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RESEARCH

Tailoring Seaweed Proteins: Impact of Extraction Techniques on Functional Properties

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Abstract

The increasing demand for food proteins caused by the exponential increase in world population has led to a deep search for novel protein sources. Seaweed emerges as a promising biomass with interesting nutritional composition and environmental performance. In this work, proteins were extracted from red seaweed *Porphyra dioica* using a sequential extraction methodology (H_2O/NaOH). Then, extracts were purified using to ultrafiltration (UF) or precipitation followed by ultrafiltration (PUF). Proteins were characterized in terms of physicochemical characteristics (molecular weight and structure) and functional properties (gelling, thickening, emulsifying, and foaming capacity). The results showed that untreated samples had good protein recoveries (23.5 ± 0.7 and $28.2 \pm 1.0\%_{\text{ Protein}}$ for H_2O and NaOH extracts, respectively) that were in the same range of the ones obtained after UF and PUF. However, the protein content in PUF extracts (81.33 ± 0.10 and $77.61 \pm 0.81\%_{\text{ Protein}}$ for H_2O and NaOH extracts, respectively) was much higher than the protein content in untreated and UF extracts (ranging from 15 to 29%), with an almost total removal of other components. On the other hand, the purification step caused changes in the structural characteristics of the extracted proteins, namely regarding the secondary structure. These did not prevent interesting functional properties from being obtained, such as emulsifying (72–100%) and foaming capacities (67–167%). Then, depending on the intended objective, different treated/untreated protein extracts can be chosen, targeting different functional properties, to be incorporated into the development of new food products.

Keywords Seaweed · Protein · Extraction · Purification · Physicochemical characteristics · Functional properties

Introduction

Seaweed are marine organisms with high value components and are increasingly recognized as a healthy and nutritious food, helping to meet the growing demand for protein driven by the exponential increase in world's population (Urlass et al., 2023). However, they have been mainly recognized for having high carbohydrate content (e.g., sulfated polysaccharides such as carrageenans) in cell walls, being widely used as a source of biopolymers with good functional properties (e.g., gelling, thickening, and stabilizing properties) (Cebrián-Lloret et al., 2024).

From an economic and sustainability point of view, in comparison to animal proteins, algae production involves much lower land (< 2.5 m^2/kg protein) than beef (144–258 m^2/kg protein), pork (47–64 m^2/kg protein), and chicken (42–52 m^2/kg protein), and involves the use of fewer resources. Thus, access to affordable, efficient, and sustainable proteins is enabled, driven by the rapid growth of the vegetarian market, by the consumers' interest in healthy and eco-friendly food options, and by climate change concerns (Cebrián-Lloret et al., 2024; Pereira & Rodrigues, 2021; Urlass et al., 2023). Also, compared to other plant-derived proteins (e.g., pea and soy), farming algae requires lower and non-arable land, reduced need for freshwater, and can be farmed in the ocean (Geada et al., 2021; Urlass et al., 2023).

Red seaweeds are the oldest eukaryotic macroalgae present in both freshwater and marine environments and are known to have a prominent feature regarding their distinctive coloration attributed to phycobiliproteins. Among them, *Porphyra* (also known as Nori) is used in Asia countries for sushi production and as a soup ingredient, providing

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different important biomolecules with low caloric value and high protein content (between 17 and 47% DW) (Rawiwan et al., 2022; Suresh Kumar et al., 2014). The recovery of proteins from red seaweeds can be done using different approaches that involve the use of conventional solvent extraction, which is directly affected by extraction conditions (e.g., time and temperature). Alkaline environments are frequently used due to the higher solubility of seaweed proteins under these conditions. However, some drawbacks are associated with high alkaline concentrations/pH, including, among other issues related to extensive extraction times and high solvent consumption, (i) protein denaturation and (ii) co-extraction of undesired compounds. To overcome these issues, distilled water and buffers can be used, but they come with lower extractability rates and lower purity extracts. In a previous work conducted by our research group, the solubilization of *P. dioica* proteins was studied in different solvents, and water and 0.1 M NaOH were the ones that showed better results (Nunes et al., 2024a). This helped to decrease both time and solvent consumption, and further application of UF techniques and protein precipitation helped to rapidly and selectively eliminate some molecules that could interfere with the functional properties of the obtained extracts. Alternatively, several technologies (e.g., high-pressure homogenization and ohmic heating) may also be combined with traditional methodologies to improve protein extractability and efficiency.

Proteins (either animal or non-animal) are recognized to have many functional properties (e.g., gelation, foaming, and emulsifying properties) that can be compared or even higher than those obtained from traditional protein isolates (e.g., whey protein). However, the need for downstream processing (extraction–fractionation–purification–concentration) to get a functional protein concentrate that can meet nutritional requirements and specific technological functions is still a challenge when the use of seaweeds is intended (Felix et al., 2021; Pereira & Rodrigues, 2021). The functionality of proteins in a food system depends on their global physicochemical properties (e.g., surface charge, hydrophobicity, molecular weight, thermal stability, among others), which arise from their structural characteristics. Also, the ability to act as emulsifiers, stabilizers, and/or gelation agents often involves protein denaturation since its unfolding results in the exposure of hydrophobic groups and free thiols that increase protein reactivity and allow the formation of new intra- and inter-molecular interactions that favor the establishment of aggregation and network formation (Pereira & Rodrigues, 2021; Wilding et al., 1984).

Since seaweed (in particular *P. dioica*) are commonly used in the food sector for their polysaccharide fraction, and despite the existence of well-established methods for protein extraction, the lack of studies regarding the influence of these methods on protein chemical and structural features, as well as on protein functionality, tends to limit the application of protein-enriched fractions of red seaweeds in foods when the final objective is to develop new products with not only improved nutritional composition but also with functional characteristics.

Thus, the main objective of this work was to assess the impact of different extraction and purification strategies on the protein recovery and structure and on the technological functionality of protein-enriched fractions obtained from *P. dioica*. In particular, this work aimed to:

1) produce protein-enriched fractions through a sequential non-thermal extraction with H_2O and NaOH, further purified by ultrafiltration or by salt precipitation followed by ultrafiltration;
2) characterize extracted *P. dioica* proteins in terms of physical (e.g., molecular size and distribution), chemical (e.g., protein solubility and surface charge), and structural (e.g., secondary structure) features;
3) study the existence of promising techno-functional properties, relevant to the possible use of *P. dioica* as a non-animal protein source for the design of novel food products; and
4) assess the impact of the extraction and purification steps on protein recovery, structure, and functionality.

Materials and Methods

Materials

The seaweed used in this work was kindly provided by ALGAplus (Ílhavo, Portugal). This biomass was produced in aquaculture performed in a land-based/on-shore system within the concept of an integrated multi-trophic aquaculture (meaning that the dissolved inorganic nitrogen input to the seaweed farm is higher due to the use of the effluent water from fish production). The biomass was washed with seawater, centrifuged to remove excess water, and then dried in a forced air-tunnel at a set temperature of 25 °C until reaching a moisture content below 12%, according to the processing methodology performed at ALGAplus.

Analytical grade chemical was purchased from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO, USA).

Methods

The applied methodology for *P. dioica* protein extraction, purification, and characterization was performed as described in Fig. 1.

The *P. dioica* used was composed of $27.68 \pm 0.05\%$ of protein, $45.69 \pm 0.65\%$ of carbohydrates, $4.25 \pm 0.79\%$ of

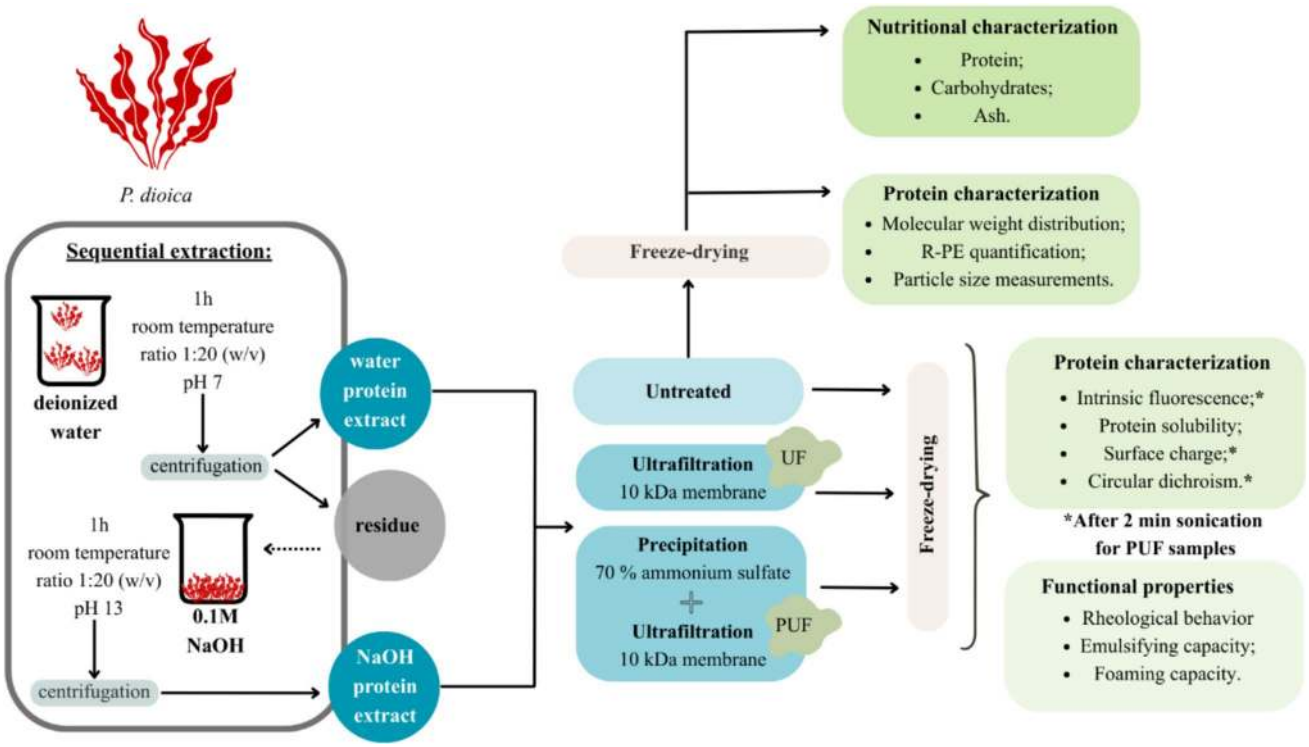

Fig. 1 Schematic workflow regarding the main stages of this work

lipids, and 28.72 ± 1.15% of ash, already used in a previous work (Nunes et al., 2024b).

Protein Extraction

The protein from red seaweed *Porphyra dioica* was extracted sequentially using distilled water (ratio of 1:20 solid/liquid, at pH 7) and 0.1 M NaOH (150 mL, pH 13) for 1 h under magnetic stirring at room temperature. After the water extraction, the sample was centrifuged at 4000 × g for 30 min, and the supernatant was collected (water protein fraction). The residue from the centrifugation step was used for the NaOH extraction. The obtained extract was separated from the final solid residue by centrifugation at 4000 × g for 30 min. Both extracts were kept at − 20 °C until further use.

