Title: Protein solubility is increased by antioxidant addition during protein extraction from the green macroalgae, *Ulva* sp.

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ABSTRACT

Ulva sp. is a cosmopolitan green macroalgae with fast growth and a well-balanced amino acid composition, making it an interesting potential protein resource. Generally, the protein digestibility in unprocessed raw seaweed is low, why extraction and concentration of the protein is needed. During protein extraction from fresh *Ulva* sp. using double screw pressing, sulfite was added as antioxidant in order to increase protein quality by inhibiting oxidative reactions. By pressing, 13-14% of the total biomass protein was extracted. Isoelectric precipitation at pH 2 resulted in a yield of 36% of the extracted protein corresponding to 5% of the total protein, independent on sulfite treatment. However, size exclusion chromatography showed that sulfite addition resulted in a higher amount of native state Rubisco protein. This was explained by inhibited redox enzyme activity and improved polyphenol levels, suggesting prevention of polyphenol-protein crosslinking, resulting in higher amount of soluble protein and improved protein quality.

Keywords: Seaweed, Rubisco, sulfite, protein quality, screw pressing, polyphenol, *Ulva*.

INTRODUCTION

The steady growth of the world human population increase the demand for food, generating an increased competition for arable land and fresh water (Godfray et al. 2010). Especially the demand for protein will grow, not only due to the increase in population, but also due to socio-demographic changes. To meet the increasing protein demand, there is a need for new and sustainable protein sources (Henchion et al. 2017). The green seaweed *Ulva* sp. (Chlorophyceae) is gaining increasing interest as a bioresource due to its cosmopolitan distribution, fast growth and high production potential, bioremediation capacity and content of particularly protein and ulvan (Kidgell et al. 2019; Bruhn et al. 2011; Neori 2007; Bikker et al. 2016; Neveux et al. 2018; Wichard et al. 2015).

The protein content and the general chemical composition of seaweeds can vary highly within species, as a result of season, geographic location, and environmental conditions, such as availability of nutrients and light (Mišurcová 2012). The crude protein content of *Ulva* sp. is often between 10-26% of dry matter (DM) (Pereira 2011; Fleurence 1999), but protein contents of >30% are also observed, especially when grown in nitrogen-rich waters (Naidoo et al. 2006; Shpigel et al. 1999), as well as protein contents of <10% can be found (Wong and Cheung 2000; Yaich et al. 2011). *Ulva* protein concentrates are documented to have a high proportion of EAA, 36-40% of total AA (Wong and Cheung 2001). Even though *Ulva sp.* in general contain all of the essential amino acids, the protein might not fulfill the EAA requirement pattern according to FAO, as the sulfur-containing amino acids are often being limiting (Vieira et al. 2018; FAO 2013; Wong and Cheung 2001). The high amount of polysaccharides present in the seaweed decrease protein digestibility (Horie et al. 1995). Extraction of the proteins has shown to improve in vitro N digestibility of *Ulva lactuca* compared to the protein in the crude biomass (Bikker et al. 2016). However, the cell wall polysaccharides may impede extraction of the proteins, due to high viscosity and ionic interactions (Jordan and Vilter 1991; Joubert and Fleurence 2008).

In addition to cell wall polysaccharides, the phenolic content in seaweeds, including in *Ulva* sp., (Farasat et al. 2014; Bleakley and Hayes 2017; Farvin and Jacobsen 2013), may impede both protein extraction and digestibility (Jordan and Vilter 1991; Wong and Cheung 2001). The phenolic compounds may bind reversibly to the proteins through hydrogen bonds, and when oxidized to quinones, they rapidly form irreversible covalent bonds with proteins (Loomis and Battaile 1966). This may further lead to the formation of cross-linked protein polymers (Rawel et al. 2001). These phenol/quinone-protein bindings decrease the digestibility and solubility of the proteins (Rawel et al. 2001; Bleakley and Hayes 2017; Wong and Cheung 2001; Amer et al. 2020). Moreover, when phenolic compounds are oxidized to quinones, the quinones can polymerize and form brown pigments (Bittner 2006; Friedman 1996). Sulfur-containing agents, such as sodium sulfite and sodium hydrogen sulfite, are documented to inhibit the enzymatic oxidation of phenols and to be able to reduce quinones to sulfo-phenolics. This way browning reactions (Narváez-Cuenca et al. 2011; Queiroz et al. 2011) and covalent binding between quinones and proteins resulting in higher polymers can be avoided (Amer et al. 2020).

So far, protein extraction from seaweeds has primarily been performed by osmotic shock, often combined with either alkaline extraction or pulsed electric field, using either dried and milled biomass or fresh grinded biomass (Harrysson et al. 2019; Vilg and Undeland 2017; Kazir et al. 2019; Mæhre et al. 2016; Postma et al. 2018; Kadam et al. 2017; Bleakley and Hayes 2017). Alkaline processing have for other proteins shown to induce amino acid racemization, resulting in decreased protein digestibility (Schwass and Finley 1984; Hayashi and Kameda 1980; Friedman 2004), why the aim of this study was to test a mechanical extraction technique, adding antioxidant during extraction to improve protein quality. The extraction was performed using a double screw press on fresh *Ulva* biomass. Antioxidant, in the form of sodium sulfite, was hypothesized to improve the quality of the extracted proteins in terms of solubility by inhibition of quinone formation.

