Food Chemistry 276 (2019) 735–744

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

In silico food allergenic risk evaluation of proteins extracted from macroalgae *Ulva* sp. with pulsed electric fields

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ARTICLE INFO

Keywords:
Food allergens
Proteins
Green macroalgae
Non-thermal pulsed electric fields
Electroporation
Sustainable food production
Ulva sp. proteome

ABSTRACT

Extraction of protein from macroalgae, currently defined as "novel food", is challenging and limited information about the health impacts of these proteins is available. Here, we report on a non-thermal, chemical-free green macroalgae *Ulva* sp. protein extraction by osmotic shock combined with pulsed electric fields (PEF) followed by hydraulic pressure. The extracted proteins were identified and annotated to allergens using sequence similarity. The allergenicity potential of PEF extracted proteins was compared to osmotic shock extracts and complete *Ulva* sp. proteome, extracted with the thermochemical method. The PEF extracts contained 'superoxide dismutase' (SOD), a known food allergen, osmotic shock extract contained 'troponin C', and thermochemical extract contained two additional potential food allergens 'aldolase A' and 'thioredoxin h'. This study shows an importance and the need for deep investigation of algal proteins and protein extraction technology health impacts prior to large-scale release to the market of "novel food" derived proteins.

1. Introduction

The world population is growing and as a result, the need for food that doesn't require arable land and fresh water is increasing too (Subasinghe, Soto, & Jia, 2009). Indeed, the food supply will have to be increased by 70% until 2050 (Godfray et al., 2010), in order to answer the whole population demand. Although the 2014 global protein consumption was approximately 473 million metric ton (MMT), the 2054 protein consumption is currently forecasted to reach 943 MMT (Stice, 2014). The current worldwide challenge is to meet this demand sustainably. This challenge is tougher than a few decades ago when agriculture intensification with synthetic fertilizers, herbicides, and pesticides was the solution for the growing food demand (Alston, Beddow, & Pardey, 2009).

However, these forms of intensifications will no longer be an option due to its severe environmental impacts, such as reducing biodiversity, increasing greenhouse gas emissions and the pollution of the terrestrial ecosystems, freshwater, and marine habitats as a result of the nutrient run-off from the fertilizers (Tilman, 1999). The increasing protein demand is expected to require an additional $100{\cdot}10^6$ ha of arable land (Stice, 2014). If the source of the required protein supply remains the terrestrial agriculture, it will magnify the negative environmental impact and cause more ecological shifts (Tilman, 1999). The world protein demand for human diet and animal feed emphasizes the importance of finding new sustainable and environmentally friendly sources (Tilman, 1999; Van Krimpen, Bikker, Van der Meer, Van der Peet-Schwering, & Vereijken, 2013).

To accommodate this growing protein demand, alternative protein sources recently have been investigated (Bleakley & Hayes, 2017; Stice & Basu, 2015). The considered alternative protein sources for human diet come from well-known plants such as pulses (pea, chickpea, lentil, and bean) (Boye, Zare, & Pletch, 2010) and more exotic options: algae, insects, and lab-grown meat. The predicted protein market share of alternative proteins is expected to increase from 2.1% to 33% of the global protein market by 2054 (Stice & Basu, 2015).

Among the alternative protein, the algae market share is predicted to be 18% (Stice & Basu, 2015). The algae consist of two main groups: plant-like organism-macroalgae (seaweed) and unicellular organisms-microalgae. Both groups are considered in the recent years as feedstock for protein supply (Becker, 2007; Bleakley & Hayes, 2017). Macroalgae and microalgae could provide higher protein yield per unit area than terrestrial plants used as protein sources such as wheat, soybean and pulse legumes (Bleakley & Hayes, 2017; Van Krimpen et al., 2013). However, to make algal protein available for human and animal

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https://doi.org/10.1016/j.foodchem.2018.09.134
Received 13 March 2018; Received in revised form 20 September 2018; Accepted 22 September 2018
Available online 29 September 2018
0308-8146/

consumption, it should be extractable, digestible, and, most importantly, to be safe. Food allergy is one of the main concerns for the food safety in novel foods (Thomas et al., 2007). However, to the best of our knowledge, the question of potential food allergy has not been addressed for extracted proteins from macroalgae. This question must be addressed due to the fact that previously was found evidence for clinical sensitivity to green algae (Bernstein & Safferman, 1973).

The goal of this work is to address the two challenges for the development of new sustainable sources of macroalgal proteins: new technologies for extraction and preliminary assessment of allergenic potential. Our model species is a green macroalga from *Ulva* sp., a promising feedstock for biorefinery (Bikker et al., 2016). In *Ulva*, the protein varies between 9 and 33 % of the dry weight, depending on the growth location, the season of the harvesting, the specific species, and the pre and post-processing procedures that were done with the algae biomass (Fleurence, 2004). In controlled, cultivation condition, yields up to 45 tons (DW) per hectare per year were reported in Denmark (Bruhn et al., 2011), suggesting a theoretical yield of 4–14.8 tons per hectare per year of protein. At the same time, the richest proteins source that comes from terrestrial plants, forage legumes, could provide only 1–2 tons per hectare per year of protein (Van Krimpen et al., 2013).

Ulva sp. biomass could be used as a protein source as the entire organism (Fujiwara-Arasaki, Mino, & Kuroda, 1984) or the protein could be extracted or concentrated in the cake after extraction of other components (Bleakley & Hayes, 2017). Different methods to increase the extraction of the protein yield for macroalgae were described: aqueous, acidic, alkaline, enzymatic, mechanical grinding, high shear force etc. (Bleakley & Hayes, 2017). Recently have been investigated new cell disruption approaches which assisting in the protein extraction such as ultrasound or microwave-assisted, high-pressure homogenization extractions (Barba, Grimi, & Vorobiev, 2015). Each method or their combinations could be used (Bleakley & Hayes, 2017; Parniakov, Apicella et al., 2015; Parniakov, Barba et al., 2015). The concentrated protein extraction potentially could be added to different food products as an ingredient (Fleurence, 1999). An extraction of water-soluble protein from *Ulva* shows efficient digestibility by human intestinal juice (Fleurence, 1999).