Protein Purification

Two different approaches were used for the purification/concentration step: (1) the extract was concentrated and purified using ultrafiltration; (2) a protein-rich fraction was precipitated using ammonium sulfate and salts were then removed using ultrafiltration.

Ultrafiltration An initial step of UF was realized for both H_2O and NaOH extracts to reduce the level of salts that were co-extracted together with other components and are also present in the seawater used in the washing step. The process was performed using Vivaflow® 50R membrane (Sartorius Stedim Lab Ltd., Stonehouse, UK) of 10-kDa molecular cut-off (MWCO) that allows the passage of salts but retains proteins with higher molecular weight. After the dialysis process for about 30 min, with solvent volume repositioning (deionized water in both cases). Extracts were allowed to pass the membrane for a time long enough to reduce to half of the initial volume and decrease the total volume of the sample. Concentrated extracts were then frozen at − 20 °C and then freeze-dried.

Ammonium-Sulfate Precipitation*Ammonium-Sulfate Precipitation* Using the same extraction basis as described above (see the "Protein Extraction" section), extracts of H_2O and NaOH were purified using ammonium-sulfate precipitation. The method used was similar to the one described by Duong-Ly and Gabelli (2014) with some modifications. Thus, after extraction, protein was removed from the rest of the molecules using 70% ammonium sulfate saturation (using 43.6 g of solid ammonium sulfate/100 mL of solution) and left under constant magnetic stirring at 4 °C overnight. The stir bar was collected, and the precipitated proteins were removed by centrifugation at 18,000 × g for 30 min. The pellet was resuspended in the same initial volume of deionized water and dialyzed with a 10-kDa ultrafiltration membrane. Extracts were frozen at − 20 °C and then lyophilized.

Extracts Chemical Characterization

Extracts obtained after UF and PUF treatments were characterized in terms of chemical composition.

Kjeldahl Method The protein content of each extract obtained was analyzed using the Kjeldahl method by quantifying the total nitrogen after sample acid digestion using an automatic Kjeldahl digestor unit (Foss Analytics, Hilleroed, Denmark), applying the nitrogen conversion factor of 4.59 (Mlambo et al., 2022). All determinations were run in triplicate, and results were reported as a percentage of protein.

High-Performance Liquid Chromatography (HPLC) The determination of sugars in the obtained extracts was carried out based on the NREL protocol (NREL/TP-510–42623) using HPLC analysis (A. Sluiter et al., 2006). The soluble sugars present in the liquid fraction were quantified directly by HPLC with no need for previous treatments. On the other hand, sugars that are present in oligomeric form were processed into their monomeric form by hydrolysis. Thus, 4 g of each liquid extract was mixed with distilled H_2O until 23.96 g of liquid was measured, and then 1 g of H_2SO_4 98% was added. The hydrolysis was performed in an autoclave at 121 °C for 15 min. Finally, the hydrolysates were filtered through 0.22-µm membranes and analyzed by HPLC with a refractive index detector using the following parameters: Aminex HPX-87H column at 60 °C; mobile phase of 0.05 M H_2SO_4 at a flow rate of 0.6 mL/min. The sugar quantification was performed using a calibration curve of each standard (glucose, galactose, xylose, arabinose, and galacturonic acid) at concentrations between 0.0625 and 2 g/L. All experiments were run in triplicate, and the results are presented as g monosaccharides equivalent per 100 g of extract.

Ash The ash content in UF and PUF extracts was determined according to NREL procedures with some modifications (NREL/TP-510–42618) (Sluiter et al. 2012). Briefly, 0.3 g of dried extract was weighed into ceramic crucibles and dried overnight at 105 °C. The crucibles were allowed to cool to room temperature and then put in a muffle furnace (ECF 12/6, Lenton, UK) at 575 °C for 14–16 h and weighed after cooling down to room temperature to determine ash content. All determinations were run in duplicate, and the results are presented as percentages.

Protein Characterization

Gel Permeation Chromatography

To assess the molecular weight distribution of proteins from each extract, an analysis using HPLC gel permeation chromatography (GPC) was performed on a PolySep-GFC-P-4000 column (300 × 7.8 mm, Phenomenex®, USA). Each lyophilized extract was redissolved in deionized water to a final concentration of 1 mg mL^{-1} and eluted in ultrapure water using a flow rate of 0.8 mL min^{-1} at 40 °C with RI and UV detection and molecular weights were calculated based on a calibration curve performed using standard pullulan kit P-82 (Shodex™, Japan) within a range of 6.3–642 kDa (Castro-Ferreira et al., 2022).

Phycoerythrin Quantification

The concentration of phycoerythrin in the extracts (which were previously set to 0.1 mg/mL of protein concentration) was determined using a UV–Vis spectrophotometer (V-560, Jasco Inc., Tokyo, Japan) at wavelengths from 450 to 800 nm. Typically, phycoerythrin has absorbance peaks at 492, 545, and 565 nm. The concentration of phycoerythrin (mg/mL) was estimated based on the Beer Eshel equation (Eq. 1) (Dumay et al., 2015).

$$[Phycoeythrin] = [(A_{565} - A_{592}) - (A_{455} - A_{592}) \times 0.20] \times 0.12 \quad (1)$$

Characterization of Protein Particle Size

The droplet size distribution and volume-weighted mean diameter of each extract (50 mg/mL) were measured using a Mastersizer 3000 Version 3.85 (Malvern Instruments, UK) (Wang et al., 2023). The refractive and absorption indexes were set to 1.5 and 0.1, respectively. Obscuration was chosen between 4 and 10%. Results are presented as an average of six measurements.

Assessment of Intrinsic Fluorescence

Fluorescence determination was assessed based on the method described by Ferreira-Santos et al. (2021). Briefly, lyophilized extracts were redissolved in water to achieve a final concentration of 0.1 mg/mL and then read with an Aqualog 800 spectrofluorometer (HORIBA-Jobin Yvon, Inc. Japan) using a 1-cm quartz cell. Samples were excited at 280 nm, and the emissions were recorded over the 250–800 range with an integration of 0.1 s and three accumulations for each sample with a 1.7-nm increment. Before measurements, PUF extracts were sonicated for 2 min in a sonication bath (200 W, 45 kHz, VRW®, Pennsylvania, USA). All determinations were run in triplicate, and the obtained spectra resulted from the average of the triplicates.

Protein Solubility

The quantity of soluble protein present in each extract was determined by measuring the ultraviolet extinction

at 280 nm as described by Avelar et al. (2024). Initially, lyophilized extracts were redissolved in deionized water to achieve a final concentration of 1% extract and left under magnetic stirring overnight. Also, both PUF extracts from H_2O and NaOH methods were put in a sonication bath (200 W, 45 kHz, VRW®, Pennsylvania, USA) for 2 min at room temperature to improve solubilization. Thus, 1 mL of each extract (total fraction) was centrifuged at 14,000 rpm for 10 min, together with 500 µL of sample added to an Amicon centrifugal tube with a 3-kDa molecular weight cut-off (Merck, KGaA, Darmstadt, Germany), and the filtrate liquid was recovered (soluble fraction). All samples were diluted (ratio 1:5) and aliquots (200 µL) were added to a UV-transparent plate, and UV measurements were performed at 280 using a UV 96 microplate reader (Synergy H1, BioTek Instruments, Inc., Winooski, VT, USA). Protein solubility was calculated according to the following Eq. 2:

$$Proteinsolubility(\%) = \frac{SFAbs_{280nm}}{TotalAbs_{280nm}} \quad (2)$$

where $SFAbs_{280nm}$ and Total Abs_{280nm} correspond to the recovered soluble fraction and the total sample, respectively.

ζ-potential

For the ζ-potential measurement, liquid extracts were dialyzed with a 3.5-kDa membrane (Spectra/Por®, Broadwick Street, Rancho Dominguez, CA) for 24 h to promote salt removal. After desalination, the charge of the extracted proteins was assessed through dynamic light scattering using a Zetasizer Nano (ZEN 3600, Malvern Instruments Ltd., Malvern, UK) equipped with a He–Ne laser at a wavelength of 663 nm (Avelar et al., 2024). Samples of 750 µL were poured into disposable capillary cells (DTS1070) and a detection angle of 173° for measurement. Cell temperature was maintained at 25 ± 0.5 °C during the measurement. Both PUF extracts from H_2O and NaOH methods were put in a sonication bath (200 W, 45 kHz, VRW®, Pennsylvania, USA) for 2 min at room temperature to improve solubilization. The ζ-potential is calculated based on the direction and velocity of the droplet's movements in the applied electric field using the Smoluchowski model (Malvern Instruments, 2007). All samples were analyzed in triplicate with three readings for each sample, and the results are expressed as the average ± standard deviation.

Circular Dichroism (CD)

The CD was used to assess protein secondary structure after the application of different treatments to the extracts obtained after sequential extraction using water and NaOH. CD spectra were recorded with a spectropolarimeter (JASCO J-1500, Jasco Corp., Tokyo, Japan) at 20 °C under a constant nitrogen gas flow (5 mL/min), using a 0.1-cm path length cell (de Figueiredo Furtado et al., 2018). The experimental parameters used in the data collection were as follows: band width of 1 nm, data pitch of 1 nm, scanning speed of 50 nm/min, response D.I.T. (Digital Integration Time) of 1 s, and three accumulations. The CD spectrum of the blank (solvent or buffer without the protein) was subtracted from each recorded spectrum to eliminate background interference.

Also, to study the behavior secondary structure of these proteins when subjected to different temperatures, a sweep between 20 and 90 °C was conducted with an increase of 5 °C in each measurement, and spectra were recorded between 190 and 260 nm. All samples were set at 0.1 mg/mL protein concentration to avoid equipment degradation. Some samples (PUF extracts) were sonicated (LBX instruments, LABBOX LABWARE S.L., Barcelona, Spain) for 30 min to help with solubilization. Results are presented as an average of three replicates.

Extracts' Functional Properties

Rheological Behavior

The gelation ability of *P. dioica* extracts was assessed using a controlled stress rheometer HR-1 (TA instruments, New Castle, USA) fitted with a plate (Ø 40 mm) and a gap of 1000 µm. For that, lyophilized extracts were redissolved in deionized water to a final concentration of 15% total extract and were stabilized at 20 °C for 10 s, and then the temperature was elevated to 90 °C with a ramp rate of 2 °C/min, a strain of 10%, and a frequency of 1.0 Hz. After 60 s at the final temperature, a temperature-decreasing ramp at the same conditions started until the sample reached 20 °C, and a frequency variation was recorded at 20 °C. To avoid evaporation, the plate was covered with silicone oil during the experiments.

The flow behavior was studied at different extract concentrations (3, 5, and 10%, redissolved in deionized water) for each condition, to assess the thickening ability. First, a conditioning sample step was introduced for 60 s, and then the first flow ramp was done for 120 s with a shear rate ranging from 0.1 to 100/s^{-1}. After another conditioning sample for 30 s, the second flow ramp was done for 120 s with a shear rate of 100–0.1 s^{-1}. All the experiments were run at 25 °C.

