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Extraction of proteins from two marine macroalgae, *Ulva* sp. and *Gracilaria* sp., for food application, and evaluating digestibility, amino acid composition and antioxidant properties of the protein concentrates

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ABSTRACT

With rising global population and decreasing available land and fresh water resources, the oceans provide an attractive domain for sourcing nutrients. The marine macroalgae *Ulva* sp. and *Gracilaria* sp. are candidate raw biomass. *Ulva* sp. has high growth rate and *Gracilaria* sp. has high protein content and their seasonal growth is complementary, allowing almost year-round high yield protein production. In this study, we aimed at developing an effective process, to yield a high macroalgae protein content concentrate suitable for food application, and studying the digestibility, amino acid composition and antioxidant properties of the obtained algal protein concentrates (APCs). We developed a new protein extraction protocol, and compared it to several published protocols. The developed protocol is food-grade, and yielded APC from *Ulva* and form *Gracilaria*, containing 70 and 86% protein respectively. The amino acid compositions of the APCs suggest their possible use as sources of essential amino acids. Simulated gastro-intestinal digestion showed that APCs proteolysis of at least 89% can be reached. We found that the APCs exhibit antioxidant activity, which is similar to that of known protein isolates in the hydrogen atom transfer mechanism, but 10 to 20 times higher in the single electron transfer mechanism. These results suggest that polyphenolic compounds might be still present in the APCs and contribute to their antioxidant activity. Our results suggest that the protein concentrates extracted from *Ulva* sp. and *Gracilaria* sp. seem to be promising sustainable sources for human nutrition thanks to their essential amino acids content, digestibility and antioxidant properties.

1. Introduction

World population is continuously growing and it is expected to reach ca. $9.6{\cdot}10^9$ by 2050 (Gerland et al., 2014). Consequently, there is also a growth in demand for food, which requires seeking new sources and new ways to increase food production. These facts together with decreasing agricultural land and dwindling fresh water resources make oceans an attractive alternative site for basing cultivation practices (known as Mariculture or Seagriculture), for human benefits.

Seagriculture can provide new sources for a wide variety and quantity of nutrients. It may be sustainable if prudently harvested and efficiently utilized, while minimizing adverse environmental impact.

Macroalgae (seaweeds) have traditionally been consumed in Asian countries for centuries (Ainis, Vellanoweth, Lapeña, & Thornber, 2014; Dillehay et al., 2008; Erlandson, Braje, Gill, & Graham, 2015; Yang, Lu, & Brodie, 2017), and are common in certain Western countries (Garcia-Vaquero & Hayes, 2016). Seaweeds may contain up to 50% protein on a dry weight (DW) basis, similarly to traditional protein sources such as meat, egg and soybean (Bleakley & Hayes, 2017; Fleurence, 1999, 2004; Harnedy & Fitzgerald, 2011). Edible algae contain a wide range of nutrients many of which have significant importance in human nutrition and great economic potential for the food industry (Plaza, Cifuentes, & Ibáñez, 2008). It would, therefore, sound reasonable to develop offshore facilities that would allow algae cultivation in the sea, alleviating the need for both additional agricultural land and freshwater for irrigation (Fernand et al., 2017; Lehahn, Ingle, & Golberg, 2016).

Algal proteins also contain significant amounts of essential amino acids (Fleurence, 1999; Wong & Cheung, 2000).

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Therefore, an integrated bio-processing, or bio-refinery that is established based on a 'zero waste' vision will allow, first, the conversion of macroalgae biomass into food or chemicals. Second, it will allow for sustainable production of a variety of biomolecules, reduced waste and lower adverse environmental impact (Ingle et al., 2017). To be able to implement biomolecule extraction procedures in an industrial process in the future, it is essential to develop fractionation protocols that are applicable in food processing.

Currently, very little is known about the functional properties of macroalgal proteins, and their digestibility. Algal proteins may be considered a by-product of polysaccharide extraction from the algae, since polysaccharides are present at higher concentrations, and their extraction is much more prevalent (Fleurence, Le Coeur, Mabeau, Maurice, & Landrein, 1995). Therefore, there is a need for food applicable protocols that not only separate the proteins from the polysaccharides, but also allow to extract both fractions as useful functional food components. This is important both economically and environmentally and is in line with the "zero waste" vision.

Species of the green marine macroalga *Ulva* spp. are very common in shallow sea areas all around the globe. *Ulva* spp. are good candidates for seagriculture due to their nutritional benefits and high protein content (Taboada, Millán, & Míguez, 2010) compared to terrestrial plants (Pirie & Pereira, 1976). *Ulva* sp. contains significant amounts of proteins (7–24% on a dry weight, DW, basis) (Gao, Clare, Rose, & Caldwell, 2017; Karray, Karray, Loukil, Mhiri, & Sayadi, 2017; Korzen, Pulidindi, Israel, Abelson, & Gedanken, 2015), and is also rich in carbohydrates (14–40% of the dry matter). Additionally, this alga contains minor fractions such as minerals, fats and phytochemicals. *Ulva* protein consists of significant amounts of essential amino acids, which make it a potentially important food protein source (Shuuluka, Bolton, & Anderson, 2013; Taboada et al., 2010). Indeed, there is considerable similarity in the total amino acid composition of the alga to that of egg ovalbumin (Shuuluka et al., 2013). In addition, the alga is also known for its high growth rate which yields more than 20 $gDWm^{-2}d^{-1}$, ranked among the highest within photosynthesizing organisms (Bolton, Robertson-Andersson, Shuuluka, & Kandjengo, 2009). An additional major advantage of *Ulva* species relates to the easy cultivation in various culture settings under high loads of nitrogen concentrations (Bolton et al., 2009). Recent works have demonstrated that its net primary productivity is higher than arable plants in the Eastern Mediterranean regions (Chemodanov et al., 2017).

The red marine macroalga *Gracilaria* thrives in tropical Atlantic waters (Melo, Feitosa, Freitas, & de Paula, 2002) and is known for its rapid growth rates (Dawes, Orduña-Rojas, & Robledo, 1999). Nowadays, this alga is mostly used as a source for commercial agar (Lai & Lii, 1997) and as a source of sulphated polysaccharides which are used in pharmaceutical and biotechnology industries (Coura et al., 2012).

For optimal year-round high yield protein production, species selection must take into account protein content, growth rate and their seasonality, hence a combination of 2–3 complementary species would be ideal. *Ulva* offers fast growth, *Gracilaria* has high protein content, and their seasonal growth periods cover almost the entire year (Chemodanov et al., 2017; Fleurence, 2004; Friedlander, 2008).

Since the extracted algal proteins are intended for human consumption, it is crucial to determine their digestibility under human gastric and intestinal conditions. High digestibility would enable greater absorption of the protein, or in fact, its amino acids or short peptides. Because human digestive proteases have their specificity to bonds near certain amino acids, it is important to verify that the algal protein sequences can be digested by the human proteases. Protein digestibility may be assessed by simulating gastro-intestinal conditions by standard protocols (Minekus et al., 2014).

In the process of extracting algal proteins, additional valuable phytochemicals may be co-extracted to increase added value, and reduce waste. For example, polyphenols, which are present in algae as in most plant materials (Sanz-Pintos et al., 2017; Zehlila et al., 2017), are bioactive molecules considered as highly beneficial for human health, thanks to their ability to serve as antioxidants (Scalbert, Johnson, & Saltmarsh, 2005). Numerous studies highlight various disease preventive attributes of algal polyphenols such as reducing the risk of inflammation (Ibañez & Cifuentes, 2013), cancer (Lee et al., 2013; Plaza et al., 2008; Thomas & Kim, 2011), cardiovascular diseases (Kumar, Ganesan, Suresh, & Bhaskar, 2008; Murray, Dordevic, Ryan, & Bonham, 2018), diabetes (Celikler et al., 2009; Murray et al., 2018; Nwosu et al., 2011), allergy (Barbosa, Valentão, & Andrade, 2014), microbial contamination (Lopes, 2014) and neurodegenerative illnesses (Barbosa et al., 2014). The high affinity of polyphenols to proteins (Papadopoulou & Frazier, 2004) facilitates their co-extraction, and their presence in the protein extract may improve its health-promoting value. Moreover, proteins have potential as antioxidant agents, due to the presence of amino acid residues with antioxidant ability (Elias, Kellerby, & Decker, 2008). Evaluation and quantification of the antioxidant activity of the algal protein extract will demonstrate this additional potential health benefit, on top of the good basic nutritional value of its amino acids.

The objectives of this study were first to develop an effective food-applicable protocol that would yield an algal protein concentrate (APC) from these two model algal species, to pave the way for their use in the food industry. The second objective was to characterize the digestibility, the amino acid composition and the antioxidant functionality of these proteins for food application, thereby, to contribute to the development of macroalgae as a new renewable and sustainable global source of extractable nutrients for the food industry.

