Cytochemical studies on portuguese carrageenophytes (Gigartinales, Rhodophyta)

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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 sulphated polygalactans, with D-galactose and anhidro-D-galactose, in contrast with the less sulphated agars, where the anhydro-L-galactose is predominant. In the scope of a larger work on the carrageenophytes of the Portuguese coast, 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*.

Key-Words: - seaweeds, carrageenan, EDX, ^{1}H-NMR, FTIR, FT-Raman, cell-wall, polymers

1 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]. 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].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, chemical analysis of the polysaccharides (spectroscopy, chromatography, enzimology, etc.) and the citochemistry, with sight to the elucidation of biosynthesis of the biological precursors of the cell wall components, with special attention given to the transport, deposition and extracellular modification [4].

2 Material and Methods

2.1 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), 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 at random, monthly. At the laboratory, carrageenophytes fronds were sorted into the different lifecycle phases and then rinsed in distilled water to eliminate debris and salt on the thalli surfaces and 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 & Mesquita [6].

2.2 Spectroscopic analysis

2.2.1 Vibrational spectroscopy

Samples of ground, dried algal material were analysed 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 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}.

2.2.2 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 Na_2HPO_4, 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 summarised by van de Velde et al. [9,10].

2.3 Cytological localization of polymers

Two methods were applied for the localisation of the cellulose (β-glycan): 1. Observation of fresh sections in fluorescence microscopy (ultraviolet light, 345-360 nm), after staining with calcofluor white (fluorescent brightner 28 of Sigma) at 0.04% [1]; 2. Observation of identical sections in polarization microscopy, with crossed polarizers (birefringence studies) to look for crystalline or para-crystalline structures [1].

For the identification and localisation of sulphated polysaccharides (carrageenans), two techni ques were also used: 1. staining of the sections with toluidine blue (0.05% in 0.1 M acetate buffer, pH 4.4) for the detection of metachromasia of acid polysaccharides [1]; 2. energy dispersive X-ray analysis (EDX), to the variation of sulphur concentration [11,12].

3 Results and Discussion

3.1 Spectroscopic analysis

The main results (lifecycle phase, % of extracted carrageenan, composition of native and alkali-

Table 1. 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.

extracted carrageenan), concerning the studied seaweed, are listed in Table 1.

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), while the tetrasporophyte stages produce carrageenans of the lambda family (hybrid ksi/tetha or ksi/lambda-carrageenan) (see Table 1). The carrageenan alkaline-extracted from female gametophytes showed lower sulphate content and a decrease in a galactose to the benefit of 3,6-anhydrogalactose. The presence of some 4-linked galactose 6-sulphate in native samples was converted to anhydro-galactose upon alkali-treatment, in sequence of the precursors conversion in kappa and iota carrageenan [6].

3.2 Cytological localization of polymers

The analysis for EDX analysis for sulphur of *G. pistillata* (female gametophyte) thallus, presented under the form of atomic percentage of sulphur, are interpreted as corresponding to the carrageenan presence [12]. In the interior of a cortical cell (cytoplasm, plastids and floridean starch grains) the sulphur levels are below the detectable level, but

