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EXTRACTION AND CHARACTERIZATION OF R-PHYCOERYTHRIN FROM MACROALGAE *Solieria filiformis* USING PRESSURIZED FLUID

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ABSTRACT

The present study investigated the extraction of R-phycoerythrin (R-PE) from the macroalgae *Solieria filiformis* using an environmentally friendly approach, precisely the pressurized water method. The study analyzed the extraction process and assessed the macroalgae's structure. Two freeze-dried and wet biomass samples were analyzed, with 1 % and 10 % m/v concentrations under pressures up to 500 bar. R-PE maximum yield was reached for lyophilized seaweed structure after 360 min, obtaining 670 μg of R-PE per gram of dry biomass. Characterization of crude and precipitated extracts included UV-VIS spectrophotometry, electrophoresis, and circular dichroism. Furthermore, the precipitated extract's cytotoxic activity was assessed through an MTT assay against tumor cell lines of colorectal cancer (HCT116), prostate cancer (PC3), glioblastoma cancer (SNB-19), and HL60 (promyeloblast leukemia). The extracted biomolecule exhibited characteristic R-PE spectra, fluorescence, and an α-helix structure as the predominant element of its secondary structure, suggesting the presence of other proteins in the precipitated extract. The most prominent in vitro results were in HL-60 and HCT-116 cell lines.

Keywords: Red macroalgae. Fluorescent protein. Pressurized extraction. Extraction yield.

1 INTRODUCTION

Solieria filiformis, a red macroalgae found throughout the Brazilian coast, has garnered significant scientific interest because of its high added value as a source of bioactive compounds[1]. Its composition has sulfated polysaccharides, secondary metabolites, and phycobiliproteins, with R-phycoerythrin (R-PE) being the predominant protein[2]. R-PE is a fluorescent molecule highly water-soluble with a molecular weight of 240 kDa, exhibiting absorption peaks at 498, 540, and 565 nm and fluorescence emission at 575 nm[3]. Once extracted, R-PE finds broad application across industrial sectors such as pharmaceuticals, cosmetics, and food. This is owing to its anti-inflammatory, antioxidant, and anticarcinogenic capacities and its role as a natural, non-toxic dye[4–6]. Efficient extraction of R-PE involves breaking the cell wall to release the intracellular protein into the aqueous environment. Several extraction methods are available in the literature to obtain phycobiliproteins. However, because of its complex structure (cell wall polysaccharides, properties of bioactive compounds, and chemical structure), these methods may not produce high purity or yield extracts.

Some of these methods involve using toxic solvents and high energy consumption[5,7,8]. Therefore, extracting high-value biocompounds using green technologies is particularly appealing, with techniques like pressurized liquid extraction (PLE) offering environmentally friendly and efficient extraction means. PLE is an advanced solid-liquid extraction method that enhances the diffusivity and solubility of target molecules in solvents, thereby accelerating the process of bioactive removal into the aqueous medium[8]. Numerous studies have been carried out in the literature on removing phycobiliproteins using pressure. However, data regarding R-PE protein from macroalgae, especially without any grinding process, are scarce. Based on this premise, this study aimed to investigate the R-PE extraction process from the macroalgae *Solieria filiformis* using pressurized water at 500 bar to obtain crude extracts with a higher yield. The study considered two different samples (wet and lyophilized macroalgae) and focused on evaluating extraction time and protein biofunctionality.

2 MATERIAL & METHODS

Solieria filiformis macroalgae was cultivated on the beach of Flecheiras in Trairi (Ceará). The seaweed was harvested and thoroughly rinsed with running and distilled water to eliminate sand and other unwanted contaminants. Subsequently, the macroalgae was frozen, and some biomass was subjected to lyophilization for 72 h. The high-pressure apparatus comprises a syringe pump (Teledyne ISCO 260D, USA) and a low-friction, high-pressure floating piston transfer cylinder (Vinci Technologies/France). Here, this equipment is named extraction cell[9]. The wet (10 % m/v) and lyophilized (1 % m/v) macroalgae, used without any grinding process, were independently suspended in ultrapure water at room temperature (25 °C) and pressure of 500 bar. Samples were collected at specified time intervals ranging from 15 to 360 min. Following each extraction step, the samples were centrifuged at 9500 ×*g* for 30 min at 4 °C. The resulting supernatants were crude wet seaweed extract (WCE) and crude lyophilized seaweed extract (LCE). They were analyzed to quantify total proteins[10], purity index (PI), and R-PE yield[11]. From the crude extracts (CE), a protein precipitation process was performed using 90 % (m/v) of $(NH_4)_2SO_4$. These results lead to the formation of the wet seaweed precipitated extract (WPE) and the lyophilized seaweed precipitated extract (LPE). Electrophoresis and cellular dichroism (CD) analysis techniques characterized the R-PE extracts obtained. Furthermore, the precipitated extract's

cytotoxic activity was analyzed through an MTT assay against tumor cell lines of colorectal cancer (HCT116), prostate cancer (PC3), glioblastoma cancer (SNB-19), and (promyeloblast leukemia).