2. Materials and methods

2.1. Materials

We used two marine macroalgal species currently cultivated at the seaweed unit of Israel Oceanographic & Limnological Research, Haifa, Israel, *Ulva sp.* and *Gracilaria sp.*. *Ulva* sp. is a green seaweed of worldwide distribution commonly found within the Israeli Mediterranean Sea intertidal zone. We used a cultivated mixture of two morphological and genetically similar types, *Ulva rigida* and *Ulva fasciata* (Krupnik et al., 2018), and for the purpose of this study we have collectively called them *Ulva* sp.. *Gracilaria* sp. (in past studies described as *Gracilaria conferta*, yet recently suggested as *Gracilaria dura*), is a rather seasonal red seaweed commonly used for the extraction of agar. Both seaweeds were cultivated in 40 L outdoor tanks constantly aerated and supplied with running seawater, pumped from a nearby seashore. The seaweeds were fertilized once a week with a mix of 0.2 mM NaH_2PO_4 and 2.0 mM NH_4Cl for several weeks to keep up optimal growth and physiological conditions before being used for the experiments.

Iron(II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ), Fluorescein, 2,2′-Azobis [2-methyl-propionamidin] dichloride (AAPH 97%), Pefabloc® SC, o-phthaldialdehyde (OPA) and Folin-Ciocalteu reagent were purchased from Sigma-Aldrich Co. (Rehovot, Israel). Ferric chloride ($FeCl_3$ 60%) was purchased from Spectrum Chemical Manufacturing Corp. (Gardena, CA). Other reagents used were of analytical grade.

2.2. Methods

2.2.1. Protein extraction

Dried and ground algae were prepared as follows: Raw biomass was oven dried (60 °C for 48 h) and milled manually with the use of mortar and pestle. The dried and ground biomass was kept at −20 °C until use.

Protocol 1 (*P1*): The extraction procedure was performed as described by (Kandasamy, Karuppiah, & Subba Rao, 2012) with modifications. Dried and ground algae were suspended in deionized water (5% w/v) and the suspension was gently stirred overnight at 4 °C. Then,

the suspension was centrifuged (10,000 × *g*, 20 min, 4 °C) and the supernatant was collected. The sediment was treated with β-mercaptoethanol (0.5% v/v), and the pH was adjusted to 12 using 1 M NaOH. After 2 h of stirring at room temperature, the mixture was centrifuged (10,000 × *g*, 20 min, 4 °C). The supernatants from both centrifugation cycles were combined and the pH was adjusted to 7 using 1 M HCl. Solid ammonium sulfate was added to the supernatant (to 85% saturation) to induce salting out. The solution was kept for 1 h at room temperature, then centrifuged (10,000 × *g*, 20 min, 4 °C). The sediment was resuspended in deionized water, dialyzed (2 kDa) against deionized water and freeze-dried.

Protocol 2 (*P2*): The extraction procedure was performed as described for P1, except this time no β-mercaptoethanol was added.

Protocol 3 (*P3*): The extraction procedure was performed as described by (Galland-Irmouli et al., 1999) with slight modifications. Briefly, dried and ground algae leaves were suspended in deionized water (1% w/v) and placed in an ultrasonic bath (Elmasonic S10, Elma Schmidbauer GmbH, Singen, Germany) for 1 h. The mixture was then gently stirred overnight at 4 °C. After centrifugation (10,000 × *g*, 1 h, 4 °C), the supernatant was collected and deionized water was added to the sediment. The mixture went through another cycle of sonication, stirring overnight and centrifugation. The supernatants from both cycles were combined and solid ammonium sulfate was added (to 85% saturation) to induce salting out of the protein. After 1 h at room temperature and centrifugation (10,000 × *g*, 1 h, 4 °C), the sediment was resuspended in deionized water, dialyzed (2 kDa) against deionized water and freeze-dried.

Protocol 4 (*P4*): The extraction procedure was performed as described by (Postma et al., 2015) with slight modifications. Dried and ground algae leaves were suspended (at 0.6% w/v) in lysis buffer (60 mM Tris, 2% SDS, pH 9) in lysing tubes (KT03961-1-303.7, Bertin Instruments) containing zirconium oxide beads. The tubes were bead-milled for 3 cycles of 60 s at 6500 rpm, with breaks of 120 s between cycles, using a bead miller (Precellys 24, Bertin Technologies). The supernatant was incubated at 100 °C for 30 min, dialyzed (2 KDa MWCO) against deionized water and freeze-dried.

Protocol 5 (*P5*): This protocol is a food-grade process developed herein as follows: dried and ground algae leaves were suspended in NaOH (10% w/v) and placed in an ultrasonic bath (Elmasonic S10, Elma Schmidbauer GmbH, Singen, Germany) for 2 h. After centrifugation (10,000 × *g*, 10 min, 4 °C), the same volume of NaOH was added to the sediment and another cycle of sonication and centrifugation was applied. The supernatants from both cycles were combined, filtered through 0.45 μM filter, dialyzed (2 kDa) against deionized water and freeze-dried.

2.2.2. Protein purification by ion exchange

The dried protein-enriched powder (P5) was resuspended in Tris buffer (pH 9.5) and added to TOYOPEARL® DEAE-650S resin to bind and remove (without degrading) the negatively charged polysaccharides. The mixture was gently shaken (40 rpm) at room temperature for 1 h. Supernatant was kept aside and the proteins, which were more weakly bound, were eluted from the loaded resin using an elution buffer (Tris buffer (pH 9.5), 1 M NaCl). The supernatant and eluent were combined, dialyzed against deionized water and freeze-dried, to form an APC.

2.2.3. Elemental composition

To determine the protein content, total nitrogen in the samples was quantified using CHNS element analyzer (Flash2000, Thermo Fisher Scientific). Acetanilide (C = 71.09%; H = 6.71%; N = 10.36%; S = 0%) was used for calibration and Helium was used as a carrier gas.

2.2.4. Chemical composition analysis

Protein: To determine protein content in the purified sample, Lowry method was applied (Lowry, Rosenbrough, Farr, & Randall, 1951), with slight modifications suggested by Waterborg (2002). Briefly, 100 μl of 2N NaOH were added to 100 μl of sample or standard and the mixture was hydrolyzed at 100 °C for 10 min in a boiling water bath. 1 ml of freshly prepared complex forming reagent (2% w/v of Na_2CO_3, 1% w/v of $CuSO_4 \cdot 5H_2O$ and 2% w/v of sodium potassium tartrate, in the proportion 100:1:1 (by vol.)) was added to each hydrolysate. After incubation (10 min at room temperature), 100 μl of Folin reagent were added. Absorbance at 750 nm was measured after 30 min of reaction at room temperature. Results were expressed as equivalent Bovine serum albumin (BSA).

Carbohydrates: To determine total carbohydrates content in the purified sample, phenol-sulfuric acid method was applied as described by (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956), and modified to microplate format by (Masuko et al., 2005). Briefly, 150 μl of concentrated sulfuric acid were added rapidly to 50 μl of sample or standard in a 96 well microplate, along with 30 μl of 5% phenol. After 5 min of incubation in 90 °C water bath, the microplate was cooled down to room temperature and absorbance at 490 nm was measured. Results were expressed as equivalent glucose.

Phenolics: To determine total phenolic content, the Folin-Ciocalteau method was used, as described by (Borochov-Neori et al., 2015). Briefly, 900 μl of reaction solution (consisting of 0.2N Folin-Ciocalteau reagent, 20% Na_2CO_3 and deionized water, 2:1:6 v/v) were added to 100 μl sample. After 1 h of incubation at room temperature, the absorbance at 765 nm was measured, using Gallic acid as a standard (results were expressed as mg of equivalent Gallic acid).

2.2.5. Amino acid composition

Total amino acid analysis was carried out by High Pressure Liquid Chromatography, according to Application note 163 “Determination of Protein Concentrations Using AAA-Direct™” (Dionex Corporation, 2004) from Dionex Inc. (Thermo Fischer Scientific, MA, USA) with some modifications. Briefly, 1 ml 6 M HCl was added to 1 mg of protein extract and thermochemical deconstruction was conducted in a dry bath (Bio-Base, China) (16 h, 112 °C). At the end of the thermochemical deconstruction, the samples were dried by nitrogen. The dry samples were reconstituted with ultrapure water, vortexed multiple times, and kept at rest for at least 1 h in sealed vials. Diluted solutions of each sample (1/10 and 1/50) were then filtered (0.22 μm) into HPIC (High Pressure Ion Chromatography) vials.

Total amino acid content was analyzed by HPAEC-PAD (High Pressure Anion-Exchange Chromatography coupled with Pulsed Amperometric Detection) using a Dionex ICS-5000 platform (Dionex, Thermo Fischer Scientific, MA, USA) with an analytical column (Aminopack 10) and its corresponding guard column. An electrochemical detector with a gold AAA™ electrode and an AgCl reference electrode was used for detection. The eluent gradient program and the waveform for the electrochemical detector used were as described in the above mentioned Application Note 163 (Dionex Corporation, 2004), other conditions were as follows: flow rate = 0.25 ml/min, injection volume = 10 μl, column temperature = 30 °C, autosampler temperature = 5 °C. The program was validated by using a commercial amino acid mix (AAS18, Sigma Aldrich, MO, USA) and four dilutions of the mix (1/50, 1/100, 1/250 and 1/1000) were used to build a calibration curve for 17 amino-acids (Alanine, Arginine, Aspartate, Cystine, Glutamate, Glycine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylalanine, Proline, Serine, Threonine, Tyrosine, and Valine) with a correlation factor $R^2 > 99\%$ for each of them. As methionine, cysteine and its dimer cystine are sensitive to acid hydrolysis, extensive degradation could occur and thus the measured value for methionine and cystine are underestimated.

