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Ulva fenestrata protein – Comparison of three extraction methods with respect to protein yield and protein quality

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ABSTRACT

Seaweed is gaining attention as a possible alternative and sustainable source of proteins. This study investigates three protein extraction methods and their effect on protein yield and quality when applied to *Ulva fenestrata*. Two of the methods included alkaline extractions (pH-shifts); one version solubilizing the proteins at pH 8.5 and one solubilizing them at pH 8.5 followed by pH 12 (pH 8.5 + 12). The third method was a mechanical pressing, using a double screw press. All extraction methods were followed by isoelectric precipitation to concentrate the proteins. Extraction at pH 8.5 gave the significantly highest total protein yield after the isoelectric precipitation, followed by extraction at pH 8.5 + 12 and lastly mechanical extraction gave the lowest yield. Proteins extracted with both alkaline methods had a significantly higher solubility at pH 7 and pH 9, compared to proteins from the mechanical pressing. There were no significant differences between the three methods in total D/L-amino acid ratio. Amino acid cross-links measured as lysinoalanine (LAL) and lanthionine (LAN) where found in significantly higher amounts in alkali-extracted proteins compared to mechanically extracted, however not to a degree that expect to compromise functional or nutritional quality. Further, no significant difference in protein in vitro digestibility was found between extraction methods. In conclusion, results indicated that protein extraction at pH 8.5 can be recommended, especially regarding total protein yield and solubility of the final protein extract.

1. Introduction

There is a need for new and sustainable protein sources generated by the increasing world population [1,2]. Seaweed is one of the potential alternatives to land-based sources, particularly due to the ability of seaweed to provide several ecosystem services, such as nutrient remediation and carbon sequestration; improving water quality where it is grown and harvested, and supporting climate change mitigation [3–7]. The high productivity as well as a relative high content of essential amino acids (EAA) (36–42% of total amino acids) [8–10] makes e.g. green seaweeds of the genus *Ulva* interesting for protein extraction. Based on a biomass productivity of 70 t dry matter (DM) ha^{-1} $year^{-1}$ for commercially cultivated *Ulva ohnoi* in a land-based integrated aquaculture facility, *Ulva* spp. have been described as having a protein production potential competitive to production of protein rich soybean meal [11]. The protein content in *Ulva* depends on several factors, such as season, nutrient availability and geographic location [12] and is often between 10 and 26% (DM) [9,13], which is a relatively low protein amount compared to e.g. soybean. Further, the high content of fibers and phenolic compounds in seaweed negatively affect the protein digestibility [8,14,15], as well as it impedes the protein extractability [8,9,15,16]. However, *Ulva* is still interesting as a source of protein upon concentration or extraction of the protein [17]. Extraction of protein from *Ulva* spp. has been shown to increase in vitro protein digestibility

Abbreviations: CML, carboxymethylysine; DHA, dehydroalanine; FUR, furosine; LAN, lanthionine; LAL, lysinoalanine.

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compared with protein digestibility in the crude biomass [18,19]. Several different methods for protein extraction from seaweed have been evaluated during the past years [20–24]. Harrysson et al. [20] and Wong and Cheung [8] show some of the highest protein yields for *Ulva* sp., using alkaline extraction followed by isoelectric or ammonium sulfate-induced precipitation, respectively, the former referred to as the pH-shift method. A total protein yield of 36% was shown by Wong and Cheung [8], but the use of 2-mercaptoethanol to cleave disulfide bonds makes the process non-suitable for food. Using the food-grade pH-shift method on dry, grinded *Ulva* including an osmotic shock at start, followed by two consecutive incubation steps at pH 8.5 and pH 12 for protein solubilization, Harrysson et al. [20] showed a total protein yield of 29% after precipitation. High pH during processing has, however, earlier shown to induce cross-linking of aminoacyl residues and racemization of amino acids, especially in combination with heat, in protein isolates from other biomasses, e.g. soybean and alfalfa [25,26]. When protein is exposed to high pH and/or heat, β-elimination of cysteine and serine can occur forming dehydroalanine (DHA), which can further react with lysine and cysteine to form the aminoacyl cross-links lysinoalanine (LAL) and lanthionine (LAN), respectively [27]. These types of cross-links can, just like racemization of L-amino acids to D-amino acids, decrease protein quality and digestibility [28,29]. Not all proteins are equally susceptible towards racemization and crosslinking, and further concentration and availability of the reactants play important roles [25,27]. Furthermore, carboxymethyl lysine (CML) and furosine are well-characterized markers in the Maillard reaction [30,31]. The aim of this study was to investigate whether these alkaline-induced reactions occur during protein extraction from *Ulva fenestrata* Postels & Ruprecht 1840, by testing different protein extraction methods and their effect on the protein quality. It was hypothesized that protein exposed to pH 12 during extraction would generate an increased amount of D-amino acids and a higher degree of cross-linked aminoacyl residues, and therefore would lower the protein quality, compared to protein extracted at pH 8.5 or by mechanical pressing at native pH. By looking into both protein yield and process-induced modification effects caused by the different extraction methods, this study wish to contribute to an important discussion on the possible trade-off between protein yield and protein quality, an important focus point in designing future seaweed protein biorefineries.

2. Methods

2.1. Biomass

Ulva fenestrata was harvested from a long-term indoor tank culture at the Tjärnö Marine Laboratory (58°52′36.4" N, 11°6′42.84″ E), November 1st 2019. The seaweed was grown in 90 L cultivation tanks in a flow-through system with filtered seawater (5 μm filter and UV filter) (flow = 10–14 L h^{-1}) under permanent aeration. Salinity and temperature fluctuated depending on the surrounding weather and seasonal conditions. The *Ulva* was grown in a green house with a 16:8 h light:dark cycle at an irradiance of 140 μmol m^{-2} s^{-1} and light source INDY66 LED 60 W 4000 K 6000 lm. Detailed information on the molecular identification of the biomass used in this study can be found in Toth et al. [32] (GenBank accession numbers: MN240309-MN240311). After harvesting, biomass was stored at −80 °C prior to extraction. The frozen biomass was roughly chopped with a knife and then mixed before being divided into batches for the different protein extraction techniques, to secure homogeneity between batches. Biomass for pH-shift was grinded with a Titracarne grinder C/E22N (Minerva Omega, Italy) with a 4.5 mm hole plate.