However, current methods used for protein extraction often involve thermal or chemical procedures that could affect the nutritional value of the extracted proteins and peptides, and unwanted chemicals also could remain. Moreover, these methods may alter the allergenic properties of the food proteins (Thomas et al., 2007). To address these problems non-thermal, chemical-free protein extraction methods from macroalgae are needed. Pulsed electric field (PEF) is an emerging method for that is already used as an energy-efficient extraction of proteins from microalgae and plants (Bluhm & Sack, 2009; Parniakov, Apicella et al., 2015; Parniakov, Barba et al., 2015). We recently described a water-soluble proteins extraction from *Ulva* using PEF (Polikovsky et al., 2016). We also showed that PEF enables selective protein extraction (Polikovsky et al., 2016).

In the current work, we investigated the impact of various PEF regimes on crude protein extraction. In addition, we analyzed *in silico* the potential allergenic effect of extracted *Ulva* proteins. For this analysis, *Ulva* sp. protein extractions were done with osmotic shock and mechanical press with or without PEF or thermochemically. This study will support further the integration of sustainably produced macroalgae derived proteins into the global food and feed supply chain.

2. Materials and methods

2.1. Source of Ulva sp. biomass

Biomass of macroalgae *Ulva* sp. was supplied by AlGAplus (Aveiro, Portugal). The cultivation was done in a certified facility for aquaculture. After obtaining the macroalgae, it was stored for two days in an aquarium with a volume of 400 Liter, in seawater with a salinity of about 3.5%.

2.2. Proteins extraction using pulsed electric fields, osmotic shock, and mechanical press

In order to remove the external water from the *Ulva* sp. biomass, the biomass was centrifuged three times for 1 min each, at 840 RPM. After the centrifugation, 140*g* of *Ulva* sp. biomass were weighted in a 2 Liter Becher (by using KERN balance, model 440-49N). The *Ulva* sp. biomass was loaded into the PEF treatment chamber (working volume 232 cm^3). Freshwater was added to fill the chamber completely. This fresh water created an osmotic shock. The chamber was closed and PEF were applied. After the PEF treatment, the biomass was collected and weighed again. The treatment parameters were: 0–75 pulses, 12 or 26 kV of applied voltage (1.56 or 7.26 kV cm^{-1} field strength), and pulse duration 2.2–7.2 μs, delivered at 0.5 Hz. For each pulse, voltage and current the data were collected using a high-voltage divider (Hilo-Test Company, HVT 240 RCR). The current was measured with a probe from Pearson electronics (110 A). For collecting data about the voltage and the current, the high-voltage divider and the current probe were connected to an oscilloscope (Tektronix TDS 640A). For the temperature measurements, TFA digital thermometer (30.1018) was used. In total 74 samples were treated with at least three repeats per experimental condition with at least triplicates per experimental condition.

The invested energy (E_t) was calculated using Eq. (1):

$$E_t = 0.5 \cdot C \cdot (V)^2 \cdot N \quad (1)$$

where C is the capacitance of the discharging capacitor (Farad); the applied voltage is *V* (Volt), the number of pulses is *N*. Any additional losses in the capacitor charger were neglected.

The specific energy that was invested for the protein extraction (e_p) was calculated by using Eq. (2):

$$e_p = E_t / Yield \quad (2)$$

where *Yield* (gram) is the extracted protein yield.

During the mechanical extraction with pressing, the algae biomass was wrapped in a folded cloth, for preventing the biomass escape during the process. The pressing with 45 decanewtons per square centimeter (daN cm^{-2}) was done with the mechanical press (HAPA Company (SPM 2.5S). The pressing was applied for 5 min in the automatic mode. During the extraction with the press, a juice was collected into a two Liter Becher and was weighed after the pressing process. The pressing matter that was left in the press was weighted, then after reorganizing the pressed biomass, the biomass was loaded back into the press for another pressing step. Finally, the extracted juice was frozen on a dry ice. As a control, we repeated the procedure exactly, excluding, however, the application of pulsed fields. During the control experiments, cells were broken partially by an osmotic shock.

2.3. Extracted proteins identification with LC-MS/MS

2.3.1. Thermochemical, PEF with osmotic shock and mechanical press proteins extraction for proteomic analysis

Proteins extracted by three methods were used for proteomic analysis: thermochemical extraction, PEF with osmotic shock and mechanical press and osmotic shock and mechanical press.

The thermochemical protein extraction method was done with urea buffer. 9 M urea, 400 mM Ammonium bicarbonate, 10 mM DTT were added to 50 mg (dry weight) of a sample, vortexed, and sonicated (5′, 90%, 10-10). Then, the protein reduction was done at 60 °C for 30 min.

Proteins extracted with PEF, osmotic shock, and mechanical press as described in Section 2.2 with the following specific PEF parameters:75 pulses, 24 kV (total capacitance 200 (nF)), average applied field strength of 2.964 ± 0.007 kV cm^{-1}, and pulse duration 5.70 ± 0.30 μs, delivered at 0.5 Hz.

Osmotic shock and mechanical press extraction were done as described in Section 2.2.

2.3.2. Proteolysis for proteomic analysis

The 200 µL samples algae after the osmotic shock and with PEF or without PEF was added to 8 M urea. Then the protein was in 8 M urea, and reduced by using 2.8 mM DTT (at a temperature of 60 °C, for 30 min), the modification done with 8.8 mM iodoacetamide in 100 mM ammonium bicarbonate (in room temperature for 30 min, in the dark conditions) and digested in 2 M urea, 25 mM ammonium bicarbonate.

After the protein extracted with the thermochemical method, samples modified with 37.5 mM iodoacetamide (in the dark, room temperature for 30 min) and the digestion is done in 1 M urea, 60 mM ammonium bicarbonate. An additional second digestion was done for 4 h. Modification with trypsin (Promega) at a 1:50 enzyme-to-substrate ratio done to all samples during an overnight at 37 °C. Finally, from each sample, one microgram was injected into an LC-MS/MS device.

2.3.3. Mass spectrometry analysis

The desalting of tryptic peptides was done by using C18 tips (Ultra-Micro, Harvard) then dried. The re-suspension has done in 0.1% Formic acid. The peptides resolved in reverse-phase chromatography on 0.075 × 180-mm fused silica capillaries (J&W), the capillaries were packed with 'Reprosil', a reversed phase material (Dr. Maisch GmbH, Germany). The elution of the peptides was done with Linear A gradient of 5–28% during 60 min, the gradient of 28–95% during 15 min and finally 15 min at 95% acetonitrile with 0.1% formic acid with a water flow rates of 0.15 µL/min. Mass spectrometry done with the positive mode of the 10 most dominant ions which selected from the first MS scan by using repetitively full MS scan with collision induces dissociation (HCD), in a Q Exactive plus mass spectrometer (Thermo Fischer Scientific, CA).

