Cytochemical studies on underutilized carrageenophytes (Gigartinales, Rhodophyta)

Leonel Pereira, Fred van de Velde and José F. Mesquita

***Abstract*—** In opposition to the relatively hard cell walls of other algae, those of the majority of red algae are flexible and soft, what is due to the co-existence of great quantities of amorphous material and relatively scarce fibrilar components. The intercellular matrix of carrageenophytes is mainly composed of highly sulfated polygalactans, with D-galactose and anhydro-D-galactose, in contrast with the less sulfated agars, where the anhydro-L-galactose is predominant. In the scope of a larger work on underutilized carrageenophytes (Gigartinales, Rhodophyta), chemical and spectroscopic analysis (vibrational and nuclear resonance spectroscopy) was carried out to the extracted and purified phycocolloids of these algae. As a complement of this work, the results of a cytochemical study on distribution of the main components of cell wall and intercellular matrix in the thalli are herein presented for the following carrageenophytes: *Chondracanthus teedei* var. *lusitanicus*, *Gigartina pistillata*, *Gymnogongrus crenulatus* and *Ahnfeltiopsis devoniensis.*

***Keywords*—** Carrageenan, cell-wall, EDX, FTIR, FT-Raman, ^{1}H-NMR, polymers, seaweeds.

I. Introduction

As red algae inhabit aquatic environments quite different from those typical of land plants, it is not perhaps unexpected to discover that the composition and organization of their intercellular matrix are distinct from those commonly found in the latter [1]. The red algae extracellular matrixes are composed of a cellulose microfibrils network associated with material that includes amorphous polymers of sulfated galactans, mucilage and cellulose. In contrast to the relatively rigid cell walls of other algae, those of red algae are flexible and with soft consistence due to the presence of a minor fibrilar component (cellulose) [1], [15], [16].

In the economically important marine red algae, the polysaccharide matrix is almost entirely made up of galactans (carrageenans or agars), which may be highly substituted with sulphate esters groups [2]. Carrageenans are sulfated polysaccharides present in the cell walls of members of the Gigartinales, which have been used extensively as gels and thickeners in food industry. There are about 15 idealized carrageenan structures identified by Greek letters [13]. Chemical differences are known to exist among carrageenans from different generations of the life cycle of some carrageenophytes. The gametophyte thalli of members of the Gigartinales produce a kappa or kappa/iota hybrid carrageenan and the tetrasporophyte thalli produce lambda-family carrageenan [2], [13], [17].

The study of the cell wall formation in red seaweeds is a very complex but fascinating subject [3]. This type of study involves diverse areas and techniques as molecular biology, biology of the development, physicochemical analysis of the polysaccharides (spectroscopy, chromatography, enzymology, etc.). Cytochemistry techniques, with sight to the elucidation of biosynthesis of the biological precursors of the cell wall components, are used with special attention given to the transport, deposition and extracellular modification of the polymers [4].

The aim of the current study is to: 1) analyze the native composition of the polysaccharides produced by the studied algae, through vibrational spectroscopy techniques (FTIR-ATR and FT-Raman) and ^{1}H-NMR; 2) quantify and locate, in the cell wall and intercellular matrix, the main components (carrageenans and cellulose), with cytochemical techniques (energy dispersive X-ray analysis, fluorescence, polarize and electron microscopy).

II. Material and Methods

A. Algal Material

Specimens of red algae *Chodracanthus teedei* var. *lusitanicus* (Fig. 1A), *Gigartina pistillata* (Fig. 1M), *Gymnogongrus crenulatus* (Fig. 1H) and *Ahnfeltiopsis devoniensis* (Fig. 1D) (Gigartinales, Rhodophyta), were collected in Buarcos bay, central zone of the western coast of Portugal.

