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CELL-WALL OF *Lobophora variegata* (OCHROPHYTA): EXTRACTION AND BIOCHEMISTRY OF SULFATED POLYSACCHARIDES

PAREDE CELULAR DE *Lobophora variegata* (OCHROPHYTA): EXTRAÇÃO E BIOQUÍMICA DE POLISSACARÍDEOS SULFATADOS

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Abstract Complex cell wall composition of *Lobophora variegata* (Ochrophyta) rich in sulfated polysaccharides (LvSPs) is few understood. In this study, LvSPs were sequentially extracted and compared on their yield and structural properties. Decumbent morphotype was digested with papain (3 or 24 h) and then LvSPs3h-1→4 or LvSPs24h-1→6 quantified and examined their charge/mass by electrophoreses (agarose/polyacrylamide gels) *vs.* standard glycosaminoglycans stained with toluidine blue or Stains-All. The structural features were then analyzed by infrared spectroscopy. The results showed that the time impacted on the number and total extraction yield (15.63 ± 3.41 and 30.69 ± 0.42%, w w^{-1}, for LvTSPs3h-1→4 and LvTSPs24h-1→6, respectively, $p < 0.05$). Chemical analyses reflected marked differences (LvTSPs3h-1→4 *vs*. LvTSPs24h-1→6) in charge showing a system of heterofucan and alginic acid (<100kDa), but without presenting degradation of the polymers coextracted from the algal cell wall. Therefore, complex wall of *L. variegata* is rich in acid polysaccharides with 24 h optimal extraction, suggesting a taxonomic adaptation to herbivory, when exposure to enviromental pressure.

Key Words: extracellular matrix, brown alga, structural profile, algal biology.

Resumo Composição da complexa parede celular é pouco entendida de *Lobophora variegata* (Ochrophyta) rica em polissacarídeos sulfatados (LvPSs). Neste estudo, foram extraídos sequencialmente e comparados LvPSs sobre seus rendimentos e propriedades estruturais. Foi digerido com papaína (3 ou 24 h) morfotipo decumbente e, em seguida, quantificados e examinados de LvPSs3h-1→4 ou LvPSs24h-1→6 suas cargas/massas por eletroforeses (géis de agarose/poliacrilamida) *vs*. padrões de glicosaminoglicanos corados com azul de toludina ou "*Stains-All*". Foram analisadas posteriormente as características estruturais por espectroscopia de infravermelho. Os resultados mostraram que o tempo impactou sobre o número e rendimento de extração total (15,63 ± 3,41 e 30,69 ± 0,42%, m m^{-1}, para LvPSTs3h e LvPSTs24h, respectivamente, $p < 0,05$). Diferenças marcantes (LvPSs3h-1→4 *vs*. LvPSs24h-1→6) nas análises químicas refletiram na carga mostrando um sistema de heterofucana e ácido algínico (<100kDa), porém sem os polímeros coextraídos da parede celular algal apresentarem degradação. Portanto, parede complexa de *L. variegata* é rica em polissacarídeos ácidos com extração ótima de 24 h, sugerindo uma adaptação taxonômica à herbivoria, quando exposta a pressão ambiental.

Palavras-Chave: matriz extracelular, alga parda, perfil estrutural, biologia algácea.

Introduction

Algae are photosynthetic multicellular Eukaryotes that have a land plants-associated phylogenetic history (Popper et al., 2011). The algal diversity of benthic forms (known as seaweeds or macroalgae: Chlorophyta - green algae, Rhodophyta - red algae and Ochrophyta - brown algae) makes part of the coastal structure of marine aquatic life (Joly, 1965; Marinho-Soriano et al., 2009). They are important taxonomic entities in ecological and physiological terms due to their large degree of morphological plasticity adapted to survival a range of marine enviromental conditions (spatial and temporal variability), but with coexistence of different groups of individuals occuping a common region and showing differences in biochemical composition or, until, life strategies (Stengel et al., 2011).

Cell-walls of seaweeds have been of substancial interest by researchers toward understanding of their complex anatomies and multifatious functions involved in separation between morphotypes and species from ancient photosysnthetic sources. Wall biochemical varies in composition depending on the algal cycle (developmental stage), cell type and climatic fluctuations (season). Studies on this topic have brought questions on the adaptation of the seaweeds to enviromental and evolutionary pressures based on cell-wall components (Popper et al., 2011; Stengel et al., 2011). Not least, as their architectures also vary with the algal species (Reviers, 2006), studies have focused on the high economic value (*e.g.*, paper, food and fiber industries) of their chemical constituints (mainly proteins and sulfated polysaccharides-SPs) for future use as biofuels, nutraceuticals and pharmaceuticals (Marinho-Soriano et al., 2009; Popper et al., 2011); and scientific knowledge regarding the chemodiversity and how it could generate of beneficial to human and animal health is also of interest by glycobiologists (Pomin & Mourão, 2008; Pomin, 2012).

Well-known matrixes have revealed the occurrence of specific wall components in major algal taxa (Popper et al., 2011). These scenarios already allowed to analyze the richness of algae species as commercially-valuable sources in SPs accounting >40% of the algal weight. SPs occur in high concentrations in the algae extracellular matrixes, being their major features conserved among phyla, revealing complexity, heterogeneity and structural diversity; and showing intriguing biological functions among species, including evolutionary questions (Pomin & Mourão, 2008). SPs have usually >100 kDa of molecular weight and a highly electrostatic character due to their high content of sulfate groups on their backbone chains capable of binding with health/diseases-linked basic proteins generating, therefore, specific affinities that lead to pharmacological effects (*e.g.*, anti-inflammatory, anticoagulant, anti-angiogenesis, anticancer, antioxidant and antival effects) in a multitude of biological systems (Farias et al., 2000; Pomin, 2012). Rhodophyta produces sulfated galactans (carrageenans and agarans), while in Ochrophyta fucans or fuicodans depending on the algal species (Pomin & Mourão, 2008). In Chlorophyta, ulvan-like sulfated hetropolysaccharides (Yaich et al., 2013; Rodrigues et al., 2017) occur in minor concentration among the species (Pomin & Mourão, 2008). Novel SPs-based agents have already been isolated and/or characterized (Zibetti., 2005; Rodrigues et al., 2009a; 2011, 2016), although they have been still few understood and explored in algal wall biology (Stengel et al., 2011; Popper et al., 2011).

