactopyranosyl residues detected through the linkage analysis (9.5 mol%) could come from the change of pyruvate acetal-bearing 3-linked galactopyranosyl units into 4′,6′-*O*-(1-carboxyethylidene)-galactopyranosyl residues. GC–MS analysis also showed the existence of a heterogeneous mixture composed of substituted and linked residues, including 3-, 4-, 4,6-, 3,6-, 2,3,4-, 2,3,4,6-linked galactopyranosyl residues, in low amounts (<4 mol%) (Table 3).

A comparison of the derivatives obtained by methylation of the native polysaccharide with CD_3I showed a good correlation with the one using CH_3I (SC-CH_3I and SC-CD_3I, Table 3). The use of CD_3I allowed the separation of 3,4-linked galactopyranosyl residues from 3,4-linked 6′-*O*-methyl galactopyranosyl ones. The former were issued from the 3-linked galactopyranosyl-4-sulfate, whereas the latter came from the 3-linked 6′-*O*-methyl-galactopyrannosyl-4-sulfate. The finding of 8 mol% of 3-linked-Gal6Me4S residues in this polysaccharide is in agreement with the results of the composition- and ^{13}C NMR-analyses.

The linkage analysis of the desulfated polysaccharide allowed us to determine the positions of sulfates (Table 3, SCDS). The apparition of 4-linked anhydrogalactopyranosyl residues (17 mol%) in the desulfated product suggest the presence of a sulfate group on the C2 of the 4-linked anhydrogalactopyranosyl residues in the native polysaccharide. According to the literature (Stevenson & Furneaux, 1991), the high amount of 4-linked galactopyranosyl residues found after desulfation (23.5 mol%) could both be explained by the removal of the sulfate groups of 4-linked,galactopyranosyl-2,6-disulfate residues and also the cleavage of the 3,6-anhydro bridge and the removal of the sulfate of the 4-linked,anhydrogalactopyranosyl-2-sulfate residues. The finding of a higher content in 3-linked galactopyranosyl residues in the desulfated product compared to the native one (21.5 mol% compared to 4) together with the concomitant decrease of 3,4-linked galactopyranosyl residues are in favor of the presence of 3-linked galactopyranosylglycerol residues with sulfate groups in *O*-4. The low amount of 3,4-linked galactopyranosyl residues after desulfation (6.5 mol%) may result from an incomplete reaction (4.19 ± 0.30 wt.% of remaining sulfate determined by turbidity). As the content in 3,4,6-linked galactopyranosyl residues was alike before and after the desulfation steps, this residue was attributed to the 3-linked 4′,6′-*O*-(1-carboxyethylidene)-galactopyranosyl residues with no sulfate group. Furthermore, the lack of 4-linked anhydrogalactopyranosyl in the carrageenan before the desulfation steps confirmed the presence of a pyruvated α-carrageenan (i.e. 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate) already described in carrageenans from the indopacific species of *Solieria* (Chiovitti et al., 1999).

3.2. Characterization of low molecular weight fractions from carrageenans

Further to the mild-acid hydrolysis and free-radical depolymerizations, the HA and F hydrolysates were obtained in yield of 25 and 35 wt.%, respectively. Table 4 gives the average molecular weight of each of the six LMWFs issued from HA or F hydrolysates after fractionation and desalting (respectively denoted from HA1 to HA6 and F1–F6) and shows that all of them lie within >35–2 kDa.

The chemical analyses of the LMWFs (Table 4) showed quite similar contents in sulfate ester, pyruvate acetal and monosaccharides in the F fractions (except for F1). One should note that this was not the case for the HA fractions: indeed, the fractions with the lowest molecular weights had the highest contents in substituents (sulfate ester, pyruvate acetal). Though the F fractions had different weights, their chemical compositions were very similar, conversely to those of HA fractions.

The ^1H NMR spectra recorded from each LMWF were alike and showed similar ^1H signals of variable intensities. This is exemplified by Fig. 2 about HA4. This fraction was subjected to 2D NMR analyses (COSY, Fig. 3), HMQC and HMBC (not shown) for assignment of the ^1H NMR signals. Assignment of the visible spin systems was made from the literature (Chiovitti, Bacic, Craik, Munro, et al., 1998; Falshaw et al., 1996). Briefly, the anomeric resonances between 5.0 and 5.6 ppm were attributed to the 4-linked residues. The anomeric region of the HMQC spectrum connectivities exhibited four ^{13}C–^1H systems located and attributed as follows: (i) 5.3/94.5 ppm to the C-1/H-1 of AnGal*p*2S in ι-carrageenan, (ii) 5.5/93.5 ppm to the C-1/H-1 of AnGal*p*2S in 4′,6′-*O*-(1-carrboxyethylidene) carrabiose 2-sulfate (i.e. pyruvated α-carrageenan), (iii) 5.12/97 ppm to the C-1/H-1 of AnGal*p* linked to Gal*p*4S (κ-carrageenan), and (iv) the small shoulder on the signal at 5.25 ppm to the H1 of AnGal*p*2S linked to Gal*p* (α-carrageenan). Three-linked galactopyranosyl residues were also observed on the NMR spectra and assigned as follows: (i) the Gal*p*4′,6′pyr displayed C-1/H-1 at 4.70/105 and (ii) the Gal*p*4S in κ-carrageenan at 4.65/105 ppm. About the Gal*p*4S in ι-carrageenan and Gal*p* in α-carrageenan, the C-1/H1 signals looked alike and were located at 4.68/105 ppm. The 2D NMR analyses allowed the complete assignment of ^1H for the anhydrogalactopyranosyl residues and the assignments of H-1, H-2, H-3 and H-4 for the galactopyranosyl residues present in this fraction (Fig. 3). The coupling constant between H-4 and H-5 in the galactopyranose unit was too small to give correlations. So, the NMR analyses carried out on the HA4 fraction showed the presence of the carrabiose 2,4′-disulfate of ι-carrageenan, the 4′,6′-*O*-(1-carboxyethylidene)carrabiose 2-sulfate of pyruvated α-carrageenan and the 6-methylated carrabiose 2,4′-disulfate of 6′-*O*-methylated ι-carrageenan. Moreover, κ-carrageenan and α-carrageenan proved also to be present in low amounts in the LMWFs; they likely resulted from the loss of sulfate groups on ι-carrageenan over the depolymerization process since these residues were missing on both the ^{13}C NMR spectrum of the native polysaccharide and linkage analysis result.