The dry weight and carrageenan content were evaluated. For these determinations, 100 individuals of each species greater than 3 cm were collected monthly and at random. At the laboratory, carrageenophytes fronds were sorted into the different lifecycle phases and then rinsed in distilled water to

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eliminate debris and salt on the thalli surfaces and subsequently dried, in ventilated oven, to constant weight at 60 ºC. Carrageenan extraction was carried out according to the process described by Pereira et al. [5] and Pereira and Mesquita [6]. For alkaline-extraction (resembles the industrial method), the samples were placed in a solution (150 mLg^{-1}) of NaOH (1 M) at 80 ºC for 3 h and neutralized to pH 8 with HCl (0.3 M) [5], [6].

B. Spectroscopic Analysis

Vibrational spectroscopy

Samples of ground, dried algal material were analyzed by FTIR and FT-Raman for determination of natural phycocolloids composition, according to the method described by Pereira [7,8]. The FTIR spectra of ground dried seaweed, native and alkali- modified carrageenan, were recorded on an IFS 55 spectrometer, using a Golden Gate single reflection diamond ATR system, with no need for sample preparation. All spectra reported in this paper are the average of two counts, with 128 scans each and a resolution of 2 cm^{-1}. The room temperature FT-Raman spectra were recorded on a RFS-100 Bruker FT-spectrometer using a Nd:YAG laser with excitation wavelength of 1064 nm. Each spectrum was the average of two repeated measurements, with 150 scans at a resolution of 2 cm^{-1}.

NMR spectroscopy

^{1}H-NMR spectra were taken on a Bruker AMX600 spectrometer operating at 500.13 MHz at 65 °C. Typically 64 scans were taken with an interpulse delay of 5 s (T_1 values for the resonance of the anomeric protons of κ- and ι-carrageenan are shorter than 1.5 s). Sample preparation for the ^{1}H-NMR experiments involved dissolving the carrageenan sample (5 mg mL^{-1}) at 80 °C in D_2O containing 1 mM TSP (3-(trimethylsilyl) propionic-*2,2,3,3-d*$_4$ acid sodium salt) and 20 mM Na2HPO4, followed by sonication for three times 1 h in a sonicator bath (Branson 2510). Chemical shifts (δ) are referred to internal TSP standard (δ = -0.017 ppm) relative to the IUPAC recommended standard DSS for ^{1}H according to van de Velde et al. [10]. Assignments of the ^{1}H-NMR spectra were based on the chemical shift data summarized by van de Velde et al. [9], [10].

C. Cytological Localization of Polymers

Two methods were applied for the localization of the cellulose (β-glucan): a - observation of fresh sections in fluorescence microscopy (ultraviolet light, 345-365 nm, Nikon Diaphot microscope equipped with UV 2A filter), after staining with calcofluor white (fluorescent brightener 28 of Sigma) at 0.04% [1]; b - observation of identical sections in polarization microscope (Nikon Optiphot), with cross polarization filters (birefringence studies) to look for crystalline or paracrystalline structures [1].

For the identification and localization of sulfated polysaccharides (carrageenans), two techniques were also used: a - staining of the sections with toluidine blue (0.05% in 0.1 M acetate buffer, pH 4.4), in light microscopy, for the detection of metachromasia of acid polysaccharides [1]; b - energy dispersive X-ray analysis (EDX), to the variation of sulfur concentration [11], [12], made in Hitachi H900 electron microscope, associated with an x-ray spectrometer and respective detector.

III. Results and Discussion

A. Spectroscopic Analysis

The main results of the analyses of the studied seaweeds are listed in Table I. These analyses include lifecycle phase, fraction of extracted carrageenan, and composition of native and alkali-extracted carrageenan.

In relation to the phycocolloid nature, our spectroscopic analysis showed that the studied carrageenophytes seem to present a variation similar to that existing in other species of Gigartinales family [13]. The gametophyte and non-fructified stages of *C. teedei* var. *lusitanicus*, *G. pistillata*, *A. devoniensis* and *G. crenulatus* produces carrageenans of the kappa family (hybrid kappa/iota/mu/nu-carrageenan). The tetrasporophyte stages produce carrageenans of the lambda family (hybrid xi/theta or xi/lambda-carrageenan). The carrageenan alkaline-extracted from female gametophytes showed lower sulfate content and a decrease in a galactose to the benefit of 3,6-anhydrogalactose. This corresponds to the conversion of the 4-linked galactose-6-sulfate in native samples to anhydro-galactose in the alkaline modified samples. Thus the biological precursors mu- and nu-carrageenan were converted into kappa and iota carrageenan, respectively [6].