Ochrophyta Cavalier-Smith (1995) is a monophyletic taxonomic group of brown seaweeds that evoluted from divergences of fossilized algae found in Siberia. Species are multicellulars, rich in natural products (*e.g.*, carotenoids and polyphenols) and their phylogenetics reviewed continually (Reviers, 2006). They have SPs (Leite et al., 1998; Wang et al., 2008, Ananthi at el., 2010) known as fucans (rich in sulfated L-fucose) or heterofucans (known as fucoidans) showing extremely variable molecular weights, when extracted by different protocols (Leite et al., 1998; Yoon et al., 2007; Pomin & Mourão, 2008); and alginic acids, known as alginates, a linear carboxylated polymer formed by β-D-mannuronic acid and α-L-guluronic acid. Alginate has no nutritional value, but it is commonly used to stabilize the texture of foods (Torres et al., 2007) and in chemical

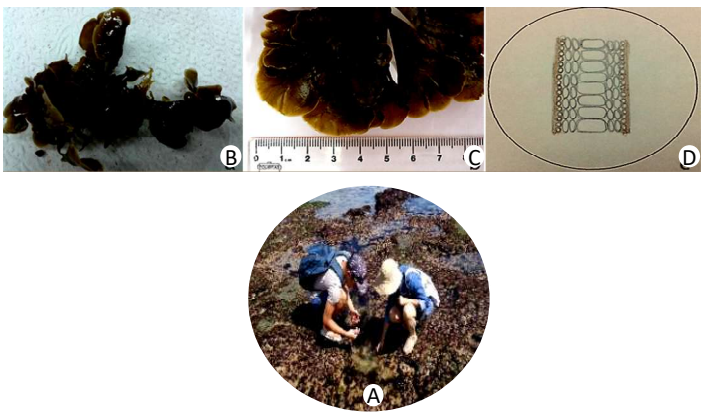
processes (Ronghua et al., 2003). *Lobophora variegata* J. V. Lamouroux (1817) (Phaeophyceae, Dictyotales) has three morphotypes (ruffled, decumbent and encrustant) (Coen & Tanner 1989) and is distributed on the Brazilian coast (Joly, 1965; Marinho-Soriano et al., 2009). Studies performed by different groups have revealed sucessibility to herbivory (Coen & Tanner 1989) and SPs with antioxidant (Paiva et al., 2011), anti-inflammatory (Paiva et al., 2011; Siqueira et al., 2011) and antiviral (Kremb et al., 2014) properties; and as protectors in saline stress-induced fishes (Rodrigues et al., 2009b). However, its system of SPs has been few explored along the wall matrix.

The current research was to sequentially extract (3 or 24 h) and evaluate the total yield of fucan-type SPs and the presence of alginic acid from the flat decumbent form (largely non-adherent) of *L. variegata,* as well as compare physically-chemically / structurally by analytical techniques of electrophoresis and Fourier Transform Infrared (FT-IR) spectroscopy, respectively, contributing to a more detailed knowledge regarding to the biochemical mapping of SPs (LvSPs) and the change cell-wall composition.

Material and Methods

Brazilian samples of *L. variegata* collection and taxon identification

Flat decumbent fresh material of *L. variegata* was manually collected at low tide from different sites of a tropical region characterized by a calcarium reef located at Pacheco beach (northeast Brazilian coast) during a field expedition carried out in Caucaia, Ceará state. It was pre-selected within other algae species occuping the same intertidal area before transported in plastic bags to the Marine Biochemistry laboratory of the Aquaculture Biotechnology Center, Department of Fishing Engineering, Federal University of Ceará (DEP-FE/FUC) (Figure 1A). After collection, specimens were washed with distillated water to remove salt, sand and shells; and necrotic parts before stored -20°C. Then, a specimen voucher (no. 1809) was reposted in the Marine Sciences Institute Herbarium/FUC, Brazil. *L. variegata* was also authorized through our registration with SISGEN (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado).



Kingdom Plantae
Phylum Ochrophyta
Order Dictyotales
Family Dictyotaceae
Genus *Lobophora* J. Agardh
Species *L. variegata* (J. V. Lamouroux) Wonmersley ex E. C. Oliveira (Kützing)

Figure 1. *L. variegata* of decombent form collected in open field attached to the substract (A) showing greenish-brown thallus in expanded shape (B, C) and representation of its wall matrix formed by seven cellular layers (D).

Taxon characterization was to a greenish-brown thallus of expanded and lobed leaf shape with unrolled margin as catalogued by Marinho-Soriano et al. (2009). It had unregular branches and consistence like plastic and fragile from the examined sample. Regarding light microscopy (Figure 1D), specimen had its wall matrix designed and identified by seven cellular layers, with differences in terms of cell shape with presence or not of pigments. Rectangular cells (located in the central layer), oval or medullar cells (located in the intermediate layers) and pigmented circular or cortical cells (located in the peripheral layers) characterized the *L. variegata* cell wall matrix, as visualized from the cross section of the thallus (Joly, 1965).