All samples were hydrolyzed in triplicate and each hydrolysate was injected twice for HPIC analysis. All data were reported as percentage of the specific amino acid (AA) out of total AA.

2.2.6. Simulated gastro-intestinal digestion

Simulated gastric and intestinal digestion (SD) was based on the protocol described by (Minekus et al., 2014), with slight modifications. Simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared as mentioned by (Minekus et al., 2014). Four parts of SGF were added to five parts of APC (4 mg/ml protein in deionized water), followed by the addition of one part containing pepsin (2000U/ml), $CaCl_2$ (0.075 mM) and HCl. After 2 h of incubation at 37 °C, five parts of the gastric phase were mixed with four parts of SIF and with one part containing NaOH, Trypsin (100U/ml), Chymotrypsin (25U/ml) and 5 mM bile salts (Taurocholic acid and sodium glycodeoxycholate). The mixture was incubated at 37 °C for 2 h. Samples were taken during both phases and the enzymatic reaction was stopped by the addition of Pefabloc® SC.

2.2.7. Proteolysis progression determination

The progress of proteolysis was measured using the o-phthaldialdehyde (OPA) assay (Goodno, Swaisgood, & Catignani, 1981). Briefly, 1600 µl of freshly prepared OPA reagent (50 ml of 0.1 M sodium tetraborate, 80 mg of OPA in 2 ml of 95% ethanol, 5 ml of 20% SDS, 0.2 ml of β-mercaptoethanol and 42.8 ml deionized water) were added to 40 µl of sample from SD and the mixture was gently stirred. Absorbance at 340 nm was measured after 2 min.

2.2.8. SDS-PAGE

The degradation of the extracted proteins during the SD was studied using Tricine-SDS-PAGE based on the method described by (Schagger, 2006). The protein bands were stained by coomassie brilliant blue. The image was filtered using a 9 by 4 median filter, to remove dust spots using Matlab® (The MathWorks, Inc, Natick, Massachusetts, USA). To quantify the percentage of digested APC as a function of SD time, gel image analysis and band densitometry was performed using ImageJ software (Schneider, Rasband, & Eliceiri, 2012).

2.2.9. Antioxidant activity

The ferric reducing antioxidant power (FRAP) assay was used to examine the antioxidative activity of the APC. The assay was based on the method described by (Benzie & Strain, 1996), with slight modifications. FRAP reagent was freshly prepared by dissolving 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) (10 mM in 40 mM HCl) and $FeCl_3{\cdot}6H_2O$ (20 mM in deionized water) solutions in acetate buffer (300 mM, pH 3.6), at 1:1:10 v/v ratio. 210 µl of FRAP reagent were transferred into each sample well of a 96 well microplate, containing 7 µl of each tested sample. To create a calibration curve for $FeSO_4$, a solution of $FeSO_4{\cdot}7H_2O$ (2000 µM) was diluted in deionized water for a concentration range of 0–2000 µM and 210 µl of FRAP reagent were added to 7 µl of each diluted sample. The absorption at 593 nm was measured, for all samples, 4 min after adding the FRAP reagent, using a plate reader (Eon™, BioTek).

The oxygen radical absorption capacity (ORAC) assay was used to examine the free radical quenching activity of the APC and based on the method described by (Folch-Cano, Jullian, Speisky, & Olea-Azar, 2010), who based it on (Ou, Hampsch-woodill, & Prior, 2001), with slight modifications. Fluorescein and 2,2′-Azobis (2-methylpropionamidine) dihydrochloride (AAPH) were dissolved in potassium phosphate buffer (75 mM, pH 7.4) and diluted to final concentrations of 30 ng/ml and 21.7 mg/ml respectively. At the first step, 25 µl of each tested sample were added to a well of a 96 well microplate, containing 150 µl of fluorescein (30 ng/ml). The 96 well microplate was placed in a fluorescence plate reader (Varioskan™ Flash, Thermo Scientific) and incubated for 15 min at 37 °C. Then, 25 µl of AAPH (21.7 mg/ml) were added to each well and the microplate was shaken for 20 s at 180 rpm. Fluorescence readings were taken every 25 s for 90 min with an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

Table 1
Composition of Carbon (C), Hydrogen (H), Sulfate (S) and Nitrogen (N) and the protein estimation for the two algal species investigates based on a DW basis.

% of DW	C	H	S	N	Protein
Ulva sp.	19.7 ± 0.6	4.8 ± 0.1	6.0 ± 0.1	1.7 ± 0.1	9 (Nx5.12)[a]
Gracilaria sp.	27.1 ± 1.1	4.9 ± 0.1	1.8 ± 0.1	5.4 ± 0.0	25 (Nx4.59)[b]

[a] Shuuluka et al., 2013.
[b] Lourenço et al., 2002.

2.2.10. Statistical analysis

Results were obtained from two or more independent experiments performed on separate days. Error bars in all figures represent standard error. The statistical significance ($p < 0.05$) of the differences was evaluated using the unpaired two-sided student's t-test.

3. Results and discussion

3.1. Algal protein extraction and purification

The main objectives of this work were to develop a food grade macroalgal protein extraction process and characterize the digestibility, amino acid composition and antioxidant properties of the protein concentrates obtained from two algae studied. Our ultimate long-term goal is to develop algal proteins as ingredients for the food industry and as part of human nutrition.

According to the elemental analysis, using the CHNS element analyzer (Table 1), the nitrogen percentage was 1.7–5.4% of the dry weight of the alga. Seaweeds and other plant materials contain considerable concentrations of non-protein nitrogenous substances, such as nucleic acids, pigments and inorganic nitrogen (Conklin-Brittain, Dierenfeld, Wrangham, Norconk, & Silver, 1999). Thus, the nitrogen-to-protein conversion factor used in the calculations should be lower than the traditional conversion factor of 6.25 (Salo-Väänänen & Koivistoinen, 1996). The conversion factors that were found to provide a more accurate estimation of the protein content for the green and the red algae were 5.12 and 4.59, respectively (Lourenço, Barbarino, De-Paula, Pereira, & Lanfer Marquez, 2002; Shuuluka et al., 2013). When using these conversion factors of nitrogen-to-protein, the protein content in the *Ulva sp.* and *Gracilaria sp.* were 8.7% and 24.8% of the DW, respectively. These values are within the ranges reported in previous works, and are greatly correlated to environmental factors such as temperature and irradiance, which vary along the seasons (Fleurence, 1999; Foster & Hodgson, 1998; Galland-Irmouli et al., 1999; Korzen et al., 2015; Shuuluka et al., 2013). For example, previous works (Chemodanov et al., 2017), showed that growth of *Ulva* sp. was observed from January to June and from October to December. Other works (Villares, Puente, & Carballeira, 1999, pp. 337–342), found that minimum contents of nitrogen, were observed in spring and summer and maximum levels in winter. In contrast, *Gracilaria sp.* grows faster during the summer when water temperatures are between 20 and 25 Celsius, yet protein contents are not necessarily at their peak (Friedlander, 2008).

To achieve a final product defined as a ‘protein concentrate’ and to be able to attribute the functional properties mainly to the protein, we obtained an extract in a form of powder comprising above 50% protein on a DW basis. Protein and carbohydrates contents of the concentrates obtained using the various protocols are shown in Fig. 1. The procedure used for protein extraction greatly affected the extract's chemical composition. Protein content of the powders varied between 20% and 62% of the DW while the carbohydrates percentage varied between 5% and 37%. The remaining non-protein and non-carbohydrate fraction in all extracts comprised phenolic compounds and most probably minerals and other phytochemicals, such as pigments, which might be bound to polysaccharides or to proteins (McManus et al., 1985).

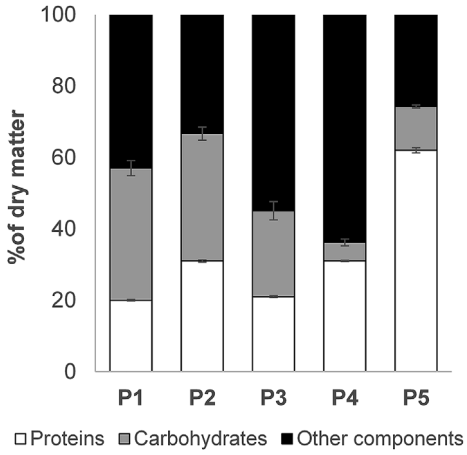

Fig. 1. Chemical composition of *Ulva sp.* extract powders, obtained using different protocols (P1-P5). Error bars represent standard error of two repeats, each performed in triplicate.

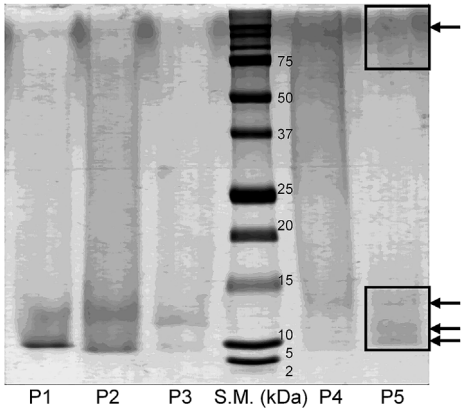

Fig. 2. SDS-PAGE analysis of *Ulva sp.* extract powders. Lane 4 contained the size marker. The arrows point at protein bands observed in lane 6 (extraction using protocol 5). The image was filtered using a 9 by 4 median filter, to remove dust spots using Matlab®.