3.3. Bioactivities of the low molecular weight fractions of carrageenans

In this study, as HA1 proved to have a heterogeneous molecular weight (MW estimated by LP-GPC to lie within 120 and 35 kDa) and, thus, a high dispersion coefficient, its biological activities were not assayed conversely to the other LMWFs of carrageenan.

3.3.1. Anti-tumoral assays

Fig. 4A illustrates the results of the assays about the cytotoxic activities by the LMWFs from carrageenans against the Daudi, Jurkat and K562 tumoral cell lines in the case where the positive control is actinomycine D. None of them showed significant cytotoxic activities against Daudi, Jurkat and k562 cell lines ($p < 0.01$), except for HA3 on K562 cells.

3.3.2. Phagocytosis assay

As illustrated in Fig. 4B, the LMWFs under test showed great immunostimulating properties by significantly enhancing phagocytosis by neutrophils. Indeed, the rate of ingestion of fluorescent bacteria by untreated neutrophils was 52% against 78–94% after pre-treatment of the cells with an LMWF.

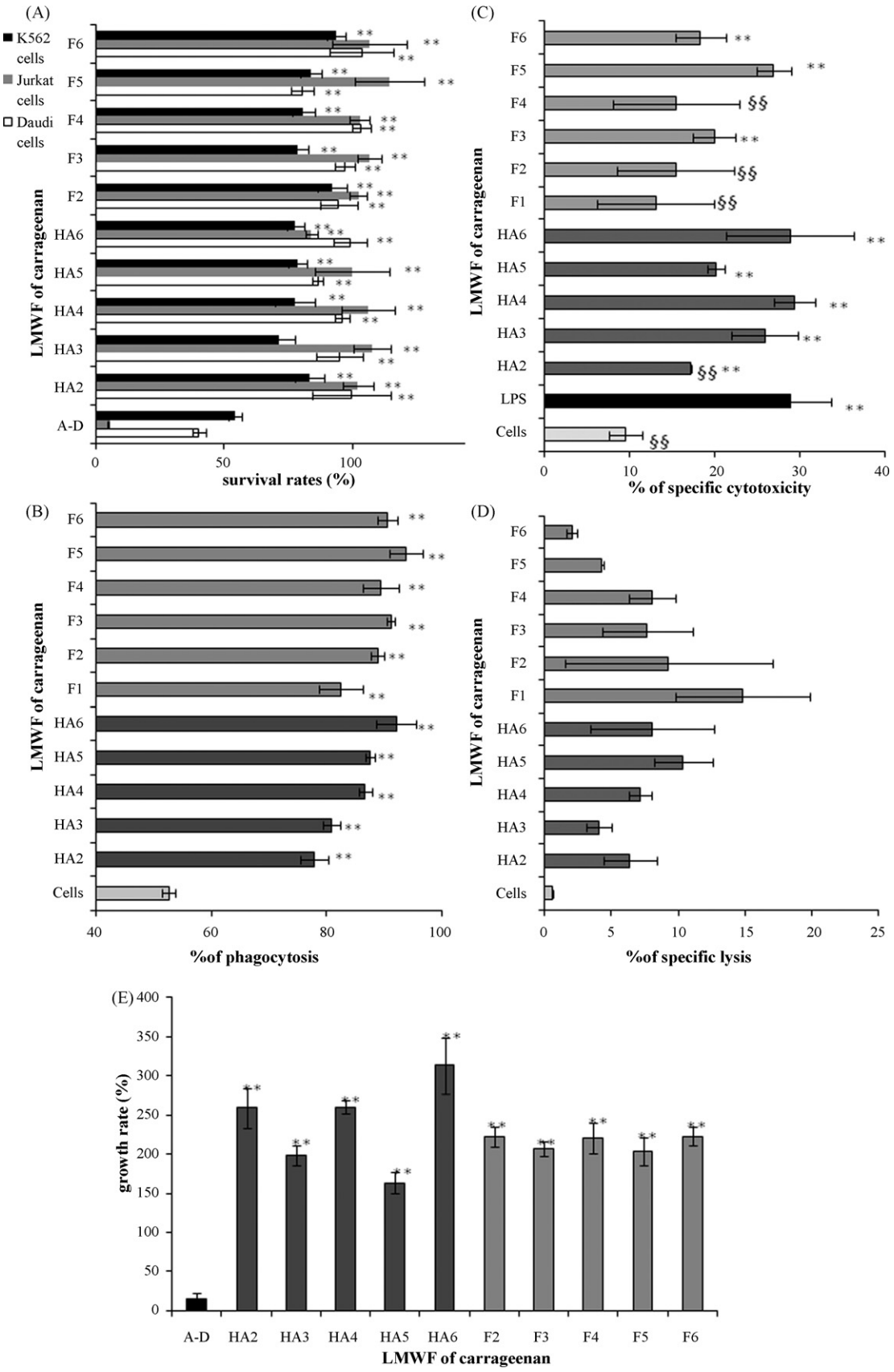

Fig. 4. Biological activities of the low molecular weight fractions (LMWF) of carrageenans from *S. chordalis*. (A) Effects of the LMWF on the fluorescence-activated sorting analysis of annexin-V-FITC/PI for quantification of apoptosis in Daudi, Jurkat and K562 tumor cells. Mean values and standard deviations for three similar experiments. (A–D) Actinomycine D; **significant difference from positive control ($p < 0.01$). (B) Effects of the LMWF on the phagocytosis of *Escherichia coli* in neutrophil-segmented cells (NSCs). Results are the mean values of incorporated *E. coli* in NSC from three healthy donors after pre-incubation with low molecular weight fractions of carrageenans from *S. chordalis* compared to the untreated cells. Mean values and standard deviations for three similar experiments. **Significant difference from positive control ($p < 0.01$). (C)

Table 4
Yield, molecular weight and composition of the low molecular weight fractions issued from the free-radical depolymerization (from F1 to F6) and from the acid hydrolysis (from HA1 to HA6) of the polysaccharide from *S. chordalis*.