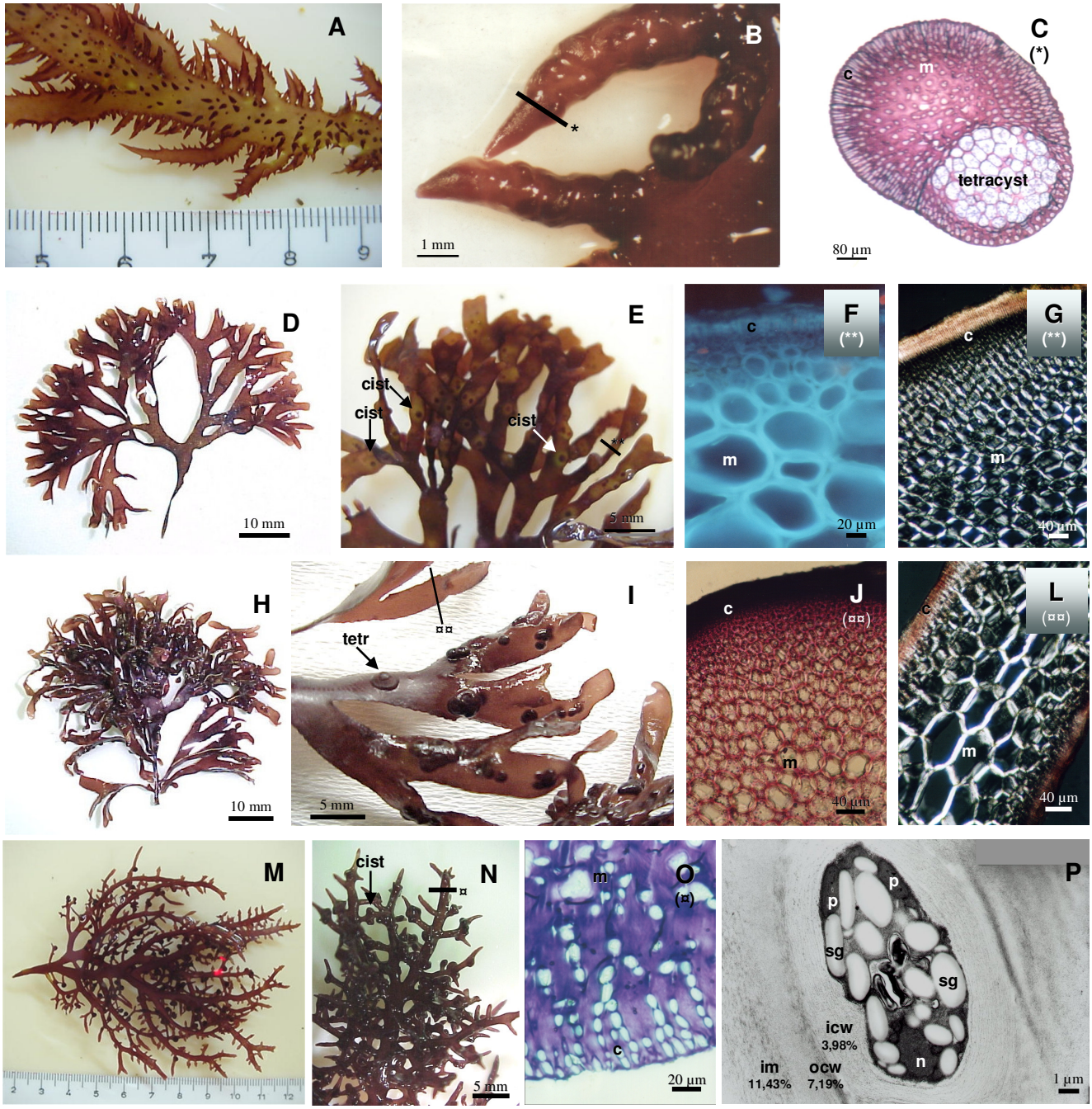

Figure 1. Identification and localisation of the cellulose in fluorescence microscopy after staining with calcofluor white (**F**) and in polarization microscopy with crossed polarisers (birefringence) (**G, L**). Localisation of sulphated polysaccharides (carrageenans), in light microscopy, following 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.

significant amounts of this element are present in the cell wall, as well as in the intercellular matrix (Fig.1P). The detected amounts of sulphur denounce the existence of a gradient of concentration, from the

inner part of the cellular wall until the intercellular matrix: interior part of the cell wall (3.98%); exterior part of the cell wall (7.19%); zone of transition between the wall and the matrix (10.39%); intercellular matrix (11.43%).

4 Conclusion

To conclude, the presented results show that the combination of FTIR and Raman spectroscopy allows identifying the natural composition of the phycocolloids presents in the seaweed. But the ^{1}H-NMR spectroscopy is necessary for the quantitative analysis of the different components of the hybrid carrageenans, extracted from the studied algae [6].

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 sulphated galactans). At the level of the thallus, the concentration of this sulphated polysaccharides decreases from the cortex to the medulla, while the cellulose gradient is inversed. At the cellular level, similarly to what happens with the high commercial value carrageenophytes (*Chondrus crispus*, *Kappaphycus alvarezzi*), the cellulose is the main fibrilar component of the cell walls, and its crystallinity changes in parallel with the cell size [14,1]. As to carrageenans, the EDX analysis for sulphur has shown (like in *C. crispus*) the existence of an increasing gradient of these phycocolloids from the innermost region of the cell wall to the intercellular matrix [1].

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