Proteolytic extraction of LvSPs

Flat decumbent material of *L. variegata* collected and identified from the litoral (Figure 1) was dehydrated in plastic trays for ~24 h under sunlight, manually cut into small pieces (Siqueira et al., 2011) and then stored in hermetic recipients in a dark place at laboratory temperature (25-28°C) (Yaich et al., 2013). Extraction of the algal-processed material was performed to obtain the crude extracts with basis on Siqueira et al. (2011) from the Farias et al. (2000)' enzymatic method, with some modifications. For this (Figure 2), the tritured algal tissues (5 g) were suspended in 100 mL of 100 mM sodium acetate buffer (pH 5.0), containing EDTA/cysteine (both 5 mM), and then heated in glass reactor flasks using a thermostatic bath applying digestion processes at 60°C by adding of crude papain (~5.1 mg mL^{-1}) for 3 or 24 h, yielding LvSPs3h-1 or LvSPs24h-1 as extracted samples.

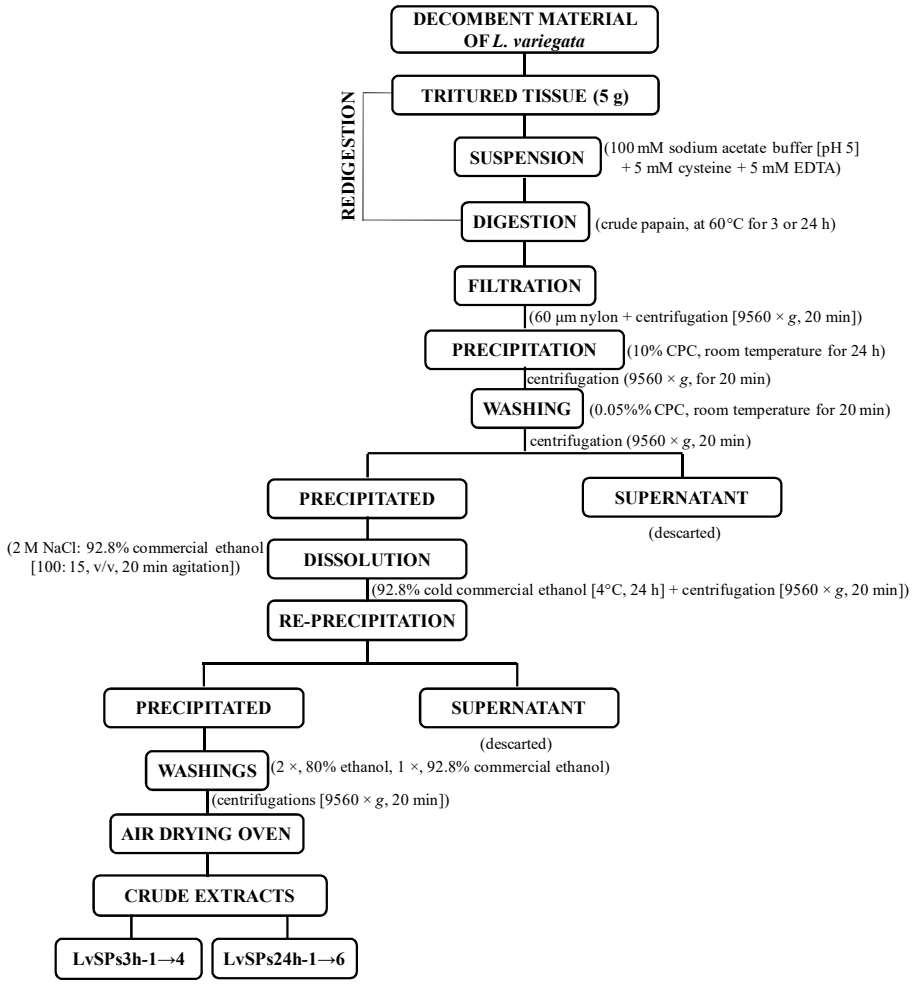


Figure 2. Sheme of sequential extraction (3 or 24 h) of cell-wall SPs from the brown seaweed *L. variegata*.

After that, the digested mixture was filtered using a nylon screen and the supernatant was recovered. LvSPs3h-1 or LvSPs24h-1 that were present in solution were precipitated with 16 mL of a 10% cetylpyridinium chloride (CPC) solution at laboratory temperature (at 25-28°C) for 24 h and the material then collected by centrifugation (9.560 × *g*, for 20 min). The *pellet* containing LvSPs3h-1 or LvSPs24h-1 was washed with 100 mL of 0.05% CPC solution, dissolved under constant agitation (for 20 min at 25-28°C) in 100 mL of a 2 M NaCl:ethanol (100:15 ratio, v:v) solution, and then precipitated for 24 h at 4°C with addition of 100 mL of cold commercial ethanol (92.8%). Then, the precipitate was centrifugated (9.560 × *g* for 20 min) followed by washing twice with 100 mL of 80% ethanol, and once with the same volume of 92.8% commercial ethanol (for 10 min at 25-28°C each step). Sequentially the *L. variegata* residues were redigested twice in the presence of crude papain following the same procedure above yielding LvSPs3h-2→4 or LvSPs24h-2→6, respectively (Rodrigues et al., 2009a, 2011, 2016; Siqueira et al., 2011). Finally, all the ethanol-washed materials were dried using an air drying oven (60°C, 6 h) and the total extraction yields (LvSPs3h-T or LvSPs24h-T) also quantified (%) (Eq. *1*) and the values expressed as the percentage (%, n = 3) of the dehydrated matter.

$$\text{Yield (\%)} = \text{dry material of LvSPs dehydrated algal material}^{-1} \times 100 \qquad (1)$$

LvSPs monitored by metachromasia

The LvSPs detection, present in the tritured tissue digested for 3 or 24 h, was monitored by metachromatic property using the 1,9-dimethylmetilene (DMB) blue dye as cationic agent that indicate for sulfated polyanions by complex formed in violet color (Farndale et al., 1976). For this, samples of LvSPs3h-1→4 or LvSPs24h-1→6 were prepared (~30 µg) and the *in vitro* tests evaluated in glass tubes (n = 3), being the color observed to be specific for SPs *vs*. the reagent property. The results were recorded by photographic images from a portable device after the assays.

