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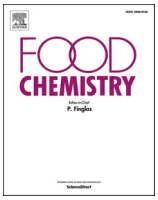

A new method for protein extraction from sea lettuce (*Ulva fenestrata*) via surfactants and alkaline aqueous solutions

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ABSTRACT

Alternative protein sources such as seaweed can help relieve the pressure on land-based protein supply. This proof-of-concept study developed an extraction method to recover soluble and lipophilic proteins from the seaweed *Ulva fenestrata*. The method consisted of processing *U. fenestrata* with 0.1–0.5 % aqueous Triton X-114 solution and reprocessing the pellet with an alkaline aqueous solution. Then, the solubilized proteins were precipitated via acidification. The new method extracted 3.4-times more protein, measured as total amino acids, compared to the control with two alkaline aqueous extraction cycles. Triton disrupted the chloroplasts and likely solubilized lipophilic membrane proteins as supported by microstructure and polypeptide pattern analysis. Triton-derived protein extracts contained lipids inside the precipitates/aggregates and were richer in fatty acids typical of photosynthetic membranes. The higher extraction yields are proposed to result from membrane charge neutralization upon acidification, triggering interactions between the membrane lipids and their subsequent precipitation with the lipophilic membrane protein.

1. Introduction

As the global population rises and the demand for protein increases, both plant- and animal-based terrestrial protein sources may face supply constraints (de Celente et al., 2023). To address this challenge, alternative protein sources such as seaweed offer nearly zero-carbon footprint protein that contains all essential amino acids, while requiring no irrigation, pesticides, or arable land (de Celente et al., 2023; Terme et al., 2020). However, cultivated seaweed typically contains 5–15 % protein on a dry weight (dw) basis, which is lower than traditional terrestrial protein crops e.g. soybean (35 % dw) (Steinhagen et al., 2021; Steinhagen et al., 2022; Trigo et al., 2023; USDA, 2018). On this matter, we successfully demonstrated that post-harvest cultivation of *Ulva fenestrata* in onshore tank systems with the addition of nutrient-rich seafood process waters increased the total amino acid content 3 to 5-fold, up to 21 % protein on dw basis (Stedt, Steinhagen, et al., 2022), thereby closing the gap to conventional plant protein sources. However, as with soybeans, peas, and other plant-based sources, seaweed requires additional concentration via extraction processes to serve as a protein source in the form of ingredients containing >50% protein (dw basis) such as isolates, concentrates, or flours.

Industrial processes to extract protein from seaweed are still in their early stages of development due to low extraction yields (usually 1–29 %, based on Lowry measurements) and/or low protein purities (Trigo, 2023). To tackle these challenges, some laboratory methods resort to combining polysaccharide-degrading enzymes with alkaline aqueous solutions to improve protein solubilization yields (Fleurence et al., 1995; Harrysson et al., 2019), which can diminish the potential of extracting intact valuable polysaccharides in additional downstream steps. Additional strategies include (i) adding a freeze-thawing cycle to maximize protein precipitation during pH-shift processing, i.e., alkaline solubilization followed by isoelectric precipitation, which in Abdollahi et al. (2019) raised total extraction yields by 2.4-times to 26 % and (ii) directly freeze-drying aqueous protein extracts (Connor et al., 2020; Kazir et al., 2019), although this process is costly. Despite these yield improvements, reported data are still far below the total protein extraction yields of >50 % usually delivered during industrial pH-shift-based protein extraction from common terrestrial crops such as soybean

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(Verfaillie et al., 2023). For other alternative protein sources such as insects and fish rest raw materials, extraction yields with the pH-shift principle can be even higher, reaching >60 % and > 70 %, respectively (Abdollahi et al., 2020; Mintah et al., 2020).

The relatively low extraction yields in seaweed can be partially attributed to its unique cell anatomy. Another key difference, especially compared to pulses, is that seaweed is rich in metabolic entities and poor in storage domains e.g., globulins. This leads to a higher degree of protein heterogeneity, which can be broadly categorized into water-soluble and water-insoluble proteins (Tenorio et al., 2018). The soluble fraction consists of e.g., the enzymes Rubisco, NADP reductase, as well as plastocyanin and ferredoxin. On the other hand, the insoluble fraction is mainly composed of integral thylakoid membrane proteins, such as photosystems I and II (PS I and PS II), Cyt b_6f, ATP synthase, and light-harvesting complexes associated with PSI and PSII (Buchanan et al., 2015; Rantala et al., 2020). While the soluble fraction is often targeted with traditional extraction processes (e.g., the pH-shift method), the aqueous-insoluble fraction can be solubilized using surfactants - a common approach in cell biology and proteomics. Surfactants are amphipathic molecules capable of disrupting lipid bilayers and surrounding the hydrophobic regions of the integral proteins, thus enabling their solubilization (Orwick-Rydmark et al., 2016). The non-ionic surfactant Triton X-114, which is widely available, relatively inexpensive, and non-denaturing to proteins, has proven effective in solubilizing thylakoid proteins from spinach, sugar beet leaves, and cyanobacteria (Bricker et al., 2001; Tamayo et al., 2017; Yu et al., 2014). Hence, despite not being considered food-grade, this surfactant was identified as a suitable candidate for a proof-of-concept study to increase protein extraction yields from seaweed.

To the best of the authors' knowledge, a complete extraction process for photosynthetic biomasses targeting both water-soluble and lipophilic proteins has yet to be reported. Based on this, a new extraction method was developed that was hypothesized to solubilize and recover both soluble and aqueous-insoluble proteins from *U. fenestrata*. The process involved a two-step protein solubilization with Triton X-114 followed by an aqueous alkali solution. The solubilized proteins were then recovered via isoelectric precipitation. Specific objectives were to (i) determine in which sequence the surfactant solution and the alkaline solution should be employed for maximal protein solubility yield; (ii) test the effect of surfactant concentration on protein solubilization and precipitation yields; (iii) map the polypeptide pattern in the seaweed pellets and supernatants obtained from the control treatment and the sequence that achieved the highest protein solubility yield; (iv) reveal the microstructure of the biomass, seaweed pellets, and protein extracts; and (v) assess how surfactant concentration influenced the composition of the final protein extracts in terms of total amino acids, fatty acids, ash, and surfactant levels.

2. Materials and methods

2.1. Production of protein-enriched U. fenestrata

2.1.1. Collection and characterization of seafood process water

Saturated salt brine from the maturation of herring fillets (SAL) was collected in February 2022 at a secondary herring processor (Klädesholmen Seafood AB, Rönnäng, Sweden). After collection, coarse particles (> 300 um) were removed by filtration and the SAL-water was then stored at −60 °C. The total ammonia content was quantified as described by Stedt, Trigo, et al. (2022).

2.1.2. Post-harvest cultivation to increase natural protein levels

The seaweed used in this study was obtained from a long-term indoor tank culture located at Tjärnö Marine Laboratory. *U. fenestrata* gametophytes were cultivated in 90 L tanks, maintained at a temperature of 14 °C, under a 16:8 h (L:D) light cycle with an irradiance of 100 µmol m^{-2} s^{-1}. The seawater used for cultivation was supplemented with 1:100 Provasoli Enriched Seawater (Provasoli, 1968). The biomass was harvested in February 2022 and promptly transferred to a 500 L tank