B. Cytological Localization of Polymers

The sulfated polysaccharides distribution in the thalli of *C. teedei* var. *lusitanicus* (Fig.1C), *G. pistillata* (Fig.1O) and *G. crenulatus* (Fig.1J), presents a similar gradient to that observed in several other carrageenophytes, particularly in *Chondrus crispus* [14], [18], [19] and agarophytes such as *Pterocladiella capillaceae* [20]. The analyzed seaweed thalli present a greater concentration of sulfated polysaccharides in the external region, especially in the cortical area, and low concentration in the internal region (medullar area) (see Fig.1M, J and O).

In the cross sections of *A. devoniensis* (Fig.1F), the staining with calcofluor white allowed us to that the walls of the medullar cells present a more intense staining than the cortical cell walls. This observation is supported by the strong birefringence presented by the walls of the medullar cells (see Fig.1G and Fig.1L, *G. crenulatus*), when observed under polarized light, denoting the presence of a highly ordered structure in the core of the cell walls (core walls). The cortical area shows a low birefringence and a weaker reaction to the calcofluor white, indicating a lower concentration of ß-glucan than in the medullar zone. This organization of the cell wall is in accordance with that seen in high commercial value

Table I. Carrageenan composition determined by vibrational spectroscopy (FTIR-ATR and FT-Raman) and ^{1}H-NMR.

Species	Lifecycle phase	Date of harvest	Carrageenan		
			Yield[1]	Alkali extracted[2] (%mol)	Native[3]
Gigartina pistillata	Female gametophyte	Mar. 2002	49.8 %	48.7 κ, 44.5 ι	κ - ι (μ/ν)
Chondracanthus teedei var. *lusitanicus*	Non-fructified	Jun. 2001	35.0 %	55.8 κ, 44.2 ι	κ - ι (μ/ν)
C. teedei var. *lusitanicus*	Female gametophyte	Jun. 2001	43.6 %	58.1 κ, 41.9 ι	κ - ι (μ/ν)
Ahnfeltiopsis devoniensis	Gametophyte	Jul. 2001	13.6 %	16.7 κ, 81.1 ι, 2.2 ν	ι - κ (ν)
A. devoniensis	Non-fructified	Aug. 2001	11.5 %	19.8 κ, 80.2 ι	ι - κ (ν)
Gymnogongrus crenulatus	Tetrasporoblastic	Apr. 2002	9.7 %	64.1 κ, 30.8 ι	κ - ι (μ)
A. devoniensis	Gametophyte	Dec. 2001	11.5 %	22.3-34.7 κ, 65.3-77.7 ι	ι - κ (ν)
G. crenulatus	Tetrasporoblastic	Nov. 2001	11.0 %	60.0 κ, 28.9 ι	κ - ι (μ)
G. pistillata	Tetrasporophyte	Apr. 2002	55.6 %	ξ, λ	ξ - λ
C. teedei var. *lusitanicus*	Tetrasporophyte	Jun. 2001	36.6 %	67.0 ξ, 37.0 θ	ξ - θ

[1]expressed in percentage of dry weight; [2]composition determined by ^{1}H-NMR; [3]composition determined by FTIR-ATR and FT-Raman analysis of ground seaweed samples; the carrageenans are identified according to the Greek lettering system [13]; the letters between parenthesis ( ) correspond to the biological precursors of the carrageenans, present in native samples.

carrageenophytes [1], as with the *Kappaphycus alvarezzi*, a major industrial source of kappa carrageenan [21]. The carrageenophytes analyzed in this work (*G. pistillata*, *G. crenulatus* and *A. devoniensis*) have, in cellulose, the main fibrilar polysaccharide component of the cell wall (see Fig.1F, G and L), varying the level of their organization (related with the level of cristalinity) according to the size of the cells [22], [23], [24].