Physical-chemical properties of LvSPs

In order to examine the charge/mass of LvSPs3h-1→4 or LvSPs24h-1→6, aliquots of each sample (~30→ µg), prepared in destilated water, were applied in two electrophoretic systems using distinct polymeric gels. As known markers of molecular mass, chondroitin-6-sulfate (C-6-S,~ 60 kDa), chondroitin-4-sulfate (C-4-S,~ 40 kDa), sulfated dextran (DexS,~ 8 kDa), dermatan sulfate (DS,~ 40 kDa) and/or UHEP (~ 15 kDa) were used as animal polysaccharides (Dietrich & Dietrich, 1976; Andrade et al., 2017).

Agarose gel

The extracted products were identified and compared on the polydispersion pattern and the charge density in a system of 0.5% agarose gel with ~0.2 cm thick (Dietrich & Dietrich, 1976). All the test samples applied in the gel, prepared with 0.05 M 1,3-acetate diaminopropane buffer (pH 9.0), were then submitted to at constant voltage (100 V) for ~1 h. After this procedure, the LvSPs were fixed in the gel with a 0.1% *N*-cetyl-*N*,*N*,*N*-trimethylammonium bromide solution for ~24 h and then dehydrated by heating at 50-55°C during ~ 6 h using an air drying oven. Finally, the dried gel was treated with a 0.1% toluidine blue or Stains-All solution to reveal the LvSPs after ~30 min or 24 h embedded in the respective dyes and, then, it was destained with a solution containing absolute ethanol, distilled water and acetic acid or destilated water until to visualization of distinct bands.

Polyacrylamide gel (PAGE)

The LvSPs present in the samples were also estimated and compared by molecular mass distribution in an electrophoretic system made of a 6% polyacrylamide gel with ~0.1 cm thick. All the test samples carefully applied (~30 µg) in the gel, prepared in 0.02 M Tris/HCl buffer (pH 8.6), were then sujected to an at constant voltage (500 mA) for ~1 h (Yoon et al., 2007). After run, the LvSPs were stained in the gel with Stains-All alone to reveal the LvSPs after ~30 or 24 h min in the presence of the respective dyes. Then, the gel was destained with distilled water until to identifying of distinct bands (Andrade et al., 2017).

Both gels that revealed the LvSPs and other acid residues were separatelly scaned and the images saved in Windows file to construct the integraded figure.

Spectral characterization of LvSPs by FT-IR technique

The structural features of LvSPs3h-1 and -4 or LvSPs24h-1 and -6 were obtained by FT-IR using a spectrometer (IRPrestige-21 Shimadzu, Japan). For this, each sample was separatelly weighed (~10 mg) and then pressed in potassium bromide (KBr). The measurements were generated from *pellets* at a resolution of 4 cm^{-1}, with 64 scans min^{-1} at wavenumber 500-4000 cm^{-1}. The spectral data and the graphicals were assigned and represented, respectively, using the Origin software version 8.0 as the Statistical Analysis Software (USA). All the graphicals of the samples were separatelly saved in Windows file to construct the integraded figure.

Statistical analyses

All the values were expressed as mean ± standard deviation (n = 3). For yield comparison among samples, statistical analysis was done by one-way ANOVA, followed by Tukey' test, applying $p < 0.05$ as significant. To examine the total yield between both extraction times, it was applied the *Studant*'-t test for unpaired consideranting $p < 0.05$. The statistical analyses were performed using the GraphPad Prism® version 5.01 for Windows (GraphPad Software, 1992-2007, San Diego, CA; www.graphpad.com).

Results and Discussion

Cell-wall and total yield of LvSPs

The complex wall matrix of flat decumbent material (*L. variegata*) showed in figure 1D that its cellular arrangement was to be a natural reservoir in SPs where their bioavailability varied or not along the sequential extraction process of the tritured algal tissue sujected to protease during the differential application of two periods (3 or 24 h) of proteolytic digestion (Figure 3).

As can be seen, the yield profile of LvSPs was drastically impacted by determined condition of time as the parameter of extraction used (Yaich et al., 2013). This significant effect on the yield of LvSPs was observed, not only among the reextracted materials, but also by number of reincubations performed to obtain the crude extracts of LvSPs between the two periods (Figure 3). Under 3 h digestion time (Figure 3A), this condition allowed us to obtain four different materials, yielding a highest extractable rate on average 7.62 ± 0.80% for first extraction (LvSPs3h-1) which significantly differed from LvSPs3h-2 (1.94 ± 0.19%), but not from other reextracted materials of the same algal residue (4.96 ± 2.29 (LvSPs3h-3) and 3.76 ± 1.94% (LvSPs3h-4), respectively, $p > 0.05$), on the basis of the dehydrated matter (w w^{-1}).

LvSPs3h-1 had highest amount in crude extract from tritured algal samples in according to other seaweeds species that sequentially yielded for cell-walls SPs, such as from Chlrophyta *Caulerpa cupressoides* var. *lycopodium* by Rodrigues et al. (2011), from Rhodophyta *Halymenia pseudofloresia* and *Acanthophora muscoides* by Rodrigues et al. (2009a, 2016); and from the same Ochrophyta *L. variegata* by Siqueira et al. (2011), although varying in yield, extended time of

extraction (24 h) and number of materials from the respective seaweeds. It is difficults to determine an analytical basis because the heterogeneity of seaweeds SPs and the diverse scientific groups using a variety of methodologies for extraction of these compounds (Leite et al., 1998; Zibetti et al., 2005; Yoon et al., 2007; Wang et al., 2008; Rodrigues et al., 2009a, 2017; Ananthi et al., 2010; Paiva et al., 2011; Yaich et al., 2013; Fidelis et al., 2014).