MATERIALS AND METHODS

Materials

Deionized (18.2 MΩ) filtered water (0.22 μm) came from a Milli-Q system, Millipore SAS (Molsheim, France). Methanol, Folin Ciocalteu's reagent, sodium carbonate, sodium bicarbonate, sodium sulfite, sodium chloride, sodium hydrogen phosphate, sodium dihydrogen phosphate, D-ribulose 1,5-diphosphate carboxylase (Rubisco) from spinach, 1,4-dithioerythritol (DTE), HCl 37%, pyrocatechol, tyrosinase from mushroom, and gallic acid, were purchased from Sigma-Aldrich (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), ethylenediaminetetraacetic acid (EDTA) and trisaminomethan (Trizma Base) from Merck (Germany), coomassie brilliant blue G-250 (Serva, Heidelberg, Germany), glycin (Applichem, Darmstadt, Germany), Precision Plus Protein Kaleidoscope Prestained Protein Standards (Bio-Rad), PageRuler Plus, and Criterion Tris-HCl gels were from Bio-Rad (Hercules, USA).

Raw material

Ulva sp. was collected at Klosterbugten, Nykøbing Mors, Limfjorden on 2 August 2018. The collected *Ulva* was stored in plastic bags at 4 °C until processing the next day.

Protein extraction

Two different treatments, control and sulphite, were used during the protein extraction. Treatments were performed by using either milli-Q water (control) or 0.2 M Na_2SO_3 (sulfite) as treatment solution. One kg fresh *Ulva* sp. (83.1±0.5% moisture content) was juiced into 100 mL treatment solution with a double screw press juicer (Angel Juicer 8500S, Domotech, Denmark) at room temperature. The double screw press was fed continuously with biomass into the feeding chute of 4.32 cm in diameter and the double screw running at 86 rpm. Juicing of the fresh *Ulva* was called

press 1. For the sulfite treated juice press 1, the resulting sulfite concentration was 36 mM. A second juicing (press 2) was performed after suspending the pulp fraction from the first press in the treatment solutions in a weight-weight ratio of 1:1 for 10 minutes. Extracts were cleared by centrifuging for 15 minutes at 1700 x *g*, 4 °C (SL 40 centrifuge, Thermo Scientific) to remove non-soluble fibers. Centrifuged extracts was kept in brown bottles at -20 °C until analysis. Only one replicate extract of each treatment was made in order to have sufficient sample material from a homogeneous batch for the subsequent processes. A flowchart of the process is shown in Figure 1.

Protein precipitation

Protein was precipitated from aliquots of juices by adjusting to pH 2.0, 3.0, 4.0, 4.5 and pH 5.0 by adding HCl (0.05 M-6 M). Acidified juices were left at 4 °C over night and centrifuged at 4816 x *g* for 30 min at 4 °C. Protein pellets were freeze dried and stored at -20 °C until analysis. Protein precipitation was performed in triplicate.

Protein content

Protein content of biomass, juices and precipitation products were measured by determination of total N content using DUMATHERM (Gerhardt Analytical Systems, Königswinter, Germany) using an external standard curve prepared from tris-base and EDTA. For liquid samples, the setting was 1.55 mg O_2 mg^{-1} sample, an O_2 flow rate of 200 mL min^{-1} and nitrogen-to-protein conversion factor 5. For fresh, crude biomass, settings were 1.4 mg O_2 mg^{-1} sample, an O_2 flow rate of 300 mL min^{-1} and nitrogen-to-protein conversion factor 5 (Angell et al. 2016). For freeze dried protein pellets the setting was 1.4 mg O_2 mg^{-1} sample, an O_2 flow rate of 300 mL min^{-1} and a nitrogen-to-protein conversion factor of 6.25 (FAO 2003), as it was assumed that the nitrogen being acid precipitated was mainly

protein nitrogen. DM of crude biomass was determined with a HR73 Halogen Moisture Analyzer (Mettler Toledo). Protein and DM determinations were performed in triplicates.

Protein yield

Protein yields were defined and calculated by following equations:

1) $Extraction\ yield = \frac{[protein]_{juice} \times mass_{juice}}{[protein]_{biomass} \times mass_{biomass}} \times 100\%$

2) $Precipitation\ yield = \frac{[protein]_{protein\ pellet} \times mass_{protein\ pellet}}{[protein]_{juice} \times mass_{juice}} \times 100\%$

3) $Total\ yield = precipitation\ yield \times extraction\ yield$

SDS-PAGE

SDS-PAGE was carried out using Criterion TGX stain-free precast gels (Bio-Rad Laboratories Inc., Hercules, CA, USA) of three different polyacrylamide levels; 4-15%, 12% and 8-16%. The technique described by Laemmli (Laemmli 1970) was used under reducing conditions. Samples were mixed with SDS-PAGE sample buffer in a 1:1 ratio from which 20 µL was loaded onto gels. Sample buffer contained 20 mM Tris, 2% SDS, 20% glycerol, 0.1 mg mL^{-1} bromophenol blue, 20 mM dithioerythritol (DTE) and was adjusted to pH 6.8. Samples were heated at 90 °C for 3-5 minutes before electrophoresis. Electrophoresis was run 30-45 minutes at 200 V. Fixation was performed with 50% ethanol and 8% phosphoric acid for staining with colloidal Coomassie Brilliant Blue (Kang et al. 2002) and with 50% and 5% acetic acid for silver staining. Silver staining was performed as described by Shevchenko (Shevchenko et al. 1996), but with the use of ethanol instead of methanol and formaldehyde instead of formalin.