3 RESULTS & DISCUSSION

Figure 1 shows the concentration of total proteins (1a) and R-PE yield (1b) obtained at different extraction times under pressure of 500 bar. The concentration of total soluble proteins ranged from 6 to 13 μg mL^{-1} and 27 to 53 μg mL^{-1} for the WCE and LCE, respectively. In the LCE, the yield ranged from 408 to 661 μg g^{-1} of dry biomass, whereas in the WCE, the yield ranged from 13 to 57 μg g^{-1} of wet biomass. Such results demonstrate that the structure of freeze-dried algae exhibits higher extraction efficiency, both for total proteins and R-PE, compared to the structure of wet algae. In the lyophilization process, the seaweed is frozen, and the ice crystals sublimate, suggesting a weakening cell wall connectivity, thus allowing the release of otherwise difficult proteins to extract[12]. The cell wall rupture without a lyophilization process (WCE) proves less effective, considering the low concentration of total proteins and the R-PE pigment obtained (Figure 1, white columns). However, LCE demonstrates the extraction of other phycobiliproteins, such as phycocyanin and other protein families. Thus, LCE exhibits a lower purity index (PI=0.15) when compared to WCE (PI=0.41).

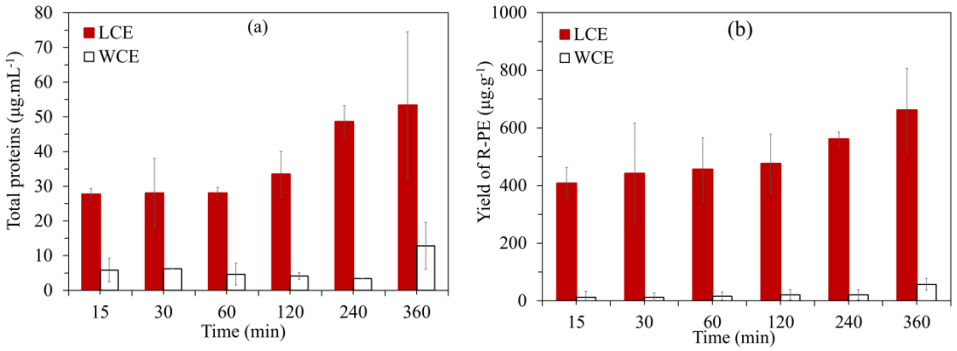

Figure 1 Effect of time on total protein concentration (a) and R-PE yield (b) obtained by pressurized extraction of *Solieria filiformis* over 360 min, under 500 bar pressure, for wet and lyophilized biomass.

The time and structure of the macroalgae (wet or freeze-dried) under pressure play a crucial role in R-PE extraction. The whole structure, without any grinding process, is a critical factor to be studied as it may eliminate a step in the extraction system. Bastos Filho[13] analyzed the extraction of R-PE from wet and ground *Solieria filiformis* macroalgae, reaching 312 μg g^{-1} in 6 hours of extraction. In this study, employing a pressurization system (500 bar) and utilizing the structure of the entire freeze-dried biomass, an R-PE yield of 441 μg g^{-1} was achieved in 30 min of extraction. These results allow for the observation of the effect of pressure on reducing extraction time. The pressure treatment at 500 bar did not show significant changes in the absorption spectrum. The WCE exhibited absorption peaks at 499 nm, between 540 and 564 nm, and a fluorescence peak at 576 nm. At the same time, the LCE showed absorption peaks at 499 nm, between 540 and 563 nm, and a fluorescence peak at 575 nm. Both extracts exhibited characteristics consistent with R-PE[13].

To confirm the purity of R-PE extracts, the electrophoretic profile of CE (WCE/LCE) and PE (WPE/LPE) were analyzed under denaturing conditions in the presence of the reducing agent β-mercaptoethanol. R-PE molecule has three different subunits in its structure (α, β, and γ)[6]. Following ammonium sulfate pre-purification, a significant increase in purity index occurred only for LPE from 0.14 to 0.25 and remained at 0.41 for WPE. A strong band at 66.0 kDa is observed in the LPE characteristic of the dimer (αβ). Discrete bands between 45.0 kDa and 35.0 kDa are observed in PE, which may represent the γ band. Bands between 30.0 and 20.1 kDa correspond to the α and β bands. Different bands in the 14.4 and 20.1 kDa range are present in all samples, which characterizes the presence of other phycobiliproteins (18 kDa) and other protein families[14].