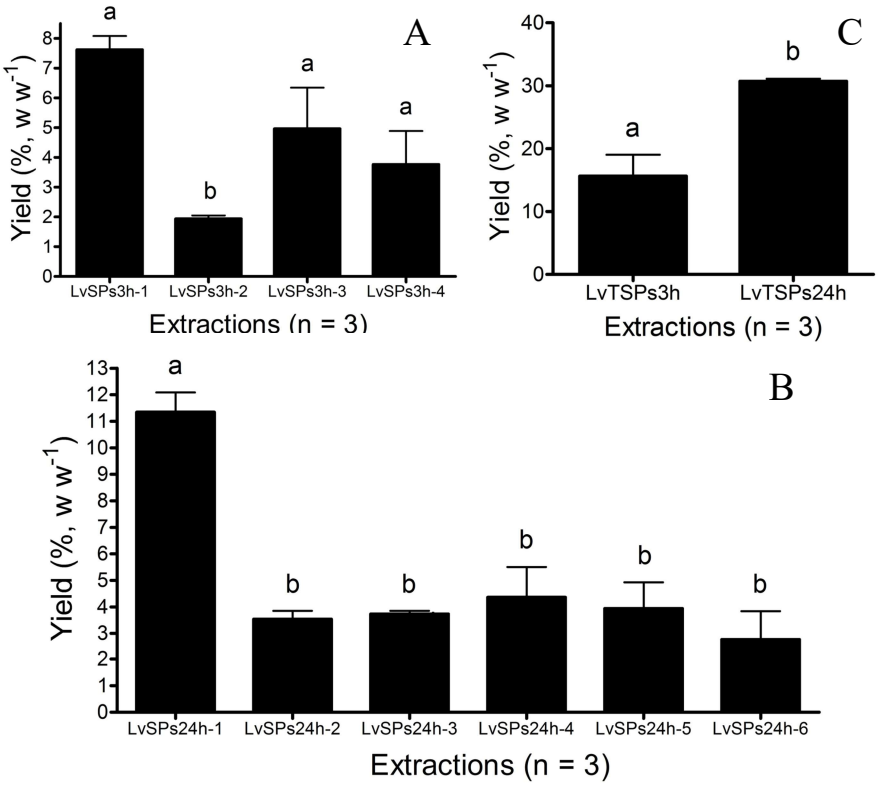


Figure 3. Effect of the extraction period (3 [A] or 24 [B] h) on the total yield ([C] %, w w^{-1}) of cell-wall SPs from the brown seaweed *L. variegata*. Different letters among LvSPs3h-1→4 or LvSPs24h-1→6 indicate differences at level of 5% (ANOVA, Tukey test, $p < 0.05$) or between LvTSPs3h and LvTSPs24h revealing difference at level of 5% (*Studant*'-t test, $p < 0.05$).

On a mass-to-mass basis, LvSPs3h-1 yielded ~3.92-fold higher than LvSPs3h-2 and, at least, ~1.53-fold higher than both LvSPs3h-3 and LvSPs3h-4 extracts. Therefore, there was a differential role of the protease action on the matrix structure to release a high concentration of SPs mainly located in the surface texture (Rodrigues et al., 2009, 2011, 2016) occuped by peripheral cellular layers (pigmented cells) that play important roles (Figure 1D), as the photosynthetic activity and the enviromental pressure (Stengel et al., 2011) where the seaweed was collected (Figure 1A). Reextractions with papain abruptally attacked the wall organization of *L. variegata* where the central cells (incolors cells) (Figure 1D) would play a function related to matrix structure on a biochemical composition of SPs which suitable the thallus (Zibetti et al., 2005; Pomin & Mourão, 2008; Rodrigues et al., 2016). Such properties could characterize a biological and biomechanical role of the LvSPs in the extracellular matrix (Pomin & Mourão, 2008; Popper et al., 2011).

Regarding higher extraction time (24 h), the prolongated digestion of tritured *L. variegata* tissue increased ($p < 0.05$) the yield about 1.48-fold higher (11.48 ± 1.28% yield for LvSPs24h-1) than LvSPs3h-1 (7.62 ± 0.46% yield) between times (Figures 3A, B), as expected (Zibetti et al., 2005; Yaich et al., 2013). More relevant was to the absence of influence of this extraction time on the yield among other five materials (3.54 ± 0.53 (LvSPs24h-2), 3.73 ± 0.21 (LvSPs24h-3), 4.36 ± 1.97 (LvSPs24h-4), 3.94 ± 1.71 (LvSPs24h-5) and 2.77 ± 1.84% (LvSPs24h-6), respectively ($p > 0.05$),

from the dehydrated matter (w w^{-1}), possibly due to the almost equal bioavailability of SPs embbeded in the cell matrix made of massive structure (Figure 1D).

These scenarios showed to a "homogeneous" profile of proteolytic action on the wall matrix composition, requiring an increased resilience to obtain and better characterize the yield of SPs present in *L. variegata*. Siqueira et al. (2011) sequentially extracted SPs from samples collected of *L. variegata* and found different five materials by papain method. These combined observations suggested that the bioavalilability of these polymers depended on the sample, time of collect and/or enviromental factors (Pomin & Mourão, 2008). Brazilian samples of Rhodophyta *H. pseudofloresia* (Rodrigues et al., 2009a) and *A. muscoides* (Rodrigues et al., 2016); and of Chlorophyta *C. cupressoides* (Rodrigues et al., 2011) yielded only different three materials, when a marked decrease in the yield of SPs was observed by papain method after 24 h digestion.