2.2. Protein extraction techniques

Protein extraction was performed by three different processes, two of the three being versions of the pH-shift technique, using alkaline pH for protein solubilization, and the third process comprising mechanical pressing with a double screw press. Extractions were performed at 8 °C. An overview of the protein extraction techniques is provided in Fig. 1. Extractions were performed in duplicates.

2.2.1. Protein extractions using the pH-shift process

Following thawing under cold running water, protein was extracted from wet, grinded biomass using a modified pH-shift process protocol [20]. Biomass was mixed with de-ionized water in a ratio of 1:6 and homogenized with a Silverson L5M mixer at 8000 rpm for 2 min on ice. This homogenate was incubated 60 min at 8 °C with stirring to subject the biomass to osmotic shock. Afterwards pH was adjusted to pH 8.5 with 1 M NaOH and incubated upon stirring at 8 °C for either 60 min (process 1) or 80 min (process 2) (Fig. 1). For process 1, after incubation at pH 8.5, the pH was adjusted to 12 with 1 M NaOH and incubated 20 min at 8 °C upon stirring. Hence, total incubation time was equal between the two pH-shift processes. These incubation steps were performed to solubilize the proteins. After the solubilization of the proteins, the homogenates were centrifuged at 8000 x*g*, 10 min, 8 °C. The supernatant was adjusted to pH 3 with 1 M HCl and immediately frozen at −20 °C over-night. The acidified supernatant was thawed in a bag in cold water and centrifuged at 8000 x*g*, 20 min, 8 °C. The resulting pellet; the protein extract, was recovered for freeze drying and further analyses.

Pre-studies (supplementary material), investigating optimal pH for protein solubilization were made by incubating homogenate for 20 min at different pH levels before centrifugation, calculating highest protein solubility yield by Eq. (1). This showed the highest solubility being at pH 12. Optimal pH for protein precipitation were made as well, determining pH 3 to give the highest isoelectric precipitation yield (Supplementary data).

2.2.2. Protein extraction using mechanical pressing

In the third extraction process, protein was extracted using mechanical pressing at native pH; pH 4.6–4.8. Biomass was pressed with a double screw press (Angel Juicer 8500S, Domotech, Denmark), separating the biomass into juice (juice 1, Fig. 1) and pulp. A second pressing was performed on the pulp, after mixing the pulp with deionized water (1:1) for 10 min, resulting in a second press juice (juice 2, Fig. 1). Each juice was centrifuged at 8000 x*g*, 10 min, 8 °C. The supernatant was collected and adjusted to pH 3 with 1 M HCl and frozen immediately at −20 °C over-night. The acidified supernatant was thawed in a bag in cold water and centrifuged at 8000 x*g*, 20 min, 8 °C. Juice 1 and 2 were processed separately, until a final step where the two protein extracts (Fig. 1) were mixed and freeze dried for further analyses.

2.2.3. Calculations of protein yield

Samples were withdrawn from biomass, homogenates, juices and supernatants (sup) for protein analysis. The following calculations (Eqs. (1)–(3)) were made to determine the protein yield, [protein] being the protein concentration of the given matrix.

$$\text{Protein solubility yield } (\%) = 100 \times \frac{sup\ 1\ [\text{protein}] \times \text{mass } (sup\ 1)}{\text{biomass } [\text{protein}] \times \text{mass } (\text{biomass})} \quad (1)$$

$$\text{Protein precipitation yield } (\%) = 100 \times \left(1 - \frac{sup\ 2\ [\text{protein}] \times \text{mass } (sup2)}{sup1\ [\text{protein}] \times \text{mass } (sup1)}\right) \quad (2)$$

$$\text{Total protein yield } (\%) = \text{protein solubility yield} \times \text{protein precipitation yield} \quad (3)$$

2.3. Protein content

For solid samples; the raw biomass, pulp (mechanical pressing) and protein extracts, protein content was determined by combustion using a

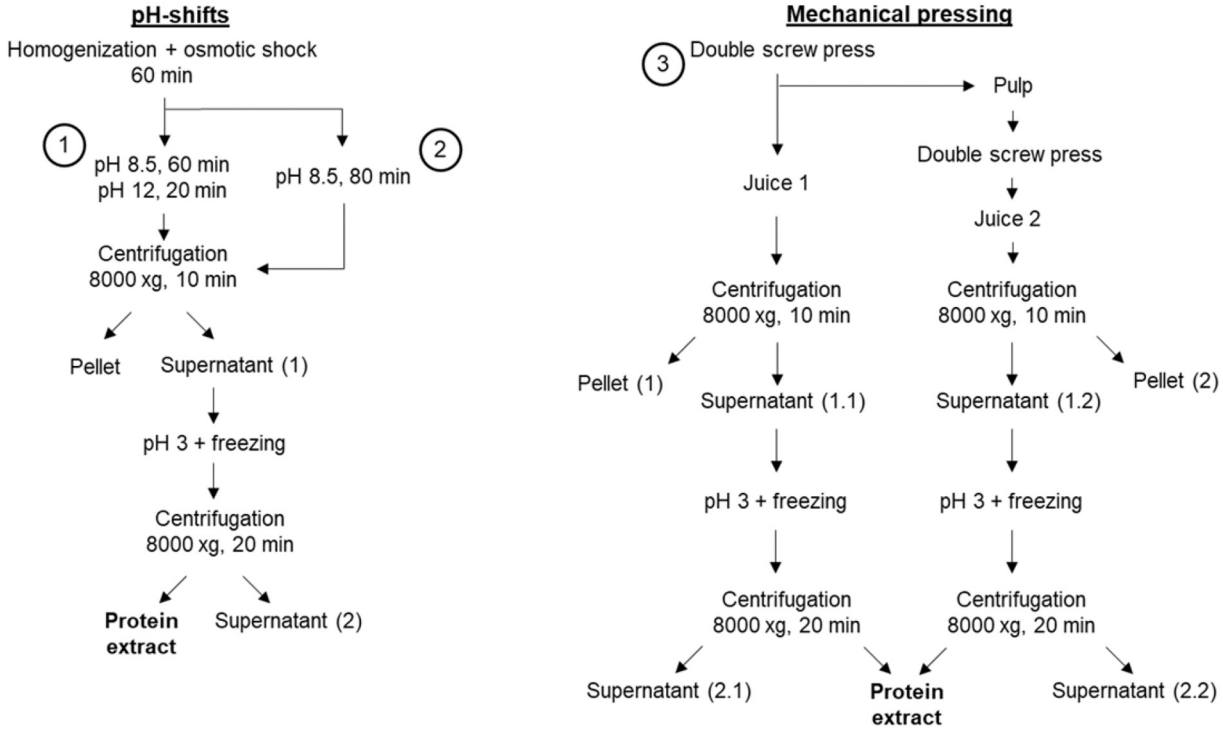

Fig. 1. Flow chart of the protein extractions performed in this study. The numbers 1, 2 and 3 denote the three different extraction processes investigated.