To characterize the extracted proteins, all powders, from all protocols, were analyzed using SDS-PAGE (Fig. 2). Lane 4 contained the size marker. In all other lanes (1–3 & 5–6), one can identify two bands with a molecular weight of 10 and 12 kDa, in agreement with the results reported in previous works on *Ulva rigida* (Fleurence et al., 1995; Rouxel et al., 2001). Differences may be attributed to seaweed cultivation carried out at different geographic locations and seasons (Fleurence, Chenard, & Luçon, 1999). Across all lanes, some smearing was observed, possibly due to covalent linking between the proteins and polysaccharides, the latter having much higher molecular weights than the proteins (Fleurence et al., 1995; Paradossi, Cavalieri, Pizzoferrato, & Liquori, 1999).

Since carbohydrates constitute a major fraction of the dry weight of the alga and may interact with algal proteins, it is a major challenge to separate them from the proteins (Fleurence et al., 1995), particularly when trying to keep both fractions usable avoiding polysaccharides degradation. For that reason, we added another step in the extraction procedure which involved separation by ion exchange. When using a cationic resin, negatively charged molecules bind to the resin and may later be released by elution with rising salt concentrations. Basic pH ensures the negative charge of both proteins and polysaccharides, which will enable their attachment to the resin but also cause an ionic repulsion between proteins and polysaccharides to facilitate their separation (Jungbauer & Hahn, 2009). The polysaccharides, which are much more negatively charged, are expected to bind more avidly to the resin, allowing the proteins to be eluted off first by the rising salt gradient.

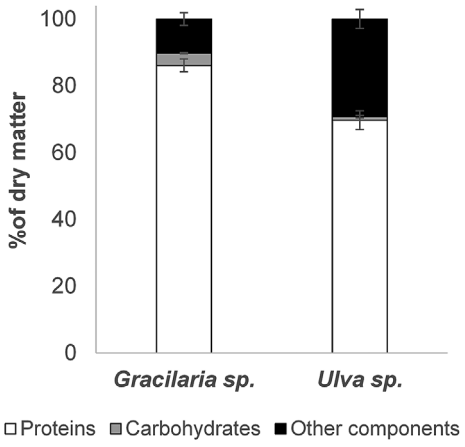

Fig. 3. Chemical composition of algal protein concentrates (APCs) obtained using protocol 5, followed by separation using ion exchange. Error bars represent standard error of two repeats, each performed in triplicate.

As can be seen in Fig. 3, the ion exchange step yielded an APC containing 70% protein and only 1% carbohydrates. The APCs obtained had a greenish hue, most likely from chlorophyll residues (Fig. 4). In this purification step, most of the carbohydrates in the extract were removed, which led to increased protein purity. In the case of *Gracilaria sp.*, the proteins extracted by using P5, followed by a purification using ion exchange, comprised 86% of the powder's dry weight (Fig. 3). These protein purity values are comparable or better than ones reported for green and red algae protein concentrates (Angell, Paul, & de Nys, 2017; Wong & Cheung, 2001), when using the correct nitrogen-to-protein conversion factors, i.e., Nx5.12 for green algae and Nx4.59 for red algae. Also, in the cited works, most of the proteins were not extracted by food grade methods, or were using enzymes to degrade the polysaccharides, which we prefer to keep as a byproduct, even at the cost of a lower protein yield.

Another challenge is that algal proteins can be found as a part of the cell membrane (Lourenço et al., 2002). This fact explains some of the difficulty in extracting algal proteins, which may lead to low yield. An even greater obstacle is the entrapment of certain proteins by cell wall and extracellular matrix (ECM) polysaccharides leading to low yield. The yield of the isolated proteins out of the total algal proteins in this preliminary study, was only 10–11%, both for *Ulva sp.* and *Gracilaria sp*. We are currently developing further improvements in the process to achieve extraction of most of the protein, to improve the economic viability of industrial production of APC.

Since the protein extract is intended for industrial food production, there was a need to develop a food-applicable extraction protocol. Such a protocol should involve only procedures and materials that are allowed in food production, and include only approved food additives, or reagents which have negligible or harmless residues, so that the final product will be considered safe for human consumption and use in the food industry. Since there are only very few food grade extraction protocols for macroalgal proteins published so far, and most of them are at lab scale, the progress reported herein, particularly in terms of purity and upscalability is very encouraging. Of all the procedures described in section 2.2.1, the protocol which yielded the highest protein purity was P5, and this was further improved by adding a purification step by ion exchange. As a whole, this procedure is upscalable and suitable for obtaining a 'food-grade' product.

3.2. Amino acid composition

Determining the amino acid composition of the APCs obtained was

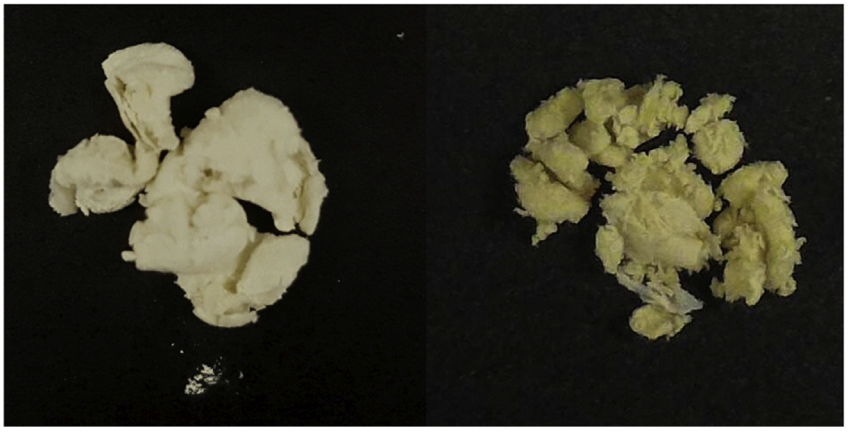

Fig. 4. Freeze-dried APCs obtained using protocol 5, followed by separation using ion exchange: L: *Gracilaria sp.*, R: *Ulva sp.*

Table 2
Amino acid composition of APCs obtained using protocol 5, followed by separation using ion exchange (% of total AA).[a]

	Ulva sp.	*Gracilaria sp.*	*Ovalbumin*[b]
Alanine	8.60 ± 0.74	10.03 ± 0.42	7.44
Arginine	23.03 ± 1.92	10.32 ± 0.45	12.99
Aspartic acid	16.11 ± 1.37	12.81 ± 0.66	6.88
Cysteine	ND	0.11 ± 0.00	1.55
Glutamic acid	12.00 ± 1.41	13.01 ± 0.57	10.99
Glycine	2.11 ± 1.08	5.98 ± 0.15	3.77
Histidine*	0.64 ± 0.04	0.98 ± 0.04	4.55
Isoleucine*	3.60 ± 0.40	4.65 ± 0.12	5.33
Leucine*	4.31 ± 0.73	8.34 ± 0.10	6.88
Lysine*	6.34 ± 1.16	6.20 ± 0.21	8.55
Methionine*	0.81 ± 0.21	0.69 ± 0.46	3.44
Phenylalanine*	2.06 ± 0.68	4.19 ± 0.12	4.55
Proline	3.32 ± 0.52	4.82 ± 0.16	3.11
Serine	6.93 ± 0.46	5.37 ± 0.15	7.55
Threonine*	4.35 ± 0.51	3.78 ± 0.08	3.33
Tyrosine	2.88 ± 0.41	1.54 ± 0.52	2.00
Valine*	2.92 ± 1.22	7.18 ± 0.17	5.99

*Essential amino acid.
[a] Values presented are means of triplicates ± SD.
[b] Fujiwara-Arasaki et al., 1984.

crucially necessary for evaluating their importance in human nutrition, and for calculating the proper nitrogen-protein conversion factors. The results are summarized in Table 2. When analyzing the amino acid composition, most important are the essential amino acids, which the human body cannot synthesize and must be consumed as part of the diet (Aristoy & Toldrá, 2012). These amino acids have an important role in metabolic pathways, growth and development (World Health Organization, 2007; Wu, 2009).

The most abundant essential amino acid in the *Ulva* APC (in terms of % of all amino acids by weight) is Lysine (6.34 ± 1.16), followed by Threonine (4.35 ± 0.51), Leucine (4.31 ± 0.73) and Isoleucine (3.60 ± 0.40). For the *Gracilaria* APC, the most abundant essential amino acid is Leucine (8.34 ± 0.10), followed by Valine (7.18 ± 0.17), Lysine (6.20 ± 0.21) and Isoleucine (4.65 ± 0.12). It is noteworthy that the amino acid Lysine is known for its relatively lower concentration in plant-based sources of protein in human diet (Young & Pellett, 1994). Hence, its significant presence in the APCs, supports the rationale in the use of macroalgae, *Ulva* sp. and *Gracilaria* sp. in particular, as a nutritional source in human diet.