Comparing both total extraction yields (Figure 3C), the first matter (*L. variegata*) sequentially digested during 24 h (considerating all extracts) confirmed to be more effective for cell wall SPs obtaining, since that the percentage recovered ($p < 0.05$) in LvTSPs24h (30.69 ± 0.42%, w w^{-1}) was 1.96-fold higher in yield than that obtained in LvTSPs3h (15.63 ± 3.41%) from the dehydrated matter (w w^{-1}). *L. variegata* was a lower source in SPs than Rhodophyta *H. pseudofloresia* (47.14%, w w^{-1}; Rodrigues et al., 2009a), but was highest than Rhodophyta *A. muscoides* (23.47%, w/w; Rodrigues et al., 2016), Chlorophyta *C. cupressoides* var. *lycopodium* (4.61%, w w^{-1}; Rodrigues et al., 2011) and other samples (about 28%, w w^{-1}) of this same Ochrophyta species collected by Siqueira et al. (2011).

Because of these marked differences (Figure 3), the biochemical characterization of the various sequentially extracted products was further analyzed regarding their molecular features by comparative analytical techniques.

Analysis by mean of metachromasia

The various LvSPs-containing materials, sequentially extracted (3 or 24 h), were further checked for metachromasia prior to the physical-chemical analyses in order to monitor the presence of sulfation from the analyzed polymer samples. In general, the *L. variegata*-derived extracts exhibited the property, but it was progressively reduced with the availability of extracted LvSPs regarding their DMB dye-binding ability (Farndale et al., 1976). In figure 4 showed marked differences on the metachromasia among the crude extracts, of which LvSPs3h-1 required a high concentration for reveal such reaction.

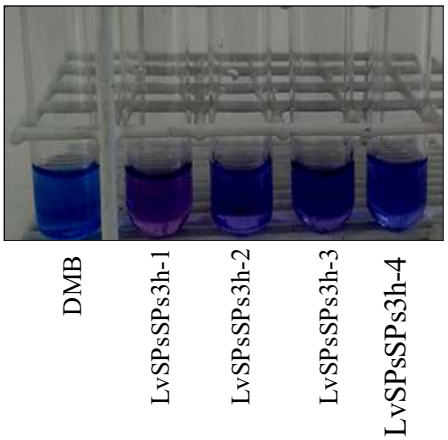


Figure 4. Metachromatic profile of the sequentially extracted crude SPs (3 h) from the brown seaweed *L. variegata*.

Extracts LvSPs3h-2→4 were almost indetectable of violet reaction for sulfated polymers, except for LvSPs3h-4 which did not has any change as the DMB control. Similar metachromatic profile was monitored for LvSPs24h-1→6, although it apparently showing more sulfated materials (data not shown). These results hypothetized on the existence to a different biochemical composition of the SPs occuping the cell wall of *L. variegata* playing different roles related to its aquatic biology (Coen & Tanner 1989; Popper et al., 2011; Stengel et al., 2011).

Charge/mass examination by electrophoreses

Electrophoretic techniques allowed us to examine the charge/mass of the LvSPs, sequentially extracted (3 or 24 h), as illustrated in figure 5. On a charge basis, all extracted materials (LvSPs3h-2→4 or LvSPs24h-2→6) were revealed on agarose gel, when treated with the toluidine blue dye, and the electrophoretic profiles confirmed those previous results found by metachromasia assay that indicated, at initial level, a marked difference among the analyzed polymer samples (Figure 4).

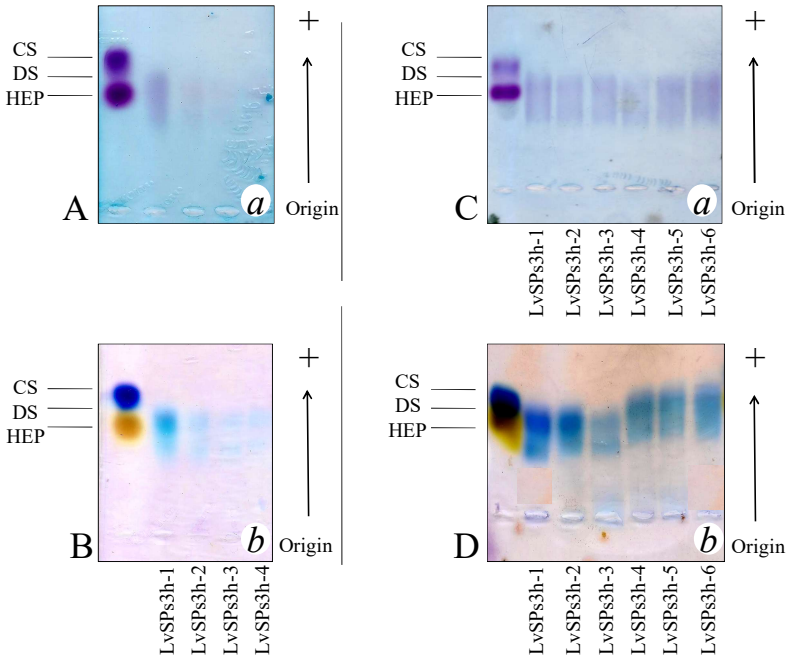


Figure 4. Agarose gel electrophoresis of *L. variegata* SPs (LvSPs3h-1→4 or LvSPs24h-1→6) and standards chondroitin-6-sulfate, dermatan sulfate (DS) and heparin (HEP) present on gels were stained with toluidine blue (*a*) or Stains-All (*b*).