Measurement of color components

158 The color of the cleared and ultracentrifuged juices was measured on 2 mL samples in a 12-well plate
159 using a handheld colorimeter (Konica-Minolta, Tokyo, Japan) to determine the color components L*
160 (black – white), a* (green – red), and b* (blue – yellow). For better determination of brown coloring,
161 chlorophyll was removed from 25 mL aliquots of extract by ultracentrifugation (34940 x *g*, 30 min,
162 4 °C), on an Optima L-80XP Ultracentrifuge (Beckman Coulter Inc., Brea, CA) with a titanium fixed-
163 angle 70-Ti Rotor (angle 23 °)(Amer et al. 2020). Color was measured in triplicates.
164
165 **Redox enzyme activity and polyphenol content**
166 Redox enzyme activity was determined by measuring conversion of an added polyphenol substrate,
167 pyrocatechol. Ultracentrifuged juices (50 µL) were added to a 96-well plate followed by 200 µL 0.05
168 M pyrocatechol in 0.1 M phosphate buffer, pH 6.5. Measurement was performed immediately after
169 addition of the substrate, measuring the change in absorbance at 420 nm every three seconds for one
170 minute using a microtiter plate spectrophotometer (BioTek Instruments Inc, Winooski, VT 05404,
171 USA). The redox enzyme activity was expressed in units mL^{-1}, where one unit corresponds to the
172 change in $A_{420\ nm}$ of 0.001 min^{-1}, when 50 µL ultracentrifuged juice was added to 200 µL substrate
173 solution. Determination of redox enzyme activity was performed in triplicate.
174 Polyphenol content was determined using a modified Folin-Ciocalteu Assay (Sánchez-Rangel et al.
175 2013). Polyphenols were extracted from juices by shaking with 80% (v/v) methanol for 60 minutes
176 shaking at 500 rpm (VIBRAX VXR basic, IKA), on the same day as the juicing. The phenolic extract
177 was centrifuged at 20800 x g for 10 minutes (Centrifuge 5417 R, F45-30-11, Eppendorf AG,
178 Germany). For each sample, 15 µL of the supernatant was added to a 96-well plate together with 235
179 µL milliQ water, 20 µL 1 N Folin-Ciocalteu reagent and 30 µL 0.5 M Na_2CO_3 and dark incubated for
180 two hours. Absorbance was read at 765 nm using a microtiter plate spectrophotometer (BioTek
181 Instruments Inc, Winooski, VT 05404, USA) and the polyphenolic content was calculated in mg gallic

182 acid equivalents per mL juice, using a gallic acid standard curve. Polyphenol extraction and analysis
183 of polyphenol content was performed in triplicate.
184
185 **Size exclusion**
186 Ultracentrifuged juices were filtered through Mini-UniPrep^{TM} filters with pore size 0.2 µm
187 (Whatman^{TM}, GE Healthcare Life Sciences) before injection into a HPLC (Agilent, United States)
188 with a Yarra 1.8 µm SEC-X150 column (size: 150 x 4.6 mm) (Phenomenex, United States). Four µL
189 of juices were injected into the HPLC and compared to a D-Ribulose 1,5-diphosphate carboxylase
190 (Rubisco) standard from spinach (Sigma-Aldrich, Germany). Protein was eluted with a 0.05 M PO_4
191 buffer with 0.3 M NaCl and pH 6.8 at a flow of 0.3 mL min^{-1}. UV absorbance was measured at 214
192 nm. Size exclusion analysis was performed in triplicate.
193
194 **Statistics**
195 Generalized linear models (McCullagh and Nelder 1989) were used to study the effects of parameters
196 of interest, with an identity link function and an interaction term between treatments (control and
197 sulfite) and presses (press 1 and 2). The Gaussian distribution was used for modelling precipitation
198 yield, total protein yield, redox enzyme activity, polyphenol content and color parameters "l" and "a".
199 A generalized linear model defined with a gamma distribution was used for juice yield and color
200 parameter "b", since these were not normally distributed. Residual analyses confirmed the adequacy
201 of the models chosen. The statistical analyses were performed with the software R version 3.5.1 (R
202 Core Team 2018). The presence of interaction between treatment and press was tested by an F-test,
203 in the case of the models based on the Gaussian distribution, and by a likelihood ratio test in the case
204 of the models based on the gamma distribution. Post-hoc analyses, including the determination of
205 statistical significance grouping, were conducted using the R-package postHoc (Labouriau 2020). P-

values were adjusted for multiple testing using the method of controlling the false discovery rate (Benjamini and Hochberg 1995). Significance level was $p = 0.05$.