2.3.4. Computational analysis

The mass spectrometry data from the biological samples were analyzed using the MaxQuant software 1.5.2.8 (Mathias Mann's group) vs. the green algae section in the NCBI-nr database using 1% FDR. Data quantification was done by label-free analysis with the same software.

2.4. Extracted protein quantification

After protein extractions were done with osmotic shock, and mechanical press with or without PEF, or thermochemical extraction method all samples are filtrated with 0.22 µm pore size filter, and the protein was quantified using Bradford buffer (Sigma-Aldrich, Israel) using EL808, BioTek spectrophotometer (Winooski, VT, USA) with an optical density (OD) of 450 nm and 590 nm. Bovine serum albumin (BSA, Amresco) was used for a standard curve.

2.5. In silico allergenic risk evaluation of macroalgal proteins

All identified proteins were evaluated for potential allergenicity using two databases: AllergenOnline database (allergenonline.org) and SDAP-Structural Database of Allergenic Proteins (fermi.utmb.edu) (Ivanciuc, Schein, & Braun, 2003). Each protein was checked for allergenicity potential using the cutoff E-scores, which indicate homology with allergens detected in other organisms, of 10^{-7} for AllergenOnline and 0.01 for SDAP (Ivanciuc et al., 2003). The complete protocol for *Ulva* proteins extraction and allergenicity determination is shown in Fig. 1 and Table S8.

2.6. Statistical analysis

For statistical analysis, a Data analysis package in Excel program (ver. 13, Microsoft, WA) was used. All samples and controls were prepared and measured, at least in triplicates, if not mentioned differently.

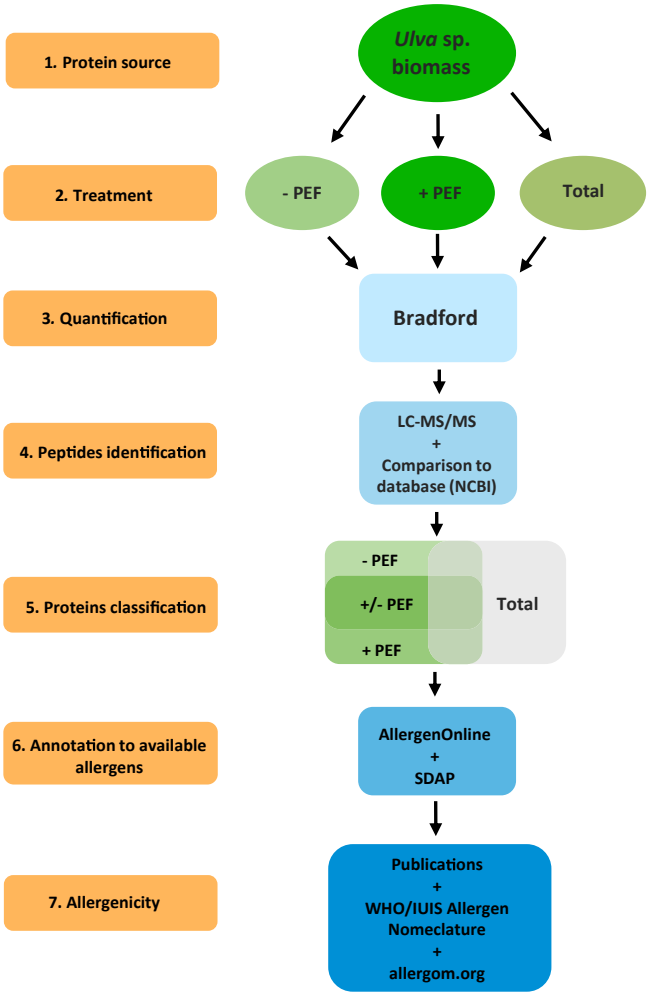

Fig. 1. Protein extraction and allergenicity determination method. The flowchart describes the allergenic identification procedure from extraction the proteins step up the functional analysis of the annotated allergens. The allergen annotation and allergenicity demined by following steps: 1. Protein source- was the *Ulva* sp. 2. Treatment- (−) PEF means treatment only with osmotic shock and mechanical press, (+) PEF means only with osmotic shock and mechanical press with PEF treatment. Method for total protein extraction that includes urea (9 M), sonication and high temperature (60 °C), as described in the methods section. 3. The quantification was done with Bradford. 4. Proteins identified after proteomic analysis. LC-MS/MS used for identifying peptides in the samples and the peptides analyzed with MaxQuant program, then the identified peptides compared vs. the green algae section in the NCBI-nr database for the proteins identification. 5. Proteins classified into groups appeared in Polikovsky et al. (2016) and in Table S8 (under the title 'Treatment') the classification done by the presence of the proteins after different proteins extraction methods. The proteins classification was to: (i) osmotic shock with the mechanical press = (−) PEF (ii) osmotic shock with the mechanical press and PEF = (+PEF). Osmotic shock with the mechanical press with or without PEF = (+/−) PEF. Total protein extraction includes urea, sonication, and heat = Total. 6. The identified proteins were annotated to allergens in two databases AllergenOnline and SDAP = Structural Database of Allergenic Proteins. 7. The identified allergens were discovered for its allergenicity effect using scientific publications, WHO/IUIS Allergen Nomenclature and allergom.org websites. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The error bars are the standard error of the mean (SEM). To compare the extracted total protein yield to the controls, a two-tailed Student's *t*-test was performed. Spearman correlation (r_s) was performed using RStudio (*RStudio Team (2015). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA URL http://www.rstudio.com/*). One-way