Interestingly, when comparing the amino acid composition in the APCs to that in egg ovalbumin (Fujiwara-Arasaki, Mino, & Kuroda, 1984), one can observe considerable similarity and even an advantage, in some cases (e.g. threonine), for the APCs (Table 2). Other essential amino acids, such as Histidine, Methionine and Phenylalanine, are also present in the APCs, but at lower concentrations.

The most abundant non-essential amino acids in the APCs (Table 2.) are Arginine, Aspartic acid and Glutamic acid. Although these can be synthesized in the human body, there is no compelling evidence for their sufficient synthesis and previous findings (Wu et al., 2013) have determined that animal, and in particular human nutrition should include both essential and non-essential amino acids.

Overall, the amino acid compositions of the APCs are relatively close to those reported in previous works (Gajaria et al., 2017; Shuuluka et al., 2013; Yaich et al., 2011). Yet, it is noteworthy that the amino acid profile reported in those works refers to the whole alga, while in this work, it refers to the APCs, which were obtained by the extraction procedure developed in this work. During this procedure, not all proteins were extracted, which may explain some of the differences between the amino acid compositions.

The amino acid compositions of the APCs obtained are in line with the FAO/WHO recommendation (FAO/WHO, 1973), regarding the suggested content of each essential amino acid in a gram of protein. These results suggest that these algae, and in particular their APCs, would be a suitable nutritional protein source for human nutrition, providing both essential and non-essential amino acids.

3.3. Simulated gastro-intestinal digestion

Gastro-intestinal digestion was simulated by imitating gastric conditions, followed by intestinal conditions, as described in section 2.2.6. We chose to stop the enzymatic reaction by adding Pefabloc®, which is a specific inhibitor of serine proteases. The progress of proteolysis during the SDn, was determined using the OPA method and is presented in Fig. 5. During the SD, as a result of enzymatic hydrolysis, primary amine groups in the protein are exposed, hence their concentration increases. The concentration of primary amine groups was monitored throughout the SD. The progress of proteolysis was described as percent from the proteolysis of Casein, which was used as digestion standard. As can be seen in Fig. 5, after 120 min of SD, i.e., the end of the gastric phase, 47.8 ± 4.3% of the *Ulva* protein and 68.1 ± 0.7% of the *Gracilaria* protein were hydrolyzed, meaning that the proteins were considerably hydrolyzed by pepsin. At the end of the intestinal phase, 89.4 ± 2.6% of the *Ulva* protein and 100% of the *Gracilaria protein* where hydrolyzed, meaning that the proteins are also hydrolysable by trypsin and chymotrypsin. The results for *Ulva* stand in correlation with the value found by (Gajaria et al., 2017), who also compared it with casein, as a digestion standard. These results indicate that the algal proteins extracted can be hydrolyzed by digestive enzymes, which may increase the possibility of its absorption in the intestine.

We further studied digestion of *Ulva* APC using SDS-PAGE and the results can be seen in Fig. 6, where lane 5 represents the size marker. A

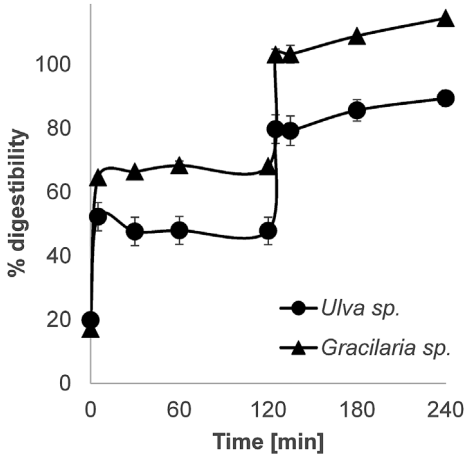

Fig. 5. Progress of proteolysis, (based on the o-phthaldialdehyde (OPA) method, relative to casein under same conditions), during simulated gastrointestinal digestion of APCs. Error bars represent standard error of two repeats, each performed in duplicate.

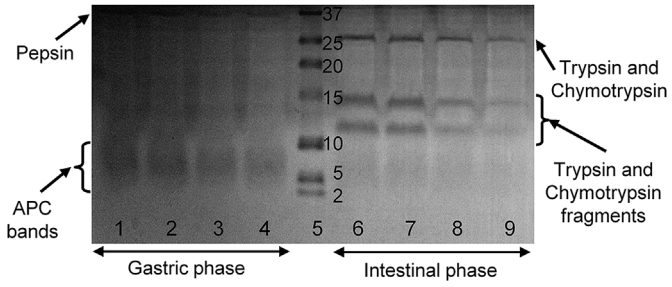

Fig. 6. SDS-PAGE analysis during SD of *Ulva sp.* APC, obtained using protocol 5, followed by separation using ion exchange. Lanes: (1) t = 0 min, (2) t = 30 min, (3) t = 60 min, (4) t = 120 min, (5) size marker: 2, 5, 10, 15, 20, 25 and 37 kDa, (6) t = 125 min, (7) t = 135 min, (8) t = 180 min, (9) t = 240 min.

band representing the pepsin (Mw = 35 kDa) appeared in all lanes of the gastric phase (1–4) and bands representing the trypsin and chymotrypsin (Mw = 23.3 and 25 kDa respectively) appeared in all lanes of the intestinal phase (6–9). Two bands with molecular weights of 12 and 15 kDa also appeared in the intestinal phase lanes. A control SDS-PAGE of the proteolytic enzymes, trypsin and chymotrypsin (data not shown), proves that these bands are fragments of these proteases. In all lanes (1–4 & 6–9), one can identify a wide band, comprising proteins with a molecular weight range of 5–10 kDa. The intensity of this band decreases with digestion time, meaning that the algal protein is degraded both by pepsin in the gastric phase and by trypsin and chymotrypsin in the intestinal phase.

The decrease in intensity was further analyzed by quantifying the bands using image densitometry analysis (ImageJ software), and the results are summarized in Table 3. The integrated color intensity diminished with SD time. It can be seen, that after 120 min of SD, i.e. at the end of the gastric phase, the value was 53% and after 240 min, at the end of the intestinal phase, the value was 5%, of that at t = 0 min. These results are compatible with the results presented in Fig. 5, indicating the good digestibility of the APC.

3.4. Antioxidant activity

Proteins and other components of living cells are sensitive to oxidation (Davies, 2005), hence food components having antioxidative activity are important for protecting membranes, proteins and other cell components from oxidation, to promote health.

In this study, we evaluated two different mechanisms of the antioxidative activity of the APCs, each was measured by a different method. We measured the antioxidant activity of the APCs before and after SD and compared that to the antioxidant activity of common proteins in human nutrition. The first method used to assess the antioxidative activity of the APCs was the oxygen radical absorption capacity (ORAC), which is based on the hydrogen atom transfer (HAT) mechanism. The results are presented in Fig. 7, using Trolox as standard. The second method used to assess the antioxidative activity of the APCs was ferric reducing antioxidant power (FRAP), which is based on the single electron transfer (SET) mechanism. The antioxidant activity is presented in Fig. 8 as equivalents of $FeSO_4$.

When comparing the antioxidative activity of the APCs, in the two different mechanisms, to the activities of several food protein isolates (β-Lactoglobulin (β-Lg), bovine serum albumin (BSA) and potato protein), we observed that the antioxidant activity of the APCs (before SD) in the HAT mechanism was similar to that of the reference proteins, but between 10 and 20 times higher in the SET mechanism. To explain that, the total phenolic content in the APCs was quantified. The results show that the APCs contained 0.7–1.0% phenolic compounds (on dry weight basis). It was not surprising that phenols were present in the APCs, since they are present in algae as in most plant materials (Sanz-Pintos et al., 2017; Zehlila et al., 2017), and are known for their high affinity to proteins (Papadopoulou & Frazier, 2004). Therefore, it is reasonable to conclude that the antioxidant activity of the APCs stems not only from the presence of amino acids with antioxidant properties, but also (and, in the SET mechanism, probably mostly) from the presence of phenols. The presence of polyphenols in the APCs may improve the oxidation tolerance of the proteins. Also, since polyphenols are bioactive molecules considered as highly beneficial for human health (Scalbert et al.,

Table 3
The integrated color intensity of the bands representing APC obtained from *Ulva sp.* using protocol 5, followed by separation using ion exchange, under SD conditions as a function of digestion time. The analysis corresponds to lanes 1–4 & lanes 6–9 of Fig. 6.

Digestion time [min]	Integrated band intensity	% of initial intensity
0	209621	100
30	193049	92
60	147971	71
120	111149	53
125	40886	20
135	33747	16
180	22065	11
240	9764	5

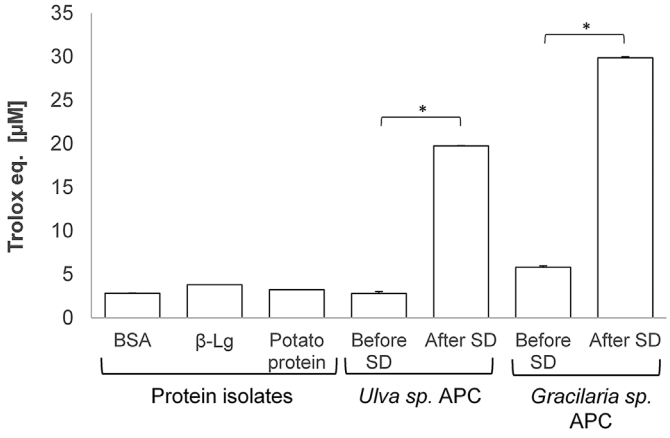

Fig. 7. Antioxidant activity by hydrogen atom transfer (HAT) mechanism before and after SD of APCs and of several food protein isolates (Bovine serum albumin (BSA), β-Lactoglobulin (β-Lg) and potato protein). All samples contained 0.01 mg/ml protein. Error bars represent standard error of two repeats, each performed in triplicate.