RESULTS

Protein yield

The isoelectric precipitation yield was investigated in a pH range from pH 2 to pH 5 on two successive presses. Protein precipitation yield increased with decreasing pH from pH 5.0 to pH 2.0 (Figure 2), especially for press 1. Therefore, pH 2 was used for isoelectric precipitation of proteins for further investigation. Press 1 juices contained 0.63% and 0.67% protein for control and sulfite samples, respectively. For press 2 juices, the protein content was 0.47% and 0.51% (Table 1). For each treatment, these protein percentages corresponded to extraction of app. 6.7 g protein from the one kg fresh *Ulva* into the juices from the two consecutive presses. Crude *Ulva* had a protein content of 15.3±2.7% based on DM. For the two presses combined, this corresponded to insignificantly different protein extraction yields (protein in juices out of total protein in crude *Ulva*) of 13.6% and 13.2% for the control and sulfite treated samples, respectively (Figure 3). Protein extraction yields were significantly higher from the first presses (7.3±0.1%) than from the second presses (6.2±0.4%) ($p < 0.001$) (Figure 3). Less than half (46.2±0.5%) of the protein was precipitated from the press 1 juices and 26.1±2.3% from the press 2 juices (Figure 3), giving a combined precipitation yield of 36.1% of the extracted protein. This resulted in total protein yields (extraction yield × precipitation yield) of 4.9% and 5.0% of total biomass protein for control and sulfite treated samples, respectively, not being significantly different. Freeze dried protein pellets had a protein content of app. 30% for first press samples and app. 20% for second press samples.

SDS-PAGE protein profile

SDS-PAGE analysis showed that lanes with press 1 juices had darker staining than lanes with press 2 juices visualizing the higher protein content of press 1 juices (figure 4a). Coomassie staining of SDS-PAGE gels with juices (Figure 4a) exhibited many bands in the range of 10-37 kDa, the most distinct band at app. 25 kDa. Around 50 kDa, a weak band appeared in sulfite treated cleared juice, which might be the large subunit of Rubisco. The silver stained gel with juices (Figure 4b) showed proteins of higher molecular weight around 100, 150 and >250 kDa. During ultracentrifugation, some of the protein was lost, which became evident by less protein in lanes with ultracentrifuged juice (figure 4a). The loss of protein during ultracentrifugation was confirmed by the total N-determination of the protein content (Table 1). For press 1, control juice lost 8.7% of protein upon ultracentrifugation and sulfite treated juice lost 9.3%. For press 2, 17.6% of the protein was lost upon ultracentrifugation, whereas just 3.7% was lost for the sulfite treated sample. Looking at the SDS-PAGE analysis (Figure 4a), it was in particular the band around 25 kDa, which was lost upon ultracentrifugation. Analyzing the protein pellets from the isoelectric precipitation with SDS-PAGE, showed that the main proteins precipitated had a molecular weight of ~25 kDa together with high molecular proteins of >250 kDa (Figure 4c). Moreover, the SDS-PAGE analysis showed that more proteins were left in the supernatant when adjusting to pH 4.0 compared to adjusting to pH 2.0 (Figure 4d). At pH 2.0, only a few high molecular weight proteins (~100 and >250 kDa) were left in the supernatant.

Enzymatic browning

Chlorophyll was removed from the *Ulva* juices by ultracentrifugation to enable observation of color differences. After the ultracentrifugation, a visual difference was observed both between treatments and presses (Figure 5a). The control juice from press 1 appeared more brown than the sulfite treated juice from press 1. No browning was observed in press 2 samples, irrespective of the sulfite treatment. Sulfite treated juices appeared yellow, especially for press 1. Color components L* (lightness), a*

(green-red) and b* (blue-yellow) were measured to get a quantitative indication of browning. The colorimetric measurement showed that redness (a*) was significantly decreased upon sulfite treatment, especially for press 1 juice ($p < 0.001$). Furthermore, lightness (L*) was significantly increased for press 1 juice upon sulfite treatment ($p < 0.001$), but reduced for sulfite treated press 2 juice ($p < 0.001$). There was an increase in yellowness for sulfite treated juices compared to control juices ($p < 0.001$) (Figure 5b).

Sulfite treatment significantly inhibited redox enzyme activity in terms of inhibiting phenolic oxidation ($p < 0.001$) when using pyrocatechol as substrate in the assay. For the control sample, the redox enzyme activity was significantly higher for the press 1 juice than for the press 2 juice ($p < 0.01$) (Figure 6). The polyphenolic content was significantly higher in sulfite treated juices ($p < 0.001$), especially in press 2 juice, where the polyphenolic content was approximately twice as high as in the control (Figure 6).

Size exclusion chromatography

When analyzing the protein content of ultracentrifuged juices by size exclusion chromatography, the protein content was higher ($p < 0.001$) for the sulfite treated juices (Figure 7), 17% higher for press 1 and 56% for press 2. A Rubisco standard was used for comparison, since seaweed extracts were expected to contain Rubisco. The Rubisco protein gave two distinct peaks, which is suggested to be native (app. 550 kDa) and larger polymers of Rubisco. A higher content of what is suggested to be the native form of the major plant protein Rubisco, was observed for sulfite treated samples, especially in press 1 (Figure 7a). For press 2 juices, the protein signal was in general much lower when compared to press 1 juices. Sulfite treated sample from press 2 contained a higher amount of especially larger polymers of the suggested protein Rubisco, and to a smaller extent native Rubisco and aggregates as well (Figure 7b).

DISCUSSION

The extraction yield (~13%) and total yield (~5%) of total biomass protein in this study were lower than what others have obtained with different methods on *Ulva* sp., extracting with e.g. NaOH or large volumes of water (Kazir et al. 2019; Harrysson et al. 2018). Robin et al. (2018) also did a mechanical extraction, but combined with pulsed electric field and with dialysis for protein purification, achieving a total protein yield of 2.9% (Robin et al. 2018). The precipitation yield obtained in this current study was within the range of what have been achieved in other studies (Harrysson et al. 2018; Harrysson et al. 2019). Harryson et al. (2019) demonstrated improved extraction yields and a total protein yield of 29% of total biomass protein using a pH-shift method, that includes step-wise incubation of homogenized *Ulva* at pH 8.5 and pH 12, and adding a freezing step during the precipitation (Harrysson et al. 2019). From a protein quality perspective, alkaline extraction may induce racemization of amino acids and cross-linking of aminoacyl residues (Schwass and Finley 1984). Whether the same accounts for *Ulva* protein will need further investigation.