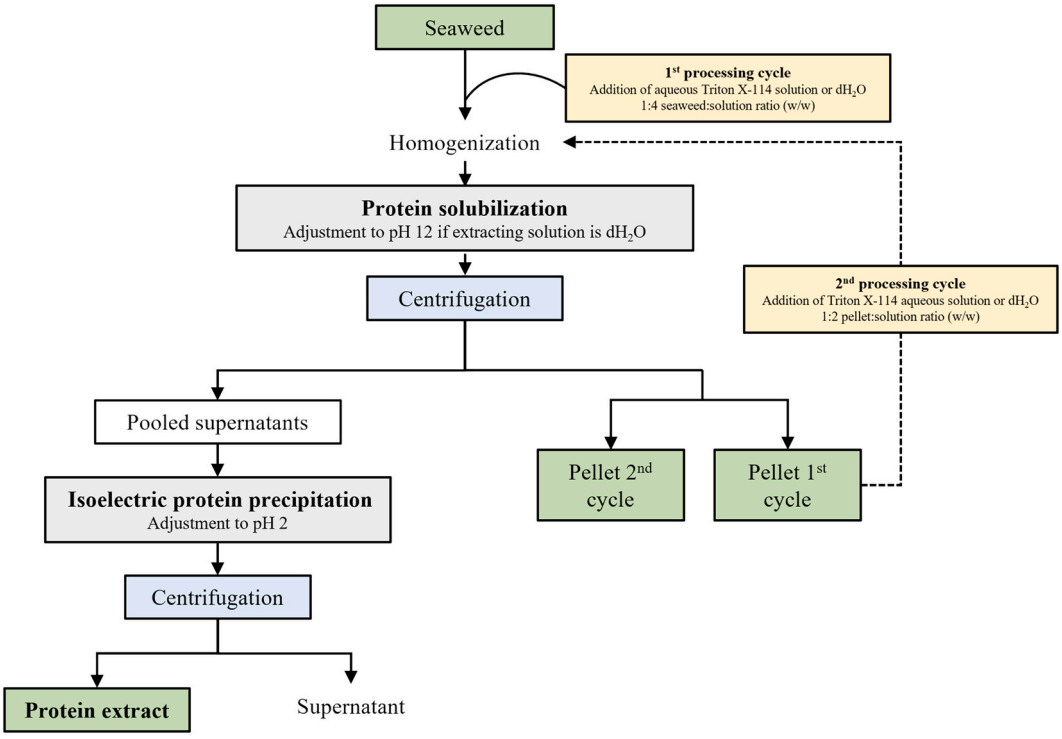

Fig. 1. - Scheme of the surfactant-based method developed to extract protein from the seaweed *U. fenestrata*. Green boxes correspond to the fractions where the total N was measured to enable the calculation of N solubilization, precipitation, and total yields. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

under the same cultivation conditions. To ensure optimal nutrient levels for seaweed protein accumulation, SAL water was added to the tank to reach a concentration of 25 μM ammonium (NH_4^+); this was repeated every third day to avoid nutrient depletion. Following a 15-day post-harvest cultivation, the protein-enriched *U. fenestrata* was collected.

2.2. Protein extraction

The fresh *U. fenestrata* was frozen at −80 °C and then minced in the frozen state using a meat grinder (Model C-E22N, la Minerva) with a 2.0 mm hole plate. Then, the biomass was divided into portions of 100 g, placed in polystyrene zip-lock bags, and stored again at −80 °C until further use. The protein extraction process, comprising a 2-cycle solubilization step and a precipitation step, was repeated in duplicate for each tested version. All processing steps were performed at 4 ± 1 °C.

2.2.1. Protein solubilization

To solubilize proteins from *U. fenestrata*, around 80 g of minced *U. fenestrata* was mixed with one of the following solutions at a 1:4 ratio (seaweed:solution; *w/w*): (i) aqueous 2 % (*w/w*) Triton X-114 based on Tamayo et al. (2017), (ii) aqueous 0.5 % Triton X-114, (iii) aqueous 0.1 % Triton X-114 or (iv) distilled water (Fig. 1). The mixtures were homogenized (LM5, Silverson) for 30 s at 6500 rpm and then increased to 8000 rpm for an additional 60 s. For the mixture containing only seaweed and distilled water, the pH was brought to pH 12 with 1 M NaOH. This pH value was chosen as earlier works have reported it to provide the highest protein solubility in *Ulva sp.* (Angell et al., 2017; Harrysson et al., 2018; Trigo et al., 2021). After incubation, all mixtures were centrifuged at 8500 ×*g* for 15 min at 4 °C (Sorvall LYNX 6000, Thermo Scientific). The recovered supernatants containing the solubilized proteins were kept in an ice-water bath, whereas the pellets were subjected to a second processing cycle. The reprocessing consisted of mixing the pellet with one of the abovementioned solutions in a 1:2 ratio (pellet:solution; *w/w*). Then, the mixture was homogenized and centrifugated as described earlier. The first and second pellets were freeze-dried and ground to a fine powder with a mortar and pestle. In one separate experiment, solubilization with 2 % Triton X-114 was combined with a 20 kHz ultrasound treatment (UIP1000hdT, Hielscher) at an amplitude of 60 % to evaluate whether extraction yields would improve sufficiently to allow omission of a second processing cycle.

2.2.2. Isoelectric protein precipitation

To precipitate the solubilized protein, the following samples were acidified to pH 2.0 with 1 M HCl: (i) supernatant from the first processing cycle; (ii) supernatant from the second processing cycle; (iii) mixture of the first and second supernatants, preserving the original ratio (*v/v*) between them. The pH value of 2.0 has been reported in several studies as the average isoelectric point of soluble *Ulva* sp. proteins (Angell et al., 2017; Harrysson et al., 2018; Trigo et al., 2021). After acidification, solutions were incubated under stirring for 15 min and were then centrifugated at 8500 ×g for 15 min at 4 °C. The resulting supernatant was discarded, and the pellet containing the recovered proteins is hereafter referred to as the *protein extract.* All obtained protein extracts were freeze-dried and ground to a fine powder with a mortar and pestle.

2.3. Protein determination

2.3.1. Total nitrogen analysis

The freeze-dried *U. fenestrata*, pellets, and protein extracts were analysed for nitrogen content through combustion elemental analysis (Elementar vario MICRO cube, Elementar Analysensysteme). The N solubility yield, N precipitation yield, and total N yield were calculated according to Eqs. 1–3.

$$N\ solubility\ yield\ (\%) = 100 - \left(\frac{N\ content\ of\ pellet\ [\%] \times Amount\ of\ pellet\ [g]}{N\ content\ of\ U.fenestrata\ [\%] \times Amount\ of\ U.fenestata\ [g]} \times 100\right) \quad (1)$$

$$N\ precipitation\ yield\ (\%) = \left(\frac{N\ content\ of\ protein\ extract\ [\%] \times Amount\ of\ protein\ extract\ [g]}{(N\ content\ of\ seaweed\ [\%] \times Amount\ of\ seaweed\ [g]\ ) - (N\ content\ of\ pellet\ [\%] \times Amount\ of\ pellet\ [g]} \times 100\right) \quad (2)$$

$$Total\ N\ yield\ (\%) = N\ solubility\ yield \times N\ precipitation\ yield \quad (3)$$

2.3.2. Total amino acids

Total amino acids (TAA) were analysed by LC-MS following an earlier protocol (Trigo et al., 2021). Briefly, 50 mg of freeze-dried *U. fenestrata* and its protein extracts were weighed into screw-cap glass tubes. To each tube, 4 mL of 6 M HCl was added and the hydrolysis occurred in a heating block at 110 °C for 24 h. The hydrolysed samples were then diluted with 0.2 M acetic acid, filtered (0.22 μm), and analysed in a LC-MS system (Agilent 1100 HPLC coupled to an Agilent 6120 quadrupole). A Phenomenex column (C18 (2) 250 μm × 4.6 μm × 3 μm), preheated to 50 °C, was used. The obtained data was compared against a set of 17 amino acid standards (Thermo Scientific) and analysed in triplicate. The total amino acid yield was calculated following Eq. 4.

$$Total\ AA\ yield\ (\%) = \left(\frac{Total\ AA\ content\ of\ protein\ extract\ [\%] \times Amount\ of\ protein\ extract\ [g]}{Total\ AA\ content\ of\ U.fenestrata\ [\%] \times Amount\ of\ U.fenestata\ [g]} \times 100\right) \quad (4)$$

2.4. Ash content

Ash content was determined gravimetrically with an analytical balance (sensitivity: 0.1 mg) by combusting 50 mg of freeze-dried seaweed pellets and protein extracts. The process consisted of the following steps: (1) gradual heating to 550 °C at a rate of 200 °C/h; (2) maintenance of the temperature at 550 °C for 6 h; (3) cooling to 200 °C. Samples were analysed in triplicate.

2.5. Total fatty acids

Fatty acids were analysed by GC–MS (Agilent 7890 A GC coupled to Agilent 5975C mass detector) on a VF-WAX column (30 cm × 0.250 mm × 0.25 μm) based on the method described by Harrysson et al. (2018).

Briefly, 25 mg of freeze-dried sample were trans-esterified with toluene containing C21:0 as internal standard and 10 % acetylchloride in methanol. A liquid-to-liquid extraction with ether was used to extract the derivatized fatty acids. After evaporating the ether extract, the residue was redissolved in isooctane and injected into the GS-MS system. Identification of fatty acids was done by using the GLC Reference Standard 463 (Nu-Chek Prep, Inc.), except for C16:1n9, C16:2n9, C16:4n3, C18:4n3, and C20:4n3, which was determined using the MS-library (NIST08). The previous fatty acids were quantified based on the area of C19:0, which was added to the samples before trans-esterification. A preliminary run revealed that C21:0 and C19:0 were not detected in the samples. All analyses were conducted in triplicate.