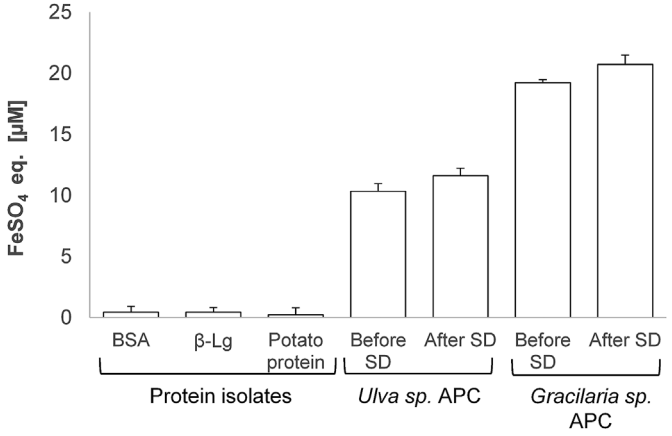

Fig. 8. Antioxidant activity by single electron transfer (SET) mechanism before and after SD of APCs and of common protein isolates (Bovine serum albumin (BSA), β-Lactoglobulin (β-Lg) and potato protein). All samples contained 1 mg/ml protein. Error bars represent standard error of two repeats, each performed in triplicate.

2005), their presence in the APCs may contribute an 'added value', which supports the beneficial use of these macroalgal APCs in human diet.

The second conclusion from Figs. 7 and 8, is that the antioxidative activity of the APCs increased significantly after SD (predominantly by the HAT mechanism). According to the literature (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005; Kong & Xiong, 2006; Liu, Kong, Xiong, & Xia, 2010), the hydrolysate often has a higher antioxidative activity than the un-hydrolyzed protein. This fact was explained by the exposure of amino acid side groups, as a result of protein hydrolysis and the main amino acids that contribute to the antioxidative activity are Trp, Tyr and Met. The high antioxidative activity of these amino acids can be explained by the capacity of their indolic and phenolic groups to serve as hydrogen donors. It is likely that the activity of free amino acids would be higher than that in the polymerized protein state. Before SD, the antioxidative activity of the amino acid residues is also limited by their accessibility within the tertiary structure of the protein. In the tertiary structure, certain amino acids with antioxidant potential are buried within the protein core. In the process of enzymatic hydrolysis, the exposure of antioxidant amino acids increases and they are more likely to donate hydrogen to the peroxyl radical (Elias et al., 2008).

4. Conclusions

We have developed a new protocol for the extraction of macroalgal proteins, which is applicable for food production. The APCs obtained were food-grade, and contained high protein content of 70–86%, and a high nutritional value of their amino acid composition. They were readily digested by SD. These APCs may thus be a valuable renewable protein source for human nutrition. Antioxidant activity experiments showed that the APCs have a relatively high antioxidative activity compared to proteins common in human nutrition, most likely due to the presence of phenolic compounds in the extract. These antioxidative properties support the beneficial use of macroalgae, particularly *Ulva* sp. and *Gracilaria* sp. in human diet. These results emphasize the feasibility of using macroalgae as a new and renewable source of proteins for human nutrition and industrial food processing.

Conflicts of interest

The authors have no conflict of interests related to this study.

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References

Ainis, A., Vellanoweth, R., Lapeña, Q., & Thornber, C. S. (2014). Using non-dietary gastropods in coastal shell middens to infer kelp and seagrass harvesting and paleoenvironmental conditions. *Journal of Archaeological Science, 49*, 343–360.

Angell, A. R., Paul, N. A., & de Nys, R. (2017). A comparison of protocols for isolating and concentrating protein from the green seaweed Ulva ohnoi. *Journal of Applied Phycology, 29*(2), 1011–1026. https://doi.org/10.1007/s10811-016-0972-7.

Aristoy, M.-C., & Toldrá, F. (2012). Essential amino acids. *Handbook of analysis of active compounds in functional foods* (pp. 3–19). .

Barbosa, M., Valentão, P., & Andrade, P. B. (2014). Bioactive compounds from macroalgae in the new millennium: Implications for neurodegenerative diseases. *Marine Drugs, 12*(9), 4934–4972. https://doi.org/10.3390/md12094934.

Benzie, I. F. F., & Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant Power": The FRAP assay. *Analytical Biochemistry, 239*(1), 70–76. https://doi.org/10.1006/abio.1996.0292.

Bleakley, S., & Hayes, M. (2017). Algal Proteins: Extraction, application, and challenges concerning production. *Foods, 6*(5), 33. https://doi.org/10.3390/foods6050033.

Bolton, J. J., Robertson-Andersson, D. V., Shuuluka, D., & Kandjengo, L. (2009). Growing Ulva (chlorophyta) in integrated systems as a commercial crop for abalone feed in South Africa: A SWOT analysis. *Journal of Applied Phycology, 21*(5), 575–583. https://doi.org/10.1007/s10811-008-9385-6.

Borochov-Neori, H., Judeinstein, S., Greenberg, A., Volkova, N., Rosenblat, M., & Aviram, M. (2015). Antioxidant and antiatherogenic properties of phenolic acid and flavonol fractions of fruits of "Amari" and "Hallawi" date (Phoenix dactylifera L.) varieties. *Journal of Agricultural and Food Chemistry, 63*(12), 3189–3195. https://doi.org/10.1021/jf506094r.

Celikler, S., Tas, S., Vatan, O., Ziyanok-Ayvalik, S., Yildiz, G., & Bilaloglu, R. (2009). Antihyperglycemic and antigenotoxic potential of Ulva rigida ethanolic extract in the experimental diabetes mellitus. *Food and Chemical Toxicology, 47*(8), 1837–1840. https://doi.org/10.1016/j.fct.2009.04.039.

Chemodanov, A., Jinjikhashvily, G., Habiby, O., Liberzon, A., Israel, A., Yakhini, Z., et al. (2017). Net primary productivity, biofuel production and CO_2 emissions reduction potential of Ulva sp. (Chlorophyta) biomass in a coastal area of the Eastern Mediterranean. *Energy Conversion and Management, 148*, 1497–1507. https://doi.org/10.1016/j.enconman.2017.06.066.

Conklin-Brittain, N. L., Dierenfeld, E. S., Wrangham, R. W., Norconk, M., & Silver, S. C. (1999). Chemical protein analysis: A comparison of kjeldahl crude protein and total ninhydrin protein from wild, tropical vegetation. *Journal of Chemical Ecology, 25*(12), 2601–2622. https://doi.org/10.1023/A:1020835120701.

Coura, C. O., de Araújo, I. W. F., Vanderlei, E. S. O., Rodrigues, J. A. G., Quinderé, A. L. G., Fontes, B. P., et al. (2012). Antinociceptive and anti-inflammatory activities of sulphated polysaccharides from the red seaweed Gracilaria cornea. *Basic and Clinical Pharmacology and Toxicology, 110*(4), 335–341. https://doi.org/10.1111/j.1742-7843.2011.00811.x.

Davies, M. J. (2005). The oxidative environment and protein damage. *Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics, 1703*(2), 93–109. https://doi.org/10.1016/j.bbapap.2004.08.007.

Dawes, C. J., Orduña-Rojas, J., & Robledo, D. (1999). Response of the tropical red seaweed Gracilaria cornea to temperature, salinity and irradiance. *Journal of Applied Phycology, 10*, 419–425.

Dillehay, T. T. D., Ramirez, C., Pino, M., Collins, M. B., Rossen, J., & Pino-Navarro, J. D. (2008). Monte Verde: Seaweed, food, medicine, and the peopling of south America. *Science, 320*(5877), 784–786. https://doi.org/10.1126/science.1156533.

Dionex Corporation (2004). *Determination of protein concentration using AAA-direct (Application Note 163)*. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/AN-163-LC-Protein-AAA-Direct-LPN1634-EN.pdf.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*(3), 350–356. https://doi.org/10.1021/ac60111a017.

Elias, R. J., Kellerby, S. S., & Decker, E. A. (2008). Antioxidant activity of proteins and peptides. *Critical Reviews in Food Science and Nutrition, 48*(5), 430–441. https://doi.org/10.1080/10408390701425615.

Erlandson, J., Braje, T., Gill, K., & Graham, M. H. (2015). Ecology of the kelp highway: Did marine resources facilitate human dispersal from Northeast Asia to the Americas? *Journal of Island and Coastal Archaeology, 10*(3), 392–411.

FAO/WHO (1973). *Energy and protein requirements: Report of a joint FAO/WHO Ad Hoc expert committee.* Geneva: World Health Organization. [meeting held in Rome from 22 March to 2 April 1971] http://www.who.int/iris/handle/10665/41042.