Emulsifying Properties

The emulsifying capacity of each protein-enriched extract was assessed based on the method described by Castro-Ferreira et al. (2022) with some modifications. Lyophilized samples were redissolved in deionized water to achieve a 10 mg/mL protein concentration. Then, 7.5 mL

of each extract solution was mixed with 7.5 mL of sunflower oil. The emulsion was formed after homogenization at 18,000 rpm for 5 min using a high-shear blender (UltraTurrax T25, Ika-Werke, Germany). The emulsion capacity was calculated by measuring the height of the emulsion after 15 min and dividing it by the total height of the samples. Moreover, the emulsion stability was assessed following the same methodology used for the emulsifying capacity for 7 days. All assays were run in triplicate, and results are expressed as a percentage.

Foaming Capacity

The ability of the H_2O and NaOH extracts to form and stabilize a foam was measured based on the volume produced after bubbling. For that, samples were set at 1% protein to a final volume of 15 mL. The foam was created using a high-shear blender (UltraTurrax T25, Ika-Werke, Germany) at 18,000 rpm for 6 min and allowed to rest for 3 min. The height of the foam was measured after that time and compared to the initial volume of the mixture. All assays were run in duplicate, and results are expressed as a percentage.

Statistical Analysis

All of the results were presented as mean ± standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, Inc., San Diego, CA) (version 9.5.1). One-way ANOVA was used to test differences between samples, considering statistically different values of $p < 0.05$.

Results and Discussion

Extracts' Chemical Characterization

After the protein-enriched fractions production, extracts were subjected to different treatments and further characterized in terms of chemical composition, and the results are presented in Table 1. Six different extracts were obtained, labeled taking in account the type of treatment applied: H_2O and NaOH untreated, for samples extracted with water or with NaOH from the water extraction solid residue (these samples were not subjected to any further treatment after extraction); UF H_2O and UF NaOH for the ultrafiltrated extracts produced after aqueous or alkaline extraction, respectively, and PUF H_2O and PUF NaOH, for the purified extracts obtained from the two different extraction environments.

The produced extracts were characterized in terms of chemical composition. Results are presented in Table 1.

The results showed that untreated samples had good protein recoveries (23.5 ± 0.7 and 28.2 ± 1.0% $_{Protein}$ for H_2O and NaOH extracts, respectively) that were in the same range as the ones obtained after UF and PUF. The application of a sequential H_2O/NaOH methodology resulted in extracts with 14.75 ± 0.43 and 21.22 ± 0.81% (g protein/100 g extract) for H_2O and NaOH, respectively. However, for both fractions, a high percentage of carbohydrates (mostly galactose and/or xylose, since both have similar retention times) was also extracted (around 50 and 25% for H_2O and NaOH extracts, respectively, obtained by the sum of each carbohydrate in its isolated form). Also, when ultrafiltration (UF) with a 10-kDa membrane was applied to concentrate samples, the protein content did not show to increase in the extracts from the first extraction

Table 1 Chemical characterization of the obtained extracts from *P. dioica* after application of sequential **(1)** H_2O/NaOH extraction, **(2)** with UF treatment, and **(3)** precipitation with ammonium sulfate (70% saturation) and UF (PUF)

Extraction sample	Extraction yield ($g_{solids}/100g_{seaweed}$)	Protein recovery ($g_{protein}/100\ g_{algae\ protein}$)	Protein content (%)	Carbohydrates (%)				Ash (%)
				Total glucose**	**Total Ac. galacturonic****	**Total xylose + galactose****	**Total arabinose****	
H_2O	34.83 ± 0.35^{f}	23.53 ± 0.74^{b}	14.75 ± 0.43^{a}	8.49 ± 0.00^{c}	10.63 ± 0.01^{d}	27.82 ± 0.30^{c}	5.19 ± 0.43^{a}	16.74 ± 0.13^{c}
NaOH	28.88 ± 0.47^{e}	28.16 ± 1.00^{c}	21.22 ± 0.81^{b}	2.63 ± 0.00^{b}	2.66 ± 0.06^{b}	16.05 ± 0.45^{b}	4.81 ± 0.12^{a}	17.78 ± 0.09^{d}
UF H_2O	22.17 ± 0.20^{c}	16.37 ± 0.37^{a}	16.41 ± 0.37^{a}	45.93 ± 3.34^{e}	9.89 ± 0.70^{c}	45.06 ± 3.71^{d}	n.d	16.47 ± 0.32^{c}
UF NaOH	24.97 ± 0.00^{d}	32.32 ± 0.08^{c}	28.76 ± 0.07^{c}	9.97 ± 0.48^{d}	0.87 ± 0.11^{a}	26.46 ± 0.32^{c}	n.d	12.56 ± 0.07^{b}
PUF H_2O	5.77 ± 0.23^{a}	17.44 ± 0.02^{a}	81.33 ± 0.10^{e}	0.90 ± 0.00^{a}	0.32 ± 0.04^{a}	n.d	n.d	0.49 ± 0.10^{a}
PUF NaOH	6.95 ± 0.08^{b}	24.29 ± 0.25^{b}	77.61 ± 0.81^{d}	0.44 ± 0.00^{a}	n.d	5.29 ± 0.01^{a}	n.d	0.31 ± 0.01^{a}

*__UF__—extracts after the application of the ultrafiltration technique; __PUF__—extracts after ammonium sulfate precipitation followed by UF; within each column, different letters indicate significant differences ($p < 0.05$)

**The content of each sugar is presented in equivalents of each monosaccharide

with water, but a significant increase of the carbohydrates content (which constituted around 50% of the water extracts) was observed, probably related to the porphyrans content (composed mainly by galactose). Further, for the extracts from the following extraction step with NaOH, UF resulted only in a 30% concentration of the protein content but in an 80% concentration of the carbohydrates with xylose and/or galactose in their composition. This behavior was expected since UF is often used to isolate proteins along with other macromolecules with sizes within the range of 1–200 kDa which can include carbohydrates (de Souza Celente et al., 2023; Geada et al., 2021). Thus, to promote the break of polysaccharide-protein interactions and reach high protein recovery in a cost-effective and not time-consuming way, a step of ammonium sulfate precipitation (70% saturation) was used to increase the ionic strength of the sample and decrease the protein solubility followed by an ultrafiltration step to remove the remaining salts. From Table 1, it is possible to verify that the protein content of precipitated extracts (PUF) is significantly higher when compared to the ones obtained from both non-treated and UF extracts making it possible to achieve protein concentrations higher than 70% for both H_2O and NaOH extracts, while only slightly affecting the protein recovery yield. On the other hand, while protein increased, the content of carbohydrates and ashes descended abruptly, to values lower than 6% and 0.5%, respectively, which indicated a successful isolation of proteins and a good purity index. Together with this, the UF technique promoted the removal of ash from non-treated to ultrafiltrated samples, but only for the NaOH extracts coupled with UF and both PUF extracts. This may suggest that some ash coming from the seaweed may be initially bound or trapped with the protein and/or polysaccharide, making it more difficult to separate; the decrease was more significant after purification with ammonium sulfate, as expected. Although the lipid content was not calculated, it was expected to be low (lower than 2.5% dw) (O' Connor et al., 2020). In a recent study conducted by O'Connor et al. (2020), different protein extraction techniques (e.g., water extraction, high-pressure processing, and autoclaving) were applied to red seaweed, and the fat content of the extracts was quantified. In these, the percentage of lipid did not surpass 1.5% for the water extraction of *P. palmata*, and *C. crispus* (O' Connor et al., 2020). Also, the fat content present in protein-enriched extracts from *Gelidium corneum* produced using aqueous and alkaline environments was determined to be 0.9% (Mateus et al., 2024). Thus, the missing fraction needed to reach 100% in the total component analysis may be ascribed to incomplete carbohydrate hydrolysis and/or the presence of additional monosaccharides that could not be quantified.

Proteins' Molecular Weight

The assessment of protein molecular weight presented in each obtained fraction was realized using gel permeation chromatography. Figure 2 demonstrates the chromatograms for each extraction condition (non-treated, UF, and PUF extracts for water and alkali extraction solvents). The extracts that were not subjected to any further treatments after the extraction process were revealed to have unique peaks of 71 and 282 kDa for H_2O and NaOH, respectively. The homogeneous size associated with water extraction was predictable since only water-soluble proteins (related to phycobiliproteins in the case of seaweeds) were expected. On the other hand, the use of alkali conditions in the second part of the extraction process should lead to the presence of more size variability due to the hydrolytic effect on the biomass that could liberate proteins that were retained in the seaweed structure (Kadam et al., 2017). Contrary to the predictions, an isolated peak corresponding to proteins with a molecular weight of 282 kDa was obtained, indicating the presence of aggregation between proteins with different molecular weights. This peak can also suggest the formation of polysaccharide-protein complexes that elevate the size of the molecule and appear as a single peak in the chromatogram (Braspaiboon & Laokuldilok, 2025). The utilization of the UF technique after extraction lowered the molecular size observed in each fraction demonstrated that some complexes were undone, and the previously expected NaOH peaks appeared in the chromatogram in the range of 3–21 kDa. Harnedy and Fitzgerald (2011) showed that for macroalgae proteins that were soluble in alkaline solvents, the average molecular size and the bands attributed to water-soluble proteins larger than 20 kDa were not present, which is to the obtained results.