LECO Trumac nitrogen analyzer, using EDTA as standard. A nitrogen-to-protein conversion factor of 5 [33] was used for biomass and pulp, whereas a factor 6.25 [34] was used for protein extracts, as it was expected that the nitrogen being acid-precipitated was primarily protein nitrogen. For liquid samples, a modified Lowry method [35] was used. Samples were diluted 20–100 times in 0.1 M NaOH. For homogenates, 900 µL homogenate was mixed with 100 µL 1 M NaOH before further dilutions. One milliliter of the diluted sample was mixed with 3 mL of freshly made reagent of 1 part 4% $CuSO_4 \cdot 5H_2O$ (Fluka, Switzerland) and 100 parts mixed 2.0% Na_2CO_3, 0.40% NaOH, 0.16% Na-K-tartrate, and 1% SDS, and incubated 30 min at RT. Three hundred microliter freshly made phenol reagent (1 part 2 N FC reagent (Sigma-Aldrich, Germany) with 1 part distilled water) was added to the sample and incubated 45 min at RT in the dark. Absorbance was measured at 750 nm with a Cary 60 UV–Vis spectrophotometer (Agilent technologies). A bovine serum albumin (BSA) standard curve (10–100 µg mL^{-1}) was used for quantification. All protein determinations were carried out in minimum triplicates.

2.4. Protein solubility of protein extracts

Protein solubility was determined by dissolving 0.5 mg mL^{-1} of freeze dried protein extract in 30 mL MilliQ water. The initial pH of the protein suspensions were pH 4.1. The protein suspensions were divided into five aliquots, four of the tubes being adjusted to pH 5, 7, 9 and 11, respectively, with NaOH. After pH adjustment, samples were incubated 30 min on a magnetic stirrer before being centrifuged at 4000 xg for 10 min at RT. Protein concentrations of the resulting supernatants and the initial protein suspensions were analyzed by the Lowry method. Protein solubility was calculated according to Eq. (4).

$$\text{Protein solubility } (\%) = \frac{\text{supernatant [protein]} \times \text{V (suspension} + \text{NaOH}_{\text{added}})}{\text{suspension [protein]} \times \text{V (suspension)}} \times 100, \quad (4)$$

where V is volume and [protein] is the protein concentration of the given solution, either being the suspension, which is the protein extract mixed with the water, or the supernatant, being the resulting supernatant following centrifugation of the suspension.

2.5. Sodium dodecyl sulphate-polyacrylamid gel electrophoresis (SDS-PAGE)

Proteins were analyzed by SDS-PAGE using Criterion TGX Stain-free precast gels with 12% polyacrylamide (Bio-Rad Laboratories Inc., USA), following the technique described by Laemmli [36] under reducing conditions. Freeze dried protein extracts as the suspension made in the solubility test at native pH, was mixed with sample buffer (20 mM Tris, 2% SDS, 20% glycerol, 0.1 mg mL^{-1} bromophenol blue and 20 mM dithioerythrito (DTE)) in ratio 1:1. Liquid samples were normalized with deionized (18.2 MΩ) filtered water (0.22 µm) (MilliQ) water (Millipore SAS, France) to a protein content of 1.3 mg mL^{-1}, which was the lowest protein concentration found among the supernatant 1 and juice samples, and then mixed with sample buffer in a 1:1 ratio. Gels were stained with colloidal Coomassie Brilliant Blue [37], or by silver staining following the protocol according to Shevchenko et al. [38] with the use of ethanol instead of methanol and formaldehyde instead of formalin. Coomassie Brilliant Blue G-250 from Serva (Germany), PageRuler Plus Prestained Protein Ladder and Spectra Multicolor Broad Range Protein Ladder was from Thermo Scientific, and Criterion Tris-HCl gels from Bio-Rad (USA).

2.6. D/L-amino acids

The relative ratio of D- and L- amino acids was determined by liquid

chromatography mass spectrometry (LC-MS) analysis of hydrolyzed protein extracts according to Danielsen et al. [39]. In brief, 10 mg protein extract was hydrolyzed into amino acids using deuterated hydrochloric acid (DCl) (20 wt% solution in D2O, Acros organics, New Jersey, USA) in 1 mL Vacuum Hydrolysis Tubes (Thermo Scientific, IL, USA). The use of DCl avoided a possible bias from hydrolysis-induced racemization occurring during sample preparation, ensuring that the results represents the D- and L-amino acid ratio of the protein powder prior to hydrolysis (see [39] for further details). Then, a chiral derivatization with (*S*)-N-(4-nitrophenoxycarbonyl) phenylalanine methoxyethyl ester (S-NIFE) (Santa Cruz Biotechnology, Dallas, TX, USA) of the hydrolyzed samples allowed a subsequent separation of the D- and L-enantiomers on reverse phase HPLC. LC–MS/MS analysis was performed on a triple quadrupole tandem mass spectrometer (6460 TripleQuad LC/MS, Agilent Technologies, Santa Clara, CA, USA) coupled to a 1290 Infinity LC system (Agilent Technologies, Santa Clara, CA, USA). Chromatographic separation was carried out on a Luna Omega C18 column (100 × 2.1 mm, 1.6 μm, 100 Å) (Phenomenex, Torrance, CA, USA). The relative ratio of D-enantiomers was calculated based on the integration of the D- and L-enantiomer peaks in the same MS spectrum. Standard solutions and calibration standards were prepared as decribed in [39]. All solvents for LC–MS analysis were hypergrade (Merch, Darmstadt, Germany). L- and D-amino acid standards were obtained from Sigma-Aldrich (Darmstadt, Germany). An amino acid standard H (an 18 amino acid mix, Thermo Fisher Scientific, Waltham, MA, USA), was used as QC sample. Internal standards (IS) were purchased as a "cell-free" amino acid mix of 20 stable isotope-labelled amino acids (Cambridge Isotope Laboratories Inc., Andover, MA, USA).