The precipitation yield was significantly higher for the press 1 juices than the press 2 juices, suggesting that the protein fraction extracted during the second press is more soluble than the proteins from the first press in the pH-range of pH 2.0-5.0. The precipitation yields of 46% and 26% (of extracted protein), respectively for press 1 and press 2 juices, indicated that the proteins in *Ulva* have a high solubility, even at low pH, as most of the proteins remain in the supernatant. However, the SDS-PAGE analysis of the supernatants (only conducted for press 1) did not show many protein bands, most likely due to aggregated proteins not entering the gel, as supported by the size exclusion analysis. The high amount of low-pH soluble proteins could make the *Ulva* proteins interesting as an application e.g. in the food industry for protein enrichment of products with low pH. The high protein solubility, however, can also be due to a high salt content in the seaweed (Magnusson et al. 2016),

302 possibly pushing the pI of the protein downwards. A closer examination the proteins by SDS-PAGE
303 analysis, revealed many proteins in the weight range of 10-35 kDa, with the most pronounced band
304 being around 25 kDa. The identities of the proteins in the bands are not known. The band around 25
305 kDa disappeared upon ultracentrifugation together with the green color, which could indicate
306 association with the chlorophylls and along with the size could be suggested to be light harvesting
307 proteins (Hanelt et al. 2003; Jansson 1994; Zhang et al. 2013). The 25 kDa band was also seen in the
308 SDS-PAGE analysis of the precipitated protein along with bands >250 kDa. This indicated that the
309 major proteins being precipitated were in particular these proteins at 25 kDa and >250 kDa. As the
310 photosynthetic enzyme Rubisco is one of the major proteins in many plants and algae, and Rubisco
311 activity indeed has been measured in *Ulva* sp. (Beer et al. 1991; Yeoh et al. 1981; Cabello-Pasini and
312 Alberte 2001; Bischof et al. 2002), it might be suggested that the bands >250 kDa could be
313 polymerized or cross-linked Rubisco. The large subunit of Rubisco have a size of ~55 kDa (Barbeau
314 and Kinsella 1988; Wang et al. 2011) and this might be what is seen as the dim band around 55 kDa
315 in the sulfite treated samples. After precipitation at both pH 4 and pH 2, bands were visible in the
316 SDS-PAGE silver staining of supernatant at 100 kDa and to some extent at >250 kDa, which suggest
317 that these were primarily the proteins soluble in the low pH range.

318 The redox enzyme activity was measured on a polyphenolic substrate, which indicated that sulfite
319 inhibited oxidation of polyphenols, and hereby preserved the polyphenols. When phenols are
320 oxidized, they turn into reactive quinones, which can polymerize into polymeric browning products
321 (Lee et al. 1990; Dong et al. 2016). The browning of the ultracentrifuged press 1 control juice was
322 most likely a result of this. The quinones may also react with proteins making the proteins more prone
323 to aggregation or polymerization through cross-linking leading to formation of high molecular protein
324 complexes with decreased solubility and functionality (Kroll and Rawel 2001; Rawel et al. 2001).
325 The loss of 17.6% protein during ultracentrifugation for the control press 2 juice indicated that the

protein extracted in the press 2 control juice had lower solubility than the protein in the sulfite treated press 2 juice. This difference between the treatments could be due to sulfite inhibiting oxidative reactions thereby reducing polymerization and improving protein solubility. The lower ratio formation of high molecular weight polymers was also supported by the size exclusion chromatography results. Two peaks in the size exclusion chromatogram from the samples eluted at the same time points as the Rubisco protein from spinach, which was used as a standard. Therefore, the protein creating these peaks in the samples was suggested to be Rubisco. The size exclusion chromatograms also showed a broad peak/simultaneous peaks eluting 4-5 minutes into the analyses (not shown), corresponding to protein sizes of ~10-150 kDa. Sulfite addition kept Rubisco in its native state and prevented polymerization and aggregation. However, the total protein yield was not different in control and sulfite treated samples. The sulfite might therefore not enhance solubility during the extraction, but keep the proteins soluble afterwards as a result of inhibition of oxidative reactions. Therefore, another approach is needed if focusing on the protein yield, possibly drawing the attention towards the cell wall polysaccharides. The higher ratio of soluble protein seen for sulfite treated *Ulva* juice, though, could have a positive effect on the nutritional value, as well as the sulfite might protect essential amino acids from oxidation. Whether this can balance out the sustainability impairment and economical cost of using antioxidant during extraction will depend on the protein quality enhancement and will need further investigation. Furthermore, in future studies sulfite content in the protein extracts needs to be measured, as, even though sulfite is widely used in food products as preservatives, adverse effects have been observed from sulfite exposure (Vally and Misso 2012).