R-Phycoerythrin Content

R-Phycoerythrin (R-PE) is a water-soluble protein composed of a 240-kDa oligomeric chromoprotein characterized by its absorption spectrum between 400 and 650 nm (Dumay et al., 2015; Tan et al., 2023; Ulagesan et al., 2021a). In this work, the presence of R-PE in the obtained extracts after UF and PUF was determined using spectrophotometry assays, and the results are presented in Fig. 3. The absorbance in the UV–visible wavelengths was recorded in the range of 450–800 nm to detect the main native R-PE peaks at 492, 545, and 565 nm, and based on the absorbance values, using Eq. 1, the R-PE concentration (μg/mL) was calculated (Aguiar et al., 2023). As expected, in Fig. 3, it is possible to identify the typical R-PE peaks in the UF H_2O and PUF H_2O and not in the NaOH extracts, which indicates the success of R-PE extraction in the initial phase of the extraction process. In terms of concentration, R-PE

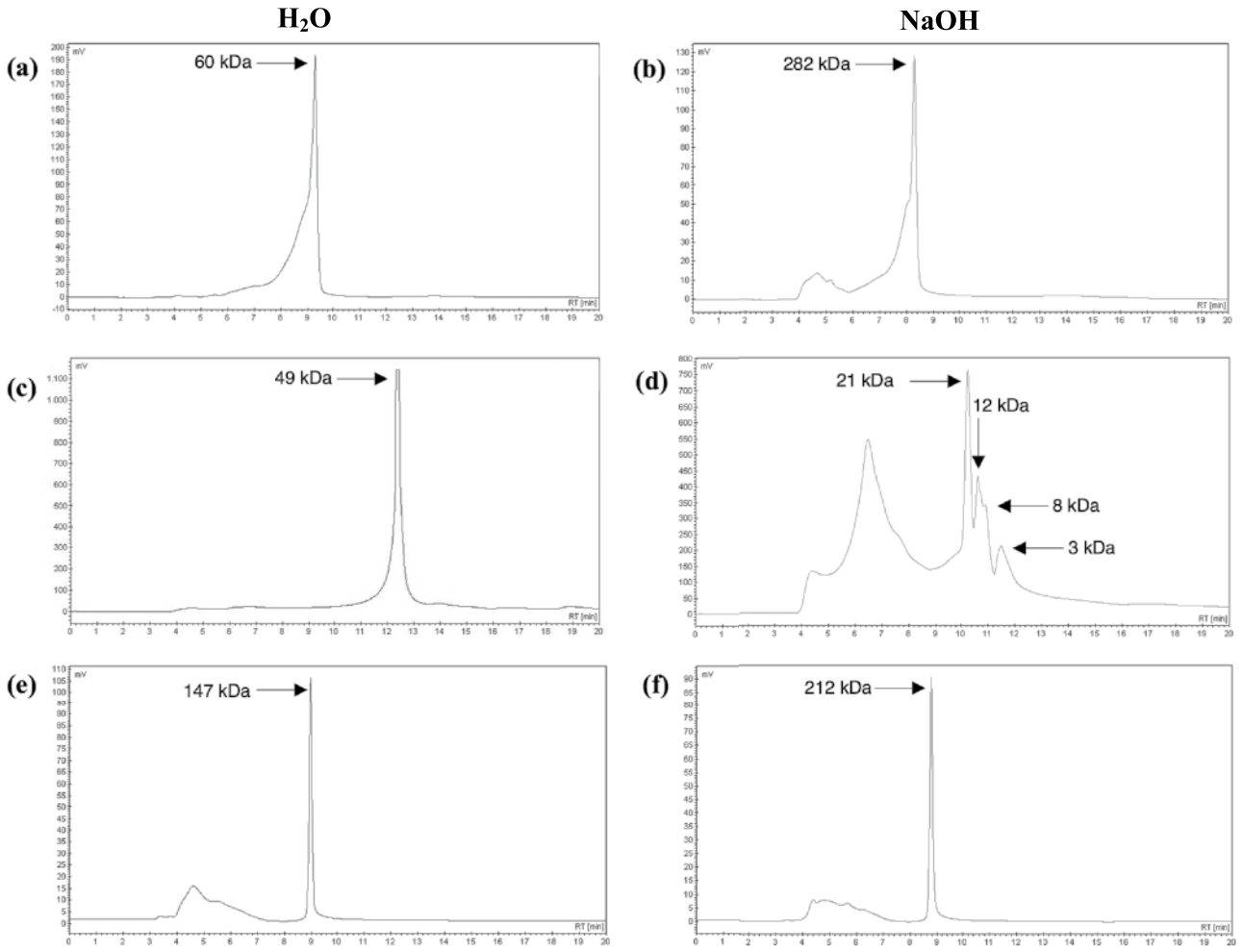

Fig. 2 Representation of the main molecular weight sizes from protein extracts obtained using sequential H_2O/NaOH extraction and application of UF and/or PUF. The molecular size of each identified peak is presented in all figures. **a** H_2O untreated extract, **b** NaOH non-treated extract, **c** H_2O UF extract, **d** NaOH UF extract; **e** H_2O PUF extract, **f** NaOH PUF extract. Untreated**,** no treatment; UF, ultrafiltration; PUF, purification and ultrafiltration

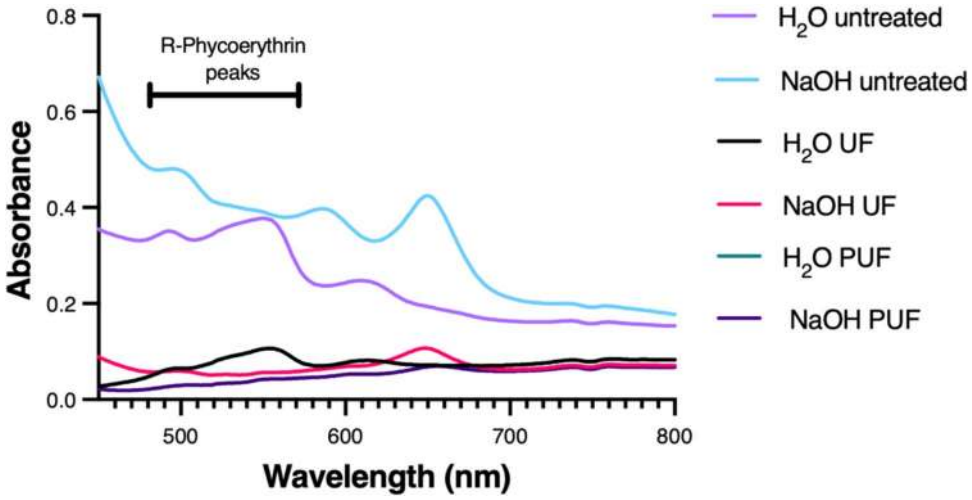

Fig. 3 Spectrophotometric behavior of *P. dioica* extracts (non-treated, UF, and PUF) was recorded within the range of 450–800 nm for the identification of R-PE typically absorbance peaks at 492, 545, and 565 nm. Light purple line, H_2O untreated; blue line, NaOH untreated; black line, H_2O UF; pink line, NaOH UF; green line, H_2O PUF; dark purple line, NaOH PUF. Untreated, no treatment; UF, ultrafiltration; PUF, purification and ultrafiltration

is found in higher quantities after the application of ammonium sulfate precipitation (36 µg/mL) when compared to the only UF (29.9 µg/mL). However, a low amount of R-PE was detected in the PUF NaOH extract (0.5 µg/mL). Since *P. dioica* absorbs a high quantity of water, the application of a sequential extraction methodology and the use of the water extraction residue for the next step of extraction allows the transference of residual amounts of water extracts for the NaOH phase, which explains the presence of R-PE in PUF NaOH extracts. In general, the applied extraction method of R-PE is a critical step that limits the quantity of protein that is extracted, and higher extraction yields and purities are often associated with wall-breaking methods such as freeze-thawing. Also, some pretreatments (e.g., ultrasound, addition of chemicals such as lysozyme, acetone, and Tryton X-100) could be applied for better results (Niu et al., 2010). In some red seaweeds (such as *Gracilaria* sp.), which are good sources of agar, studies have been made for the recovery of both fractions using a sequential extraction, allowing the recovery of R-PE that would lose its applicability in high temperatures (80–120 °C) that are normally used in traditional agar extraction methodologies (Aguiar et al., 2023).

The most recent reported values of R-PE concentration are normally higher (in the range of mg/mL) than the ones obtained in this work, since strong treatments (both in solvent and time) are used in the extraction process, contrary to our methodology. This does not limit the applicability of our extracts since low amounts of R-PE are needed when its use as a natural colorant is intended; otherwise, some additional steps of purification (e.g., RP-HPLC) must be applied (Aguiar et al., 2023). More recently, emerging technologies have been studied to overcome the drawbacks of the conventional extraction methods. Braspaiboon and Laokuldilok (2025) investigated the potential of high-pressure processing as an improved technology for protein and phycobiliprotein extraction from *Porphyra* sp. In this work, the combination of pressure (600 MPa), time (28 min), and pH (7.8) allowed for to maximization of the nitrogen and phycobiliproteins recovery in 31% and 1.57 g/100 g, respectively, which was significantly higher when compared to the alkaline method that allowed the recovery of 27% of nitrogen. Also, protein denaturation was reduced, and the denaturation temperature was similar to the untreated samples (151–154 °C) and higher than alkaline treatments (138 °C).

Particle Size Measurements

The particle size of each protein extract was assessed using Mastersizer® 3000 to determine the size pattern of each sample that could justify further results. The obtained results are presented in Fig. 4. From Fig. 4a, it is possible to observe that only H_2O extracts that were not subject to any further treatment present a heterogeneous size pattern, with small (< 1.0 µm) and larger (> 1000 µm) being the most representative peaks in the range of 10–100 µm, which was also confirmed by the main volume deviation

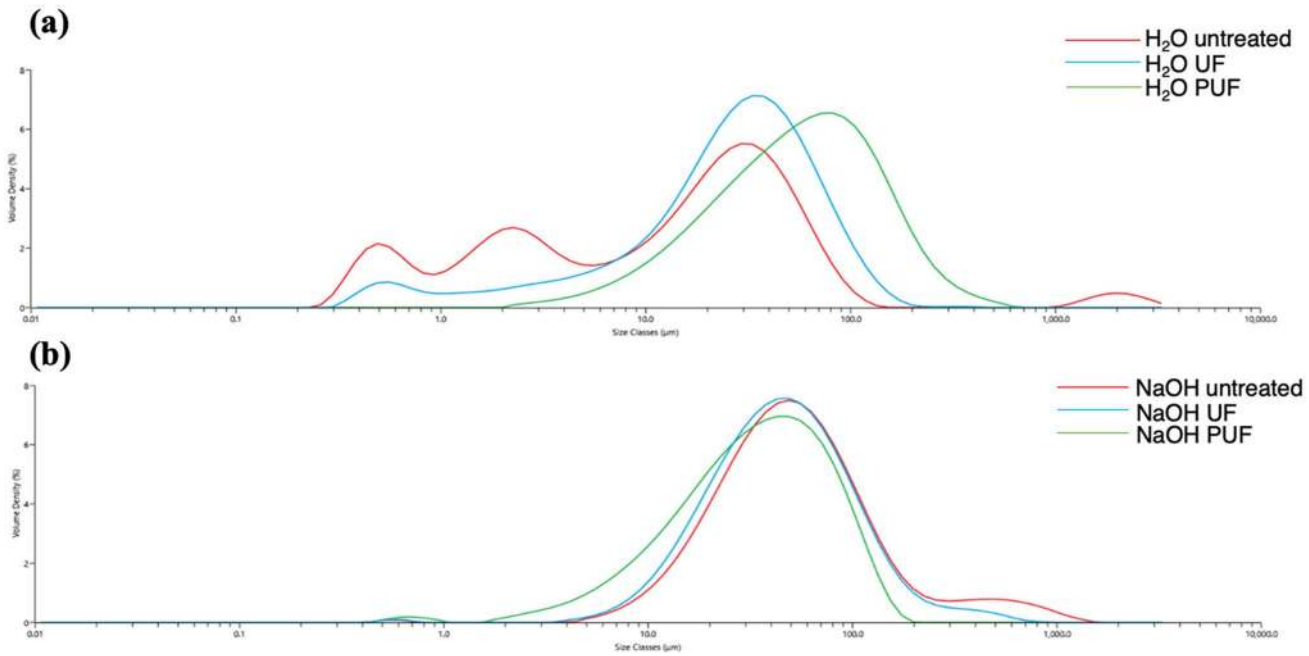

Fig. 4 Particle size distribution analysis of *P. dioica* extracts with and without the application of further treatments for **a** H_2O untreated extracts and **b** NaOH extracts. Untreated extracts are represented by a red line, followed by UF extracts in blue and PUF extracts in green. Untreated, no treatment; UF, ultrafiltration; PUF, purification and ultrafiltration

parameters (see supplementary material Table S1). On the other hand, the use of UF/purification and UF treatments leads to the decrease of small particle signals and a more homogeneous volume concentration related to the presence of molecular aggregates that elevated the D [4,3] number (see supplementary material Table S1). Moreover, similar to H_2O untreated extracts, NaOH without treatment and after UF also showed the presence of a signal for larger particles (around 1000 µm) (Fig. 4b). However, these features were not found in the purified samples (both H_2O and NaOH) that showed a principal peak between 10 and 100 µm, which is by the other extracts.