The analysis for sulfur (EDX) of *G. pistillata* (female gametophyte) thallus is presented as the atomic percentage of sulfur (mole sulfur per mole monosaccharide). The sulfur percentages are interpreted as corresponding to the presence of carrageenan in the thallus [12]. In the interior of a cortical cell (cytoplasm, plastids and floridean starch grains) the sulfur levels are below the detectable level. Significant amounts of this element are present in the cell wall, as well in the intercellular matrix (Fig.1P). The detected amounts of sulfur denounce the existence of a concentration gradient from the inner part of the cellular wall (3.98%) to the intercellular matrix (11.43%).The results of analysis by EDX show that the highest concentrations of sulfur, and thus of carrageenan, are located in the cell walls and intercellular matrix, with a gradient of carrageenan increasing from the inner side of the cell wall towards intercellular matrix, as in *Chondrus crispus* [7].

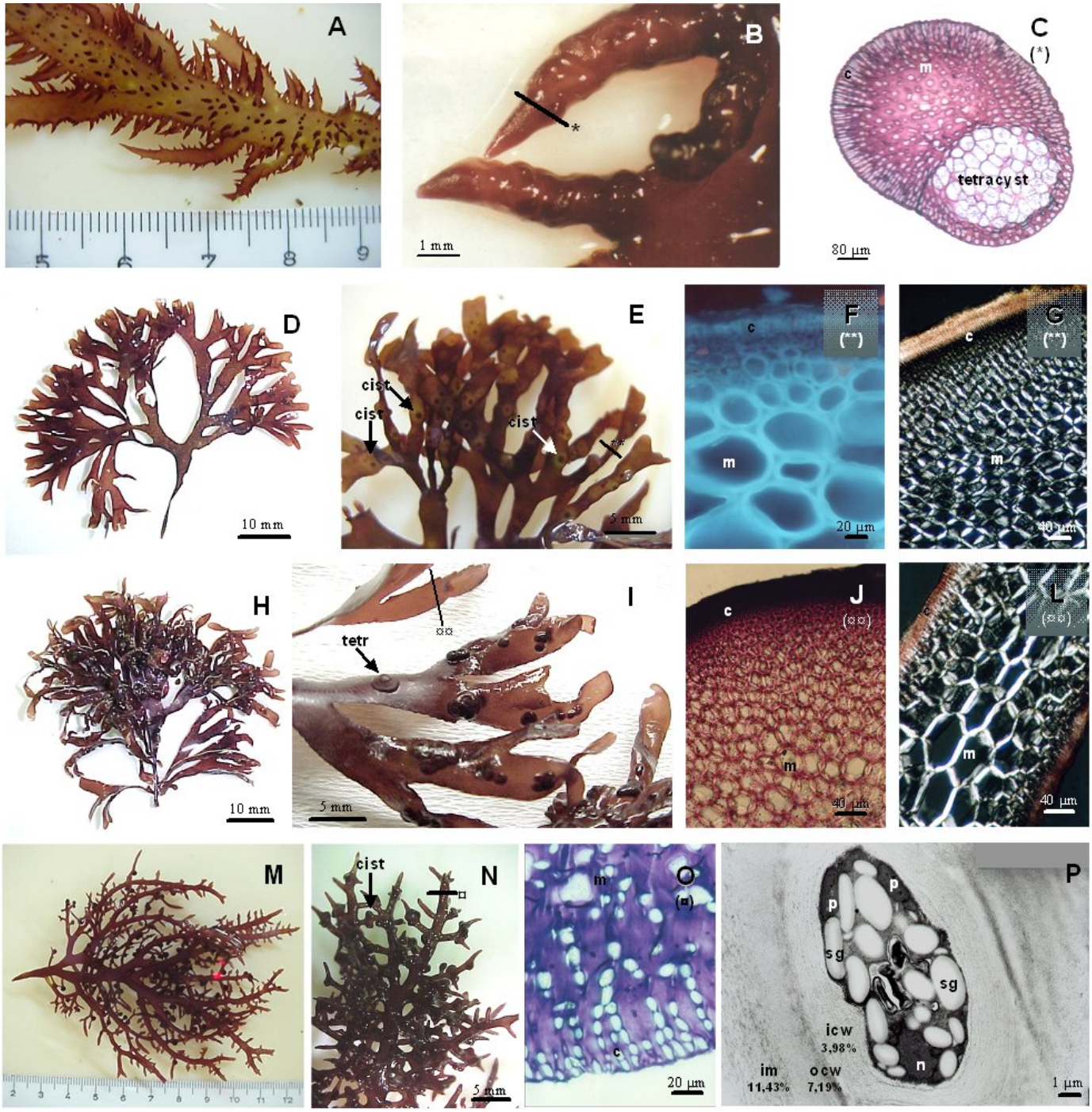

Figure 1. Identification and localisation of the cellulose in fluorescence microscopy after staining with calcofluor white (**F**) and in polarization microscopy with crossed polarisers (birefringence) (**G, L**). Localisation of sulphated polysaccharides (carrageenans), in light microscopy, following staining with toluidine blue (metachromasia) (**C, J, O**) and in electron microscopy (EDX analysis for sulphur) (**P**).

A-C. *Chondracanthus teedei* var. *lusitanicus* (tetrasporophyte); **D-H**. *Ahnfeltiopsis devoniensis* (female gametophyte); **H-L**. *Gymnogongrus crenulatus* (tetrasporoblastic thallus) **M-P**. *Gigartina pistillata* (female gametophyte).

c, cortex; **m**, medulla; **cist**, cystocarp; **tetracyst**, tetrasporocyst; **tetr**, tetrasporoblast; **icw**, inner zone of cell wall; **ocw**, outer zone of cell wall; **im**, intercellular matrix; **n**, nucleus; **p**, plastid; **sg**, floridean starch grain.

IV. Conclusion