2.7. Process-induced modifications

Process-induced changes on amino acids, carboxymethyllysine (CML), lanthionine (LAN), lysinoalanine (LAL) and furosine (FUR), were analyzed after hydrolysis of 50 mg protein extract using 1 mL 1% (*V*/V) mercaptoethanol, 3% (*W/V*) phenol in 6 M HCl in vacuum hydrolysis tubes (Thermo Scientific prod. 29,570) at 110 °C for 20 h. The samples were cooled at 4 °C and transferred to Eppendorf tubes, centrifuged for 5 min at 20800 x*g* (Eppendorf 5417 R centrifuge). The samples (500 μL) were neutralized with 350 μL 6 M NaOH, filtered through Whatmann Mini-UniPrep 0.2 filter vials and analyzed with LC-MS.

The quantitative analysis was performed on an 8050 triple quadrupole mass spectrometer (Shimadzu, Kyoto, Japan) coupled to a Nexera X2 LC system (Shimadzu, Kyoto, Japan). The LC was equipped with an Intrada column, Amino Acid, 150 × 3 mm, Imtakt (Prod. WAA35) purchased from Biolab DK. Solvent A was 0.1% formic acid in acetonitrile. Solvent B was 100 mM ammonium formate. The compounds were eluted from the column using a flow rate of 0.6 mL min^{-1} with a linear gradient 17% B at 0 min to 100% B at 16 min. To avoid carryover, solvent B was at 100% until 23 min where after 17% B was reached at 25 min and equilibrated for 10 min (35 min) at 17% B before next injection. The oven temperature was 35 °C. The electrospray ionization was set as followed; heating gas flow 10 mL min^{-1}, interface 300 °C, desolvation line 125 °C, heat block 250 °C, and drying gas flow 10 L min^{-1}. The precursor ions were identified running standards and the fragment ions were automatically generated in the LCMS solution 5.97 SP1 software. MRM transitions are presented in Table 1. The quantification was obtained from external calibration curves (10–2500 ng mL^{-1} for CML and LAN, 100–25,000 ng mL^{-1} for LAL, and 1–250 ng mL^{-1} for FUR) containing the same internal standard (IS) mix as for the sample preparation; carboxyethyllysine (CEL) D_4 IS was used for LAN, CML D_2 IS was used for CML, FUR D_4 IS was used for FUR and LAL. IS, CML, FUR, and LAL standards were from Iris Biotech GMBH (Marktredwitz, Germany), and LAN was from Sigma Aldrich.

Table 1
m/z for precursor, quantifier and qualifier ions for analytes. IS: Internal standard.

Component	Precursor	Quantifier ion	Qualifier ion
CEL D4 IS	223.0500	88.0000	134.0000
LAN	209.1500	119.9000	74.1000
CML D2 IS	206.9500	84.0500	130.2500
CML	205.0000	84.1500	130.3000
FUR D4 IS	259.2000	88.3000	134.1000
FUR	255.1000	84.0500	130.3000
LAL	234.1500	84.0500	130.2000

2.8. In vitro protein digestibility

In vitro digestibility was performed following the INFOGEST 2.0 protocol [40]. Enzymatic activity of amylase, pepsin and pancreatin (trypsin activity) was determined according to Brodkorb et al. [40]. Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were also prepared following the protocol by Brodkorb et al. [40]. Enzyme solutions were prepared just before use and kept on ice. Upon preparation of the pancreatin solution, pancreatin was mixed with SIF for 10 min and afterwards centrifuged at 3000 x*g* for 20 min at 4 °C and the supernatant was collected and used as the pancreatin solution. The in vitro digestion included the oral, gastric and intestinal phases. The dry protein extracts were mixed with MilliQ water to a 3% protein solution.

For the oral phase, 800 μL SSF and 5 μL 0.3 M $CaCl_2$ was added to 1 mL of the 3% protein solution, afterwards adding 100 μL of salivary α-amylase (A1031, Merck Millipore, Germany) solution to a concentration of 75 U mL^{-1} in the final oral digest, adding water up to a sample volume of 2 mL. The oral digestion was then performed for 2 min at 37 °C rotating samples at 40 rpm. To each sample, 1600 μL of SGF was then added along with 1 μL 0.3 M $CaCl_2$ and 1 M HCl to reach pH 3. Pepsin (from porcine gastric mucosa, P7000, Merck Millipore, Germany) solution was added to a 2000 U mL^{-1} concentration in the final gastric digest. Water was added to reach a gastric digest volume of 4 mL. For the gastric phase, samples were incubated at 37 °C for 2 h, rotating at 40 rpm. After the gastric phase, 1700 μL of SIF was added to the samples and pH adjusted to pH 7 using 1 M NaOH. Eight μL 0.3 M $CaCl_2$ was added along with bile solution to reach a bile concentration of 10 mM and 100 U mL^{-1} pancreatin (P7545, Merck Millipore, Germany) in the final intestinal digest, and adding water to a final volume of 8 mL. Samples were then incubated at 37 °C for 2 h, rotating at 40 rpm, simulating the intestinal phase.

After digestion, enzymes were inactivated by placing samples at 100 °C in a heat block for 10 min. Samples were stored in smaller aliquots at −80 °C before analysis. Digestions were performed in duplicate ($n = 2$).

To test the effect of the enzymes on protein hydrolysis, a set of control protein extract samples were going through the same procedure, except MilliQ water was added instead of the different enzyme solutions during the different digestion phases. Moreover, an enzyme control was included, where MilliQ water was added instead of protein extract to track enzyme self-digestion.