	Yield (mg)	MW[a] (Da)	Sulfate (% w/w)[b]	Pyruvate (% w/w)[b]	Monosaccharides[c] (mol%)		
					Gal	AnGal	6-MeGal
F1	10	18,744	9.44 ± 1.05	nd	64.5	29	6.5
F2	15	10,060	26.04 ± 2.21	4.15 ± 0.77	53.5	38.5	8
F3	35	5721	27.66 ± 3.44	5.69 ± 0.36	51.5	39	9.5
F4	48	3650	26.06 ± 2.31	4.35 ± 0.83	50	40.5	9.5
F5	42	2709	27.36 ± 1.13	4.33 ± 1.27	49	43	8
F6	25	2544	27.21 ± 1.95	3.05 ± 0.4	50	46.5	3.5
HA1	28	>34,826	17.45 ± 1.56	2.89 ± 0.63	75	18.5	6.5
HA2	20	12,393	20.84 ± 1.44	4.63 ± 1.30	49	39	12
HA3	20	6358	22.95 ± 2.17	6.09 ± 1.45	40.5	46	13.5
HA4	24	3980	25.11 ± 2.54	6.10 ± 1.08	41.5	45	13.5
HA5	22	3056	30.30 ± 0.42	3.04 ± 1.19	38	53	9
HA6	11	2320	16.05 ± 1.11	nd	nd	nd	nd

nd: not determined.
[a] Molecular weight estimated by HPGPC.
[b] Calculated on the oligosaccharidic fraction.
[c] Monosaccharides: Gal = galactose, AnGal = 3,6-anhydrogalactose, 6-MeGal = 6′-*O*-methylgalactose.

3.3.3. NK cell direct cytotoxicity

As illustrated in Fig. 4C, the flow cytometry assay, based on the specific ability of NK cells to kill CFSE-labeled K562 target cells, showed an enhancement of the direct cytotoxicity by natural killers (NK) cells after exposure of PBMC to LMWFs from carrageenans. It shows that PBMC pre-treatment with F1, F2, F4 and HA2 (the fractions with the highest molecular weights) led to a significant ($p < 0.05$) decrease of NK cells toxicity against tumor cells K562 in comparison with the positive control. On the other hand, pre-treatment with F3, F5, F6, HA2, HA3, HA4, HA5 or HA6 caused a significant ($p < 0.05$) enhancement of NK cells toxicity against the same tumor cells by comparison with untreated cells.

3.3.4. ADCC assay

Fig. 4D illustrates the effect of the LMWFs from carrageenans on the antibody-dependent natural killer (NK) cells activity (ADCC). The percentages of specific lysis of Daudi cells by the NK cells was between 2.06 and 14.82% for the F fractions and between 4.10 and 10.35% for the HA fractions against 0.56% for the untreated cells by LMWFs. These results show that the ADCC specific activity is enhanced by treatment of PBMC from patient's blood with LMWFs. The ADCC mechanism involves the recognition and binding of antibody (=Rituximab)-coated target cells via the Fc receptor (FcγR) on the effector cells. However, it exists three classes of FcγR (FcγRI, FcγRII, FcγRIII) that are encoded by height genes in humans. Some of these genes display a functional allelic polymorphism generating allotypes with different receptor properties. It is the case for the receptor FcγRIIIa located on the surface of NK cells. These receptors are issued from a gene dimorphism in FcGR3A which encodes FcγRIIIa with either a phenylalanine (F) or a valine (V) at amino acid position 158. Several teams have demonstrated that NK cells from individuals homozygous for the FcγR3A 158V have a higher affinity for complexed antibody (Rituximab-Daudi cells) and are more cytotoxic toward antibody-sensitized targets (Cartron et al., 2002; Wu et al., 1997). So, the differences in antibody binding are a consequence of the linked FcγRIIIa 158 V:F polymorphism (Koene et al., 1997). The high values of deviation standard recorded in this study can be explained by the polymorphism of the FcγR receptors present on the surface of NK cells.

3.3.5. Lymphocyte proliferation

Fig. 4E shows the results of the assessment of lymphocyte proliferations after exposure of PBMCs to LMWF of carrageenans and comparison to actinomycine D as a positive control. It highlights the significant enhancement ($p < 0.01$) of the cellular growth rate of PBMCs by any of the LMWFs.

4. Discussion

The rhodophycean seaweed, *S. chordalis,* is present in large quantities in the subtidal zone of the Brittany shoreline. Because of its great production of ι-carrageenan (Deslandes, 1988; Deslandes et al., 1985), this seaweed constitutes an interesting material for investigations about the metabolic pathway of carrageenan biosynthesis (Fournet, Zinoun, Deslandes, Diouris, & Floc'h, 1999; Goulard, Diouris, Quere, Deslandes, & Floc'h, 2001) and the chemical structure of this polysaccharide. The analytical techniques used in these studies led to the elucidation of the dominant structure of this polysaccharide, but gave no information about minor structures. Over the last decades, the coupling of sophisticated analytical techniques (Cases, Cerezo, & Stortz, 1995; Falshaw & Furneaux, 1995; Jol et al., 1999) with powerful detection tools (especially in NMR Spectroscopy) in order to study red algal galactans has enabled the characterization of novel substitution patterns (Errea & Matulewicz, 2003; Falshaw et al., 1996), even in the case of substituents at trace level within the polysaccharide. Concerning *S. chordalis,* the use of these sophisticated techniques allowed us to demonstrate that its cell wall galactan is a polysaccharide composed predominantly of ι-carrageenan with some 6′-*O*-methylated ι-carrageenan, 4′,6′-pyruvated α-carrageenan and precursors of ι-carrageenans (i.e. ν-carrageenans). In our opinion, the finding of pyruvate acetal- and methyl ether-substitutions in carrabiose repeating units in the carrageenan of *S. chordalis* is not surprising since these compounds seem to be common in species belonging to the genus *Solieria.* Indeed, the polysaccharide of the indopacific alga, *S. robusta,* consists mainly of carrabiose 2,4′-disulfate (the repeating unit of ι-carrageenan) with some 4′,6′-pyruvated carrabiose 2-sulfate (Chiovitti et al., 1999). According to Deslandes et al. (1985) and Deslandes (1988), 2–5% of methylation were iden-