2.6. Triton X-114 content

The Triton X-114 content in the final protein extracts was determined according to Garewal (1973). Briefly, 10 mg of the protein extracts were resuspended in 1 mL of distilled water, followed by the addition of 0.1 mL of 1 M NaOH. Then, 0.3 mL of the solution was mixed with 0.4 mL of an ammonium cobalt-thiocyanate reagent, and the final mixture was incubated for 5 min before adding 1.5 mL of ethylene dichloride. After vigorous shaking for 2 min, the mixture was centrifuged (2000 $\times g$, 5 min) and the absorbance of the lower phase was recorded at 625 nm. The results were expressed as g Triton X-114 per100 g DW of protein extract.

2.7. Microstructure

Freeze-dried *U. fenestrata* and seaweed pellets were soaked in distilled water for one hour and then frozen in liquid nitrogen. A Leica CM3050S cryostat was used to cut samples into 7 μm sections, which were then applied to polysine-coated microscopy slides. Afterward, sections were mounted in water and examined with an Olympus BX53 light microscope (Olympus Life Science). Micrographs were captured with a CMos SC50 camera (Olympus Life Science) and processed with the Olympus software cellSense Entry.

For confocal laser scanning microscopy (CLSM) analysis, freeze-dried protein extracts were firstly resuspended in distilled water at pH 2 and were analysed: (i) for chlorophyll auto-fluorescence in an unstained state; (ii) for protein and lipids, after mixing two drops of the suspensions with 2 μL each of Texas Red (Invitrogen) and BODIPY™ FL C16 (Invitrogen), followed by incubation for 30 min; and (iii) for chlorophyll and lipids, as described in (ii), but only adding the BODIPY™ dye to the suspensions. For chlorophyll auto-fluorescence, a 488 nm argon laser and a 633 nm HeNe laser were used for excitation, and emissions were collected at 500–536 nm (auto-fluorescence in green) and 669–700 nm (auto-fluorescence of chlorophyll shown in red). For protein and lipid analysis, a 488 nm argon laser and a 594 nm HeNe laser were used for excitation; emissions were collected at 500–536 nm (lipids shown in green) and 608–639 nm (protein shown in red). Analysis of chlorophyll and lipids used 488 and 633 nm lasers and emission was collected at 500–216536 and 669–700 nm. Micrographs were captured with a Leica TCS SP5 (Heidelberg, Germany) with a HCX PL APO CS 63 × 1.20 W objective. The image format was 1024 × 1024 pixels, eight lines average.

2.8. Polypeptide pattern

The polypeptide pattern of *U. fenestrata* biomass, pellets, as well as supernatants from the first and second processing cycles were evaluated through SDS-PAGE. The presence of Triton X-114 is reported to interfere with the proper binding of SDS to proteins, potentially leading to improper protein denaturation and gel migration (Orwick-Rydmark et al., 2016). Therefore, all samples, including the surfactant-free ones, were treated with ice-cold acetone to remove the Triton X-114 following the protocol from Orwick-Rydmark et al. (2016).

For SDS-PAGE, the acetone-treated samples were combined with 5 % SDS, followed by homogenization using a polytron at 18000 rpm for 2 min. The protein content of these solutions was determined using the method described by Markwell et al. (1978). Subsequently, the samples were diluted with 5 % SDS to a final protein concentration of 4 mg/mL. The diluted samples were mixed with glycine sample buffer (with or without 2 % (*v*/v) β-mercaptoethanol) in a 1:1 ratio. The mixture was then heated to 95 °C for 5 min and centrifuged at 15000 $\times g$ for 5 min. Next, a volume of the supernatant corresponding to 20 μg of protein was loaded onto precast Tris-glycine mini gels (12 %, Bio-Rad) along with 5 μL of a protein ladder (Dual Color Standards, 10–250 kDa, Bio-Rad). Electrophoresis was conducted on ice at a constant voltage of 100 V using a Mini Protein II unit (Bio-Rad). The gel was fixed in Coomassie Brilliant Blue R-250 destaining solution (Bio-Rad) for 60 min, followed by staining with Bio-Safe Coomassie stain (Bio-Rad) for 90 min. The destaining process was done overnight with MQ-water. A gel image was captured using the GelDoc Go imaging system (Bio-Rad) and the molecular weight distribution was evaluated using Image Lab 6.1 software (Bio-Rad). Partially purified Rubisco from spinach (R8000) was acquired at Sigma-Aldrich to aid identification.

2.9. Ionic strength

The ionic strength of both supernatants, before and after acidification, was recorded using a conductivity meter (CDM210, MeterLab). Sodium chloride (NaCl) was used as a standard, and the results were reported as mM NaCl equivalents. All measurements were carried out at a temperature of 20 ± 1 °C.

2.10. Statistical analysis

Student's *t*-test was used to test statistical differences in N solubility, N precipitation, and total N yields. For the compositional data, one-way ANOVA followed by Tukey's post hoc test for pairwise comparisons was used. For data that was not normally distributed, the non-parametric Kruskal-Wallis test was used. All tests were conducted using SPSS Statistics software and differences were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1. Protein solubilization

3.1.1. Effect of sequential extraction with surfactant and alkaline aqueous solutions

Fig. 2 shows the nitrogen solubility yield after extraction of *U. fenestrata* proteins with alkaline aqueous solution (at pH 12) or with 2 % Triton X-114, followed by re-extraction of the resulting pellet with one of these solutions. The highest total N solubility yield was recorded for the treatment 2Trit + Alk i.e., processing *U. fenestrata* with Triton and reprocessing the pellet with an alkaline aqueous solution. The yield was significantly higher ($p = 0.017$) than the control treatment Alk+Alk and set a new benchmark at 62%—the highest N solubilization yield reported to date, as noted in previous studies compiled elsewhere (Trigo, 2023). Specifically, for green seaweed species, solubilization yields in literature ranged from 9 % to 46 %, whereas for red and brown species, the solubilization yields were 13 to 45 % and 10 to 60 %, respectively (Trigo, 2023). By shifting the sequence of employing the surfactant and alkaline aqueous solutions (i.e., Alk+2Trit), the total N solubility yield was similar to the control Alk+Alk. Furthermore, performing both processing cycles with surfactant solutions (2Tri + 2Tri), resulted in a statistically higher total N solubility yield ($p = 0.045$) compared to Alk+Alk. The ultrasound treatment showed no significant differences in N solubility yield (49.0 ± 1.2 %) compared to the first cycle of the 2Trit + 2Trit and 2Trit + Alk treatments.

The first processing cycle of 2Trit + 2Trit resulted in more N being

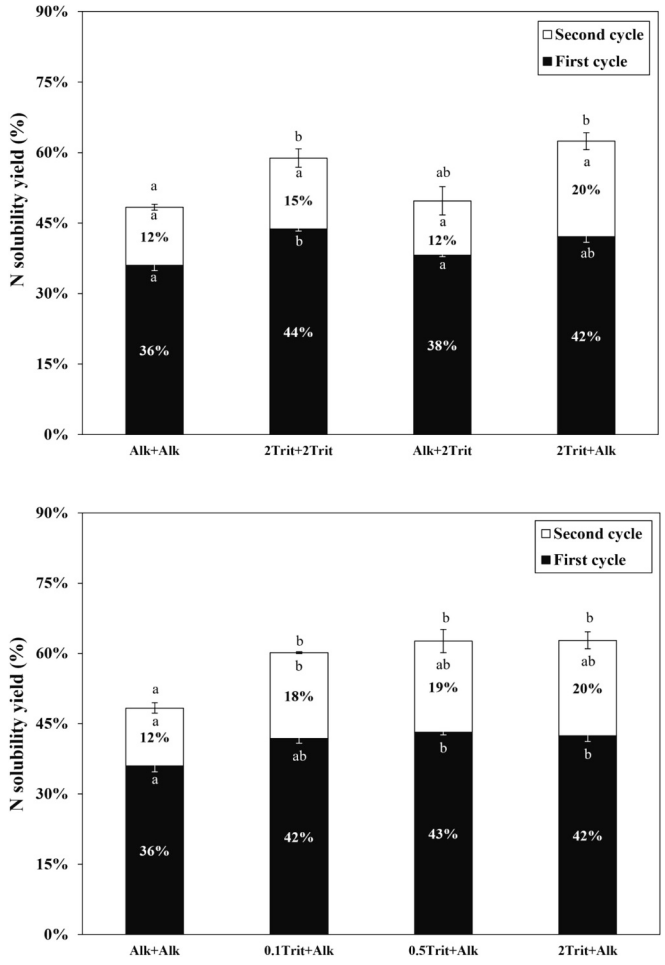

Fig. 2. – Nitrogen solubility yield (%) when (a) employing Triton X-114 in different steps during the extraction method and (b) testing different Triton X-114 concentrations in the first processing cycle, followed by reprocessing the seaweed pellet with water at pH 12. The standard deviations correspond to the yields of the first and second processing cycles (N = 2). Different letters (a-b) mean statistical differences ($p < 0.05$) within yields of first cycle, second cycle, and total sum. Alk – Alkaline; 2Trit – 2 % Triton; 0.1Trit - 0.1 % Triton; 0.5Trit – 0.5 % Triton.