Before analysis of the extent of protein hydrolysis, 50 μL of the digests was mixed with 80 μL 5% trichloroacetic acid (TCA) (Sigma-Aldrich, Germany) incubating 30 min on ice and centrifuging 13,000 x *g* for 10 min at 4 °C. Supernatant was collected and the extent of protein hydrolysis of the samples during the in vitro digestion was analyzed by determining the concentration of free N-terminals. This was done using an *o*-phthaldialdehyde (OPA) spectrophotometric assay. Ten μL sample was incubated with 200 μL OPA-buffer for 15 min at room temperature in a 96-well plate before absorbance reading at 340 nm using a microtiter plate spectrophotometer (BioTek Instruments Inc., USA). OPA-buffer consisted of 18 mL 0.1 M $Na_2B_4O_7$, 0.1%SDS, and 5.7 mM DTE in MilliQ water added 2 mL 96% ethanol with 16 mg OPA (Merck, Germany). L-leucin (Sigma-Aldrich, Germany) (0–20 mM) in 1 mM HCl was used as a standard curve, expressing the extent of protein hydrolysis

of samples in L-leucin equivalents. OPA spectrophotometric assay was performed in triplicate for all digests, controls and standards ($n = 3$). Calculations of extent of protein hydrolysis in terms of freed N-terminals during digestion was done according to Eq. (5).

$$\Delta\text{N-terminals} = \text{n(sample)} - \text{n(sample control)} - \text{n(enzyme control)}, \quad (5)$$

where n is the concentration of free N-terminals expressed in L-leucin equivalents.

2.9. Statistics

The statistical analyses were performed in the software R, version 4.0.3 [41]. To study the effects of the different extraction methods, generalized linear models [42] with an identity link function was used. The Gaussian distribution was used for modelling all analytical data except for data on process-induced LAL formation, protein precipitation yield and total protein yield, which was defined with a gamma distribution, as this data was not normally distributed. Moreover, a logarithmic link function was used for the protein precipitation yield and total protein yield. Adequacy of models was tested by residual analysis. Post hoc analyses were performed using the R package postHoc [43]. *p*-values were adjusted for multiple testing by the method of controlling the false discovery rate [44] and significance level was set to $p = 0.05$.

3. Results

3.1. Protein yield

The *Ulva fenestrata* biomass had a DM content of 15.77 ± 0.48% and a protein content of 18.02 ± 0.73% (of DM). Protein extraction using the pH-shift method where proteins were solubilized at pH 8.5 gave the statistically highest total protein yield of 8.95 ± 0.79%. Mechanical pressing gave a total protein yield of 5.30 ± 0.59%, whereas the pH-shift method comprising solubilizing at pH 8.5 + 12 gave a total yield of 6.85 ± 0.18% (Fig. 2). The main difference between pH-shift and mechanical pressing was in the amount of protein coming out in solution during alkalization vs. pressing, with the former solubilizing significantly more protein (Fig. 2). The concentration of protein in the precipitated freeze dried protein extracts differed significantly between the mechanical pressing and pH-shift extracts. The concentration of protein in the protein extract from pressing was 48.1 ± 1.7% (of DM), whereas it was 60.0 ± 0.5% and 62.3 ± 2.5% (of DM) for the pH-shift extracts solubilizing at pH 8.5 and pH 8.5 + 12, respectively.

3.2. Solubility of protein extracts

Testing the solubility at different pH levels of the freeze dried protein extracts showed that protein extracted by mechanical pressing was significantly ($p < 0.02$) less soluble at pH 7 and 9, than the protein extracted by the pH-shift methods. At native pH, the protein extracted by mechanical pressing had a very low solubility, ~6%. The solubility increased with increasing pH, solubility being ~27% at pH 7, ~45% at pH 9, and increasing even further to ~60% at pH 11. Solubility of protein extracted by the two pH-shift methods did not differ significantly whether extracted at pH 8.5 or at pH 8.5 + 12. At native pH, ~15% of the protein was soluble, the solubility further increasing with increasing pH, reaching 67–69% solubility already at pH 7, and as high as 73–82% at pH 11 (Fig. 3).

3.3. SDS-PAGE polypeptide profile

The most visible bands for all protein extracts in the Coomassie stained gel (Fig. 4A) were at ~20 kDa, where a double or triple band

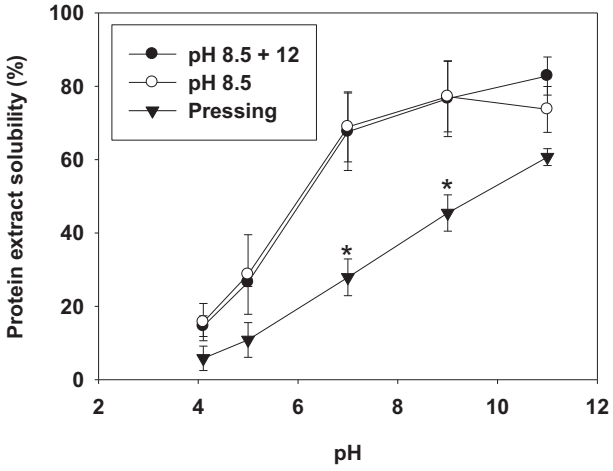

Fig. 3. Protein solubility of the freeze dried protein extracts from mechanical pressing at native pH (4.6–4.8) and the pH-shift methods comprising protein solubilization at either pH 8.5 or pH 8.5 + 12. The protein solubility was determined at different pH levels by dispersing freeze dried protein extract in MilliQ water in a concentration of 0.5 mg mL^{-1} and adjusting pH with NaOH. Data is represented as mean ± SD, n = 2. * indicates significance of difference ($p < 0.05$) between the protein extracts from mechanical pressing and the pH-shift methods at the specific pH values.