In conclusion, protein extraction by juicing showed low yields compared to studies using alkaline extraction. A comparison on protein quality between the different extraction methods would be an interesting contribution to the discussion and should be investigated. There was no significant difference on protein yield upon addition of sulfite. The addition of sulfite, however, resulted in a

higher amount of native state Rubisco protein, explained by inhibited redox enzyme activity and increased polyphenol levels, suggesting prevention of polyphenol-protein crosslinking. Thus, sulfite addition resulted in a higher amount of soluble protein and improved protein quality.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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528

529

530 **Table 1** Obtained amounts of pulp and juice, and protein content of the *Ulva* juices, for the two
531 treatments and respective presses, when juicing one kg fresh *Ulva* into 100 mL of treatment
532 solution. The numbers 1 and 2 in column 1 correspond to first and second press

Treatment/Press	Weight of pulp (g)	Weight of juice (g)	Protein% of cleared juice	Protein% of ultracentrifuged juice
Control/1	400	572	0.63	0.58
Control/2	330	691	0.47	0.39
Sulfite/1	453	552	0.67	0.61
Sulfite/2	365	571	0.51	0.49

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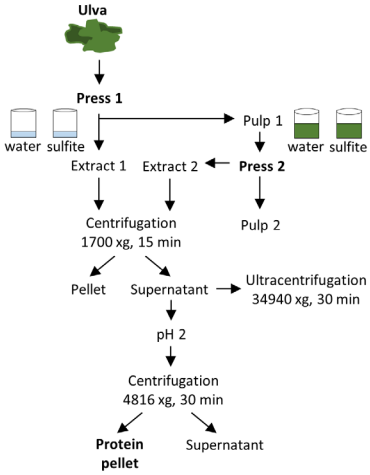

534

535 **Fig. 1** Flowchart of protein extraction and precipitation

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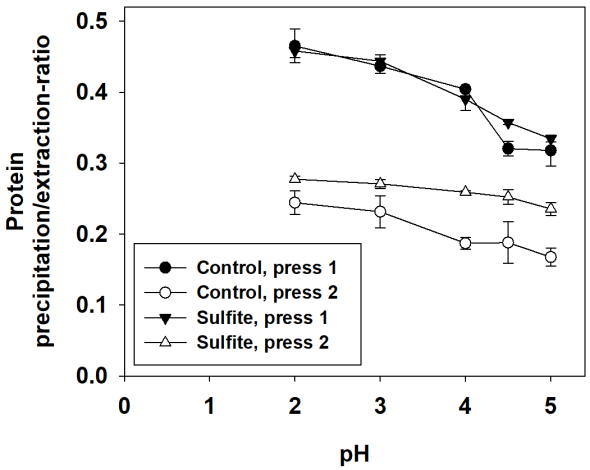

538

539 **Fig. 2** Precipitation yield of protein expressed as precipitation/extraction-ratio for the two

540 treatments, control and sulfite, and the two sequential presses. Precipitation was performed at pH

541 2.0 to pH 5.0. Data are represented as mean ± SD, n = 3

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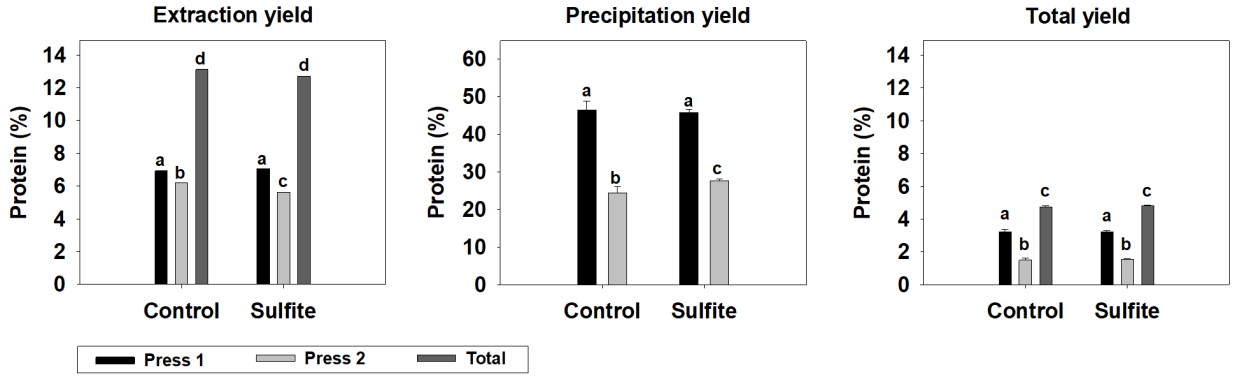

543

544 **Fig. 3** Protein yields obtained during extraction, precipitation and in total, comparing to protein
545 content in the fresh *Ulva* biomass. Protein was precipitated from cleared *Ulva* juices (extract) with
546 HCl, adjusting to pH 2. Data are represented as mean ± SD, n = 3. Different notations on bars indicate
547 significance of differences ($p < 0.05$)