solubilized when compared to the first processing cycle of Alk+Alk ($p = 0.024$) - a similar trend was observed between 2Trit + Alk vs. Alk+Alk. These findings can be related to the amphipathic nature of Triton X-114, which can solubilize membrane proteins by disrupting lipid bilayers such as those found in the envelope membranes of chloroplasts as well as in the thylakoid membranes (Buchanan et al., 2015) (see Sections 3.1.3 and 3.1.4). Additionally, the higher solubilization yield is likely due to improved cell wall permeability since surfactants can partially dissolve hemicellulose, a structural polysaccharide in *Ulva sp.* (Holdt & Kraan, 2011; Muñoz et al., 2022). Subsequent analysis of the total ash content of *U. fenestrata* and the seaweed pellets supports this hypothesis: after the first cycle of 2Trit + Alk, 75.3 ± 2.3 % of the total ash in the original biomass was released into the supernatant, compared to only 39.3 ± 0.1 % after the first cycle in Alk+Alk ($p < 0.001$). Hence, seaweed cell walls treated with Triton X-114 were probably more permeable to the extracting solvents, enabling higher N solubility yields.

In the second processing cycle, the N solubility yield was significantly higher ($p = 0.045$) for the biomasses initially treated with surfactant (2Trit + 2Trit and 2Trit + Alk) compared to the alkali-treated ones (Alk+Alk and Alk+2Trit), regardless of the solution employed in the second processing cycle. This was likely because chloroplast membranes were disrupted (refer to Section 3.1.3) leaving integral proteins more accessible for extraction and seaweed cell walls after surfactant treatment were more permeable to the extracting solution of the second cycle.

In summary, incorporating Triton X-114 in a two-step protein solubilization process proved to be an effective strategy to maximize the total N solubility yield. This effect was only seen when Triton X-114 was used in the first solubilization step, followed by an alkaline solubilization in the subsequent step. The improved total N-solubility yield is likely due to Triton X-114's ability to disrupt lipid bilayers (as explored in Section 3.1.3) and to induce structural cell wall changes.

3.1.2. *Effect of surfactant concentration*

Fig. 2b depicts the effect of different surfactant concentrations on the N solubility yield. Surfactant concentrations of 0.1 %, 0.5 %, and 2 % (*w/v*) were tested following the sequence of 2Trit + Alk as it achieved the highest total N solubility yield (Section 3.1.1). This trial was conducted to: (i) minimize the presence of surfactant in the final protein extracts; (ii) decrease costs associated with surfactant usage in the context of a hypothetical method scale-up; (iii) and enable protein precipitation as studied in **Section 3.2**. It should be highlighted that all tested concentrations were ≥ 10-fold above the critical micelle concentration (CMC) of Triton X-114 (0.01 % *w/v*, according to the manufacturer Sigma-Aldrich), which is required for effective membrane protein solubilization (Arachea et al., 2012). Reducing surfactant levels by factors of 4 and 20 times (0.5Trit + Alk and 0.1Trit + Alk, respectively) retained the total N solubility yields, which were significantly higher than those of Alk+Alk (*p*-values of 0.031 and 0.025, respectively). Therefore, surfactant concentration was minimized without compromising the total N solubility yield.

3.1.3. *Microstructure of seaweed and seaweed pellets*

Light micrographs revealed that unprocessed cells contained, as expected, a central chloroplast with the pyrenoid lying between the lobes (Fig. 3a). Alkali extraction preserved the well-defined chloroplast structure (Fig. 3b). On the other hand, extraction with 0.1, 0.5, or 2 % Triton X-114 led to the disintegration of the chloroplast structure and a reduced amount of chlorophyll within the cells of the seaweed pellets (Fig. 3c-e) compared to alkali extraction (Fig. 3b). Additionally, the amount of chlorophyll remaining inside the cells was similar across the three Triton X-114 concentrations tested. This observation aligned with the similar N solubility yields of ~43 % for each concentrations. If less chlorophyll was retained, then a change in N solubility yield would be expected since the pigment is typically associated with proteins, such as the Light Harvest Complex II (LHCII) (Buchanan et al., 2015).

3.1.4. *Polypeptide pattern in supernatants and seaweed pellets*

Fig. 4 illustrates the polypeptide patterns under reducing and non-reducing conditions of *U. fenestrata* and its supernatants and seaweed pellets obtained from Alk+Alk and 2Trit + Alk. A commercially available Rubisco from a plant was analysed as a control since plants and green algae share the same Rubisco isoform (classified as Rubisco I—B), which is composed of eight large and eight small subunits (Tabita et al., 2008).

The first processing cycle of 2Trit + Alk resulted in bands located at the top of the stacked gel and at >250 kDa, which were absent in the Alk+Alk treatment and the unprocessed *U. fenestrata*. Tamayo et al. (2017) reported similar observations when extracting leaf membrane proteins using the Triton X-114 phase-partition method. In the present work, both treatments (i.e., Alk+Alk and 2Trit+Alk) exhibited common bands at around 10, 50, and 105 kDa. The large subunit of the Rubisco standard was found at ~48 kDa (Fig. 4a), which may correspond to the band at ~50 kDa. However, in any lanes except for the Rubisco standard, it was not possible to identify the band corresponding to the Rubisco small subunit (~12 kDa), which might indicate that (i) the enzyme was present in limited quantities, (ii) Rubisco was not extracted during sample preparation, or (iii) crosslinking or endogenous

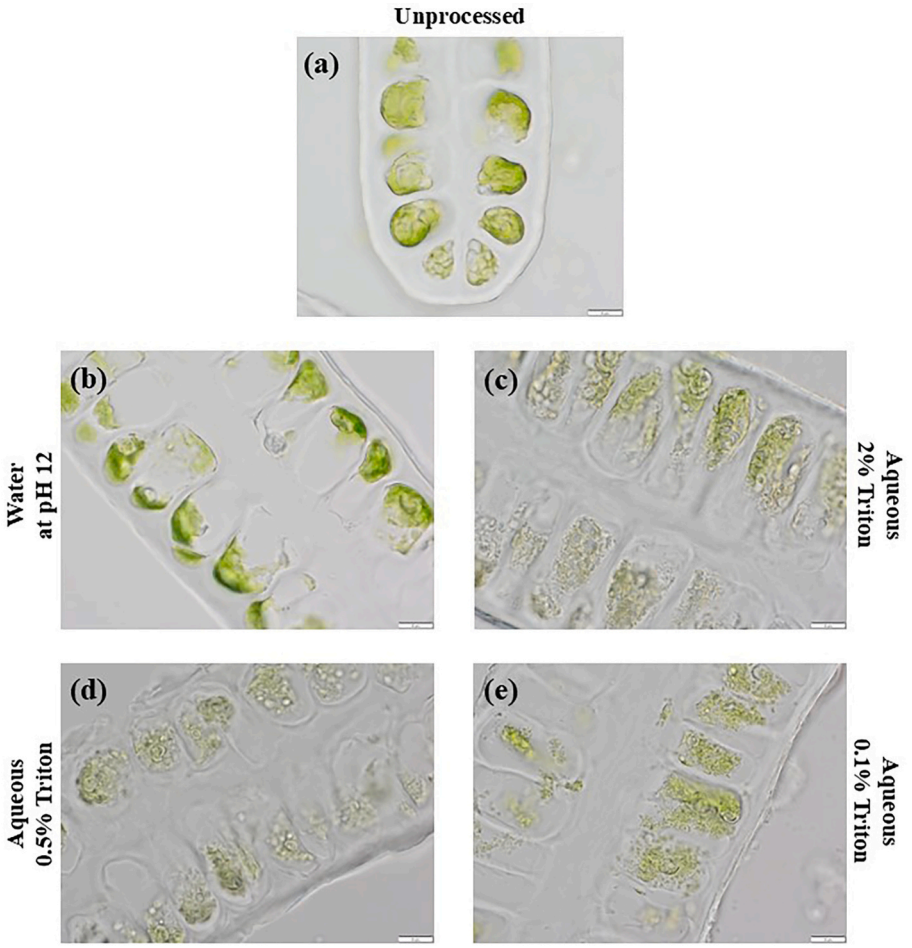

Fig. 3. - Light micrographs of cryosectioned unprocessed *U. fenestrata* (a) and seaweed pellets after treatment with alkaline aqueous solution at pH 12 (b) and 0.1, 0.5, and 2 % Triton X-114 aqueous solutions (c-e). The scale on the bottom right corner is 10 μm.