Effects of LMWF on Natural Killers cells cytotoxicity against K562 tumor cells. Mean values and standard deviations for three similar experiments. ** Significant difference from the untreated cells ($p < 0.05$); §§ significant difference from positive control ($p < 0.05$). (D) Effects of LMWF on the antibody-dependent cellular cytotoxicity (ADCC). Results are expressed as percentages of specific lysis of Daudi cells by the natural killer cells. Mean values and standard deviations for seven similar experiments. (E) Effects of LMWF on the peripherical blood mononuclear cells proliferation. Mean values and standard deviations for three similar experiments. (A–D) Actinomycine D; ** significant difference from the untreated PBMC ($p < 0.01$).

tified in the polysaccharide of the Atlantic species of *S. chordalis*. Pyruvate acetal- and methyl ether-substitutions are traditionally associated with agars (Craigie, 1990; Young, Duckworth, & Yaphe, 1971) whereas they are rare in carrageenans (Chopin, Kerin, & Mazerolle, 1999; DiNinno, McCandless, & Bell, 1979; Hirase & Watanabe, 1972; Whyte, Foreman, & DeWreede, 1984; Zablackis & Perez, 1990). However, according to the studies of the Australian species of Solieriaceae (family described in Gabrielson & Hommersand, 1982) by Chiovitti et al. (1996), Chiovitti, Bacic, Craik, Munro, et al. (1998), Chiovitti, Kraft, et al. (1998), and Chiovitti, Kraft, Bacic, Craik, and Liao (2001) some carrageenophytes belonging to the genera, *Rhabdonia, Erythroclonium*, *Austroclonium* and *Areschougia* exhibit highly methylated carrageenans (>20 mol%) and sulfate patterns of ι- and α-carrageenans. The relationships between these genera are closer than those with any other genus of Solieriaceae family, which are acknowledged as being unable to synthesize methylated carrageenans, or only at trace levels (<5 mol%). This peculiarity drove these authors to recommend, in Chiovitti, Kraft, et al. (1998), the transfer of these genera from the Solieriaceae to the family Areschougiaceae issued from the re-establishment of Rhabdoniaceae (by Kylin). The chemotaxonomic groupings based on carrageenan chemistry (especially the substitution patterns) in Solieriaceae are in line with those determined from the r*bc*L sequence analysis available in the literature (Fredericq, Wilson, & Hommers, 1999), but not with the hypotheses about algal taxonomy made from classical morphology analyses (Gabrielson & Hommersand, 1982). The screening of cell wall galactans in the Australian species of Solieriaceae also revealed the presence of pyruvated carrageenan in low and high proportions (8–10 and 50 mol%, respectively) in some genera (Chiovitti et al., 1997; Chiovitti, Bacic, Craik, Munro, et al., 1998; Chiovitti et al., 1999). However, it is worth noticing that the presence of these two substituents (methyl ether and pyruvate acetal) on a same polysaccharide backbone is rather unfrequent. To our knowledge, the present study is the first one to provide evidence of the existence of this complex substitution pattern in the carrageenan of a species belonging to the genus *Solieria*.

The protection against disease exerted by the immune system within an organism relies on the pattern recognition by its constitutive and highly complex network of cells and molecules. Indeed, the function of immune cells (macrophage, lymphocytes, natural killers, . . .) and molecules (complement and antibody) is to patrol the body and destroy, or inactivate, any foreign invaders. These molecules and immune cells express their activities through several mechanisms such as phagocytosis, the ADCC, the direct NK cytotoxicity that constitute the innate immunity, the primary defense line against tumor cells and host invasion. In this study, the immunological assays showed an enhancement of the innate immunity further to an *in vitro* exposure of immune cells to high concentrations of LMWFs from *S. chordalis* carrageenan through NK cell- and neutrophil-dependent mechanism and antibody-dependent cell cytotoxicity. Moreover, no marked direct toxic effects on tumoral cells lines were noticed. The innate immunity sounds the alarm about the occurrence of infection and initiates the specific adaptive immunity. Indeed, dendritic cells (i.e. subset of macrophages) can function as antigen-presenting cells and interact with T lymphocytes to modulate the adaptive immune response. These activated cells undergo a maturation process and express the costimulatory molecules (i.e. protein antigens in the context of class-I and class-II of the major histocompatibility complex (MHC)) needed for lymphocyte activation. The results of the present study are in line with these proven facts since the LMWFs from *S. chordalis* carrageenans exerted a mitogenic activity through a stimulation of the PBMC caused, maybe, by a polyclonal proliferation of T- and B-lymphocytes. The stimulation of macrophages, NK cells, T- and B-lymphocytes is mediated by the binding of polysaccharides to their corresponding receptors (including the family of toll-like receptors (TLRs)), which triggers cell activation by mitogen-activated protein kinases (MAPKs) (Han et al., 2003). So, the tumor-inhibition by LMWFs likely relies on an enhancement of activity by the immune system instead of cytokines. These molecules are, thus, classified as immunomodulators or biologic response modifiers and their use can constitute an alternative to classical treatments through enhancement of the host defense responses (Tzianabos, 2000). The conclusions of this study are in agreement with the immunological performances by LMWFs from other structures of carrageenan such as κ- and λ-types (Haijin et al., 2003; Hu et al., 2006; Yuan et al., 2006; Zhou et al., 2005; Zhou et al., 2006). However, one should note that these studies were about the immunological effects of a fixed molecular weight carrageenan fraction inoculated in mice with transplanted-tumors (S180 and H22). So, it is worth noting the significant impact of the molecular weight of degraded carrageenans on the immunological responses. On the other hand, the origin of immunological activity would be also in the basic structure of carrageenans as reported in Yuan et al. (2006). The basic chemical structure, or the molecular weight, of the oligosaccharide or, even, both could be at the origin of the immunomodulatory effects. It is worth recalling that the low molecular weight fractions (LMWFs) of carrageenans of *S. chordalis* were obtained by free-radical depolymerization and mild-acid hydrolysis. The free OH radical led to a non selective degradation of the polysaccharide, contrary to the acidic hydrolysis, more directed towards the less stable 3,6-anhydrogalactosyl bonds (Myslabodski, Stancioff, & Heckert, 1996). The free-radical depolymerization generated products with identical chemical structures (sulfate, methyl and pyruvate substitutions), but different molecular weights. On the other hand, the products issued from acid hydrolysis exhibited heterogeneous chemical structures together with different molecular weights. By showing that the highest activities were always produced by the lowest molecular weight fractions, our immunological assays carried out in this study suggest an implication of the molecular weight in the direct cytoxicity by NK cells. Furthermore, they also indicate an impact by the molecular weight and structure on the ADCC mechanism. In conclusion, according to the adhered-immunity cells under study, these investigations provided evidence of a synergistic, or not, impact by the molecular weight and the structure of oligo-carrageenans on the immune response. Further studies are needed to confirm and gain more insight into this impact.