Moreover, it can be observed that D10 is significantly smaller than D50 for all samples, which indicates that low percentages of < 20 µm particles are present and most of them are within the range of 20–200 µm (reported by D50 and D90). The measure of distribution width (indicated as Span) is relatively high in all samples, being more pronounced in the untreated extracts (with values of 3.50 and 3.24 for H_2O and NaOH extracts, respectively) (see supplementary material Table S1).

Protein Solubility and Charge

Protein solubility is one of the most important technological properties in food processing, sensory attributes, shelf-life, and nutritional profile of food formulated with protein-rich materials since it directly affects other properties such as emulsification, foaming, and gelation (Grossmann & McClements, 2023; Purdi et al., 2023; Rawiwan et al., 2022). The relative protein solubility of the produced extracts was calculated using Eq. 2, and the results are summarized in Fig. 5. Before the quantification, the pH of each extract was measured (see Fig. 5). The comparison of protein solubility between extraction conditions was determined using UV extinction at 280 nm. Before the protein measurements and knowing that spectroscopy approaches often involve the influence of other components (e.g., phenolic, pigments), the non-contamination of nucleic acid was performed by (i) removal of the insoluble fraction by centrifugation and (ii) study of the absorbance spectral profile at the chosen wavelength to ensure no interferences occurred (Akharume et al., 2021). The untreated samples showed to have better protein solubility (69.15 ± 1.25 and 64.91 ± 0.63% for H_2O and NaOH extracts, respectively) when compared to ultrafiltrated samples (58.37 ± 2.40 and 29.01 ± 1.21% for H_2O and NaOH extracts, respectively). This indicates the presence of a relatively low electrostatic repulsion between the protein molecules that allowed its association through van der Waals, hydrophobic, or hydrogen bonding (which normally appears when pH is near the protein's isoelectric point). These have resulted in more hydrophobic extracts with decreased affinity for water (Braspaiboon & Laokuldilok, 2024). Regarding

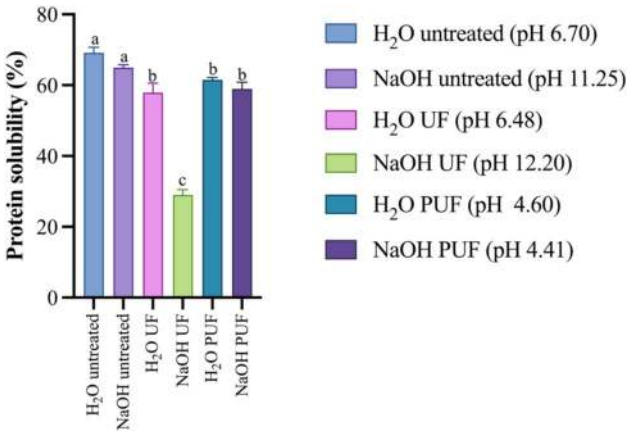

Fig. 5 Quantification of the protein solubility (%) of each produced extract with and without the application of purification treatments. Untreated, no treatment; UF, ultrafiltration; PUF, purification and ultrafiltration

the UF samples, lower solubility percentages were observed for the NaOH extracts. This was not expected since, in general, stronger alkaline environments cause an increase in protein solubility. Nevertheless, it is important to highlight that these extracts were obtained sequentially, and that the proteins more easily soluble in water environments were extracted in the first step, just with water. As previously demonstrated (see the "Proteins' Molecular Weight" section), the UF NaOH extracts showed various protein molecular weights. Thus, the decrease in protein solubility may be explained by the formation of protein aggregates (as a result of the exposition of hydrophobic sites caused by the high pH) that were not fully solubilized during quantification and did not contribute to the soluble protein fraction. On the other hand, the aggregation can also be interactions between protein and sulfated polysaccharides (e.g., carrageenans) that are co-extracted in alkaline conditions (Alavi et al., 2021; Ng et al., 2024; Wong & Cheung, 2000).

The purified samples underwent sonication to reduce protein aggregation and enhance dissolution, resulting in increased protein solubility, measured at 61.48 ± 0.55% for H_2O extracts and 58.97 ± 1.55% for NaOH extracts. However, these solubility values were still significantly lower ($p < 0.05$) compared to the untreated samples, as not all aggregates were completely broken down. This was expected since the preliminary characterization of particle size measurements (see the "Particle Size Measurements" section) indicated the presence of molecules with large molecular sizes, which would make the solubilization difficulted. Literature indicates that the protein solubility of algal proteins is generally low at low pH values, but a significant increase in solubility is observed when the pH is raised. For example, the interfacial and foaming properties of protein concentrates from seaweed *P. dioica* were studied, and poor protein

solubility was obtained at pH (< 5%), with an increase to 43.3 ± 3.3% when pH was higher (≥ 7) (Felix et al., 2021).

Protein solubility results were then corroborated by the zeta-potential analysis (Table 2). The presence of proteins with low zeta-potential values, whether positive or negative, suggests that the repulsive forces between different molecules are insufficient to prevent them from forming connections. This can lead to destabilization mechanisms, such as coalescence—where molecules merge into one another—and flocculation, where they come close together and behave as a single entity. These processes significantly impact the characteristics of the proteins, which in turn affect their functional properties (Nunes et al., 2020). In this case, results indicate that all samples presented good and stable surface charges (between − 20 and − 30 mV) that are directly related to protein solubilization and also indicate that the environmental pH was far from the isoelectric point of proteins (see Table 2). In fact, Chan et al. (2013) reported that low protein solubility typically involves low surface charges. Regarding our results, UF extracts were the ones that showed the lower absolute zeta potential values (− 24.90 ± 0.37 and − 21.77 ± 0.31 mV for H_2O and NaOH extracts, respectively), which is in accordance with the lower protein solubilities presented above. On the other hand, H_2O untreated and NaOH PUF extracts showed higher absolute zeta potential values (− 27.17 ± 0.21 and − 29.73 ± 1.72 mV, respectively) that were translated into higher solubilities.

Table 2 Zeta potential of protein-enriched extracts from seaweed *P. dioica*. Untreated, no treatment; *UF* ultrafiltration, *PUF* purification and ultrafiltration

Samples	Zeta potential (mV)
H_2O untreated	-27.17 ± 0.21^{b}
NaOH untreated	-26.43 ± 0.79^{b}
H_2O UF	-24.90 ± 0.37^{b}
NaOH UF	-21.77 ± 0.31^{a}
H_2O PUF	-25.60 ± 0.25^{b}
NaOH PUF	-29.73 ± 1.72^{c}

Secondary Structure Assessment

The CD measures conformational changes in the secondary structure of proteins, allowing for correlation with (i) protein aggregation, (ii) thermal/chemical unfolding, and (iii) binding interactions. The α-helix is characterized by the appearance of two negative peaks at 208 nm and 222 nm and an intense positive band at 195 nm, while ß-sheets typically demonstrate a broad negative band between 215 and 225 nm and a positive band at 195 nm. On the other hand, disordered structures show a unique negative band near 200 nm (Ferreira et al., 2021; Ferreira-Santos et al., 2020; Pignataro et al., 2020). In this study, the CD spectra were obtained in the far UV region (190–260 nm) for H_2O and NaOH protein extracts (normalized to 0.1 mg/mL protein concentration) with and without treatments. Figure 6 demonstrates the results obtained for each protein-enriched fraction. Regarding the H_2O extracts, the untreated sample spectra evidence the presence of a well-structured protein that was also confirmed by higher intensities in the fluorescence spectra (see supplementary material Fig. S1) when compared to the NaOH extracts, with a predominance of α-helix that unfolded with the application of purification treatments. On the other hand, the use of NaOH in the extraction led to the extraction of proteins in the form of random coils (with a relevant negative peak around 200 nm,

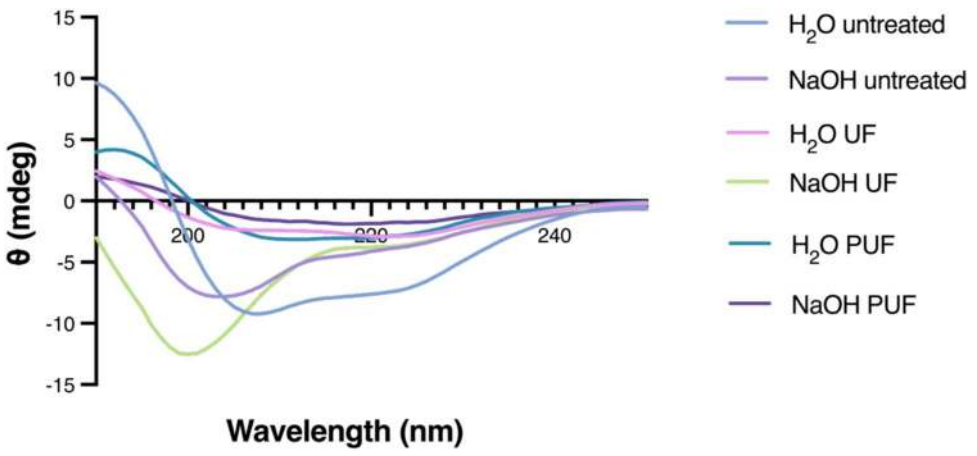

Fig. 6 Protein secondary structure analysis using circular dichroism spectra of protein-enriched fractions obtained after H_2O/NaOH sequential extraction and purification treatments from red seaweed *P. dioica*. Blue line, H_2O untreated; light purple line, NaOH untreated; pink line, H_2O UF; green line, NaOH UF; blue green line, H_2O PUF; dark purple line, NaOH PUF. Each spectrum is represented as an average of three independent measurements. Untreated, no treatment; UF, ultrafiltration; PUF, purification and ultrafiltration

attributed to disordered structures) and lower fluorescence (see supplementary material Fig. S1), although some ordered forms were also present. Further, when UF is applied, the intensity of this negative peak increases (and also the fluorescence intensity peak), ascribed to an increase in the random coil form of the proteins. Regarding the PUF extract fluorescence analysis (see supplementary material Fig. S1), the intensity of the H_2O extracts was five times higher (around 15,000 a.u.) than that observed for the untreated samples, and the fluorescence intensity of the NaOH extracts did not reveal the presence of any peak, even though the intensity of the signal was also five times higher. The peak shape for the purified water extracts is attributed to the existence of molecular aggregates and free proteins that resulted in the superposition of two different peaks. This, together with CD spectra results, supports the existence of structural loss.