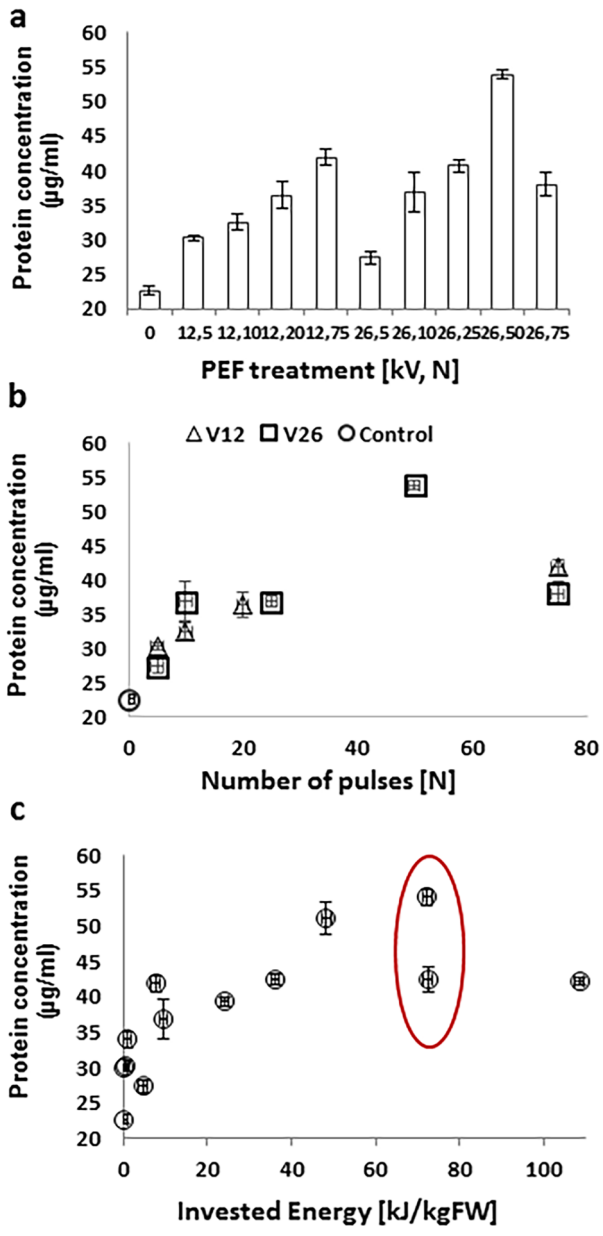

Fig. 2. The protein PEF extraction optimization from *Ulva* sp. a. Protein extraction (µg ml^{-1}) depend on PEF treatment (voltage and number of pulses). x-axis = first number (from left) is the charging voltage per stage [kV], the second number is the number of pulses. b. The protein concentration (µg/ml) dependence on a number of pulses. Triangles (V12) = PEF treatments with a voltage of 12 kV (kilovolts), squares (V26) = PEF treatments with a voltage of 26 kV. Circle = control, a protein extraction with an osmotic shock and press (without PEF). c. A protein concentration (µg ml^{-1}) dependence on the energy invested to extract the protein kJ per kg of fresh algae biomass. Detailed treatment protocols are described in Table S1, the x-axis values describe the numbers shown in the column 'spec. Energy relative to raw mass (kJ/kg)'. The dots in the figure are the averages of the PEF treatment with a difference in the range of ± ~1% in the invested energy. The y-axis of the chart displays the averages numbers of the extracted proteins for every invested energy. a–c: The protein extraction included PEF, osmotic shock, and pressure. The control was only osmotic shock and pressure without PEF. Protein quantified with Bradford assay. The columns and dotes represent averages of the biological replicates, respectively. Arrow bars = ± standard error, $n \geq 3$. Averages included at least of 95% of the biological replicas ($\mu \pm 2\sigma$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Analysis of variance (ANOVA) was done for group comparison with the significance level set up on 0.05. Identified protein is taken into consideration in the analysis, based on a peptide that appeared more than once in each sample and it detected in at least two biological replicates out of three.

3. Results and discussion

3.1. Protein extraction with pulsed electric fields from Ulva sp. biomass

The pulse shape of the voltage delivered in the first and last pulse of the 75 pulses (delivered in series) is shown in Fig. S2. Various combinations of PEF protocols for protein extraction were tested (Fig. 2 and Table S1) and showed significant differences between treated groups (ANOVA: df = between groups = 9, within groups = 41, $P = 9.17{\cdot}10^{-16}$, $n = 51$). We found that increasing the number of pulses from 5 to 75 at 12 kV led to the increase of the protein in the extract from 22.5 ± 0.64 to 41.9 ± 1.09 µg ml^{-1} monotonically (Fig. 2a, b). In addition, increasing the number of pulses from 5 to 75 at 26 kV led to the increasing of protein content in the extract until 50 pulses (from 27.3 ± 0.96 to 53.8 ± 0.69 µg ml^{-1}), further increasing of the number of pulses to 75 led to the total extracted protein yield reduction to 38 ± 1.67 µg ml^{-1} (Fig. 2a, b). This extracted protein yield reduction is in agreement with previous work that showed the effects of PEF processing on egg protein content and aggregation (Wu, Zhao, Yang, & Chen, 2014), which could prevent extraction.

The influence of the energy investment in PEF treatment for the protein extraction was calculated using Eq. (1) (Fig. 2c). After any energy investment, the extracted protein was higher than in control ($df = 7$, $P < 5.4{\cdot}10^{-5}$, two-tailed Student's t-test, $n = 4$), even after investing the lowest amount of energy (0.26 kJ kg^{-1} (Fresh Weight, FW, of *Ulva*), Fig. 2c, red circle). The extracted protein yield tended to increase with the increase of the invested energy ($r_s = 0.77$). However, the investment of 108 kJ kg^{-1} FW decreased the extracted proteins yield in comparison with 72 kJ kg^{-1} FW (Fig. 2c, Table S3, $df = 4$, $P < 0.01$, two-tailed Student's t-test, $n = 3$).

Interestingly, energy investment alone, could not explain the differences between extracted protein yields (Tables S3 and S4). For example, in samples where invested energy was 7.71 and 36.15 kJ kg^{-1} FW, no significant difference was observed in the extracted proteins yields. However, the investment of 72.29 kJ kg^{-1} FW led to 27.4% higher extracted protein yield than 72.43 kJ kg^{-1} FW (Fig. 2c red circle). In these two samples, the applied voltage was the same (26 kV) but the pulses amount and the capacitance were different, 50 or 75 pulses and the capacitance of 600 or 400 nF respectively for 72.29 kJ kg^{-1} FW and 72.43 kJ kg^{-1} respectively. These results show that the form of energy investment is critical in PEF process development.