Circular dichroism (CD) spectroscopy was used to characterize the secondary structures of the pressurized-extracted R-PE pigment, providing relevant information about the R-PE's conformation and structural organization, as shown in Figure 2. The CD spectrum for the precipitated extracts (LPE and WPE, Fig. 2A) showed negative ellipticity from 201 nm and a peak in the upbeat band at 192 nm, typical of α-helix[14]. In contrast, the CE did not show characteristic conformation of the secondary structures of R-PE. This is due to the amount of interferents present in the CE, such as sulfated polysaccharides and other families of proteins present. When subjected to different temperatures (20-90°C), the spectra revealed that the secondary structure of the pigment undergoes significant alterations from 70°C onwards, transitioning from ordered structures to disordered ones. However, they still retained α-helix characteristics for LPE. The results indicated irreversible denaturation of R-PE and a highly stable natural pigment[14].

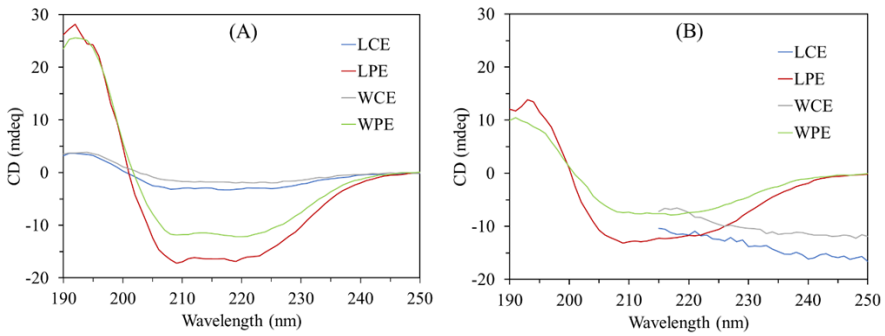

Figure 2 Circular dichroism (CD) spectrum of R-PE of crude and precipitated extracts of Solieria filiformis (A) and CD spectrum post-melting of the individual fractions (B).

The R-PE molecule cytotoxicity analysis was obtained from WPE and LPE and analyzed in four tumor cell lines: HCT-116, SNB19, PC3, and HL60. None of the samples demonstrated activity against the SNB-19 cell line. Inhibition for the promyeloblast leukemia tumor lineage (HL-60) featured the best result, with around 84 % and 85 % WPE and LPE, respectively. In this study, the LPE extract presented the lowest IC_{50} of 61.27 μg mL^{-1} in the HL60 leukemic lineage, this being the best result obtained, in addition to 153.0 and 306.2 μg mL^{-1} for PC3 and HCT116, respectively, after 72 h of incubation.

Table 1 Cytotoxic potential of R-PE extracted from the macroalgae *Solieria filiformis* evaluated by the MTT method for cancer cell growth. ND - Not determined.

Cell line tested	LPE	WPE
	500 μg mL^{-1}	500 μg mL^{-1}
Colorectal cancer (HCT-116)	69.05% ± 2.50	70.76% ± 4.78
Prostate cancer (PC3)	60.17% ± 3.95	52.93% ± 2.73
Glioblastoma cancer (SNB-19)	38.55% ± 3.96	48.35% ± 1.01
Leukemic (HL-60)	84.96% ±10.46	83.79% ± 3.16

The pressurized extraction method addressed in this study presented the advantages of the R-PE extraction, which did not involve any additional chemical solvent during the process and did not generate any other waste. The temperature conditions are mild, at room temperature (25 °C), with no temperature increase during the process, (thus) avoiding protein degradation. In this regard, it is inferred that pressurized extraction is an attractive alternative to non-aggressive extraction with biological activity of interest for the formulation of biotechnological products in different industrial sectors.

4 CONCLUSION

The extraction of R-PE from the macroalgae *Solieria filiformis* by the pressurized water process proved to be an attractive alternative for non-aggressive extraction with biological activity of interest, reaching a yield of R-PE of 0.66 mg g^{-1} of the dry algae from lyophilized biomass, being this structure more efficient in this study, under conditions of 500 bar, ultrapure water as extraction solvent and 360 min of process. Electrophoresis showed low purity rates due to the high extraction of carbohydrates, phycobiliproteins, and other proteins with similar molecular weight. The extract containing R-phycoerythrin obtained in this study is active against cancer cells (strain leukemic HL60). More studies that approach the size of the biomass structure and the pressure variation are necessary for an improvement in the yield of R-PE obtained from the macroalgae *Solieria filiformis*.

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