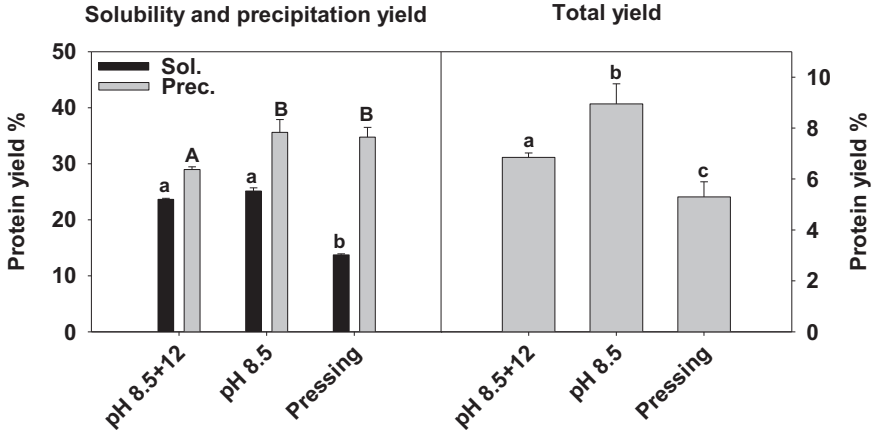

Fig. 2. Protein solubility yield (Eq. (1)), protein precipitation yield (Eq. (2)) and total protein yield (Eq. (3)). Data is represented as mean ± SD, $n = 2$. Different notations on bars indicate significance of difference between extraction methods ($p < 0.05$).

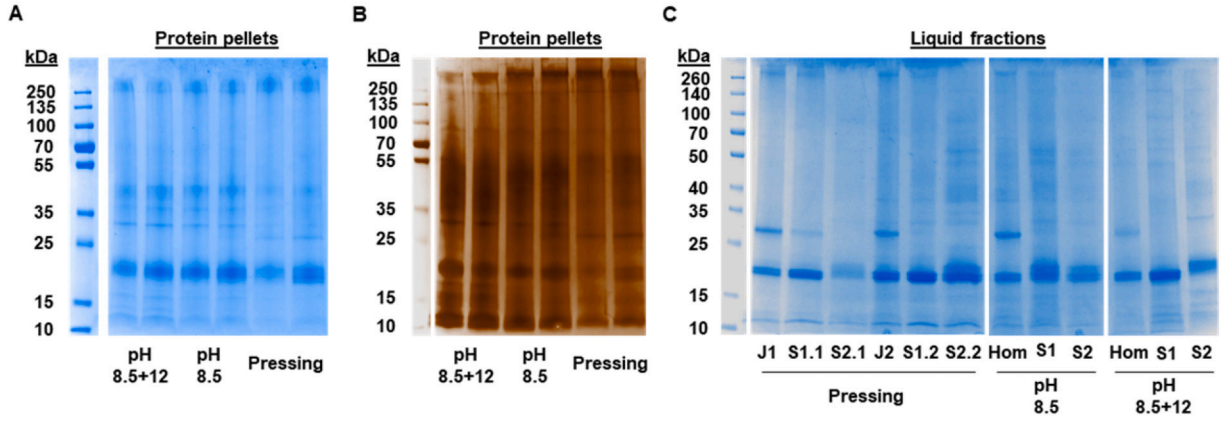

Fig. 4. Polypeptides in the different process fractions evaluated by reduced SDS-PAGE on 12% polyacrylamide gels. A) Coomassie staining of protein extracts (duplicate), B) Silver staining of protein extracts (duplicate), C) Liquid fractions from protein extractions. Pressing: J1 = juice from press 1, J2 = juice from press 2, S1.1 = supernatant 1 from press 1, S1.2 = supernatant 1 from press 2, S2.1 = supernatant 2 from press 1, S2.2 = supernatant from press 2. pH-shift methods: Hom = homogenate, S1 = supernatant 1, S2 = supernatant 2 (see Fig. 1).

appeared for all processes. Several bands were visible in the size from 25 to 50 kDa, being similar for the protein extracts from the different methods, except for a band appearing at ~33 kDa in the pH-shift extracted protein, which was less visible in the protein extract from mechanical pressing, where another band appeared at ~25 kDa. Especially in the pH-shift extracted protein, several bands were also visible in the low molecular weight range. For all samples, protein bands of >250 kDa appeared. Comparing samples between the Coomassie and silver stained gel, higher molecular weight bands became visible at ~90 kDa as well as a bands appeared at ~55 kDa upon silver staining. The band at 55 kDa seemed more pronounced in the protein extract from mechanical pressing.

For liquid samples (Fig. 4C), the triple band of ~20 kDa appeared in supernatant 1 from the pH-shift solubilizing only at pH 8.5, whereas only a double band was visible when incubation was also done at pH 12. A triple band also appeared in supernatants from mechanical pressing. Another major band showed at ~30 kDa in homogenates from pH-shifts and juice 1 and 2 from mechanical pressing, but seemed to be lost upon centrifugation as it did not appear in supernatants, except for supernatant 1.1. from mechanical pressing.

3.4. Protein quality parameters

The total ratio of D- and L-amino acids did not differ significantly between the extraction methods, the level of D-amino acids being <0.3% in average for all samples. However, for one amino acid, alanine, protein extract from mechanical pressing had a significantly higher ($p < 0.001$) D-alanine level, 0.44 ± 0.02%, compared to the pH-shift protein extracts with a level of ~0.33% (Fig. 5).

Process-induced changes on amino acids due to the different extraction methods were analyzed measuring levels of LAN, LAL, CML and FUR. Levels of LAN were significantly higher in protein extracts from the pH-shift method using solubilization only at pH 8.5, having a level of ~90 ng mg^{-1} protein, whereas for the other extraction methods the LAN content was ~62 ng mg^{-1}. Protein extract from the pH-shift method comprising solubilization at pH 8.5 + 12 had a significantly higher content of CML and LAL compared to the protein extracts from the two other methods. The LAL content of pH 8.5 + 12 protein extract was 44 ± 6.9 ng mg^{-1}, whereas it was 1.0–3.2 ng mg^{-1} in the other samples. Protein extract from mechanical pressing had a FUR content of 2.4 ± 0.3 ng mg^{-1}, which was similar to the content in protein extract from pH-shift extraction at pH 8.5 + 12 (2.0 ± 0.1 ng mg^{-1}), but significantly higher than in protein extracted only at pH 8.5, having a FUR content of 1.8 ± 0.01 ng mg^{-1} (Fig. 6).