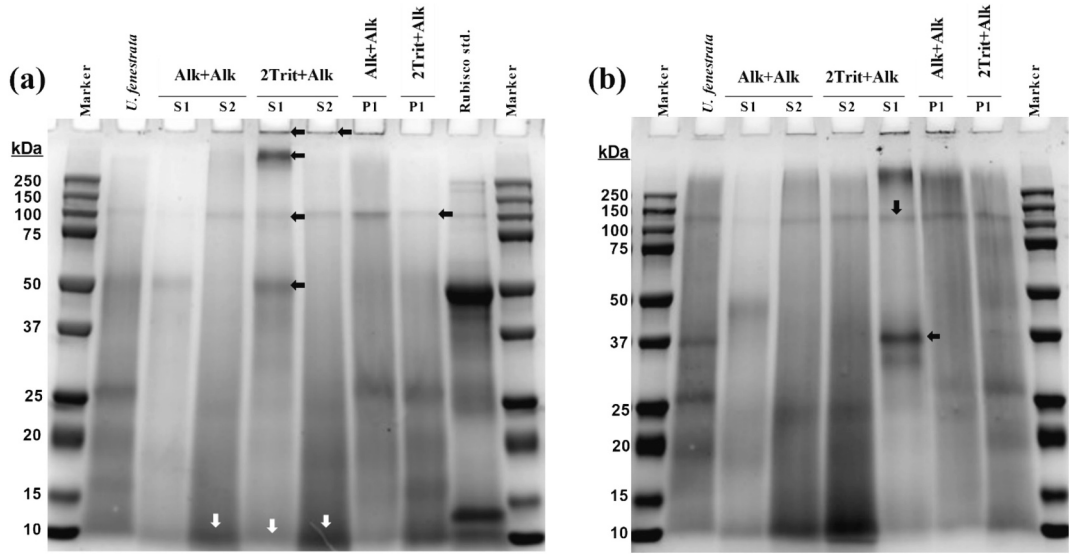

Fig. 4. – Polypeptide patterns of *U. fenestrata* biomass, supernatants, and pellets analysed through SDS-PAGE under reducing (a) and non-reducing conditions (b). Twenty micrograms of protein were loaded in each lane. Alk – Alkaline; 2Trit - 2 % Triton X-114; S1 and S2 – supernatants 1 and 2; P1 – seaweed pellet 1.

proteolytic degradations occurred, yielding larger or smaller (poly) peptides, respectively, during sample preparation. Although we did not find studies reporting on small and large Rubisco subunits following SDS-PAGE of unprocessed *Ulva* sp., previous studies have reported Rubisco activity in *Ulva* sp. (Beer et al., 1991; Bischof et al., 2002) and that Rubisco accounts for 7–37 % of the total soluble protein in seaweed

(Iñiguez et al., 2019). Another potential match for the band at ~50 kDa, as suggested by proteome analysis of the brown seaweed *Saccharina japonica*, is the ATP synthase subunit beta, which is located in the thylakoid membranes (Buchanan et al., 2015; Kim et al., 2011). The band at ~105 kDa displayed a higher intensity following Triton extraction compared to alkaline extraction; an inverse relation was observed for the pellets, with the Triton extraction yielding a less intense band at ~105 kDa, suggesting a partial extraction (Fig. 4a**)**. Under non-reducing conditions, the only high molecular weight band observed was around 129 kDa (Fig. 4b), suggesting that the band ~105 kDa is likely a protein subunit. While proteome analysis of seaweed is still limited, studies on the plant *Nicotiana tabacum* have associated a molecular weight of ~105 kDa to the ATP-binding subunit of the ATP-dependent caseinolytic protease, which is an integral protein found in the chloroplast (Wu & Yan, 2018). Therefore, the integral nature of this protein can elucidate why Triton extraction resulted in a denser band at ~105 kDa compared to Alk+Alk. Notably, under non-reducing conditions (Fig. 4b), a sharp band at ~37 kDa, likely corresponding to a non-monomeric protein(s), was found after the first processing cycle of 2Trit + Alk, but not in the corresponding supernatant of Alk+Alk. The proteome analysis of *N. tabacum* mentioned earlier revealed that over 70 proteins had a molecular weight between 36.5 and 37.5 kDa (Wu & Yan, 2018). Thus, additional proteomic studies are necessary to identify the specific protein corresponding to the band at ~37 kDa.

In the second processing cycle, the band at >250 kDa was absent after the 2Trit + Alk treatment (Fig. 4a), while the band at the top of the stacked gel exhitited lower intensity compared to the first cycle. Regarding the comparison between the second cycle supernatants of Alk+Alk and 2Trit + Alk, no major visible differences were observed; however, the 2Trit + Alk treatment resulted in a more intense band at 10 kDa.

The first cycle of 2Trit + Alk resulted in a dark green color supernatant (**Fig. S1**), which aligns with the disruption of chloroplasts and loss of chlorophyll observed in the microstructure analysis (Fig. 3). In contrast, the first supernatant of Alk+Alk was nearly colorless (**Fig. S1**). Upon chloroplast disruption, it is plausible that aqueous-soluble or alkali-soluble proteins located in the stroma could have been solubilized with Triton X-114; however, this is not evident in the SDS-PAGE gels (Fig. 4). The second cycle of Alk+Alk and 2Trit + Alk resulted in green supernatants and the color intensity was higher in the one from 2Trit + Alk. The green coloration indicates the co-extraction of chlorophyll, which has been used as an indicator of thylakoid membrane concentration (Emek et al., 2010). This is due to the association of chlorophyll with light-harvesting complexes (LHCs), such as the trimer LHCII. The latter represents about 50 % of the total protein in the thylakoid membranes and consists of ~25 kDa monomers (Buchanan et al., 2015). While the presence of LHCII or its monomers was not detected in the SDS-PAGE gels (Fig. 4a-b), it is reported that the trimer has a strong tendency to aggregate outside its *in vivo* conditions (Schaller et al., 2011). Moreover, membrane lipids have been reported to remain attached to LHCII after Triton-based extraction (Simidjiev & Barzda, 1997). Therefore, the bands at the top of the stacked gel and at >250 kDa in Fig. 4 probably correspond to aggregated LHCs associated with membrane lipids.

Overall, surfactant- and alkaline-based extractions yielded different polypeptide patterns of proteins extracted from *U. fenestrata.* Specifically, Triton X-114-based extraction appeared more selective towards membrane proteins like LHCII, though further confirmation is needed.

3.2. Isoelectric protein precipitation

Protein precipitation was induced by acidifying the obtained supernatants to pH 2, but this only occured at lower Triton X-114 concentrations i.e., 0.1 % and 0.5 %. Although non-ionic surfactants bind weakly to proteins through hydrophobic interactions (Dickinson, 1993), we hypothezise that at relatively high concentrations (i.e., 2%) they

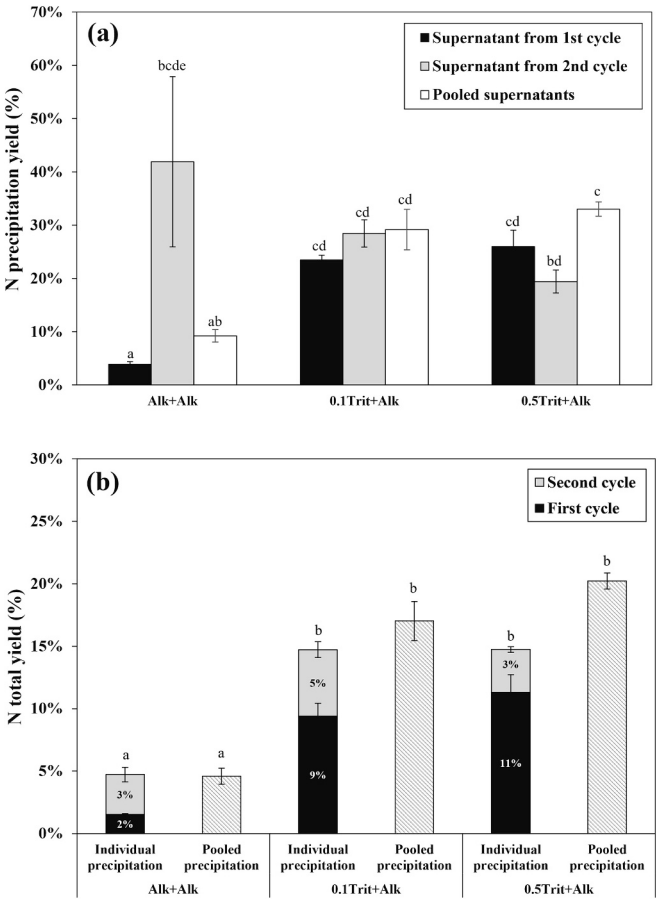

Fig. 5. - Nitrogen (a) precipitation and (b) total yields (%) of supernatants obtained after the first and second processing cycles as well as the pooled supernatant ($N = 2$). Proteins were solubilized by processing *U. fenestrata* with an alkaline solution at pH 12 or with solutions containing 0.1 or 0.5 % Triton X-114 and reprocessing the resulting seaweed pellet with an alkaline solution at pH 12. Different letters (a-d) mean statistical differences ($p < 0.05$) across all samples. Alk – Alkaline; 0.1Trit - 0.1 % Triton; 0.5Trit – 0.5 % Triton.