In order to verify the impact of temperature on the secondary structure of proteins from the obtained H_2O and NaOH extracts, a temperature interval measurement was realized between 20 and 90 °C, and the CD spectra for each sample were recorded between 190 and 250 nm. Figure 7 demonstrates that for the H_2O untreated samples, a clear transition and loss of protein secondary structure affected by the temperature was visible. These results are also in accordance with CD spectra that had already demonstrated the presence of a more closed-to-native structure in the H_2O untreated samples. Therefore, this was expected, as these extracts had the most structured proteins. This effect was also visible, though in a less pronounced way, in the untreated NaOH extracts and in the UF NaOH extracts, which is also in accordance with their less structured form. On the other hand, all of the other samples showed similar behavior in all temperatures tested, indicating that the extraction and purification procedures had already caused a significant degree of denaturation and thus no transitions were observed. There are already some published works that emphasize the impact that the extraction methodology and parameters have on the secondary structure of proteins. After the protein extraction from *Porphyra* sp. through traditional alkaline treatment and high-pressure processing (Braspaiboon & Laokuldilok, 2025), the loss of native protein structure under alkaline conditions was not observed after extraction using a non-thermal high-pressure processing method. On the other hand, this method caused a partial protein unfolding while maintaining structural integrity under the extraction process.

Rheological Behavior

Gelling Capacity

Different plant-based protein sources have already been studied as an alternative to animal proteins in the design of modern foods due to their functional properties (Batista et al., 2005). The potential of these proteins to form a stable network like a gel structure is related to their ability to unfold and is driven by a certain degree of thermal denaturation. The exposure of functional groups (i.e., hydrophobic and sulfhydryl groups) allows the establishment of disulfide bonds and hydrophobic interactions to achieve a state of minimal energy of the system (Batista et al., 2005). However, gel formation is only possible when the protein concentration is above its critical gelling point. Otherwise, there are not enough groups to establish molecular interactions, and the gel is not formed. Figure 8 presents the results obtained from the analysis of the gelling capacity of each extract, using both H_2O and NaOH, with and without the application of different purification treatments. All extracts were redissolved in deionized water to a final concentration of 15% total extract concentration since the gelling or thickening capacity may be attributed to synergistic interaction between protein carbohydrates in the sample. Moreover, the gap between storage and G″ is higher in all NaOH extracts when compared to H_2O extracts, demonstrating that its behavior is more stable in these samples. Nevertheless, G′ was equal at some points between 80 and 100 °C for the H_2O untreated and UF (Fig. 8a and Fig. 8c, respectively), which may be related to protein denaturation and aggregation (though not sufficient for effective gelling). In seaweeds, the gelling behavior is typically associated with the presence of a high quantity of carbohydrates in the extracts, which is also visible in the H_2O samples that were subjected to the UF technique since they revealed to have a content of total carbohydrates higher than 90% (see the "Extracts' Chemical Characterization" section) (Gómez-Mascaraque et al., 2021). However, in seaweeds, this functional property is mostly attributed to alginates that are found in the cell wall of brown seaweeds or agars and carrageenans for red seaweeds. However, *P. dioica* is rich in porphyran (a sulfated galactan with no gelling properties) and floridean starch, which explains why these extracts did not demonstrate gelling properties (Gómez-Mascaraque et al., 2021; Torres et al., 2019). On the other hand, the capability to form gels was already seen within protein-concentrated samples obtained from microalgae. Bashir et al. (2016) studied the functional properties and amino acid profile of *Spirulina platensis* protein isolates and showed that for samples with a protein content of 74%, the least gelling concentration was about 18%, which is high when compared to the ones needed to form gels with typical protein sources (e.g., whey protein isolates). Thus, in this work, the extracts that were expected to show some gelling properties were the ones that passed through UF, and PUF led to the presence of high protein content. However, as shown above, the PUF extracts (from either H_2O or NaOH extraction) revealed to have protein aggregates that resulted from an aggressive treatment with

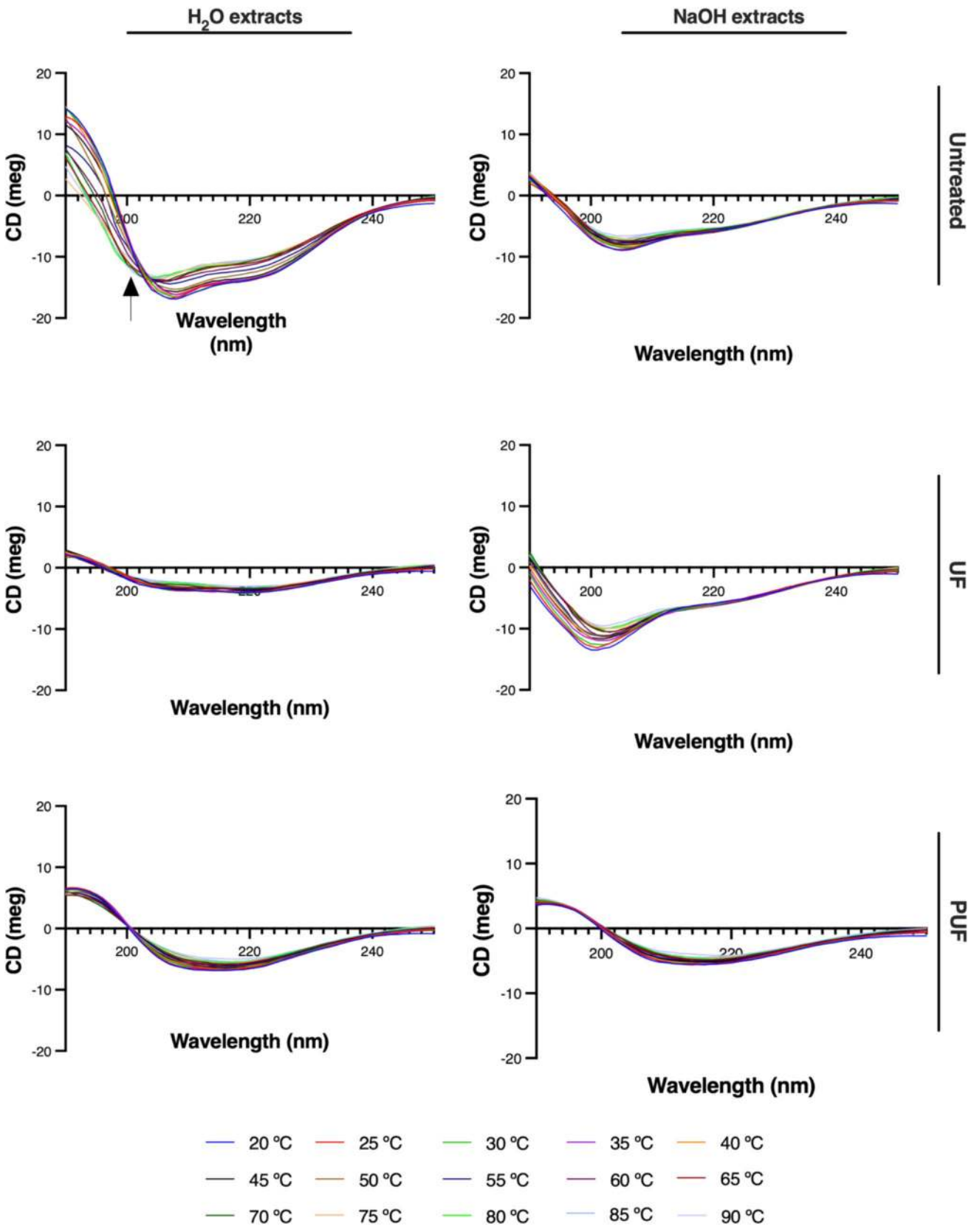

Fig. 7 Far-UV CD spectra of *P. dioica* protein-enriched extracts obtained after H_2O/NaOH sequential extraction and application of purification techniques. Lines in the spectra were collected with a 5 °C increment between 20 and 90 °C. Results are presented as an average of three measurements. Untreated, no treatment; UF, ultrafiltration; PUF, purification and ultrafiltration

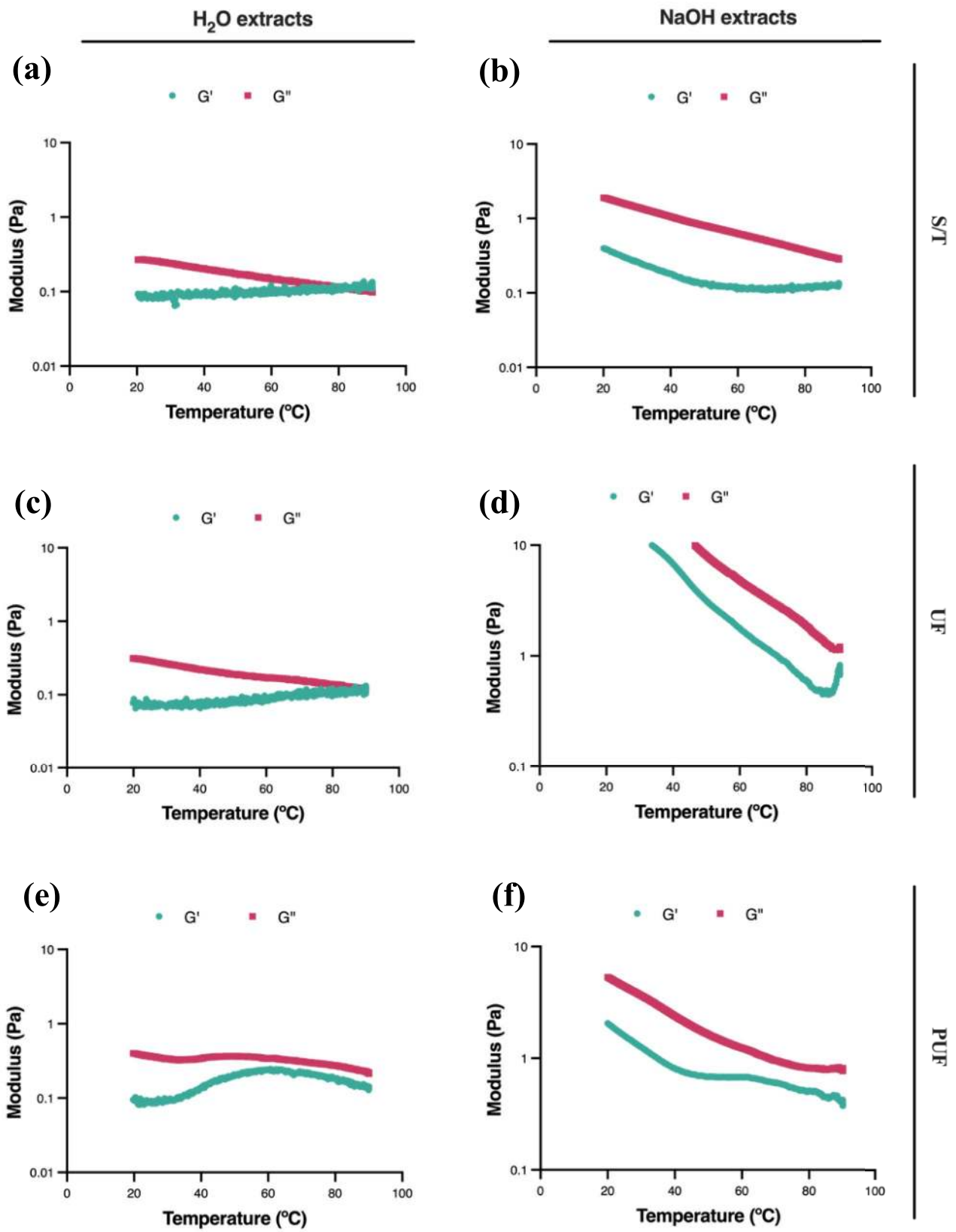

Fig. 8 Rheological properties of *P. dioica* extracts after sequential H_2O and NaOH with and without the application of further treatments. **a**, **b** Untreated extracts; **c**, **d** UF extracts; **e**, **f** PUF extracts. Untreated, no treatment; UF, ultrafiltration; PUF, purification and ultrafiltration