The highest extraction yield of 53.8 ± 0.69 µg ml^{-1} was obtained with 50 pulses with 2.3 µs duration, applied at 26 kV, 7.26 kV cm^{-1} field strength. The final temperature after extraction with these parameters was 26.9 ± 0.4 °C. The energy investment was 72.29 kJ kg^{-1} FW or 1.5 ± 0.5 kJ $mg_{extracted\_protein}^{-1}$. It is important to emphasize that Bradford assay with the BSA standard curve, done in this work for protein quantification has limitations. When quantifying algae proteins and other stains reported in the literature, it showed significantly higher protein yields on the same samples probably because of the variation in the amino acid composition (Barbarino & Lourenço, 2005).

3.2. Protein quantification for proteomic analysis

The PEF method allowed to extract proteins but not all of them, for the comparison a method for total protein extraction including urea (9 M), sonication and heat (60 °C) was used. This method used before for proteomic analysis (Levitan et al., 2015). By using that method 738.1 ± 51.5 µg protein was extracted out of 50 mg dry weight (DW)

Table 1
Estimation of the potential allergenicity of proteins extracted from *Ulva* sp. biomass with osmotic shock and mechanical press (Bauermeister et al., 2011; Chen, Yang, Wei, & Tao, 2014; De Coaña et al., 2010; Hindley et al., 2006; Jeong et al., 2010; Ledesma, Villalba, & Rodríguez, 2000; Tinghino et al., 1998).

Allergen name	Organism	Sequence Link in SwissProt /NCBI/PIR	GI source	Database	Identity %	*E* score (full FASTA)	Allergenicity
Tyr p 24.0101	*Tyrophagus putrescentiae*	ACL36923	219815476	AO	45.6	2.30E-21	Sera from 5 of the 47 subjects displayed positive IgE responses to the recombinant troponin C (Jeong et al., 2010).
Tyr p 24.0101	*Tyrophagus putrescentiae*	ACL36923	219815476	SD	44.97	2.50E-26	
Bla g 6.0101	*Blattella germanica*	ABB89296	82704032	AO	42.9	5.70E-19	**Not food allergen** Troponin allergen with a calcium-dependent IgE reactivity that may be involved in muscle contraction (Hindley et al., 2006).
Bla g 6.0101	*Blattella germanica*	ABB89296	82704032	SD	42.28	1.90E-23	
Per a 6	*Periplaneta americana*	Q1M0Y3	60678791	AO	41.5	6.40E-19	**Not food allergen** Per a 6 allergen was predicted to have nine strongly binding nonamer core epitope sequences and 28 weakly binding sequences (Chen, Yang, Wei, & Tao, 2014).
Per a 6	*Periplaneta americana*	Q1M0Y3	60678791	SD	40.94	2.20E-23	
Bla g 6.0301	*Blattella germanica*	ABB89298	82704036	AO	42.1	1.40E-18	**Not food allergen** Troponin allergen with a calcium-dependent IgE reactivity that may be involved in muscle contraction (Hindley et al., 2006).
Bla g 6.0301	*Blattella germanica*	ABB89298	82704036	SD	42.95	5.30E-23	
Bla g 6.0201	*Blattella germanica*	ABB89297	82704034	AO	41.5	1.90E-18	**Not food allergen** Troponin allergen with a calcium-dependent IgE reactivity that may be involved in muscle contraction (Hindley et al., 2006).
Bla g 6.0201	*Blattella germanica*	ABB89297	82704034	SD	40.94	8.20E-23	
MLC-1	*Gallus gallus*		55584149	AO	46.6	2.60E-18	
Cra c 6.0101	*Crangon crangon*		238477333	AO	40.8	2.00E-17	**Food allergen** 6/25 (24%) of shrimp-allergic patients had IgE that reacted with Cra c 6 in IgE immunoblotting (Bauermeister et al., 2011).
Hom a 6.0101	*Homarus americanus*	P29291		SD	39.6	1.50E-22	**Food allergen** 6/25 (24%) of shrimp-allergic patients had IgE
							that reacted with Hom a 6 in IgE*
Pen m 6	*Penaeus monodon*	ADV17344	317383200	SD	39.6	8.50E-22	**Food allergen***
Jun o 4	*Juniperus oxycedrus*		5391446	AO	44.4	1.3E-16	**Not food allergen** Of 41 human sera from subjects allergic to Cupressaceae, 6 displayed IgE binding to run o 4 on immnublot (Tinghino et al., 1998)
Jun o 4	*Juniperus oxycedrus*	O64943	5391446	SD	42.28	1.30E-20	
Ole e 8	*Olea Europea*		6901654	AO	37	2.00E-15	**Not food allergen** The recombinant protein binds IgE antibodies from patients allergic to olive pollen (Ledesma, Villalba, & Rodríguez, 2000).
Ole e 8	*Olea europea*	AAF31151	6901654	SD	36.24	3.40E-19	
Amb a 10.0101	*Ambrosia artemisiifolia*	Q2KN25	AY894659**	SD	35.57	5.00E-19	**Not food allergen***
Cup a 4	*Cupressus arizonica*		261865475	AO	42.2	2.00E-15	Sera from 9.6% *Cupressus arizonica* allergic patients contain specific IgE antibodies against recombinant Cup a 4 (De Coaña et al., 2010)
Cup a 4.0101	*Cupressus arizonica*	ABP87672	145581052	SD	39.6	5.40E-19	

Database "AO" = AllergenOnline; "SD" = SDAP, *E* score $< 10^{-7}$ indicates significant homology for AllergenOnline. *E*-score $< 10^{-2}$ indicates significant homology for SDAP (Ivanciuc et al., 2003). Description of evidence for allergenicity is shown. Allergens annotated in both databases are highlighted in grey. In the allergenicity description all allergens that describe as 'not food allergen' or a food allergen, that information was taken from allergen.org site. In case of this information is not described this means that it was not described at allergen.org site. Asterisk = ALLERGEN NOMENCLATURE WHO/IUIS Allergen Nomenclature Sub-Committee – www.allergen.org. Two asterisks = No GI number. GenBank nucleotide number (NCBI).