Testing the protein digestibility with the INFOGEST method did not show any significant differences between the different protein extraction methods. The digestibility was measured by the occurrence of free N-terminals in the TCA soluble phase of the digests. The concentration of

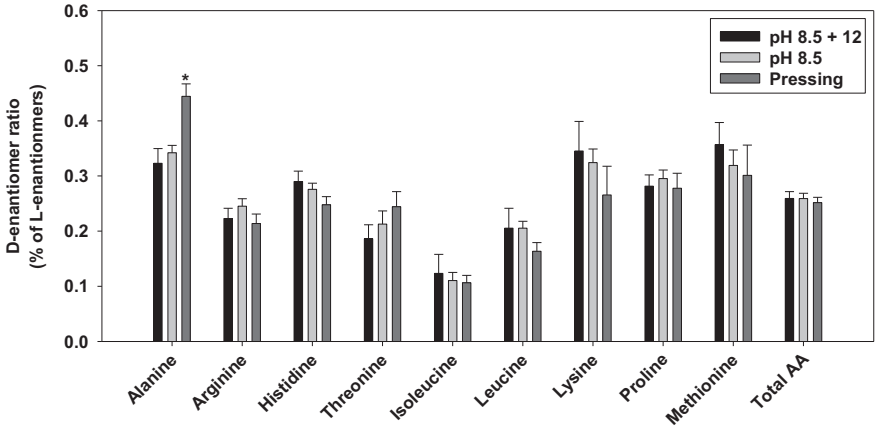

Fig. 5. Ratio of D-enantiomers of 9 given amino acids. The Total AA shows the mean D-amino acid level across the 9 amino acids shown in the figure. Data is represented as mean ± SD, $n = 2$. * indicate significance of difference between the extraction methods.

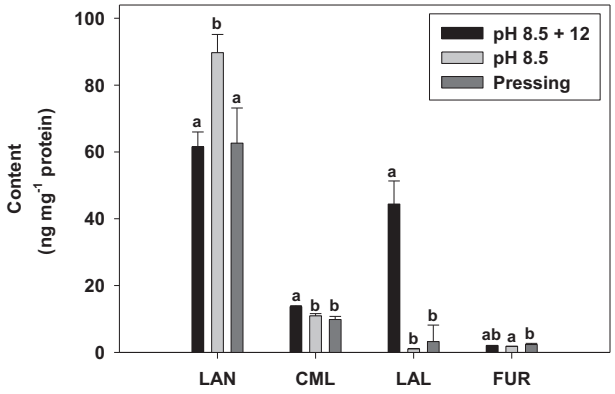

Fig. 6. Process-induced formation of lanthionine (LAN), carboxymethyllysine (CML), lysinoalanine (LAL) and furosine (FUR). Data is represented as mean ± SD, n = 2. Different notations on bars indicate significance of difference ($p < 0.05$).

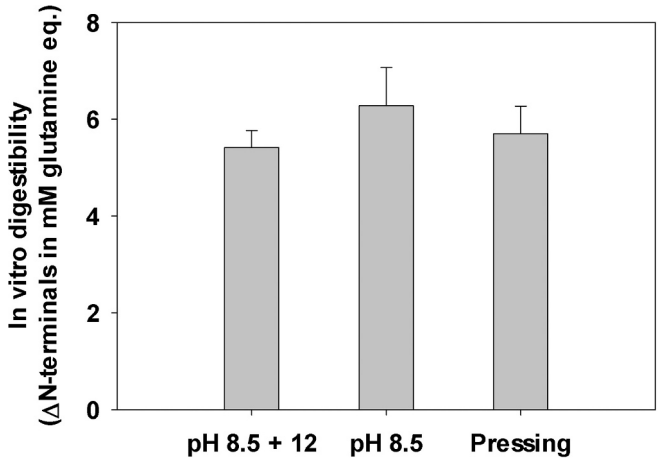

Fig. 7. Digestibility of proteins expressed as formation of free N-terminals in the TCA soluble phase of the digests resulting from in vitro digestion of protein extracts. Data is represented as mean ± SD, n = 2.

free N-terminals was 5–6 mM glutamine equivalents (Fig. 7), being similar to the digestibility of BSA, which was used as a control protein.

4. Discussion

The pH-shift method with protein solubilization only at pH 8.5 gave the significantly highest total protein yield. However, the protein yields using the two pH-shift methods (pH 8.5 and pH 8.5 + 12) were only a third as high as has earlier been observed by Harrysson et al. [20] who showed a total protein yield of 29% by using the same procedure (pH 8.5 + 12). Several factors might have influenced the large observed difference between the two studies, even though the input material of both studies originated from a long-term aquaculture of *Ulva fenestrata* which was maintained by parthenogenetic proliferation (gametophytic strain). It cannot be excluded that seasonal variation and stage of maturation of the biomass affect the general extractability of proteins. Such potential differences could result from the translocalization of proteins within the cell, for example when the vegetative thallus undergoes maturation and during formation of gametangia. It has been observed, investigated for certain red and brown seaweed species though, that the relative expression level of the photosynthetic protein Rubisco was higher in gametophytes as compared to sporophytes [45]. Moreover, in the study by Harrysson et al. [20] the biomass was freeze dried prior to extraction, which could allow milling of the biomass to finer particle size. According to Wijers et al. [46], the drying in itself should not improve the extraction. Further, the protein content (% of DM) of the biomasses differed between the two studies, 12.8 ± 1.5% (determined by Lowry assay) and 18.02 ± 0.73% (determined by LECO) in Harrysson et al. [20] and this study, respectively. Differences between protein determination methods may also add to different results [47]. The total protein yield was, however, more similar to the yield observed by Trigo et al. [19], who obtained a total protein yield of 11.1 ± 3.7% using the same extraction method (pH 8.5 + 12), pretreatment (freezing at −80 °C) and biomass species as in this study.