Acknowledgements

The authors would like to thank M.P. Friocourt for English reviewing and V. Stiger-Pouvreau for advices in statistical analyses. This work was supported by a fellowship from the French Ministry of Education and Research to Dr Bondu. This work was also supported by the FEDER, Région Bretagne, Conseil général du Finistère, Brest Métropole Océane and Bretagne Valorisation.

References

Alves de Sousa, A. P., Torres, M. R., Pessoa, C., Odorico de Moraes, M., Rocha Filho, F. D., Negreiros, A. P., et al. (2007). *In vivo* growth-inhibition of Sarcoma 180 tumor by alginates from brown seaweed *Sargassum vulgare*. *Carbohydrate Polymers*, *69*, 7–13.

Bellion, C., Brigand, G., Prome, J. C., Welti, D., & Bociek, S. (1983). Identification et caractérisation des précurseurs biologiques des carraghénanes par spectroscopie RMN-^{13}C. *Carbohydrate Research*, *119*, 31–48.

Ben, J., & Quah, C. (2007). Monitoring lymphocyte proliferation *in vitro* and *in vivo* with the intracellular fluorescent dye carboxyfluorescein diacetate succinimidyl ester. *Nature Protocols*, *2*, 2049–2056.

Carlucci, M. J., Pujol, C. A., Cianca, M., Noseda, M. D., Matulewicz, M. C., Damonte, E. B., et al. (1997). Antiherpetic and anticoagulant properties of carrageenans from the red seaweed *Gigartina skottsbergii* and their cyclized derivatives: Correla-