Table 2
Estimation of the potential allergenicity of proteins extracted from *Ulva* sp. biomass with either only osmotic shock or PEF and mechanical press with osmotic shock (Achatz et al., 1995; Aki et al., 1994; An et al., 2013; Andersson et al., 2004; Crameri, 1998; Cui et al., 2016; De Vouge et al., 1998; Gruehn, Suphioglu, O'Hehir, & Volkmann, 2003; Miao & Gaynor, 1993; Postigo et al., 2011; Rihs, Chen, Rozynek, & Cremer, 2001; Shen et al., 1997; Wagner et al., 2001).

	Organism	Sequence Link in SwissProt/ NCBI/PIR	GI source	Database	Identity %	*E* score (full FASTA)	Allergenicity
Tyr p 28	*Tyrophagus putrescentiae*		1055365842	AO	73.2	2.60E-179	**Not food allergen** 8 of 17 dust mite allergic subjects with IgE binding to *E. coli* synthesized recombinant protein (Cui et al., 2016).
	Cladosporium herbarium		729764	AO	70.70	1.30E-168	**Not food allergen** Of 62 *C. herbarium* sensitized patients (positive immunoblot of C.herbarum extract), 22% showed IgE binding to Cla h 4 on immunoblot (Achatz et al., 1995).
Cla h 5	*Cladosporium herbarum*	P40918, P42039		SD	70.44	5.10E-170	
Der f 28	*Dermatophagoides farinae*		685432788	AO	69.50	7.40E-166	**Not food allergen** Serum IgE binding in 28 of 41house dust mites allergic subjects reacted to Der f 28. In Skin Prick Testing detected 7 of 10 (70%) dust mite allergic patients showed a positive reaction to Der f 28 (An et al., 2013).
Der f 28	*Dermatophagoides farinae*		442565876	AO	67.30	8.50E-136	
Pen c 19	*Penicillium citrinum*	Q92260.1	14423733	AO	74.70	1.30E-131	**Not food allergen**
Pen c 19	*Penicillium citrinum*	Q92260		SD	54.75	9.40E-133	Sera from 14 (41%) of 34 *Penicillium*- allergic patients showed IgE-binding to the recombinant Pen c 19 in dot immunoassay (Shen et al., 1997).
Mala s 10	*Malassezia sympodialis ATCC 42132*		465797105	AO	29.80	2.40E-46	**Not food allergen** 69% of 28 atopic dermatitis patients had IgE to rMala s 10 by ELISA. ~35% inhibition of IgE binding to recombinant heat shock protein when rMala s 10 (heat shock protein) was in a concentration of 0.25 mg/ml and ~86 inhibition in the concentration of 3mg/ml of rMala s 10 (Andersson et al., 2004).
Putative heat shock protein	*Malassezia sympodialis*		28564467	AO	29.60	9.20E-46	
Mala s 10	*Malassezia sympodialis*	CAD20981	28564467	SD	29.11	3.20E-46	
Alt a 3	*Alternaria alternata*		14423730	AO	62.70	2.10E-30	**Not food allergen** rAlt a 3 recognized on immunoblot by 5% of the sera from *Alternaria alternata* sensitive patients (De Vouge et al., 1998).
Alt a 3	*Alternaria alternata*	P78983		SD	15.54	7.10E-31	
Cor a 10	*Corylus avellana*	CAC14168		SD	59.43	3.60E-145	**Not food allergen** Five of seven allergic patient sera demonstrated IgE binding to the Cor a 10 in the purified protein fraction as well as in the crude hazel pollen extract (Gruehn, Suphioglu, O'Hehir, & Volkmann, 2003).

(*continued on next page*)

Table 2 (*continued*)

Der f mag29	*Dermatophagoides farinae*	BAA04556		SD	12.37	7.40E-26	**Not food allergen** A 9.8% binding frequency of IgE in mite-allergic sera (41 cases) to a blot of Mag29 (Aki et al., 1994).
Amb a 4.0101	*Ambrosia artemisiifolia*	CBK52317		SD	4.51	1.8e-03	
Superoxide dismutase	*Hevea brasiliensis*		348137	AO	37.30	7.80E-28	
Hev b 10.0101(Mia o & Gaynor, 1993)	*Hevea brasiliensis*	AAA16792	348137	SD	37.66	5.80E-28	
IgE-binding protein MnSOD	*Hevea brasiliensis*		10862818	AO	39.80	1.40E-27	**Not food allergen** rHev b 10 able to abrogate 93% of IgE binding to rHev b 10 at a concentration of 1 µg/ml, and almost 100% of binding at concentrations greater than 50 µg/ml (Wagner et al., 2001).
Hev b 10.0103	*Hevea brasiliensis*	CAC13961	10862818	SD	34.2	1.00E-27	
MnSOD	*Hevea brasiliensis*		5777414	AO	39.30	3.80E-27	**Not food allergen** Two sera (1.27 kU/l and 0.43 kU/l) from 20 SB patients tested showed specific IgE antibodies to recombinant latex MnSOD (Rihs, Chen, Rozynek, & Cremer, 2001).
Hev b 10.0102	*Hevea brasiliensis*	CAB53458	5777414	SD	33.77	2.80E-27	
Manganese superoxide dismutase-like protein	*Pistaci*		149786150	AO	35.30	1.90E-26	**Food allergen***
Pis v 4.0101	*Pistacia vera*	EF470980	149786150	SD	35.06	1.40E-26	
Manganese superoxide dismutase	*Aspergillus fumigatus*		1648970	AO	34.70	1.90E-25	**Not food allergen** Of 54 patients with allergic bronchopulmonary aspergillosis (ABPA), 30 (56%) showed IgE binding to r Asp f 6 in ELISA. Of 35 A.fumigatus-sensitized patients without ABPA, 0 (0%) showed IgE binding to rAsp f 6 (Crameri, 1998). 1 µg of Asp f 6 inhibit ~ 8% of the IgE and 100 µg of Asp f 6 inhibit ~ 35% of IgE (Wagner et al., 2001).
Asp f 6	*Aspergillus fumigatus*	Q92450, AAB60779	1648970	SD	31.6	7.20E-25	
Asp f 6	*Aspergillus fumigatus*		83305645	AO	36.70	1.40E-24	
Manganese superoxide dismutase	*Malassezia sympodialis*		28569698	AO	33.50	2.50E-24	**Not food allergen** 75% of 28 atopic dermatitis patients had IgE to rMala s 11 by ELISA (Andersson et al., 2004)
Mala s 11	*Malassezia sympodialis*	CAD68071	28569698	SD	31.17	1.90E-24	
Allergen	*Malassezia sympodialis ATCC 42132*		465795607	AO	34.20	7.30E-24	
Alt a 14.0101	*Alternaria*		529279957	AO	34.20	5.20E-20	**Not food allergen** 28 from 30 patients with the previous diagnosis of respiratory allergies caused by *Alternaria* showed positive IgE (Postigo et al., 2011)