The protein yield obtained by mechanical pressing agrees to previous studies using mechanical extraction [21]. It was expected that the protein yield would be higher for the pH-shift method comprising protein solubilization at pH 8.5 + 12 compared to only solubilizing at pH 8.5, as pre-studies solubilizing at different pH levels showed a higher protein solubility of the *Ulva* biomass with increasing pH (supplementary data). This has also been shown by Harrysson et al. [48]. That there was no significant difference in protein solubility yield between the two pH-shift methods could be due to the relatively long incubation time of 1 h at pH 8.5 compared to a shorter solubilization time used in the pre-study. Moreover, certain proteins being extracted at pH 8.5 might have lost their solubility when increasing the pH to pH 12, which could be an explanation for the incubation step at pH 12 not enhancing the solubility yield further. This is supported by the SDS-PAGE analysis of the liquid samples, where an extra band was visible at ~20 kDa in supernatant 1 from the pH-shift extraction at pH 8.5, creating a triple band, whereas only a double band was visible for the supernatant 1 from pH-shift extraction at pH 8.5 + 12. This could be an indication of different solubility of individual proteins at different pH values. Otherwise, the polypeptide composition of the liquid samples looked similar regardless of extraction method. The SDS-PAGE analysis of the protein extracts also showed similar polypeptide composition regardless of extraction method, except of a band of ~25 kDa which was only visible in the protein extract from the mechanical pressing. Otherwise, it seemed that the main polypeptides being precipitated had a size of ~20 kDa. However, bands of >250 kDa were also visible, which most likely illustrated protein aggregates.

The protein extraction method did not seem to influence the ratio of D/L-amino acids. Thus, contradictive to the hypothesis, the extraction at pH 8.5 + 12 did not induce racemization from L- to D-amino acids compared to mechanical pressing and extraction at pH 8.5. The level of D-enantiomer amino acids was in general low, being <0.3% of total analyzed amino acids. The highest amount of a D-amino acid was found to be 0.44 ± 0.02% of D-alanine in the protein extract from mechanical pressing. This is, however, a lower amount than what can be found in some often-consumed fruits and vegetables [49], why the D-amino acid content in the seaweed-derived protein extracts in this study is not expected to compromise functional or nutritional quality. Extracting at pH 8.5 + 12 induced an increased ($p < 0.05$) amount of LAL, which is formed by cross-linking between DHA (derived from cysteine or serine) and lysine, whereas protein extracted at pH 8.5 alone showed a higher ($p < 0.03$) content of LAN, which is DHA cross-linked with cysteine [27]. LAN formation has shown not to be as pH dependent as the β-elimination forming DHA [50]. The higher concentration of LAL that was formed in protein extracted at pH 8.5 + 12 was most likely due to the pH 12 exposure, deprotonating the amino group of the lysine, which has a pK_a-value around 10. This means that more reactive ε-NH_2 groups are available for reaction with DHA at pH 12 than at pH 8.5, increasing the possibility of LAL formation [27]. Moreover, LAL formation is favoured above LAN formation at higher pH levels [51]. The content of LAL, even in the pH 8.5 + 12 protein extracts, can however be considered low, the amount being less than what is observed in regularly consumed foods, such as different milk and cereal products [27,52]. In the *Ulva* protein extracts, CML, a compound formed from the Amadori product in the glycation pathway of the Maillard reaction [53], is in the range of what is found in vegetables and fruits [54], and the content of FUR is less than

10% of what can be found in e.g. pasteurized milk [30]. FUR is often used as a marker for Maillard reactions in thermally treated foods, as well as CML also can be used as a marker of heat treatment [30,31], why it makes sense that the content of these crosslinking products were very low in the protein extracts in this study. Further, FUR and CML are known to have toxic effects, e.g. liver and kidney toxicity and induced development of diabetes have been proved in rodents, along with an increased level of pro-inflammatory markers have been observed in humans with increased intake [55–57]. In general, none of the protein extraction methods seemed to induce amino acid racemization or cross-linking to a level of concern for food safety or reduced nutritional quality, and the produced protein extracts were not significantly different regarding the level of in vitro protein digestibility. However, the protein quality is not only determined by the protein digestibility, but also factors such as the AA composition and the bioavailability. The bioavailability, just as the protein digestibility, can be highly influenced by anti-nutritional factors [28]. Furthermore, the AA composition is important for the overall protein score, determined by the limiting AA. It would be highly relevant to perform an AA analysis both of the initial biomass and the protein extract, as well as conducting a proximal analysis, to address the nutritional quality and how it may change during extraction. Unfortunately, it was not possible in the current setup, but such analyses should be included in future studies. *Ulva* spp. are in general known to possess a relatively high amount of EAA [8–10]. The protein extracts from the pH-shift methods had a higher solubility in water at pH 7–9 (adjusted with NaOH) than the protein extract from mechanical pressing. Since the SDS-PAGE analysis showed similar polypeptide composition regardless of extraction method used is this study, the solubility difference must be due to something else than protein composition. This could be related to the protein partially unfolding and re-folding during the pH-shifts, creating a so-called molten globule state, with other protein conformations and three-dimensional structures [58–60]. Molten globule-like conformation has also been observed for e.g. soy protein isolate upon alkali treatment at pH 12 and re-adjustment to neutral pH, which showed to increase protein solubility 2.5-fold [61]. The same has been shown for e.g. barley protein isolate, even treated at milder alkaline conditions; pH 9, and readjusting to pH 7, increasing the protein solubility [62], and for pea protein [63].

The *Ulva* protein extracted with the pH-shift methods performed best regarding protein yield and protein solubility, otherwise the protein quality seemed similar between the different extraction methods. Therefore, based on especially the protein yield, the pH-shift methods, and especially with protein solubilization at pH 8.5, can be recommended for extraction of *Ulva* protein. However, it should be kept in mind that temperature can affect racemization and cross-linking to a high extent especially combined with high pH [25], when setting the parameters for protein extraction methods. Thus, cold temperatures are suggested if economically possible.

Statement of informed consent

No conflicts, informed consent, or human or animal rights are applicable to this study.

CRediT authorship contribution statement

Louise Juul: Methodology, Investigation, Formal analysis, Visualization, Writing – Original Draft, **Marianne Danielsen:** Methodology, Investigation, **Caroline Nebel:** Methodology, Investigation, **Sophie Steinhagen:** Resources, Writing – Review and Editing, **Annette Bruhn:** Supervision, Writing – Review and Editing, **Søren Krogh Jensen:** Supervision, Writing – Review and Editing, **Ingrid Undeland:** Conceptualization, Supervision, Writing – Review and Editing, **Trine Kastrup Dalsgaard:** Conceptualization, Resources, Supervision, Writing – Review and Editing.

Declaration of competing interest

The authors 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.algal.2021.102496.

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