shield the hydrophobic regions of proteins. Therefore, it is plausible that such a shielding effect prevented protein-protein hydrophobic interactions, known to be the primary forces driving protein aggregation and precipitation. This effect can potentially be monitored by calculating the surfactant-to-N molar ratio, which was 0.01 and 0.06 for the 0.1 % and 0.5 % Triton treatments, respectively, compared to a ratio of 0.24 for the 2% Triton treatment.

Fig. 5a depicts the N yield after isoelectric protein precipitation of the supernatants obtained from the first and second processing cycles as well as the pooled mixture of these two supernatants. The reason to test the mixture was that, on an hypothetical industrial scale, using a single pooled supernatant for isoelectric protein precipitation would be more cost-effective and time-saving than performing two acidifications in parallel. Furthermore, it was hypothesized that the IS and Triton-concentration of the first supernatant would be further diluted into the second supernatant, leading to higher N precipitation yield and lower amounts of Triton in the final protein extract, respectively (Section 3.4).

According to **Fig. 5a**, N precipitation yields of the pooled supernatants containing Triton (i.e., 0.1Trit + Alk and 0.5Trit + Alk) were more than 3 times higher ($p < 0.05$) compared to the pooled supernatants from Alk+Alk, with no statistical differences observed between 0.1Trit + Alk and 0.5Trit + Alk. Precipitation yields reported elsewhere for seaweed protein were reviewed by Trigo (2023). Unfortunately, those studies (7 in total) only quantified protein via the Lowry method, thus

impairing direct comparison with data from the present study.

In general, pooling supernatants in Alk+Alk, 0.1Trit + Alk, and 0.5Trit + Alk did not yield significantly higher precipitation yields compared to the individual precipitation of supernatants from the first and second cycles (Fig. 5a). However, it is important to note that the 0.5Trit + Alk treatment showed a significant difference, with pooled supernatants resulting in a higher precipitation yield compared to the second supernant alone ($p = 0.033$).

In an earlier work with *Saccharina latissima*, we demonstrated, via a dialysis model, that relatively low IS after acidification contributed to higher protein precipitation yields (Trigo et al., 2023). In the present work, IS after acidification in treatment Alk+Alk was 198, 129, and 172 mM NaCl eq. for the first, second, and pooled supernatants, respectively. The corresponding data for treatment 0.1Trit + Alk were 166, 123, and 156 mM, respectively, while for 0.5Trit + Alk, IS values were 173, 146, and 172 mM, respectively. The contribution of the second supernatant to the volume of pooled mixture was 41 ± 1 % for Alk+Alk and between 22 and 25 % for 0.1Trit + Alk and 0.5Trit + Alk. Within the treatment Alk+Alk, it is plausible that low IS values contributed to the two highest N precipitation yields, particularly in the case of the second supernatant alone. However, in the presence of Triton X-114 (0.1Trit + Alk and 0.5Trit + Alk), no significant correlation was detected between N-precipitation yield and IS (Pearson correlation, $R = 0.16$, $p > 0.05$, $N = 6$), leading us to reject our initial hypothesis. Therefore, other factors are likely influencing the higher N precipitation yield, as explored in Sections 3.4 and 3.5.

3.3. Total protein yield based on N and amino acid analysis

The treatments 0.1Trit + Alk and 0.5Trit + Alk resulted in significantly higher total N-yields ($p < 0.001$) when compared to Alk+Alk (Fig. 5b). The statistical significance was maintained whether protein precipitation was performed on individual or pooled supernatants. Additionally, in all three treatments, pooling the supernatants before protein precipitation resulted in a similar total N yield compared to processing them separately.

Given the documented fluctuation of the nitrogen-to-protein conversion between the original biomass and the produced protein extracts (see Section 3.4), it was important to quantify the total amino acid content for an accurate estimation of the total protein yield. For this purpose, amino acid analysis was performed on the protein extracts obtained from the pooled supernatants, given that pooling offers higher

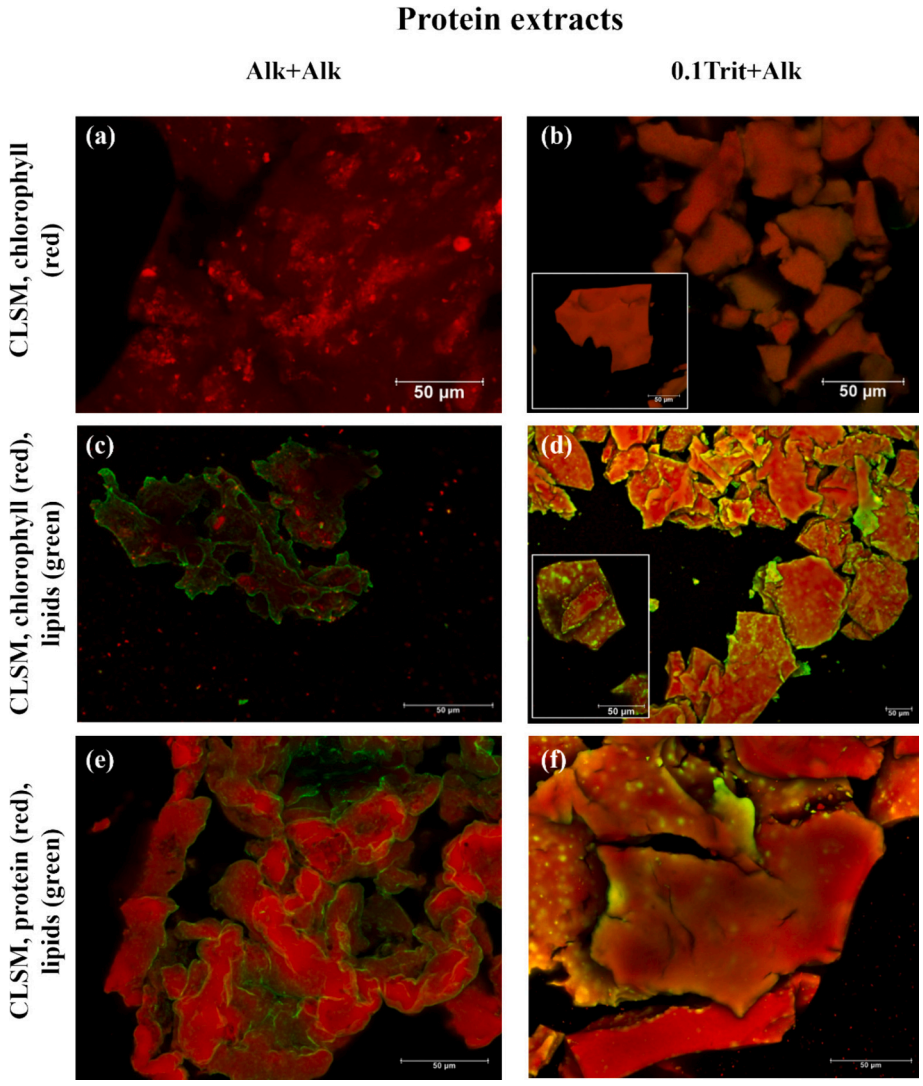

Fig. 6. – Confocal laser scanning micrographs of protein extracts from Alk+Alk and 0.1Trit + Alk resuspended at pH 2. Samples were unstained for chlorophyll autofluorescence and stained for protein and lipid with Texas Red and BODIPY™, respectively. All scale bars correspond to 50 µm; note that panel (d) is visualized in a different magnification. Alk - Alkaline; 0.1Trit - 0.1 % Triton X-114. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

scalability potential. The total amino acid yield of 0.1Trit + Alk was 22.6 ± 1.5 %, which represented a 3.4-fold increase when compared to Alk+Alk (p = 0.01). Furthermore, no statistical differences in total amino acid yield were found between 0.1Trit + Alk and 0.5Trit + Alk. In earlier works dealing with protein extraction from *U. fenestrata,* total amino acid yields ranged from 5 to 11 % (Harrysson et al., 2018; Trigo et al., 2021), thus placing the new extraction method around 2-fold above the maximum range of this interval. Other works on protein extraction testing the same *Ulva* species reported total protein yields based on the Lowry analysis method (Harrysson et al., 2019; Juul et al., 2021), thus hindering direct comparisons with data from the present study. However, it should be noted that the aforementioned studies maximized protein precipitation through a freeze-thawing cycle as part of the pH-shift method (Harrysson et al., 2018, 2019; Juul et al., 2021; Trigo et al., 2021) or ammonium-sulphate induced precipitation as part of a traditional protein extraction using sonication in water (Harrysson et al., 2018). While both strategies are relevant for academic purposes, their scalability is limited due to the elevated time and energy consumption as well as the extra steps required to remove ammonium sulfate.