Fernand, F., Israel, A., Skjermo, J., Wichard, T., Timmermans, K. R., & Golberg, A. (2017). Offshore macroalgae biomass for bioenergy production: Environmental aspects, technological achievements and challenges. *Renewable and Sustainable Energy Reviews, 75*, 35–45. https://doi.org/10.1016/j.rser.2016.10.046.

Fleurence, J. (1999). Seaweed proteins: Biochemical, nutritional aspects and potential uses. *Trends in Food Science & Technology, 10*(1), 25–28. https://doi.org/. https://doi.

org/10.1016/S0924-2244(99)00015-1.
Fleurence, J. (2004). Seaweed proteins. *Proteins in food processing* (pp. 197–213). Woodhead Publishing Limited. https://doi.org/10.1533/9781855738379.1.197.
Fleurence, J., Chenard, E., & Luçon, M. (1999). Determination of the nutritional value of proteins obtained from Ulva armoricana. *Journal of Applied Phycology, 11*, 231–239.
Fleurence, J., Le Coeur, C., Mabeau, S., Maurice, M., & Landrein, A. (1995). Comparison of different extractive procedures for proteins from the edible seaweeds Ulva rigida and Ulva rotundata. *Journal of Applied Phycology, 7*(6), 577–582. https://doi.org/10.1007/BF00003945.
Folch-Cano, C., Jullian, C., Speisky, H., & Olea-Azar, C. (2010). Antioxidant activity of inclusion complexes of tea catechins with β-cyclodextrins by ORAC assays. *Food Research International, 43*(8), 2039–2044. https://doi.org/10.1016/j.foodres.2010.06.006.
Foster, G. G., & Hodgson, A. N. (1998). Consumption and apparent dry matter digestibility of six intertidal macroalgae by Turbo sarmaticus (Mollusca: Vetigastropoda: Turbinidae). *Aquaculture, 167*(3–4), 211–227. https://doi.org/10.1016/S0044-8486(98)00315-9.
Friedlander, M. (2008). Israeli R & D activities in seaweed cultivation. *Israel Journal of Plant Sciences, 56*(1–2), 15–28. https://doi.org/10.1560/IJPS.56.1-2.15.
Fujiwara-Arasaki, T., Mino, N., & Kuroda, M. (1984). The protein value in human nutrition of edible marine algae in Japan. *Hydrobiologia, 116*(1), 513–516.
Gajaria, T. K., Suthar, P., Baghel, R. S., Balar, N. B., Sharnagat, P., Mantri, V. A., et al. (2017). Integration of protein extraction with a stream of byproducts from marine macroalgae: A model forms the basis for marine bioeconomy. *Bioresource Technology, 243*, 867–873. https://doi.org/10.1016/j.biortech.2017.06.149.
Galland-Irmouli, A.-V., Fleurence, J., Lamghari, R., Luçon, M., Rouxel, C., Barbaroux, O., et al. (1999). Nutritional value of proteins from edible seaweed Palmaria palmata (dulse). *The Journal of Nutritional Biochemistry, 10*(6), 353–359. https://doi.org/10.1016/S0955-2863(99)00014-5.
Gao, G., Clare, A. S., Rose, C., & Caldwell, G. S. (2017). Reproductive sterility increases the capacity to exploit the green seaweed Ulva rigida for commercial applications. *Algal Research, 24*, 64–71. https://doi.org/10.1016/j.algal.2017.03.008.
Garcia-Vaquero, M., & Hayes, M. (2016). Red and green macroalgae for fish and animal feed and human functional food development. *Food Reviews International, 32*(1), 15–45. https://doi.org/10.1080/87559129.2015.1041184.
Gerland, P., Raftery, A. E., Ševčíková, H., Li, N., Gu, D., Spoorenberg, T., et al. (2014). World population stabilization unlikely this century. *Science, 346*(6206).
Goodno, C. C., Swaisgood, H. E., & Catignani, G. L. (1981). A fluorimetric assay for available lysine in proteins. *Analytical Biochemistry, 115*(1), 203–211. https://doi.org/10.1016/0003-2697(81)90547-9.
Harnedy, P. A., & Fitzgerald, R. J. (2011). Bioactive proteins, peptides, and amino acids from macroalgae. *Journal of Phycology, 47*(2), 218–232. https://doi.org/10.1111/j.1529-8817.2011.00969.x.
Hernández-Ledesma, B., Dávalos, A., Bartolomé, B., & Amigo, L. (2005). Preparation of antioxidant enzymatic hydrolysates from α-lactalbumin and β-lactoglobulin. Identification of active peptides by HPLC-MS/MS. *Journal of Agricultural and Food Chemistry, 53*(3), 588–593. https://doi.org/10.1021/jf048626m.
Ibañez, E., & Cifuentes, A. (2013). Benefits of using algae as natural sources of functional ingredients. *Journal of the Science of Food and Agriculture, 93*(4), 703–709. https://doi.org/10.1002/jsfa.6023.
Ingle, K., Vitkin, E., Robin, A., Yakhini, Z., Mishori, D., & Golberg, A. (2017). Macroalgae biorefinery from kappaphycus alvarezii: Conversion modeling and performance prediction for India and Philippines as examples. *BioEnergy Research,* 1–11. https://doi.org/10.1007/s12155-017-9874-z.
Jungbauer, A., & Hahn, R. (2009). Chapter 22 ion-exchange Chromatography. In R. R. Burgess, M P B T-M, & E. Deutscher (Vol. Eds.), *Guide to protein purification: Vol.463*, (pp. 349–371). Academic Press. 2nd Edition https://doi.org/https://doi.org/10.1016/S0076-6879(09)63022-6.
Kandasamy, G., Karuppiah, S. K., & Subba Rao, P. V. (2012). Salt- and pH-induced functional changes in protein concentrate of edible green seaweed Enteromorpha species. *Fisheries Science, 78*(1), 169–176. https://doi.org/10.1007/s12562-011-0423-y.
Karray, R., Karray, F., Loukil, S., Mhiri, N., & Sayadi, S. (2017). Anaerobic co-digestion of Tunisian green macroalgae Ulva rigida with sugar industry wastewater for biogas and methane production enhancement. *Waste Management, 61*, 171–178. https://doi.org/10.1016/j.wasman.2016.11.042.
Kong, B., & Xiong, Y. L. (2006). Antioxidant activity of zein hydrolysates in a liposome system and the possible mode of action. *Journal of Agricultural and Food Chemistry, 54*(16), 6059–6068.
Korzen, L., Pulidindi, I. N., Israel, A., Abelson, A., & Gedanken, A. (2015). Single step production of bioethanol from the seaweed Ulva rigida using sonication. *RSC Advances, 5*(21), 16223–16229. https://doi.org/10.1039/C4RA14880K.
Krupnik, N., Paz, G., Douek, J., Lewinsohn, E., Israel, A., Mineur, F., et al. (2018). Native, invasive and cryptogenic Ulva species from the Israeli Mediterranean Sea: risk and potential. *Mediterranean Marine Sciences, 19*(1), 132–146. https://doi.org/10.12681/mms.2104.
Kumar, C. S., Ganesan, P., Suresh, P. V., & Bhaskar, N. (2008). Seaweeds as a source of nutritionally beneficial compounds - a review. *Journal of Food Science & Technology, 45*(1), 1–13. Retrieved from http://eprints.uni-kiel.de/29698/.
Lai, M.-F., & Lii, C.-Y. (1997). Rheological and thermal characteristics of gel structures from various agar fractions. *International Journal of Biological Macromolecules, 21*, 123–130.
Lee, J. C., Hou, M. F., Huang, H. W., Chang, F. R., Yeh, C. C., & Tang, J. Y. (2013). Marine algal natural products anti-oxidative, anti-inflammatory, and anti cancer properties. *Ncbi,* 1–7. https://doi.org/10.1186/1475-2867-13-55.
Lehahn, Y., Ingle, K. N., & Golberg, A. (2016). Global potential of offshore and shallow waters macroalgal biorefineries to provide for food, chemicals and energy: Feasibility and sustainability. *Algal Research, 17*, 150–160. https://doi.org/10.1016/J.ALGAL.2016.03.031.
Liu, Q., Kong, B., Xiong, Y. L., & Xia, X. (2010). Antioxidant activity and functional properties of porcine plasma protein hydrolysate as influenced by the degree of hydrolysis. *Food Chemistry, 118*(2), 403–410. https://doi.org/10.1016/j.foodchem.2009.05.013.
Lopes, G. L. L. (2014). *Seaweeds from the Portuguese Coast: Chemistry, antimicrobial and anti-inflammatory capacity*. University of Porto. Retrieved from https://repositorio-aberto.up.pt/bitstream/10216/71802/2/27749.pdf.
Lourenço, S. O., Barbarino, E., De-Paula, J. C., Pereira, L. O. D. S., & Lanfer Marquez, U. M. (2002). Amino acid composition, protein content and calculation of nitrogen-to-protein conversion factors for 19 tropical seaweeds. *Phycological Research, 50*(3), 233–241. https://doi.org/10.1046/j.1440-1835.2002.00278.x.
Lowry, O. H., Rosenbrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin. *Journal of Biological Chemistry, 193*(1), 265–275. https://doi.org/10.1016/0304-3894(92)87011-4.
Masuko, T., Minami, A., Iwasaki, N., Majima, T., Nishimura, S. I., & Lee, Y. C. (2005). Carbohydrate analysis by a phenol-sulfuric acid method in microplate format. *Analytical Biochemistry, 339*(1), 69–72. https://doi.org/10.1016/j.ab.2004.12.001.
McManus, J. P., Davis, K. G., Beart, J. E., Gaffney, S. H., Lilley, T. H., & Haslam, E. (1985). Polyphenol interactions. Part 1. Introduction; some observations on the reversible complexation of polyphenols with proteins and polysaccharides. *Journal of the Chemical Society, Perkin Transactions 2, 0*(9), 1429. https://doi.org/10.1039/p29850001429.
Melo, M. R., Feitosa, J. P., Freitas, A. L., & de Paula, R. C. (2002). Isolation and characterization of soluble sulfated polysaccharide from the red seaweed Gracilaria cornea. *Carbohydrate Polymers, 49*(4), 491–498. https://doi.org/10.1016/S0144-8617(02)00006-1.
Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., et al. (2014). A standardised static in vitro digestion method suitable for food - an international consensus. *Food Funct, 5*(6), 1113–1124. https://doi.org/10.1039/c3fo60702j.
Murray, M., Dordevic, A. L., Ryan, L., & Bonham, M. P. (2018). An emerging trend in functional foods for the prevention of cardiovascular disease and diabetes: Marine algal polyphenols. *Critical Reviews in Food Science and Nutrition, 58*(8), 1342–1358. https://doi.org/10.1080/10408398.2016.1259209.
Nwosu, F., Morris, J., Lund, V. A., Stewart, D., Ross, H. A., & McDougall, G. J. (2011). Anti-proliferative and potential anti-diabetic effects of phenolic-rich extracts from edible marine algae. *Food Chemistry, 126*(3), 1006–1012. https://doi.org/10.1016/j.foodchem.2010.11.111.
Ou, B., Hampsch-woodill, M., & Prior, R. L. (2001). Development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent probe development and validation of an improved oxygen radical absorbance capacity assay using fluorescein as the fluorescent. *Journal of Agricultural, 49*, 4619–4626. and, 2001 https://doi.org/10.1021/jf010586o.
Papadopoulou, A., & Frazier, R. A. (2004). Characterization of protein–polyphenol interactions. *Trends in Food Science & Technology, 15*(3–4), 186–190. https://doi.org/10.1016/j.tifs.2003.09.017.
Paradossi, G., Cavalieri, F., Pizzoferrato, L., & Liquori, A. M. (1999). A physico-chemical study on the polysaccharide ulvan from hot water extraction of the macroalga Ulva. *International Journal of Biological Macromolecules, 25*(4), 309–315. https://doi.org/10.1016/S0141-8130(99)00049-5.
Pirie, N. W., & Pereira, H. C. (1976). Food protein sources. *Philosophical Transactions of the Royal Society of London B Biological Sciences, 274*(934), 489–498.
Plaza, M., Cifuentes, A., & Ibáñez, E. (2008). In the search of new functional food ingredients from algae. *Trends in Food Science & Technology, 19*(1), 31–39. https://doi.org/10.1016/j.tifs.2007.07.012.
Postma, P. R., Miron, T. L., Olivieri, G., Barbosa, M. J., Wijffels, R. H., & Eppink, M. H. M. (2015). Mild disintegration of the green microalgae Chlorella vulgaris using bead milling. *Bioresource Technology, 184*, 297–304. https://doi.org/10.1016/j.biortech.2014.09.033.
Rouxel, C., Bonnabeze, E., Daniel, A., Jérôme, M., Etienne, M., & Fleurence, J. (2001). Identification by SDS PAGE of green seaweeds (Ulva and Enteromorpha) used in the food industry. *Journal of Applied Phycology, 13*(3), 215–219. https://doi.org/10.1023/A:1011161505839.
Salo-Väänänen, P. P., & Koivistoinen, P. E. (1996). Determination of protein in foods: Comparison of net protein and crude protein (N x 6.25) values. *Food chemistry*. *Vol.57*. *Food chemistry* (pp. 27–31). Elsevier. https://doi.org/10.1016/0308-8146(96)00157-4.
Sanz-Pintos, N., Pérez-Jiménez, J., Buschmann, A. H., Vergara-Salinas, J. R., Pérez-Correa, J. R., & Saura-Calixto, F. (2017). Macromolecular antioxidants and dietary fiber in edible seaweeds. *Journal of Food Science, 82*(2), 289–295. https://doi.org/10.1111/1750-3841.13592.
Scalbert, A., Johnson, I. T., & Saltmarsh, M. (2005). Polyphenols: Antioxidants and beyond. *American Journal of Clinical Nutrition, 81*(1 Suppl), 215S–217S.
Schagger, H. (2006). Tricine-SDS-PAGE. *Nature Protocols, 1*(1), 16–22.
Schneider, C. A., Rasband, W. S., & Eliceiri, K. W. (2012). NIH image to ImageJ: 25 years of image analysis. *Nature Methods, 9*(7), 671–675. https://doi.org/10.1038/nmeth.2089.
Shuuluka, D., Bolton, J. J., & Anderson, R. J. (2013). Protein content, amino acid composition and nitrogen-to-protein conversion factors of Ulva rigida and Ulva capensis from natural populations and Ulva lactuca from an aquaculture system, in South Africa. *Journal of Applied Phycology, 25*(2), 677–685. https://doi.org/10.1007/s10811-012-9902-5.
Taboada, C., Millán, R., & Míguez, I. (2010). Composition, nutritional aspects and effect on serum parameters of marine algae Ulva rigida. *Journal of the Science of Food and*