tion between structure and biological activity. *International Journal of Biological Macromolecules*, *20*, 97–105.
Cartron, G., Dacheux, L., Salles, G., Solal-Celigny, P., Bardos, P., Colombat, P., et al. (2002). Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcγRIIIa gene. *Blood*, *99*, 754–758.
Cases, M. R., Cerezo, A. S., & Stortz, C. A. (1995). Separation and quantitation of enantiomeric galactoses and their mono-*O*-methylethers as their diastereomeric acetylated 1-deoxy-1-(2-hydroxypropylamino)alditols. *Carbohydrate Research*, *269*, 333–341.
Cérantola, S., Marty, N., & Montrozier, H. (1996). Structural studies of the acidic exopolysaccharide produced by a mucoid strain of *Burkholderia cepacia*, isolated from cystic fibrosis. *Carbohydrate Research*, *285*, 59–67.
Chen, D., Wu, X. Z., & Wen, Z. Y. (2008). Sulfated polysaccharides and immune response: Promoter or inhibitor? *Panminerva Medica*, *50*, 177–183.
Chiovitti, A., Bacic, A., Craik, D. J., Kraft, G. T., & Liao, M.-L. (2004). A nearly idealized 6′-*O*-methylated ι-carrageenan from the Australian red alga *Claviclonium ovatum* (Acrotylaceae, Gigartinales). *Carbohydrate Research*, *339*, 1459–1466.
Chiovitti, A., Bacic, A., Craik, D. J., Kraft, G. T., Liao, M.-L., Falshaw, R., et al. (1998). A pyruvated carrageenan from Australian specimens of the red alga *Sarconema filiforme*. *Carbohydrate Research*, *310*, 77–83.
Chiovitti, A., Bacic, A., Craik, D. J., Munro, S. L. A., Kraft, G. T., & Liao, M.-L. (1997). Cell-wall polysaccharides from Australian red algae of the family Solieriaceae (Gigartinales, Rhodophyta): Novel, highly pyruvated carrageenans from the genus *Callophycus*. *Carbohydrate Research*, *299*, 229–243.
Chiovitti, A., Bacic, A., Craik, D. J., Munro, S. L. A., Kraft, G. T., & Liao, M.-L. (1998). Carrageenans with complex substitution patterns from red algae of the genus *Erythroclonium*. *Carbohydrate Research*, *305*, 243–252.
Chiovitti, A., Bacic, A., Kraft, G. T., Craik, D. J., & Liao, M.-L. (1999). Pyruvated carragenans from *Solieria robusta* and its adelphoparasite *Tikvahiella candida*. *Hydrobiologia*, *398–399*, 401–409.
Chiovitti, A., Kraft, G. T., Bacic, A., Craik, D. J., & Liao, M.-L. (2001). Chemistry, properties, and phylogenetic implications of the methylated carrageenans from red algae of the genus *Areschougia* (Areschougiaceae, Gigartinales, Rhodophyta). *Journal of Phycology*, *37*, 1127–1137.
Chiovitti, A., Kraft, G. T., Bacic, A., Craik, D. J., Munro, S. L. A., & Liao, M.-L. (1998). Carrageenans from Australian representatives of the family Cystocloniaceae (Gigartinales, Rhodophyta), with description of *Calliblepharis celatospora nov*., and transfer Austroclonium to the family Areschougiaceae. *Journal of Phycology*, *34*, 515–535.
Chiovitti, A., Liao, M.-L., Kraft, G. T., Munro, S. L. A., Craik, D. J., & Bacic, A. (1996). Cell wall polysaccharides from Australian red algae of the Solieriaceae (Gigartinales, Rhodophyta): Highly methylated carrageenans from the genus *Rhabdonia*. *Botanica Marina*, *39*, 47–59.
Chopin, T., Kerin, B. F., & Mazerolle, R. (1999). Gigartinales symposium: Phycocolloid chemistry as a taxonomic indicator of phylogeny in the Gigartinales, Rhodophyceae: A review and current developments using Fourier transform infrared diffuse reflectance spectroscopy. *Phycological Research*, *47*, 167–188.
Ciucanu, I., & Kerek, F. A. (1984). *S*imple and rapid method for the permethylation of carbohydrates. *Carbohydrate Research*, *131*, 209–217.
Craigie, J. S. (1990). Cell walls. In K. M. Cole, & R. G. Sheath (Eds.), *Biology of the red algae* (pp. 221–257). Cambridge: Cambridge University Press.
Deslandes, E. (1988). *Etudes biochimiques des polysaccharides extraits de quelques algues carraghénophytes des côtes de Bretagne*. Doctoral Thesis. University of Western Brittany, Brest, France, p. 280.
Deslandes, E., Floc'h, J. Y., Bodeau-Bellion, C., Brault, D., & Braud, J. P. (1985). Evidence of ι-carrageenan in *Solieria chordalis* (Solieriaceae) and *Calliblepharis jubata*, *Calliblepharis ciliata*, *Cystoclonium purpureum* (Rhodophyllidaceae). *Botanica Marina*, *28*, 317–318.
DiNinno, V. L., McCandless, E. L., & Bell, R. A. (1979). Pyruvic acid derivative of carrageenan from a marine red alga (*Petrocelis* species). *Carbohydrate Research*, *71*, 1–4.
Doty, M. S. (1988). Prodromus ad systematica Eucheumatoideorum: A tribe of commercial seaweeds related to *Eucheuma* (Solieriaceae, Gigartinales). In I. A. Abbott (Ed.), *Taxonomy of economic seaweeds with reference to some Pacific and Caribbean species* (pp. 159–207). La Jolla: California SeaGrantCollege Program, University of California.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry*, *28*, 350–356.
Errea, M. I., & Matulewicz, M. C. (2003). Unusual structures in the polysaccharides from the red seaweed *Pterocladiella capillacea* (Gelidiaceae, Gelidiales). *Carbohydrate Research*, *338*, 943–953.
Falshaw, R., & Furneaux, R. H. (1994). Carrageenan from the tetrasporic stage of *Gigartina decipiens* (Gigartinaceae, Rhodophyta). *Carbohydrate Research*, *252*, 171–182.
Falshaw, R., & Furneaux, R. H. (1995). The structural analysis of disaccharides from red algal galactans by methylation and reductive partial-hydrolysis. *Carbohydrate Research*, *269*, 183–189.
Falshaw, R., & Furneaux, R. H. (1998). Structural analysis of carrageenans from tetrasporic stages of the red algae, *Gigartina lanceata* and *Gigartina chapmanii* (Gigartinaceae, Rhodophyta). *Carbohydrate Research*, *307*, 325–331.
Falshaw, R., Furneaux, R. H., Wong, H., Liao, M.-L., Bacic, A., & Chandrkrachang, S. (1996). Structural analysis of carrageenans from Burmese and Thai samples of *Catenella nipae* Zanardini. *Carbohydrate Research*, *285*, 81–98.