Clearly, as already expected for unfractionated LvSPs (Paiva et al., 2011; Siqueira et al., 2011), agarose analysis of LvSPs3h-1 characterized as a polydisperse band containing negatively charged polysaccharides with electrophoretic mobility as HEP standard (Figure 4A), typical for seaweeds SPs (Rodrigues et al., 2009a; Fidelis et al., 2014), but the resolution pattern of SPs could vary among seaweeds species (Rodrigues et al., 2011, 2016). Although with coincident bands on gel, the staining pattern of other blots (LvSPs3h-2→4) progressively reduced on the extraction process. As the diamine buffer interacted with the sulfate groups of the LvSPs (Fidelis et al., 2014; Rodrigues et al., 2016) similar to animal SPs (Dietrich & Dietrich, 1976), it was speculated to an important difference on the charge density of sulfate ester groups in the chemical structures of these molecules, since that was possible identify the different LvSPs extracts (Fidelis et al., 2014). Therefore, such observations were peculiar for the LvSPs, when variably obtained after the time of extraction applied sucessively (3h) (Figure 2).

In fact (Figure 4C), the sequentially digested tissue with the protease (papain) for 24 h led to a molecular profile of metachromatic bands coincidents among each material (LvSPs24h-1→6) as commonly found for other seaweeds SPs extracted using this same technique, such as in Rhodophyta *H. pseudofloresia* (Rodrigues et al., 2009a) and *A. muscoides* (Rodrigues et al., 2016); and Chlorophyta *C. cupressoides* var. *lycopodium* (Rodrigues et al., 2011), although varying in the resolution pattern of the matrix SPs.

Treatment with Stains-All dye led to an increased visualization of bands and standards in the gel (Rodrigues et al., 2017) (Figures 4B, D), whose algal materials (LvSPs3h-1→4 or LvSPs24h-1→6) comigrated as polydisperse components, suggesting that they have same charge/mass ratio and structural conformation on the algal matrix (Fideli et al., 2014) and the cyan-like coloring deduced that *L. variegata* synthetized non sulfated residues (like uronic acid) that were not observed after treatment with toluidine blue from the samples (Rodrigues et al., 2017). Acid sugar residues are commonly found in Ochrophyta SPs (Leite et al., 1998; Yoon et al., 2007; Wang et al., 2008; Pomin & Mourão, 2008; Paiva et al., 2011) as part of their native structures. Therefore, both cationic dyes separately characterized, at initial level, the LvSPs on a basis of electrostatic density in the matrix composition.

In order to elucidate the molecular mass profile, PAGE clearly showed LvSPs, stained with Stains-all alone, to be of wide dispersion in their molecular sizes (Figure 5). Comparison among LvSPs3h-1→4 observed mobilities these LvSPs extracts showing apparent mass distribution from ~8 to < 100 kDa based on standards (Andrade et al., 2017), as also found for a SPs fraction from Chlorophyta *C. cupressoides* var. *lycopodium* (Rodrigues et al., 2017) and for those from Ochrophyta *L. cichorioides* (Yoo et al., 2007), denoting SPs with extremely variable molecular weights among seaweeds species. Since that LvSPs3h-1→4 exhibited a pattern of detection for cyan in the gel like animal hyaluronic acid (Andrade et al., 2017), it also supported the agarose gel analysis (Figures B, D) (Rodrigues et al., 2017). Similar results were found for LvSPs24h-1→6 regarding electrophoretic behaviour and intense cyan propriety in the gel (data not shown).

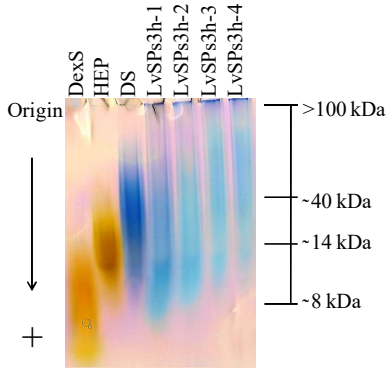


Figure 5. PAGE of *L. variegata* SPs (LvSPs3h-1→4) and standards dermatan sulfate (DS, ~40 kDa), heparin (HEP, ~14 kDa) and dextran sulfate (DexS, ~8 kDa) present on gel were stained with Stains-All.

SPs from red seaweeds have been usually described as high molecular masses (Pomin & Mourão, 2008; Pomin, 2012), but variations in size for those isolated from brown and green seaweeds have been fractionated by chromatographic columns which separate the material into polisaccharidic fractions of <100kDa (depending on the structural complexity), when identified by PAGE (Yoon et al., 2007; Rodrigues et al., 2016, 2017), or by other extraction conditions (Fidelis et al., 2014). Findings were intriguing because the cell wall of *L. variegata* preponderantly constituted by acid sugars residues in high concentrations and of low molecular sizes embbeded in the complex amorphous matrix, since that both time of extraction (3 or 24 h) did not degrade the LvSPs to other fragments, based on ulvan-type polysaccharides which are rich in uronic acid (Yield et al., 2014).

On the acidic composition, alginic acid could be coextracted, since Ochrophyta are also natural sources these compounds (Torres et al., 2007). The prolonged use of extraction (24 h) suggested as the better way to partially characterize LvSPs, on their biochemical characters (charge/mass), derived from the wall matrix. Studies by mean of FT-IR spectroscopy was further carried out to explore the system of SPs present in *L. variegata* cell wall on the basis of their structural composition.

Structural analysis by FT-IR

In order to study the functional groups present in LvSPs, extracts LvSPs3h-1 and -4 or LvSPs24h-1 and -6 were chosen and compared for structural analysis by FT-IR spectroscopy. Results were also compared to other polysaccharides isolated and characterized as a qualitative basis of various chemical features (Ronghua et al., 2003; Torres et al., 2007; Ananthi et al., 2010; Paiva et al., 2011; Fidelis et al., 2014; Rodrigues et al., 2017). Overral, it was found a structural regularity among LvSPs 3 or 24 h samples, which supported their electrophoretic differences, especially in sulfation and acid sugars residues after 3 h of the extraction process (Figure 6).