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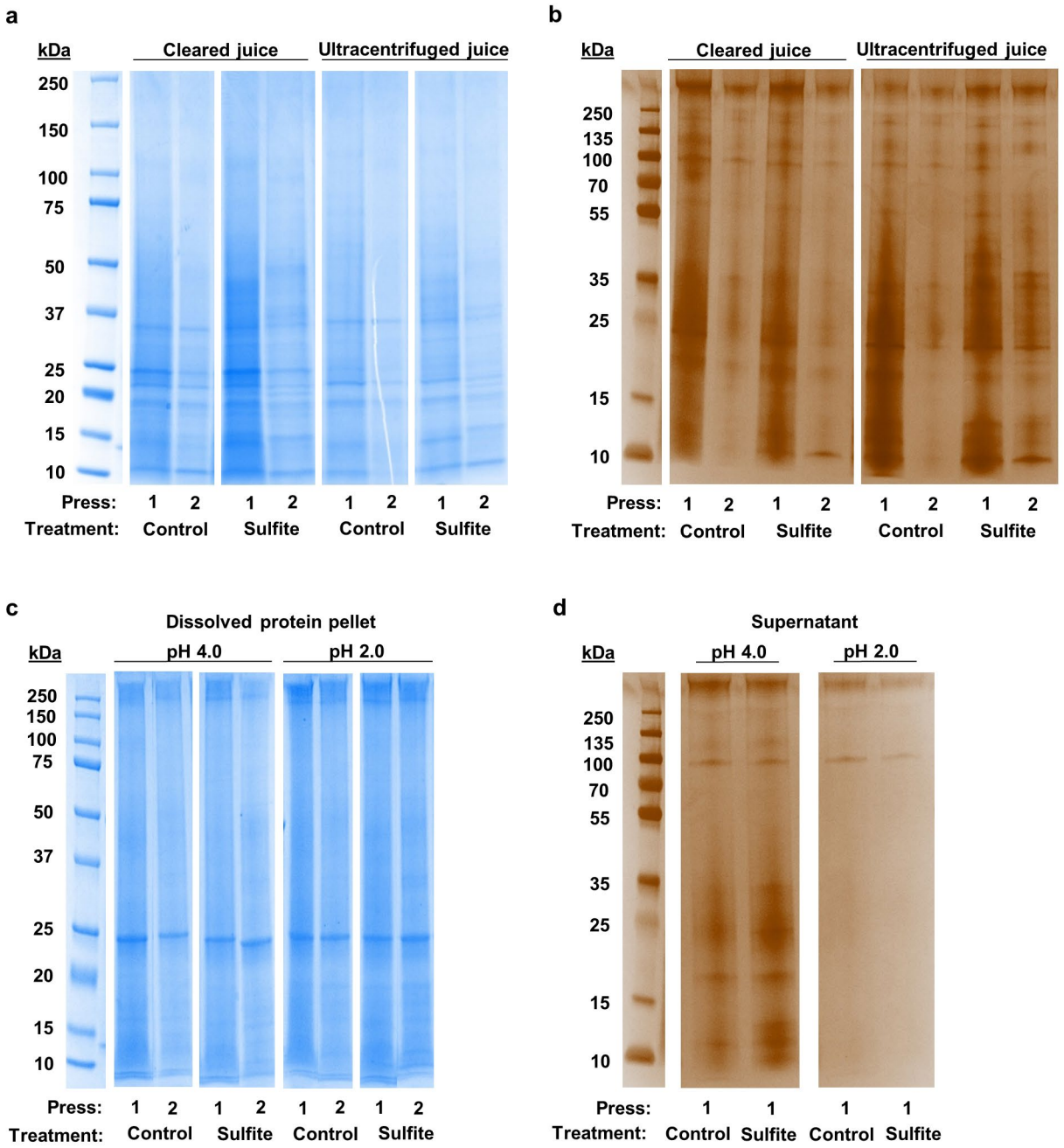


549

550 **Fig. 4** Protein in *Ulva* juices (cleared and ultracentrifuged), precipitated protein and supernatant
551 analyzed by reduced SDS-PAGE. Samples were mixed with sample buffer containing 20 mM DTE
552 in a ratio of 1:1. a) Coomassie stained 4-15% polyacrylamide gel. b) Silver stained 8-16%

polyacrylamide gel. c) Protein pellet (6 mg mL^{-1} in samplebuffer) from acid precipitation at pH 4.0 or pH 2.0 on a coomassie stained 12% polyacrylamide gel. d) Supernatant from acid precipitation at pH 4.0 or pH 2.0 on a silverstained 8-16% polyacrylamide gel

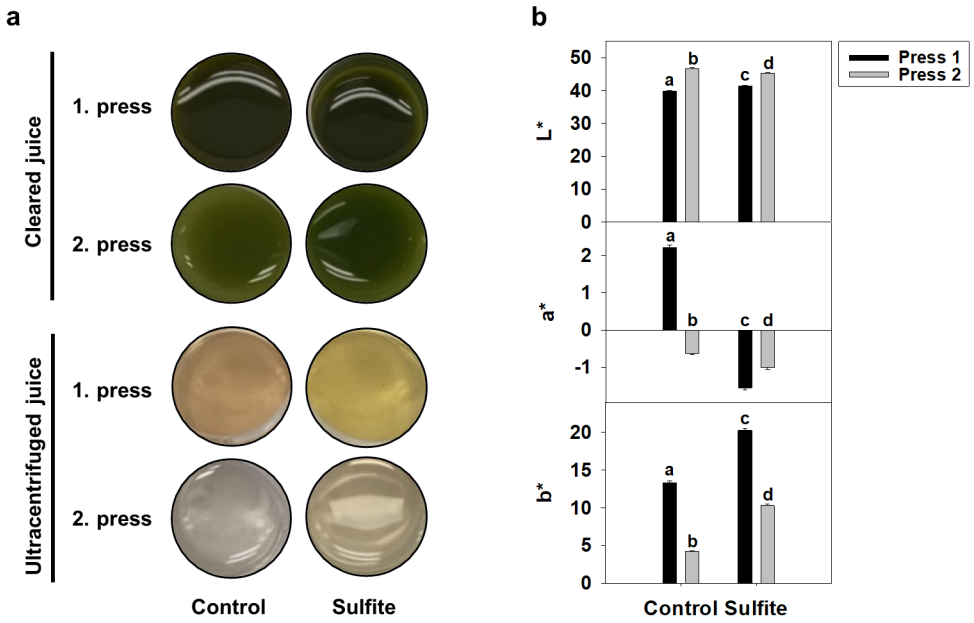
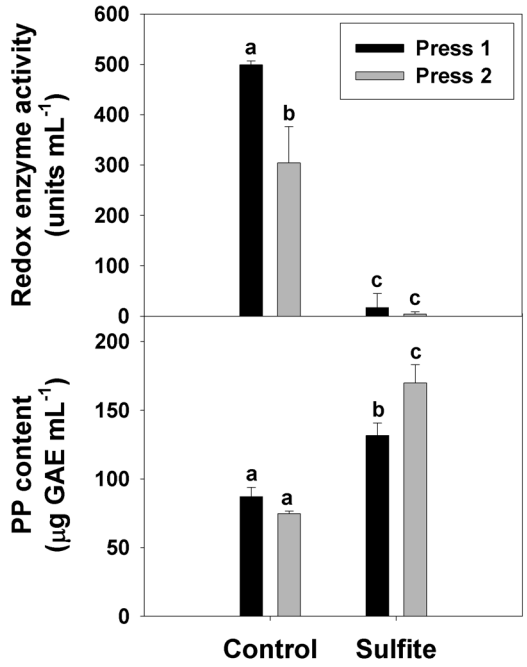