salts that are exposed to several sites of proteins and led to the formation of crosslinking with other proteins and prevented these sites from being able to form new molecular bonds upon the denaturation process and therefore preventing the gel formation (Edward et al., 2023).

Thickening Capacity

It was already established in this work that the application of different UF treatments caused the formation of protein aggregates and loss of secondary structure, which will affect the existence of functional properties and also their mechanical properties. Figure 9 shows the flow curves of shear stress as a function of shear rate for the untreated protein-enriched extracts and the extracts obtained after UF and/or PUF at 3% total extract concentration. It is possible to see that all extracts (treated and untreated) presented a pseudoplastic behavior (Rodrigues et al., 2020). Despite these measurements being done for 5% and 10% total extract concentration, the behavior was similar, and thus the data is not presented to facilitate observation. Moreover, to have a better understanding of the flow behavior of the samples, the power-law model was adjusted to the experimental data obtained through Eq. (3).

$$\sigma = K.\gamma^{n} \quad (3)$$

where σ is the shear stress (Pa), γ is the shear rate (s^{-1}), K is the consistency index (Pa s^{-1}), and n is the flow behavior or power-law index.

Table 3 summarizes the obtained results for each fraction and demonstrates the existence of different flow behaviors. In general, the consistency index (related to the solution's viscosity) was low for all 3 and 5% extracts, indicating the absence of a structured system and thus interactions between the solution constituents (in agreement with the lower content of molecular aggregates). On the other hand, for higher extract concentration of the NaOH samples, higher values of K were found, which were related to the high level of protein aggregation that was more evident when the concentration increased. Regarding the n values, all samples provided an $n < 1$, indicating the presence of a pseudo-plastic nature of the fluids. Despite that, the NaOH extracts were the ones that showed n values close to 1 and then demonstrated an almost linear behavior characteristic of Newtonian fluids (Rodrigues et al., 2020).

Table 3 Power law fitted parameters to the flow curves of treated and untreated protein samples. Untreated, no treatment; *UF* ultrafiltration, *PUF* purification and ultrafiltration

Samples	Parameters	
	K $(Pa\ s^{-1})$	n
H_2O untreated 3%	0.0099 ± 0.0027^{a}	0.575 ± 0.073^{a}
H_2O untreated 5%	0.0124 ± 0.0026^{d}	0.556 ± 0.050^{a}
H_2O untreated 10%	0.0178 ± 0.0000^{b}	0.534 ± 0.000^{a}
NaOH untreated 3%	0.0155 ± 0.0003^{b}	0.788 ± 0.001^{c}
NaOH untreated 5%	0.0359 ± 0.0028^{c}	0.823 ± 0.024^{c}
NaOH untreated 10%	0.1979 ± 0.0000^{f}	0.796 ± 0.000^{c}
H_2O UF 3%	0.0079 ± 0.0012^{a}	0.638 ± 0.030^{b}
H_2O UF 5%	0.0073 ± 0.0002^{a}	0.681 ± 0.012^{b}
H_2O UF 10%	0.0136 ± 0.0003^{b}	0.602 ± 0.002^{b}
NaOH UF 3%	0.0277 ± 0.0011^{b}	0.839 ± 0.017^{c}
NaOH UF 5%	0.0688 ± 0.0018^{e}	0.864 ± 0.012^{c}
NaOH UF 10%	0.9983 ± 0.0017^{g}	0.838 ± 0.162^{b}
H_2O PUF 3%	0.0139 ± 0.0001^{b}	0.684 ± 0.017^{b}
H_2O PUF 5%	0.0213 ± 0.000^{b}	0.644 ± 0.000^{b}
H_2O PUF 10%	0.0389 ± 0.0003^{c}	0.756 ± 0.002
NaOH PUF 3%	0.0188 ± 0.0014^{b}	0.712 ± 0.026^{b}
NaOH PUF 5%	0.0524 ± 0.0056^{d}	0.811 ± 0.035^{c}
NaOH PUF 10%	0.2105 ± 0.0133^{f}	0.813 ± 0.010^{c}

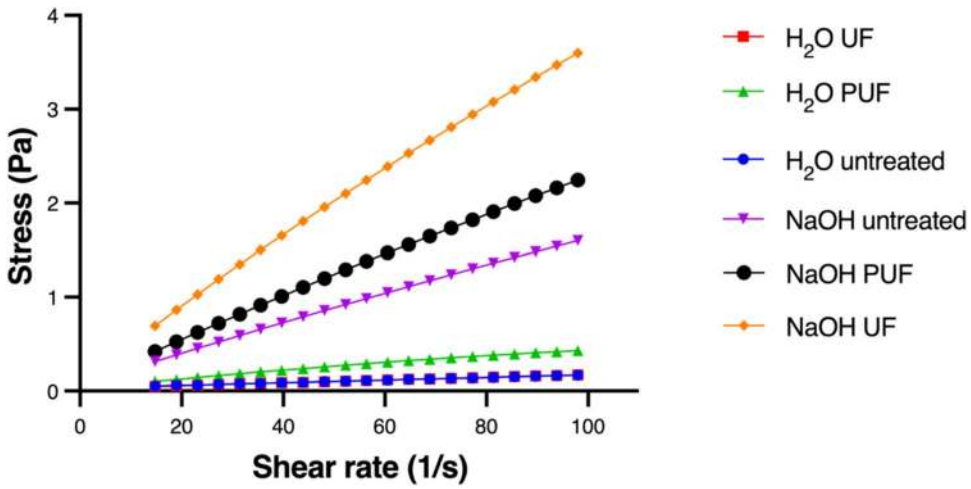

Fig. 9 Flow curves of treated and untreated *P. dioica* protein fraction obtained after the application of H_2O/NaOH sequential extraction at 3% extract. Lines correspond to experimental data fitting. Untreated, no treatment; UF, ultrafiltration; PUF, purification and ultrafiltration

Moreover, values of *K* increased for higher extracts concentrations due to the presence of more particles (and thus higher viscosity), but the *n* values did not change in a significant way. Thus, the thickening capacity is apparently higher for the NaOH extracts (following the order non-treated, UF, and PUF), indicating that eventually protein has a significant role in the development of this functional property. Furthermore, some synergistic effect with other molecules (e.g., polysaccharides such as porphyrans) can help to improve the thickening capacity by increasing the apparent viscosity, as it becomes evident from the lower thickening ability of the NaOH PUF samples in comparison with the NaOH UF samples (Table 3), in spite of their much higher protein content (Table 1).

Emulsifying Capacity and Stability

In the food sector, the primary function of proteins is to stabilize the interface between these two distinct phases, which helps to prevent phenomena such as coalescence, creaming, flocculation, or sedimentation over time. The emulsifying capacity and emulsion stability are critically important characteristics of proteins that significantly influence their interaction with food matrices (Cano-Medina et al., 2011; Geada et al., 2021).

The emulsifying capacity and stability of all extracted samples are summarized in Fig. 10. For the H_2O extracts, it was observed that the samples subjected to UF and PUF, as well as the untreated samples, exhibited higher emulsifying capacities (94% for UF and 100% for PUF samples) compared to those that were only ultrafiltrated, which had an emulsifying capacity of 84% ($p > 0.05$), measured after 15 h of stabilization (referred as day 0). Furthermore, over 7 days, the H_2O PUF extracts demonstrated greater stability, with only a 25% loss on the first day that remained consistent over time. In contrast, the untreated extracts experienced a more significant decline, dropping from 94% on day 0 to 54% by day 7 ($p > 0.05$). For the NaOH extracts, both the untreated and PUF samples exhibited the highest emulsifying capacities (around 95%), while the UF samples reached only 72%. The stability of all NaOH extracts decreased over time, with PUF and untreated samples achieving approximately 70% final emulsifying capacity, compared to 60% for the UF samples ($p > 0.05$). The fact that the most treated samples (PUF extracts that underwent UF and PUF) presented both higher emulsification capacity and stability was expected since this treatment had a major impact on protein physicochemical characteristics. Despite the decrease in the emulsion stability of all NaOH samples, high stabilities were found, which can be explained by the presence of stable molecular aggregates (confirmed by the presence of low IF that resulted from the low exposition of amino acids to the environment) (see supplementary material Fig. S1) that were formed along with the occurrence of some denaturation degree (confirmed by circular dichroism in the "Secondary Structure Assessment" section). The treatment caused some protein denaturation that favored the emulsifying capacity of the samples, which usually does not occur when the denaturation is caused by heat (Y. Guo et al., 2024a, 2024b; Li et al., 2021). By favoring the formation of protein–protein interactions and molecular aggregation, a strong network was formed, helping to stabilize the water–oil interface.