Both spectra (LvSPs3h-1 and -4) at 500-4000 cm^{-1} revealed a structural composition of the extracellular matrix SPs, but showing more detailed information that in study of Paiva et al. (2011) for SPs from samples of *L. variegata* collected on the northeast coast of Brazil, although using maxatase digestion. The 3398-3412, 2935, 1612-1615 and 1415 cm^{-1} bands were assigned to the axial deformation of O-H, C-H for the secondary (-CH_2-) and primary (-CH_3) bonds and angular deformation of the C-H bond of the both primary and secondary carbons, respectively, in conformity with those found by Paiva et al. (2011). Intense signals at 1612-1615 and at 1415 cm^{-1} were to be uronic acids from LvSPs3h-1 and -4 samples confirming the presence acidic sugar residues (Ananthi et al., 2010; Fidelis et al., 2014), as suggested by electrophoretic analyses (Figure 4B, D and Figure 5) (Rodrigues et al., 2017).

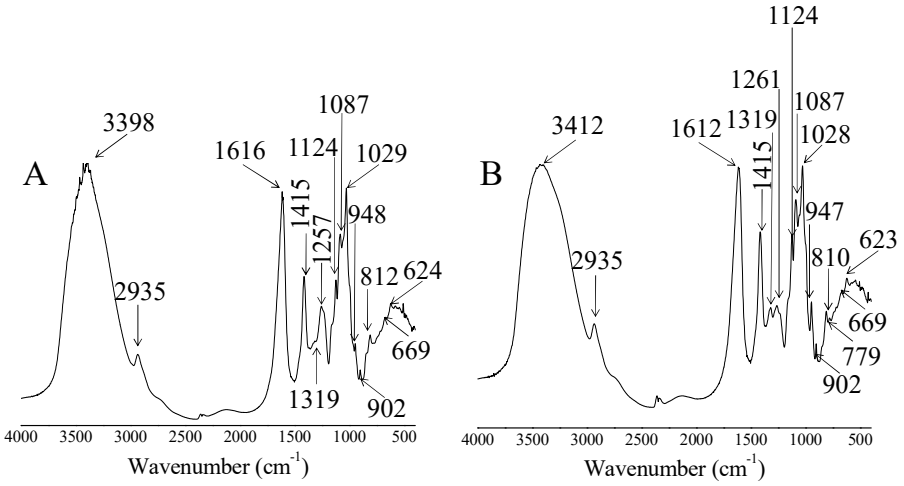


Figure 6. FT-IR spectra of the *L. variegata* SPs (LvSPs3h-1 [A] and -4 [B]) at 500-4000 cm^{-1}.

Interestingly, absorption values around 947-948, 902 and 810-812 cm^{-1} were associated to alginic acid in both preparations, since that respective signals were attributed to guluronic acids and mannuronic acids (Ronghua et al., 2003). This component was also coextracted with the sulfated fucan from *L. variegata* because at 1257-1261 cm^{-1} and at 810-812 cm^{-1} indicated that sulfate groups would be located at positions 2 and 3 of the polymer (Ananthi et al., 2010), however, did not reveal in LvSPs3h-1 and -4 samples sulfation around 850 cm^{-1}, demonstrating that the SPs from *L. variegata* in this study varied their sulfation regions in comparison with that isolated in another study (Paiva et al., 2011).

There was a drastical difference regarding the intensity of total sulfation (at 1257-1261 cm^{-1}) between LvSPs3h-1 and -4 (Figure 6) supporting electrophoretic analysis (Figure 4) and evidenced to a distinct electrostatic environment on the algal cell wall composition, as well as the additional presence at 779 cm^{-1} related to a discret sulfation (Ronghua et al., 2003; Rodrigues et al., 2017) in only LvSPs3h-4. Signals from 623-624 to 669 cm^{-1} were common in both spectra of acid polysaccharide. Collectively, all these functional groups were identified in both LvSPs24h-1 and -6 extracts, but with higher intensities, except to the absence at 779 cm^{-1} and to the presence at 545-586 cm^{-1} (data not shown).

Given the yield and molecular analyses, flat decumbent form of *L. variegata* has some variation in its wall composition that appers to has taxonomic value and biological relevance (Popper et al., 2011; Pomin & Mourão, 2008). This taxon has morphological and biochemical aspects that were adapted to its coexistence to herbivory (Coen & Tanner 1989) and the acidic polysaccharide-rich cell wall would shows to be determinant to enviromental pressure (*e.g*, habitat, season, and life cycle stage) (Popper et al., 2011), since that in seaweeds there are a trend that major structural features of SPs to be conserved in each phylum, although a fascinating challenge (Pomin & Mourão, 2008). As this algal species is an abundant source in SPs exhibiting various properties for biotechnological use (Rodrigues et al., 2009b; Siqueira et al., 2011; Paiva et al., 2011; Kremb et al., 2014), our study more provided information on the biochemical composition of SPs in order to contributes with the comparative research to better understanding the wall anatomy at structural and biochemical level of acidic SPs synthetized by *L. variegata* found commonly in Brazilian coastal. An in-depth analysis comparing the three morphotypes of this species deserve to be explored as an adaptative defense strategy to a better knowledge of its chemical biology.

Conclusion

The brown seaweed *Lobophora variegata* has three morphologies in nature and that of flat decumbent presents a wall anatomy formed by seven cellular layers that, when sequentially digested with papain for 3 or 24 h, releases distinct profiles in yield, expressing biochemical features of sulfated polysaccharides. The prolonged proteolytic action on the wall matrix revealed as an optimal condition to obtain and extensively characterize the yield and the molecular features of sulfated polysaccharides, without presenting chemical degradation. Analytical techniques by electrophoreses and infrared spectroscopy suggested that its cell wall was rich in both heterofucans and alginic acid-type polysaccharides of low molecular masses having small structural variation. Such complex wall composition could suggest as a taxonomic adaptation of the chemical biology of this species to herbivory, when exposure to enviromental pressure.

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