The new extraction method achieved relatively high yields, but there is still room for further improvement. Preliminary data with surfactants from the Tween family revealed that at a concentration 10 times higher than their CMC, total N yield ranged from 15.2 to 19.6 % for the same biomass used in this study (data not shown). Furthermore, different types of non-ionic surfactants have been reported to selectively target certain membrane proteins (Arachea et al., 2012), so surfactant combination could potentially improve extraction yields. Surfactants are also characterized by their hydrophilic-lipophilic balance (HLB) value which can dictate their extraction efficiency as earlier reported for e.g., mitochondrial protein and phospholipids (Egan, 1976). In the latter study, optimum extraction was found between HLB values of 12.5 and 13.5; Triton X-114 has a HLB value of 12.4.

3.4. Microstructure of protein extracts and proposed mechanism for protein precipitation

Confocal laser scanning micrographs were captured for the protein extracts from the 0.1Trit + Alk and Alk+Alk treatments (Fig. 6). The objective was to understand potential reasons for the higher total yields observed with 0.1Trit + Alk versus Alk+Alk, primarily driven by improved precipitation yields. Micrographs examining chlorophyll autofluorescence indicated that protein aggregates in the extracts from 0.1Trit + Alk were richer in this pigment (Fig. 6a,c) when compared to those from Alk+Alk (Fig. 6b,d). As previously mentioned, chlorophyll is associated with LHCs (Buchanan et al., 2015), supporting the hypothesis that membrane proteins were solubilized and precipitated to a greater extent in 0.1Trit + Alk. Texas Red staining revealed that the protein aggregates from the Alk+Alk extracts were primarily composed of protein (Fig. 6e). It also showed more compact aggregates in 0.1Trit + Alk (Fig. 6e) than in Alk+Alk (Fig. 6f). Staining with BODIPY™ indicated the presence of lipids inside the aggregates of the 0.1Trit + Alk extract (Fig. 6d,f), but not in those of Alk+Alk (Fig. 6c,e).

It is reported that membrane lipids in leaves remain bound to LHCs even after Triton-based solubilization (Simidjiev & Barzda, 1997). Moreover, membranes in *U. fenestrata*, particularly those found in the thylakoids, contain lipids with sulfonic groups (Khotimchenko & Yakovleva, 2004). Due to the very low pKa of these sulfated groups (pH ≤ 2), it is probable that acidification to pH 2 triggered charge neutralization of the membrane surfaces, followed by solubility loss, promotion of hydrophobic interactions between the membrane lipids and their subsequent precipitation together with the bounded membrane proteins (Fig. 7). To the best of the authors' knowledge, no prior peer-reviewed and patented documentation exists for the precipitation mechanism depicted in Fig. 7, which aligns with the observations in Fig. 6d,f.

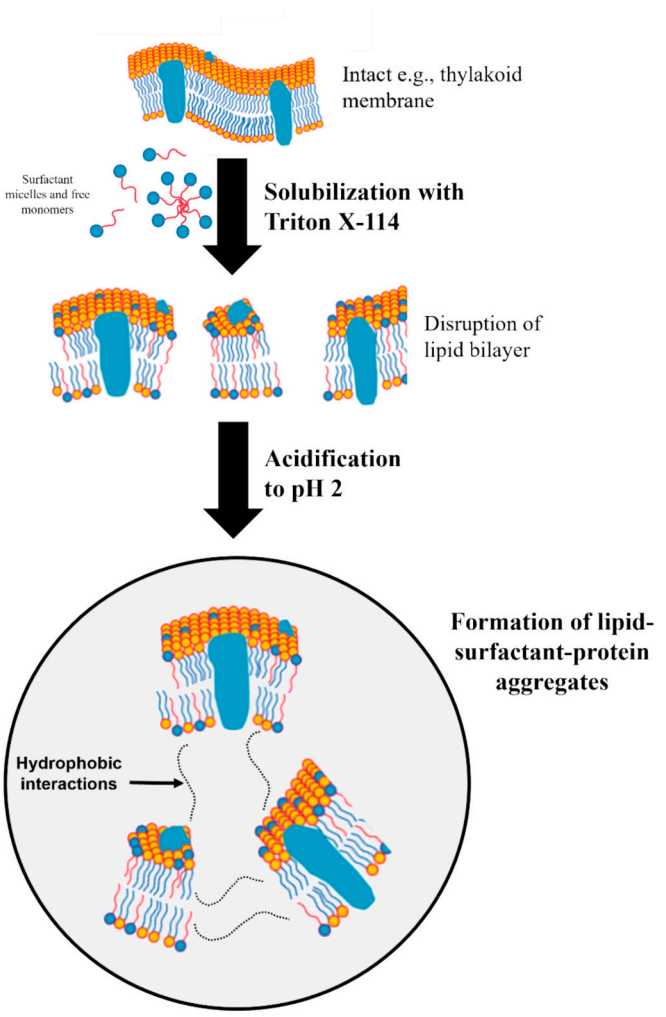

Fig. 7. – Proposed mechanism for the solubilization and precipitation steps of the new protein extraction method developed for *U. fenestrata*. Note that the role of surfactants in the solubilization of membrane proteins has been studied over the years (Kalipatnapu & Chattopadhyay, 2008).

3.5. Relationship between the fatty acid profile of the protein extracts and co-extraction of membrane lipids

Glycolipids constitute one of the main lipid classes in seaweed and terrestrial plants (Buchanan et al., 2015; Khotimchenko & Yakovleva, 2004). These lipids are the building blocks for photosynthetic membranes and are characterized by high levels of n-3 fatty acids, specifically 16:4n3, 18:3n3, and 18:4n3 in the case of *U. fenestrata* (Khotimchenko & Yakovleva, 2004). Thus, the content of n-3 fatty acids and the mentioned individual fatty acids may serve as a marker for co-extracted membrane lipids bound to the LHCs. According to Table 1, the protein extract produced in 0.1Trit + Alk had 22 % more n-3 fatty acids than the control Alk+Alk (p < 0.05). This difference was mainly attributed to a higher content in 18:3n3 and 18:4n3 (Table 1). As additional evidence of co-extraction of membrane lipids, the total n-3 fatty acids to protein ratio was similar between the extract produced with 0.1Trit + Alk and the biomass (~0.020), while in the Alk+Alk protein extract it was lower (0.013) (**Table S3**). A similar trend was observed for the ratios of 18:3n3 and 18:4n3, while the one for 16:4n3 remained consistent across all protein extracts and biomass.

3.6. Composition of the protein extracts

Table 1 presents the composition of the protein extracts, including

Table 1
– Composition of produced protein extracts ($N \geq 3$) regarding total amino acids, fatty acids, ash, and Triton X-114 content.