Also, the results suggest that the emulsifying capacity and emulsion stability of protein extracts from *P. dioica* may be comparable to those of certain plant-based matrices already known for their functional properties. Kaur and Ghoshal (2022) studied the functional properties of protein isolates extracted from sunflower seeds. They found that after extracting at pH 10 and 50 °C for 30 min, the maximum emulsifying capacity achieved for sunflower protein isolates was 74.6 ± 1.7%. This value is similar to or even lower than the emulsifying capacities reported in our study. Additionally, Santos et al. (2022) investigated various plant protein concentrates—including soy, pea,

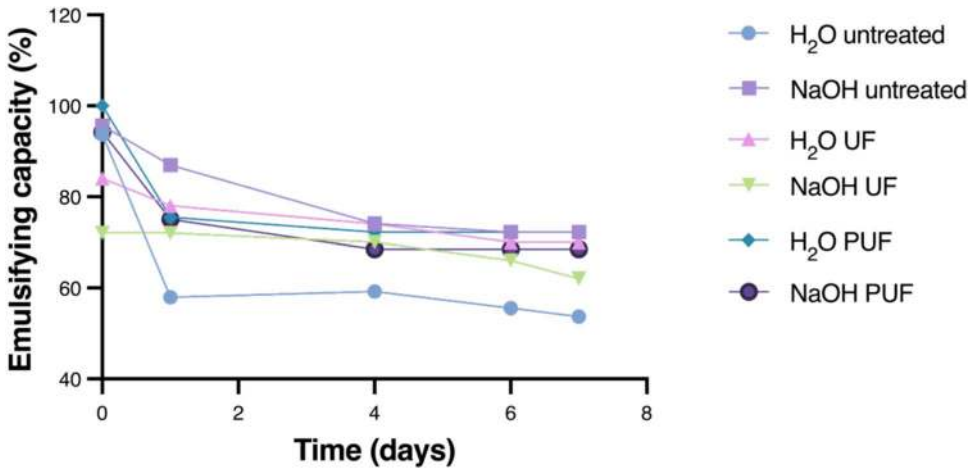

Fig. 10 Emulsifying stability of protein extracts obtained after sequential extraction with H_2O and NaOH from red seaweed *P. dioica* with and without the application of different treatments. Untreated, no treatment; UF, ultrafiltration; PUF, purification and ultrafiltration

rice, sunflower, and fava bean—as partial substitutes in hybrid meat emulsions. Their findings indicated that all plant substitutes exhibited good emulsion stability, with the minimum liquid loss being 1.73% for sunflower and the maximum being 12.5% for rice. More recently, Bojorges et al. (2025) reported emulsification capacities of around 91% for *S. latissima* and *A. nodosum* when proteins were extracted using enzymes and ultrasonication. In contrast, they observed an emulsification capacity of about 70% when a sequential extraction method (H_2O–HCl–NaOH) was applied.

Numerous studies on animal-derived proteins have demonstrated that the molecular weight of a peptide or protein is critical to its functional properties, particularly in the stability of emulsions. This is because the molecular weight can influence how proteins and peptides adsorb at the oil-in-water interface, as well as the characteristics of the interface layer (Yang et al., 2022). For example, the degree of hydrolysis obtained after enzymatic treatment of rice bran proteins strongly affected its emulsifying capacity. Using the highest level of hydrolyzation (~ 32%), the emulsion activity index decreased by more than 50% when compared to the native form, and the decrease was more evidenced when the solution pH was higher (maximum difference obtained for pH 9 with 80.6 ± 1.62 and 34.3 ± 1.19% emulsion activity index of native and high denatured samples, respectively). In our case, the existence of aggregation in NaOH extracts (untreated and PUF) allowed the stabilization of the oil-in-water interface and prevented the breaking of the emulsion, as a result of an interfacial film formation. In samples that showed some degree of denaturation with the presence of peptides of different sizes (NaOH UF extract) (see the "Proteins' Molecular Weight" section), the degree of hydrolysis was not high enough to affect the formation or stabilization of the obtained emulsion. Another protein feature that has a considerable impact on protein functionality is the degree of denaturation/unfolding. In this case study, the partial denaturation of proteins leads to the formation of molecular aggregates (in most NaOH extracts, except for NaOH UF) that, as described above, helped to stabilize the oil-in-water interface, and thus the emulsifying capacity of the samples remained high and with a good stability index. However, other studies described some influence of denaturation on protein functionality. A study conducted by Liu et al. (2011) verified that peanut protein isolate (PPI) extracted by alkali dissolution and acid precipitation showed poor functional properties when compared to native peanut protein (NPP) that was extracted by ammonium sulfate. In this work, the emulsifying capacity did not show any differences for low pH values, but significant differences were observed when pH was ≥ 7 (around 120 and 40 m^2/g for NPP and PPI, respectively).

Foaming Capacity

Foams are thermodynamically unstable systems due to the large interfacial area and influence of the film properties on the bubble surface. The presence of proteins can help to increase kinetic stability through adsorption at the air–water interface, which results in reduced interfacial tension and forms a viscoelastic interfacial layer on the bubbles that induces steric repulsion between the molecules that prevents coalescence. On the other hand, the protein interaction with gas bubbles can be affected by size, conformation, solubility, and the presence of other molecular components (e.g., lipids and carbohydrates), among others (Ramos & Vidigal, 2022).

Figure 11 shows the foaming capacity of all protein extracts obtained after a sequential extraction and application of different purification methods. It is possible to observe a major difference between the H_2O and NaOH fractions on the non-treated samples (166.7 ± 0.0 and 66.7 ± 0.0% for H_2O and NaOH fractions, respectively), which decreased with the application of UF methods and became similar for both ammonium sulfate precipitated samples (133.3 ± 0.0 and 141.7 ± 8.3% for H_2O and NaOH fractions, respectively). It is possible to verify that low foaming capacities were obtained in the untreated NaOH extracts when compared with the H_2O, but the application of UF reduced the difference between solvents, which became similar in the PUF extracts. Thus, it is possible to see that these purification treatments meant that the solvent effect was no longer a determining factor in the stabilization of the water–gas interface, possibly related to the presence of molecular aggregates after some denaturation. This also happened in the emulsifying capacity and indicates that the degree of aggregation was not sufficiently higher to become a negative factor, and the partial protein denaturation exposed polar and non-polar groups towards the water/air phase, respectively (Ding et al., 2022; Nicorescu et al., 2011). Further, in the case of NaOH, and unlike the case of the thickening properties, the presence of other small molecules and polysaccharides seems to have a deleterious effect on the foaming ability, as it is much higher for the precipitated and purified extract, with a protein content of 72%.

The foamy properties of the protein-enriched fractions may be correlated with the interfacial properties that were previously obtained using dilatational and interfacial shear rheology. Dilatational rheology was previously correlated with short-term stability, whereas interfacial shear rheology was related to long-term stability, which means that better dilatational properties indicate that foams can be formed with the systems, but stronger interfacial shear responses suggested that the interfaces formed would be more stable (Felix et al., 2021).

In a previous study conducted by Ghribi et al. (2015), chickpea protein concentrates' functional properties were

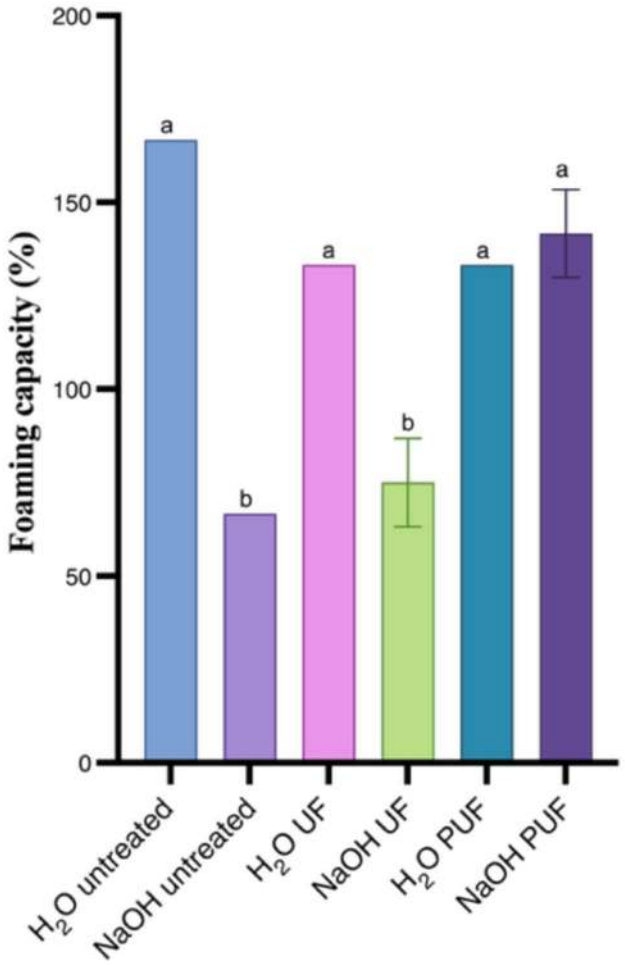

Fig. 11 Foaming capacity of different *P. dioica* protein-rich fractions with and without the application of ultrafiltration and purification techniques. Untreated, no treatment; UF, ultrafiltration; PUF, purification and ultrafiltration

studied after the application of drying methods, and a maximum value of 58.1 ± 1.6% for freeze-drying concentrates, which was smaller than the values obtained in this work, at least in the H_2O fractions. In the marine field, a recent work conducted by Guo et al., (2024a, 2024b) reported the existence of good foaming capacities (compared to commercial soy protein (SP)) of *Arthospira platensis* and *S. platensis* protein isolates extracted with phosphate buffer and purified using 30% or 50% ammonium sulfate precipitation. In this work, *A. platensis* precipitated with 30% of ammonium sulfate was allowed to obtain a foaming capability similar to SP, while the same conditions applied to SP showed to have a better foaming capacity significantly higher than the other samples (around 30%) but significantly lower than the ones obtained in our work (Jia et al., 2021). This information is quite valuable since there are not many studies regarding seaweed functional properties, and thus, microalgae and vegetable sources become a good point for comparison. In this work, foams with similar interactions in the air/water interface to some plant-based protein concentrates were obtained, confirming the applicability of all extracts in the development of new food products.

Conclusions

Results showed that proteins were successfully extracted from *P. dioica*, and the use of purification treatments (i.e., UF and PUF) helped to concentrate and purify the protein extracts with almost complete removal of the carbohydrate fraction (verified in the precipitated samples). On the other hand, these treatments caused the loss of some protein secondary structure and the formation of molecular aggregates. Chromatographic techniques demonstrated the existence of different protein size patterns, with the formation of molecular aggregates in the UF and PUF extracts that were also confirmed using Zetasizer particle measurement. However, despite the loss of structure observed with the application of the purification techniques and with the NaOH extraction, these did not prevent interesting functional properties from being obtained. The seaweed protein-rich extracts demonstrated the ability to be used as thickeners and were able to stabilize both water/oil and air/water interfaces, demonstrating to have good emulsifying (72–100%) and foaming capacity (67–167%), which were similar to the ones described for plant-based protein matrices. Therefore, the different treatments led to the extraction of protein-rich extracts with different physicochemical and functional characteristics. In line with this, useful data was generated to be used in the food sector when the development of novel food products, with strong insights on how chemical and structural characteristics of *P. dioica* proteins are affected by the processing and their implications on protein functionality. Also, the extraction process may be improved by the utilization of emerging technologies. Therefore, further studies for process intensification are needed to allow increasing extraction yields and extract functionality, making the process economically feasible. Overall, *P. dioica* extracts are a promising resource that can be studied as an alternative to animal proteins, to be used as texturizing food ingredients.

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Data Availability The authors declare that the data supporting the findings of this study are available within the paper and its Supplementary Information files. Should any raw data files be needed in another format, they are available from the corresponding author upon reasonable request.

Declarations

Competing Interest The authors declare no competing interests.

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