Database "AO" = AllergenOnline; "SD" = SDAP, *E* score < 10^{-7} indicates significant homology for AllergenOnline. *E*-score < 10^{-2} indicates significant homology for SDAP (Ivanciuc et al., 2003). Description of evidence for allergenicity is shown. Allergens annotated in both databases are highlighted in grey. In the allergenicity description all allergens that describe as 'not food allergen' or a food allergen, that information was taken from allergen.org site. In case of this not mentions meaning that not described at allergen.org site. Asterisk = ALLERGEN NOMENCLATURE WHO/IUIS Allergen Nomenclature Sub-Committee – www.allergen.org. Two asterisks = No GI number. GenBank nucleotide number (NCBI). The table derived 'Amb a 4.0101' allergen with a black line, means all the allergens above (includes this allergen) are annotated to Heat shock protein 70 and all allergens below are annotated to superoxide dismutase. kU/L = measurement of total IgEs, > 0.35 kU/L were considered positive (Inc, 2012).

algae. After PEF treatment with osmotic shock and the mechanical press 39. 04 ± 1.19 mg was extracted out of 140 mg dry weight (DW) algae while without PEF only with osmotic shock and the mechanical press was extracted 22.5 ± 0.64 mg out of 140 mg dry weight (DW) algae.

3.3. In silico estimation of the potential allergenicity of proteins extracted from Ulva sp. biomass

Extracted proteins, after identification (Table S7), were annotated to allergens from two databases (Fig. 1): AllergenOnline database (allergenonline.org) and SDAP-Structural Database of Allergenic Proteins (fermi.utmb.edu) (Ivanciuc et al., 2003). *In silico* identified potential allergens are summarized in Tables 1, 2, and S8. The allergens in Tables 1 and 2 are with significant similarity to the proteins found in samples after specific treatment, more details on those proteins described in Tables S5 and S6. All allergens found in two databases (AllergenOnline and SDAP) after comparing the sequence of calmodulin found in samples after the osmotic shock and mechanical press or with thermochemical method displayed in Table 1. The allergens presented in Table 2 are identified after sequence comparison to two databases, the proteins superoxide dismutase (SOD) and heat shock protein (HSP) found in samples either after osmotic shock with the mechanical press and osmotic shock with mechanical press including PEF treatment or after thermochemical method. All allergens identified in all treatments presented in Table S8.

The potential allergens and proteins, which were extracted only with osmotic shock and mechanical press treatments are shown in Tables 1 and S5. The protein detected only after osmotic shock or thermochemical treatment is calmodulin, annotated to 13 allergens, most of which are described as troponin C. Troponin C – is a calcium-binding domain. Troponin, actin, and tropomyosin are proteins that compose striated muscle (skeletal and cardiac). Troponin is a complex of three proteins: troponin C, troponin I and troponin T. This complex is a calcium receptive protein at the calcium regulation of muscle contraction (Grabarek, Tao, & Gergely, 1992). Troponin C protein is a calcium binding protein which is one of the most important families of allergens (Radauer, Bublin, Wagner, Mari, & Breiteneder, 2008). It has a helix–loop–helix structural motif with four EF-hand motifs (Grabarek et al., 1992). Troponin C is a parvalbumin (Grabarek et al., 1992), which is the major allergens coming from fish, ubiquitous pollen allergens (Radauer et al., 2008), mold mite (Tyr p 24.0101) (Jeong et al., 2010) and cockroach (Bla g) (Hindley et al., 2006). Bla g is troponin C mite allergen that recently reported to be a calcium-dependent (Hindley et al., 2006). Previous studies showed that 10.6% from the study group observed IgE binding to *Tyrophagus putrescentiae* recombinant troponin C. After addition of $CaCl_2$, the sera from some patients showed strong IgE responses and the effect increased approximately two-fold (Jeong et al., 2010).

The potential allergens and proteins which were extracted with either with PEF and osmotic shock and mechanical press or osmotic shock and mechanical press without PEF treatments are shown in Tables 2 and S6. The two proteins annotated to allergens are 'Heat shock protein 70' and 'Iron-superoxide dismutase 1'. These proteins were annotated mainly to the allergens described as 'Heat shock protein' and 'Superoxide dismutase' respectively.

Heat shock proteins (HSP's) are a family of proteins produced in the cells as a response to stressful conditions. Hsp-70 is recognized by antigen presenting cells (APCs) and can activate these cells (Nishikawa, Takemoto, & Takakura, 2008). All the annotated Hsp-70 allergens (Table 2) coming from dust mite (Tyr p 28, Der f 28 and Der f mag29) (Aki et al., 1994; An et al., 2013; Cui et al., 2016) and fungi (Cla h IV, Pen c 19, Mala s 10 and Alt a 3) (Achatz et al., 1995; Andersson et al., 2004; De Vouge et al., 1998; Shen et al., 1997). Previous studies described a large range of allergenic effects from approximately 5% (De Vouge et al., 1998) to 70% (An et al., 2013) (Table 2). For instance: the results of Skin Prick Test on dust mite allergic patients were that 7 of 10 (70%) showed a positive reaction to Der f 28 (An et al., 2013). The allergen.org site labels all the allergens that annotated to Hsp in Table 2 (and Table S6), as not food allergens.

Superoxide dismutase (SOD) is an enzyme that converts ion of superoxide (O_2^-) and hydrogen into peroxide (H_2O_2). This function is a defense mechanism against highly reactive oxygen spices at the cell. SODs are divided by its metal molecule in the active site Cu, Zn, Fe or Mn (Candas & Li, 2014).