Agriculture, 90(3), 445–449. https://doi.org/10.1002/jsfa.3836.
Thomas, N. V., & Kim, S. K. (2011). Potential pharmacological applications of polyphenolic derivatives from marine brown algae. *Environmental Toxicology and Pharmacology, 32*(3), 325–335. https://doi.org/10.1016/j.etap.2011.09.004.
Villares, R., Puente, X., & Carballeira, A. (1999). Nitrogen and phosphorus in Ulva sp. in the Galician rias bajas (northwest Spain): Seasonal fluctuations and influence on growth. *Boletin-InstitutoEspanol De Oceanografia, 15*(1-4), 337–341.
Waterborg, J. H. (2002). The Lowry method for protein quantitation. In J. M. Walker (Ed.). *The protein protocols handbook* (pp. 7–9). Totowa, NJ: Humana Press. https://doi.org/10.1385/1-59259-169-8:7.
World Health Organization (2007). *Protein and amino acid requirements in human nutrition technical report series No 935* https://books.google.co.il/books?id=YaYsDwAAQBAJ&lpg=PA1&ots=ZapUHrdsH5&dq=Protein%20and%20amino%20acid%20requirements%20in%20human%20nutrition&lr&hl=iw&pg=PA103#v=onepage&q=Protein%20and%20amino%20acid%20requirements%20in%20human%20nutrition&f=false.
Wong, K. H., & Cheung, P. C. K. (2000). Nutritional evaluation of some subtropical red and green seaweeds. Part I - proximate composition, amino acid profiles and some physico-chemical properties. *Food Chemistry, 71*(4), 475–482. https://doi.org/10.1016/S0308-8146(00)00175-8.
Wong, K., & Cheung, P. C. (2001). Nutritional evaluation of some subtropical red and green seaweeds Part II. In vitro protein digestibility and amino acid profiles of protein concentrates. *Food Chemistry, 72*(1), 11–17. https://doi.org/10.1016/S0308-8146(00)00176-X.
Wu, G. (2009, May). *Amino acids: Metabolism, functions, and nutrition.* Vienna: Springer. Amino Acids https://doi.org/10.1007/s00726-009-0269-0.
Wu, G., Wu, Z., Dai, Z., Yang, Y., Wang, W., Liu, C., et al. (2013). Dietary requirements of "nutritionally non-essential amino acids" by animals and humans. *Amino Acids, 44*(4), 1107–1113. https://doi.org/10.1007/s00726-012-1444-2.
Yaich, H., Garna, H., Besbes, S., Paquot, M., Blecker, C., & Attia, H. (2011). Chemical composition and functional properties of Ulva lactuca seaweed collected in Tunisia. *Food Chemistry, 128*(4), 895–901. https://doi.org/10.1016/j.foodchem.2011.03.114.
Yang, L.-E., Lu, Q.-Q., & Brodie, J. (2017). A review of the bladed bangiales (Rhodophyta) in China: History, culture and taxonomy. *European Journal of Phycology, 00*(00), 1–13. https://doi.org/10.1080/09670262.2017.1309689.
Young, V. R., & Pellett, P. L. (1994). Plant proteins in relation to human protein and amino acid nutrition. *American Journal of Clinical Nutrition, 59*(5 Suppl), 1203S–1212S.
Zehlila, A., Schaumann, A., Mlouka, A. Ben, Bourguiba, I., Hardouin, J., Masmoudi, O., et al. (2017). Glioprotective effect of Ulva rigida extract against UVB cellular damages. *Algal Research, 23*, 203–215. https://doi.org/10.1016/j.algal.2017.02.001.