Fournet, I., Zinoun, M., Deslandes, E., Diouris, M., & Floc'h, J. Y. (1999). Floridean starch and carrageenan contents as responses of the red alga *Solieria chordalis* to culture conditions. *European Journal of Phycology*, *34*, 125–130.
Fredericq, S., Wilson, D., & Hommers, M. H. (1999). Observations on the phylogenetic systematics and biogeography of the Solieriaceae (Gigartinales, Rhodophyta) inferred from rbcL sequences and morphological evidence. *Hydrobiologia*, *398–399*, 25–38.
Gábor, M. (2003). Models of acute inflammation in the ear. *Methods in Molecular Biology*, *225*, 129–137.
Gabrielson, P. W., & Hommersand, M. H. (1982). The morphology of *Agardhiella subulata* representing the Agardhielleae, a new tribe in the Solieriaceae (Gigartinales, Rhodophyta). *Journal of Phycology*, *18*, 46–58.
Garegg, P. J., Lindberg, B., & Kvarnström, I. (1979). Preparation and NMR studies of pyruvic acid and related acetals of pyranosides: Configuration at the acetal carbon atoms. *Carbohydrate Research*, *77*, 71–78.
Gonzales, M. E., Alarcon, B., & Carrasco, L. (1987). Polysaccharides as antiviral agents: Antiviral activity of carrageenan. *Antimicrobial Agents and Chemotherapy*, *31*, 1388–1393.
Gonzales, M. E., Crance, J. M., Van Cuyck-Gandre, H., Renaudet, J., & Deloince, R. (1991). Antiviral activity of carrageenan on hepatite A virus replication in cell culture. *Research in Virology*, *142*, 261–270.
Goulard, F., Diouris, M., Quere, E., Deslandes, E., & Floc'h, J. Y. (2001). Salinity effects on NDP-sugars, floridoside, starch, and carrageenan yield, and UDP-glucose-pyrophosphorylase and -epimerase activities of cultivated *Solieria chordalis*. *Journal of Plant Physiology*, *158*, 1387–1394.
Haijin, M., Xiaolu, J., & Huashi, G. A. (2003). κ-Carrageenan derived oligosaccharide prepared by enzymatic degradation containing antitumor activity. *Journal of Applied Phycology*, *15*, 297–303.
Haimin, C., Yan, X., Lin, J., Wang, F., & Xu, W. (2007). Depolymerized products of lambda carrageenan as a potent angiogenesis inhibitor. *Journal of Agricultural and Food Chemistry*, *55*, 6910–6917.
Han, S. B., Yoon, Y. D., Ahn, H. J., Lee, H. S., Lee, C. W., Yoon, W. K., et al. (2003). Toll-like receptor-mediated activation of B cells and macrophages by polysaccharide isolated from cell culture of *Acanthopanax senticosus*. *International Immunopharmacology*, *3*, 1301–1312.
Hirase, S., & Watanabe, K. (1972). The presence of pyruvate residues in λ-carrageenan and a similar polysaccharide. *Bulletin of the Institute for Chemical Research, Kyoto University*, *50*, 332–336.
Hirt, W., Nebe, T., & Birr, C. (1994). Phagotest and Bursttest (Phagoburst), test kits for study of phagocyte functions. *Wiener Klinische Wochenschrift*, *106*, 250–252.
Hoffman, R., Woodrow Burns, W., & Paper, D. H. (1995). Selective inhibition of cell proliferation and DNA synthesis by the polysulphated carbohydrate ι-carrageenan. *Cancer Chemotherapy and Pharmacology*, *36*, 325–334.
Hu, X., Jiang, X., Aubree, E., Boulenguer, P., & Critchley, A. T. (2006). Preparation and *in vivo* antitumor activity of κ-carrageenan oligosaccharides. *Pharmaceutical Biology*, *44*, 646–650.
Ishiyama, M., Tominaga, H., Shiga, M., Sasamoto, K., Ohkura, Y., Ueno, K., et al. (1995). Novel cell proliferation and cytotoxicity assays using a tetrazolium salt that produces a water-soluble formazan dye. *In Vitro Toxicology*, *8*, 187–190.
Jackson, S. G., & McCandless, E. L. (1978). Simple, rapid turbidometric determination of inorganic sulfate and/or protein. *Analytical Biochemistry*, *90*, 802–808.
Jol, C. N., Neiss, T. G., Penninkhof, B., Rudolph, B., & De Ruiter, G. A. (1999). Novel high-performance anion-exchange chromatographic method for the analysis of carrageenans and agars containing 3,6-anhydrogalactose. *Analytical Biochemistry*, *268*, 213–222.
Kamerling, J., Gerwig, G., Vliegenthart, J., & Higashi, T. (1975). Characterization by gas–liquid chromatography mass spectrometry and proton-magnetic resonance spectroscopy of permethylsilyl methyl glycosides obtained in the methanolysis of glycoproteins and glycopeptides. *Journal of Biochemistry*, *151*, 491–495.
Kane, K. L., Ashton, F. A., Schmitz, J. L., & Folds, J. D. (1996). Determination of natural killer cell function by flow cytometry. *Clinical and Diagnostic Laboratory Immunology*, *3*, 295–300.
Knutsen, S. H., & Grasdalen, H. (1992). The use of neocarrabiose oligosaccharides with different length and sulphate substitution as model compounds for ^{1}H NMR spectroscopy. *Carbohydrate Research*, *229*, 233–244.
Knutsen, S. H., Shetmoen, M., Kristensen, T., Barbeyron, T., Kloareg, B., & Potin, P. (2001). A rapid method for the separation and analysis of carrageenan oligosaccharides released by iota- and kappa-carrageenase. *Carbohydrate Research*, *331*, 101–106.
Koene, H. R., Kleijer, M., Algra, J., Roos, D., Von Dem Borne, A., & De Haas, M. (1997). FcγRIIIa-158 V/F polymorphism influences the binding of IgG by natural killer cell FcγRIIIa, independently of the FcγRIIIa-48L/R/H phenotype. *Blood*, *90*, 1109–1114.
Leung, M. Y. K., Liu, C., Koon, J. C. M., & Fung, K. P. (2006). Polysaccharides response modifiers. *Immunology Letters*, *105*, 101–114.
Lowry, H. L., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biology Chemistry*, *193*, 265–275.
Mazumder, S., Ghosal, P. F., Pujol, C. A., Carlucci, M. J., Damonte, E. B., & Ray, B. (2002). Isolation, chemical investigation and antiviral activity of polysaccharides from *Gracilaria corticata* (Gracilariaceae, Rhodophyta). *International Journal of Biological Macromolecules*, *31*, 87–95.
Moore, A. R. (2003). Pleural models of inflammation: Immune and non immune. *Methods in Molecular Biology*, *225*, 123–128.
Moreno, S. E., Alves-Filho, J. C., Bertozi, G., Alfaya, T. M., Thèze, J., Ferreira, S. H., et al. (2006). Systemic administration of interleukin-2 inhibits inflammatory