Fig. 5 Color of *Ulva* juices. a) Appearance of *Ulva* juices treated with milliQ water (control) or sodium sulfite, before (cleared juice) and after removal of chlorophyll by ultracentrifugation (34940 x *g*, 30 min, 4 °C). b) Values of color components (lightness: L*, green-red: a*, and blue-yellow: b*) of ultracentrifuged juices. Different notations on bars indicate significance of differences ($p < 0.05$). Data are represented as mean ± SD, n = 3



564

565 **Fig. 6** Redox enzyme activity and polyphenol (PP) content. Redox enzyme activity was analyzed
566 using pyrocatechol as substrate. One unit is equal to an increase in absorbance at 420 nm of 0.001
567 min^{-1}, when 50 µL ultracentrifuged juice is mixed with 200 µL substrate. Polyphenol content was
568 analyzed by Folin Ciocalteu's assay measured as gallic acid equivalents (GAE). Data are represented
569 as mean ± SD, n = 3. Different notations on bars indicate significance of differences ($p < 0.05$)

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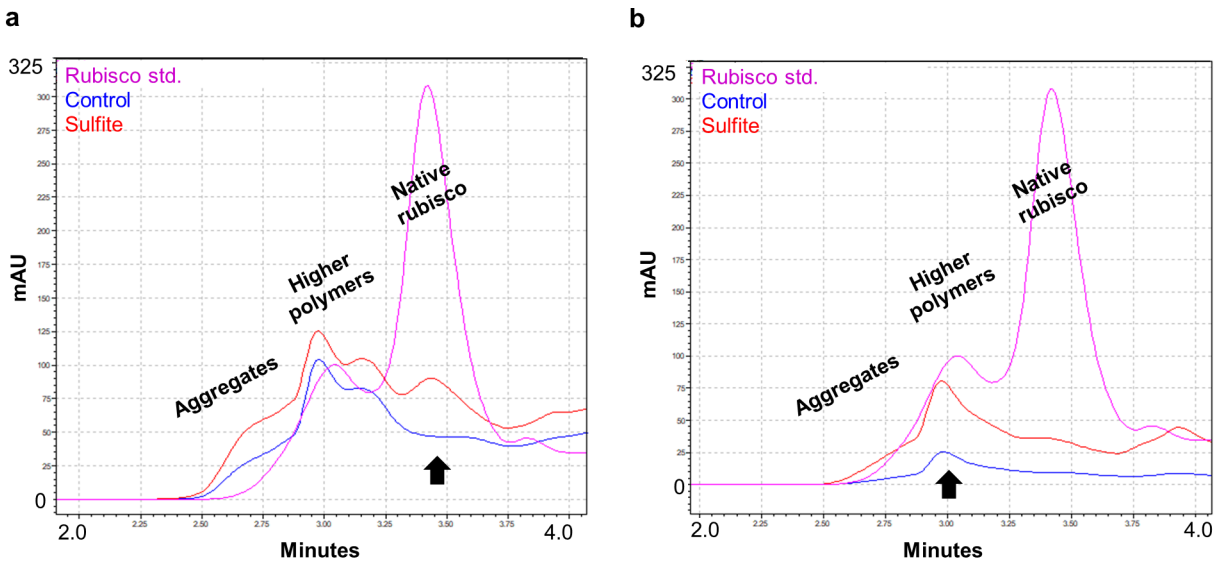

Fig. 7 Size exclusion chromatograms of ultracentrifuged *Ulva* juice. Ultracentrifuged *Ulva* juices treated with either milliQ water (control) or sulfite, were analyzed by size exclusion chromatography, measuring the absorbance at 214 nm. Rubisco protein (2.5 mg mL^{-1}) from spinach was used as standard. a) Press 1 juice. The arrow points to the native Rubisco peak. b) Press 2 juice. The arrow indicate the peak from larger polymers of Rubisco