To conclude, the presented results show that the combination of FTIR and Raman spectroscopy allows identifying the natural composition of the phycocolloids present in the seaweed [7], [8]. Since the vibrational spectrometers (FTIR and FT-Raman) are now standard equipment in many Laboratories, the techniques used in this work [6], [7] are useful for study of carrageenophyte populations, in substitution of the traditional tests of iridescence and resorcinol [25]. These techniques are also useful for the development and the implementation of strategies of sustainable seaweed harvest, the evaluation of the natural seaweed composition and the evaluation and control o the quality of the different batches of algal material harvested and/or cultivated [6], [8].

But ^{1}H-NMR spectroscopy is necessary for the quantitative analysis of the different repeating units of the hybrid carrageenans, extracted from the studied algae [6]. ^{1}H-NMR allows to identify and quantify the different carrageenan types based on the intensity and the chemical shift of the resonances of the anomeric protons [9].

Cytological localization of intercellular matrix polymers *in situ* provides valuable information complementary to chemical and physical characterization of extracted components. Thus, based on this cytochemical study, the following conclusions might be drawn: in the carrageenophytes here studied (*C. teedei* var. *lusitanicus*, *G. pistillata*, *G. crenulatus* and *A. devoniensis*) the two main components of the cell walls and intercellular matrix are the cellulose and carrageenans (polymers of sulfated galactans). At the level of the thallus, the concentration of this sulfated polysaccharides decreases from the cortex to the medulla, while the cellulose gradient is inversed. At the cellular level the cellulose is the main fibrilar component of the cell walls. This has also been observed for the carrageenophytes with a high commercial value (*Chondrus crispus* and *Kappaphycus alvarezzi*). Moreover, the cristalinity of the cellulose changes in parallel with the cell size [14, [1]. The EDX analysis for sulfur showed (like in *C. crispus*) the existence of an increasing gradient of the carrageenans from the innermost region of the cell wall to the intercellular matrix [1]. This is in good agreement with the direct analysis of the carrageenan concentration.

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