Protein extracts obtained from pooled supernatants	Amino acid analysis				Fatty acid analysis (g/100 g sample dw)					Total ash content (g/100 g sample dw)	Triton X-114 content (g/100 g sample dw)
	Total content (g/100 g sample dw)	TEAA (g/100 g amino acids)	Limiting amino acids*	N-to-Protein conversion factor	Total content	n3 PUFAs	16:4n3	18:3n3	18:4n3		
Crude *U. fenestrata*	23.6 ± 0.4^{a}	33.2 ± 0.4^{a}	Lys, Met, His	4.41	1.41 ± 0.04^{a}	0.49 ± 0.02^{a}	0.13 ± 0.01^{a}	0.22 ± 0.01^{a}	0.08 ± 0.00^{a}	26.2 ± 0.4^{a}	–
Alk+Alk	66.2 ± 2.8^{b}	36.8 ± 0.2^{b}	Lys, Met, His	6.14	2.37 ± 0.07^{b}	0.87 ± 0.01^{b}	0.40 ± 0.00^{b}	0.22 ± 0.00^{a}	0.11 ± 0.00^{b}	5.2 ± 0.1^{b}	–
0.1Trit + Alk	52.1 ± 1.9^{c}	37.4 ± 0.3^{b}	Met, His	5.76	3.16 ± 0.05^{c}	1.06 ± 0.01^{c}	0.30 ± 0.00^{c}	0.44 ± 0.01^{b}	0.17 ± 0.00^{c}	5.0 ± 0.7^{b}	10.0 ± 0.4^{a}
0.5Trit + Alk	34.1 ± 2.2^{d}	36.5 ± 0.1^{ab}	Lys, Met, His	5.37	n.a	n.a	n.a	n.a	n.a	n.a	36.9 ± 0.6^{b}

TEAA *total essential amino acids*; dw *dry weight*; n.a *not analysed.*
* Limiting amino acids according to the amino acid scoring pattern recommended for an adult by WHO/FAO/UNU (2007); Different letters (a-d) in each column indicate statistical differences ($p < 0.05$).

total amino acids, fatty acids ash, and Triton X-114 content. These parameters contribute to the evaluation of the nutritional value of the protein extracts, while also disclosing potential benefits and limitations compared to protein extracts obtained through other extraction methods.

Total amino acid content differed significantly ($p < 0.05$) between the crude *U. fenestrata* and protein extracts obtained from the pooled supernatants of Alk+Alk, 0.1Trit + Alk, and 0.5Trit + Alk. Moreover, total amino acid content ranked the protein extracts in the following order: 0.5Trit + Alk >0.1Trit + Alk > Alk+Alk, which indicates that selecting lower surfactant concentration leads to higher protein purities. Only the latter two were within or above the range of 51.2–58.4 % TAA per dw earlier reported for protein extracts from *U. fenestrata* (Harrysson et al., 2018; Trigo et al., 2021). The complete amino acid profile can be found in **Table S1**. Total essential amino acids increased from biomass to extract regardless of how proteins were extracted, which aligns with previously described protein extraction methods (Harrysson et al., 2018; Trigo et al., 2021). Among the essential amino acids for an adult, only the protein extract produced from 0.1Trit + Alk had lysine above the recommendations established by WHO/FAO/UNU (2007); other protein extraction methods applied to *U. fenestrata* and *Ulva ohnoi* produced an extract with lysine as a limiting amino acid (Angell et al., 2017; Harrysson et al., 2018). Furthermore, these cited studies also reported methionine and/or histidine as limiting amino acids, which aligns with our findings (Table 1). The nitrogen-to-protein conversion factor increased due to protein extraction, with the highest value (6.14) being recorded for Alk+Alk, followed by 0.1Trit + Alk, and 0.5Trit + Alk. This increase is consistent with data from Magnusson et al. (2019) and can be attributed to removing non-protein-bound nitrogen during protein extraction.

Compared to the crude biomass, total fatty acids were significantly up-concentrated 1.7- and 2.2-fold in Alk+Alk and 0.1Trit + Alk (Table 1), respectively, with the difference between the two treatments being statistically significant ($p < 0.05$). The result between treatments align with the increased presence of lipids in the micrographs of the protein extract from 0.1Trit + Alk (Fig. 6d,f). The fatty acid content of the crude biomass and protein extracts produced by Alk+Alk and 0.1Trit + Alk is provided in **Table S2**. The treatment 0.1Trit + Alk yielded a protein extract significantly richer in n-6 and n-3 polyunsaturated fatty acids (PUFAs), when compared to the extract produced with Alk+Alk ($p < 0.05$). Moreover, the content of the essential fatty acids C18:2n6 (linoleic acid) and C18:3n3 (α-linolenic acid) were found to be twice as high in the protein extract from the 0.1Trit + Alk treatment compared to Alk+Alk.

Protein extraction significantly decreased the total ash content ($p < 0.05$), with no differences observed between the Alk+Alk and 0.1Trit + Alk treatments. Lower ash content is an important factor associated with improved N digestibility in protein extracts derived from *Ulva* sp., as ascribed by Juul et al. (2022).

The treatment 0.1Trit + Alk resulted in a protein extract with a lower amount of Triton X-114, compared to the 0.5Trit + Alk treatment (Table 1). This was likely due to high moisture levels in the precipitated pellets (87–93 % moisture). Therefore, it is expected that further reduction of Triton X-114 content to levels closer to its CMC would result in protein extracts with higher protein, lipid, and ash content. It can also be concluded from Table 1 that the initial hypothesis - suggesting that lower amounts of Triton X-114 in the protein extracts could be achieved by diluting Triton X-114 from the first supernatant into the second supernatant - should be rejected. This rejection is based on the observation that, in the 0.1Trit + Alk treatment, protein extracts from individual precipitation of the first and second supernatants had a Triton X-114 content of 15.4 ± 2.9 % and 15.3 ± 2.6 %, respectively, which were statistically similar ($p > 0.05$) to the protein extract produced when pooling the supernatants (10.0 ± 0.4 %); similar observations were found for 0.5Trit + Alk (Table 1).

Overall, the new method resulted in total protein yields >3 times higher than the control method without surfactant. Additionally, it provided the co-benefits of producing a protein ingredient with lysine above the WHO/FAO guidelines, along with higher levels of n-6 and n-3 PUFAs and lower ash content.

4. Conclusion

This study aimed to develop a new extraction method that targeted soluble and aqueous-insoluble proteins from *U. fenestrata*. The process involved a two-step protein solubilization using Triton X-114 and alkaline aqueous solutions, followed by the recovery of soluble protein through acidification to pH 2. This study showed that:

- The treatment 2Trit + Alk, comprising a first processing cycle with 2 % Triton X-114 and then reprocessing the pellet with an alkaline aqueous solution (pH 12), achieved the highest N solubility yield.
- Triton X-114, but not alkaline extraction, disrupted the chloroplast membrane and promoted the loss of chlorophyll to the extraction solvent.
- The presence of bands at >250 kDa and at the top of the stacked gel during SDS-PAGE, indicates that chlorophyll-binding proteins (mostly lipophilic proteins) were solubilized during Triton-based extraction.
- The second processing cycle of 2Trit + Alk achieved the highest N solubility yield likely due to the action of Triton X-114 in the first cycle that disintegrated chloroplasts and made the cell walls more permeable to the second extraction solvent.

- Reducing Triton X-114 levels from 2 % to 0.5 % and 0.1 % enabled protein isoelectric precipitation.
- Once precipitation was enabled, N precipitation yields were up to 3.2–3.6 times higher compared to the control Alk+Alk.
- Pooling the supernatants before protein precipitation resulted in similar N precipitation yields compared to processing them individually, thereby enhancing the scalability potential of the method.
- Triton-derived protein extracts contained lipids inside the precipitates/aggregates, but not in those derived from Alk+Alk; the former extracts were richer in n-3 fatty acids, particularly C18:3 and C18:4, indicating that the lipophilic membrane proteins remained bound to membrane lipids during precipitation.
- The total amino acid yield was up to 3.4 times higher in 0.1Trit + Alk, compared to Alk+Alk.
- Protein extracts produced with 0.1Trit + Alk had a similar TEAA content and limiting acids as well as a higher content of the essential fatty acids linoleic acid and α-linolenic acid when compared to Alk+Alk.

In summary, this proof-of-concept study developed a more efficient extraction method that can contribute to the rising global need for more sustainable food protein. Future studies shall focus on viable food-grade alternatives to Triton X-114 and on reducing surfactant amounts in the protein extract. Moreover, it would also be valuable to test the method's efficiency in other photosynthetic biomasses.

CRediT authorship contribution statement

João P. Trigo: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sophie Steinhagen:** Writing – review & editing, Resources, Funding acquisition. **Kristoffer Stedt:** Writing – review & editing, Resources. **Annika Krona:** Writing – review & editing, Visualization, Resources, Investigation. **Simone Verhagen:** Writing – review & editing, Conceptualization. **Henrik Pavia:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Mehdi Abdollahi:** Writing – review & editing, Validation, Resources, Methodology, Conceptualization. **Ingrid Undeland:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: João P. Trigo, Ingrid Undeland, and Mehdi Abdollahi have a patent pending to Chalmers Ventures AB as a result of the work described in this article. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141839.

Data availability

Data will be made available on request.

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