neutrophil migration: Role of nitric oxide. *British Journal Pharmacology*, *148*, 1060–1066.
Morris, C. J. (2003). Carrageenan-induced paw edema in the rat and mouse. *Methods in Molecular Biology*, *225*, 115–121.
Murano, E., Toffanin, R., Cecere, E., Rizzo, R., & Knutsen, S. H. (1997). Investigation of the carrageenans extracted from *Solieria filiformis* and *Agardhiella subulata* from Mar Piccolo, Taranto. *Marine Chemistry*, *58*, 319–325.
Myslabodski, D. E., Stancioff, D., & Heckert, R. A. (1996). Effect of acid hydrolysis on the molecular weight of kappa carrageenan by GPC-LS. *Carbohydrate Polymers*, *31*, 83–92.
Opoku, G., Qiu, X., & Doctor, V. (2006). Effect of oversulfatation on the chemical and biological properties of kappa carrageenan. *Carbohydrate Polymers*, *65*, 134–138.
Papadopoulos, N. G., Dedoussis, G. V. Z., Spanakos, G., Gritzapis, A. D., Baxevanis, C. N., & Papamichail, M. (1994). An improved fluorescence assay for the determination of lymphocyte-mediated cytotoxicity using flow cytometry. *Journal in Immunological Methods*, *177*, 101–111.
Prado-Fernández, J., Rodríguez-Vázquez, J. A., Tojo, E., & Andrade, J. M. (2003). Quantitation of κ-, ι- and λ-carrageenans by mid-infrared spectroscopy and PLS regression. *Analytica Chimica Acta*, *480*, 23–37.
Pujol, C. A., Scolaro, L. A., Cianca, M., Matulewicz, M. C., Cerezo, A. S., & Damonte, E. B. (2006). Antiviral activity of a carrageenan from *Gigartina skottsbergii* against intraperitoneal murine herpes simplex virus infection. *Planta Medica*, *72*, 121–125.
Saito, R. M., & De Oliveira, E. C. (1990). Chemical screening of Brazilian marine algae producing carrageenans. *Hydrobiologia*, *204–205*, 585–588.
Sloneker, J. H., & Orentas, D. G. (1962). Pyruvic acid, a unique component of an exocellular bacterial polyssacharide. *Nature*, *194*, 478–479.
Solimabi, & Das, B. (1980). Antispasmodic and antiinflamatory activity of carrageenan from *Hypnea musciformis* wulfen. *India Journal of Pharmacology*, *12*, 259–261.
Stancioff, D. J., & Stanley, N. I. (1969). Infrared and chemical studies on algal polysaccharides. In R. Margalef (Ed.), *Proceeding of the 6th international seaweeds symposium* (pp. 595–609).
Stevenson, T., & Furneaux, R. (1991). Chemical methods for the analysis of sulphated galactans from red algae. *Carbohydrate Research*, *210*, 277–298.
Talarico, L. B., Pujol, C. A., Zibetti, R. G. M., Faria, P. C. S., Noseda, M. D., Duarte, M. E. R., et al. (2005). The antiviral activity of sulfated polysaccharides against dengue virus is dependant on virus serotype and host cell. *Antiviral Research*, *66*, 103–110.
Talarico, L. B., Zibetti, R. G. M., Faria, P. C. S., Scolaro, L. A., Duarte, M. E. R., Noseda, M. D., et al. (2004). Anti-herpes simplex virus activity of sulfated galactans from the red seaweeds *Gymnogongrus grittithsiae* and *Cryptonemia crenulata*. *International Journal of Biological Macromolecules*, *34*, 63–71.
Tobacman, J. K. (2001). Review of harmful gastrointestinal effects of carrageenan in animal experiments. *Environmental Health Perspectives*, *109*, 983–994.
Tzianabos, A. (2000). Polysaccharides immunomodulators as therapeutic agents: Structural aspects and biologic function. *Clinical Microbiology Review*, *13*, 523–533.
Van de Velde, F., Knutsen, S. H., Usov, A. L., Rollema, H. S., & Cerezo, A. S. (2002). ^{1}H and ^{13}C high resolution NMR spectroscopy of carrageenans: Application on research and industry. *Trends Food Science Technology*, *13*, 73–92.
Whyte, J. N. C., Foreman, R. E., & DeWreede, R. E. (1984). Phycocolloid screening of British Columbia red algae. *Hydrobiologia*, *116–117*, 537–541.
Womersley, H. B. S. (1994). *The marine benthic flora of Southern Australia. Rhodophyta. IIIA. Bangiophyceae and Florideophyceae (Acrochaetiales, Nemaliales, Gelidiales, Hildenbrandiales and Gigartinales sensu lato)*. Canberra: Australian Biological Resources Study., p. 508.
Wu, J., Edberg, J. C., Redecha, P. B., Bansal, V., Guyre, P. M., Coleman, K., et al. (1997). A novel polymorphism of FcyrIIIa alters receptor function and predisposes to autoimmune disease. *Journal of Clinical Investigations*, *100*, 1059–1070.
Xuelian, T., Jing, L., Xianliang, X., & Meiyu, G. (2006). A new marine-derived sulfated polysaccharide from brown alga suppresses tumor metastasis both *in vitro* and *in vivo*. *Cancer Biology and Therapy*, *5*, 1474–1480.
Yamada, T., Ogamo, A., Saito, T., Uchiyama, H., & Nakagawa, Y. (2000). Preparation of *O*-acylated low molecular-weight carrageenans with potent anti-HIV activity and low anticoagulant affect. *Carbohydrate Research*, *41*, 115–120.
Yamada, T., Ogamo, A., Saito, T., Watanabe, J., Uchiyama, H., & Nakagawa, Y. (1997). Preparation and anti-HIV cativity of low-molecular-weight carrageenans and their sulfated derivatives. *Carbohydrates Polymers*, *32*, 51–55.
Yaphe, W., & Arsenault, G. P. (1965). Improved resorcinol reagent for the determination of fructose, and 3.6-anhydrogalactose in polysaccharides. *Analytical Biochemistry*, *13*, 143–148.
Young, K., Duckworth, M., & Yaphe, W. (1971). The structure of agar. Part III. Pyruvic acid, a common feature of agars from different agarophytes. *Carbohydrate Research*, *16*, 446–448.
Yu, G., Guan, H., Ioanoviciu, A. S., Sikkander, S. A., Thanawiroon, C., Tobacman, J. K., et al. (2002). Structural studies on κ-carrageenan derived oligosaccharides. *Carbohydrate Research*, *337*, 433–440.
Yuan, H., & Song, J. (2005). Preparation, structural characterization and *in vitro* antitumor activity of kappa-carrageenan oligosaccharide fraction from *Kappaphycus striatum*. *Journal of Applied Phycology*, *17*, 7–13.
Yuan, H., Song, J., Li, X., Li, N., & Dai, J. (2006). Immunomodulation and antitumor activity of κ-carrageenan oligosaccharides. *Cancer Letters*, *243*, 228–234.
Zablackis, E., & Perez, J. (1990). A partially pyruvated carrageenan from Hawaiian *Grateloupia filicina* (Cryptonemiales, Rhodophyta). *Botanica Marina*, *33*, 273–276.
Zhou, G., Sheng, W., Yao, W., & Wang, C. (2006). Effect of low molecular lambda-carrageenan from *Chondrus ocellatus* on antitumor H-22 activity of 5-Fu. *Pharmacological Research*, *53*, 129–134.
Zhou, G., Sun, Y. P., Xin, H., Zhang, Y., Li, Z., & Xu, Z. (2004). *In vivo* antitumor and immunomodulation activities of different molecular weight lambda-carrageenans from *Chondrus ocellatus*. *Pharmacological Research*, *50*, 47–53.
Zhou, G., Xin, H., Sheng, W., Sun, Y., Li, Z., & Xu, Z. (2005). *In vivo* growth-inhibition of S180 tumor by mixture of 5-Fu and low molecular lambda carrageenan from *Chondrus ocellatus*. *Pharmacological Research*, *51*, 153–157.
Zuniga, E. A., Matsuhiro, B., & Mejias, E. (2006). Preparation of a low-molecular weight fraction by free radical depolymerisation of the sulfated galactan from *Schizymenia binderi* (Gigartinales, Rhodophyta) and its anticoagulant activity. *Carbohydrate Polymers*, *66*, 208–215.