The SOD type annotated in the current study is MnSOD (Table 2), which is a mitochondrial antioxidant encoded by genomic DNA and it's gene upregulated by oxidative stress (Candas & Li, 2014). Previous studies show that SOD activity increased by a salinity stress in *Ulva fasciata* (Lu, Sung, & Lee, 2006). MnSOD was described as an allergen in *Aspergillus fumigatus* (Asp f 6) with cross-reactivity with the human MnSOD (Crameri, 1998). The allergen sources of all the annotated MnSOD allergens (Table 2) coming from the rubber tree (Hev b) (Wagner et al., 2001), pistachio (Pis v 4.0101 from allergen.org) and fungi (Asp f 6 and Mala s 11) (Andersson et al., 2004; Crameri, 1998; Wagner et al., 2001). Previous studies describe that recombinant Mala s 10 (HSP) and Mala s 11 (MnSOD), could play role in atopic eczema/dermatitis syndrome (AEDS) (Andersson et al., 2004), both allergens were annotated at the study protein in either osmotic shock or PEF and mechanical press samples (Table 2).

The potential proteins and allergens which were extracted with thermochemical method appear in Tables S7 and S8. With thermochemical extraction, we successfully identified 98 proteins, which included 13 identified proteins extracted with PEF. Based on the correlation between the two databases for allergen identification, 13 proteins (extracted thermochemically) were identified with allergic potency, while only four of them are potential food allergens (Table S8, the details about calmodulin allergenicity displayed in Tables 1 and S5). In addition to previously found potential food allergens in PEF and osmotic shock extracts, thermochemically extracted proteins contained fructose-bisphosphate aldolase and thioredoxin. Those were annotated to allergens, aldolase A, and thioredoxin h, respectively (Table S8).

Aldolase A – known as fructose-bisphosphate aldolase – is a glycolytic enzyme that catalyzes the reversible conversion of fructose – 1,6 bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. Previous studies showed that 50% of the patients with a clinical history of reaction to fish extract were found with IgE to aldolase A (Kuehn et al., 2013). The authors mentioned the importance of IgE to aldolase when IgE to parvalbumin (Kuehn et al., 2013).

The thioredoxin, a small redox protein, plays a role in many biological processes such as redox signaling. In human, this protein is a protein involving in indirect reactive oxygen species (ROS)-mediated response (Adler, Yin, Tew, & Ronai, 1999). This protein is annotated to three thioredoxin allergens Plo i 2.0101, Tri a 25.0101 and Mala s 13. Only Tri a 25 considered as a food allergen (Weichel, Glaser, Ballmer-Weber, Schmid-Grendelmeier, & Crameri, 2006). Tri a 25 is a sequence encoding to wheat thioredoxin. In a previous study, a recombinant protein was created for immunological studies (Weichel et al., 2006). Sera of bakers with occupational asthma for IgE-binding structures were tested. The recombinant protein cause for sensitization rate of 47% among bakers. Tri a 25 is sharing 74% identity to Zea m 25 a maize allergen which previously exhibited distinct IgE cross-reactivity (Weichel et al., 2006).

The information provided in this study is the first sign of the potential existence of allergens in the proteins extracted from *Ulva* sp. Our study also shows that the extraction method affects the extraction of potentially allergenic proteins. These results are intriguing, as they suggest that a method for protein extraction with fewer allergens could be developed if the mass transport of allergens from the seaweed tissue to the solvent is understood. Future studies should provide more detailed information about the identified proteins as allergenic if are active or not. This understanding is critical before *Ulva* sp. derived proteins could be considered as a protein source for humans.

4. Conclusions

Macroalgae *Ulva* is a promising protein source. However, to be one of the sustainable alternative proteins it should have an optimal extraction process and most importantly, to be safe for a human consumption. One of the main risk assessments for human protein consumption is the allergenicity. Here we report an optimization of a water-soluble extraction method by using combinations of an osmotic shock, a mechanical press and an electroporation with PEF. The highest extraction yield of 53.8 ± 0.69 $\mu g\,ml^{-1}$ was obtained with 50 pulses with 2.3 μs duration, applied at 26 kV, 7.26 $kV\,cm^{-1}$ field strength. The final temperature after extraction with these parameters was 26.9 ± 0.4 °C. The energy investment was 72.29 $kJ\,kg^{-1}$ FW or 1.5 ± 0.5 $kJ\ mg_{extracted\_protein}^{-1}$. The proteins that were released by using PEF, without PEF or thermochemical method for protein extraction were identified. The identified proteins sequences were annotated to allergens. A PEF treatment selectively avoids releasing of calmodulin protein compared to the control without PEF. This protein annotated to allergen type troponin C, which is a calcium-binding protein and one of the most important families of allergens, includes the food allergens. From the proteins that were released none selectively; with PEF treatment but also without treatment, two proteins were detected: 'Heat shock protein 70' (HSP) and Superoxide dismutase (SOD). Only SOD was annotated to food allergens. In the proteomic analysis of the proteins extracted with a thermochemical method, four potential food allergens were detected. These included SOD, calmodulin fructose-bisphosphate aldolase and thioredoxin, annotated to SOD (Hev b), troponin C, aldolase A and thioredoxin h (Tri a 25), respectively. To the best of our knowledge, the first evidence for macroalgae proteins to be a potential cause an allergic reaction done *in silico*. Nevertheless, more research on this topic should be conducted to get more practical information about the human immune system allergic reaction to the proteins extracted from macroalgae.

5. Author's contributions

MP quantified the protein content and did bioinformatics work on proteins identification and allergy estimation, and drafted the paper. FF, MS, FF, and GM did the PEF extraction experiments. AG conceived the study, analyzed the data and drafted the paper. All authors read and approved the paper.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The fellowship to MP for this study was partially funded by the Israel Ministry of Science and Technology and space. AG and MP acknowledge Israel Ministry of Economy – 'Kamin' program and 'Rieger' foundation for the support of the study. All authors thank the members of Smoler Proteomics Center Faculty of Biology especially to Keren Bendalak from the Technion, for her help with the LC-MS/MS analysis.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2018.09.134.

References

Achatz, G., Oberkofler, H., Lechenauer, E., Simon, B., Unger, A., Kandler, D., ... Breitenbach, M. (1995). Molecular cloning of major and minor allergens of *Alternaria alternata* and *Cladosporium herbarum*. *Molecular Immunology, 32*(3), 213–227.

Adler, V., Yin, Z., Tew, K. D., & Ronai, Z. (1999). Role of redox potential and reactive oxygen species in stress signaling. *Oncogene, 18*(45), 6104.

Aki, T., Fujikawa, A., Wada, T., Jyo, T., Shigeta, S., Murooka, Y., ... Ono, K. (1994). Cloning and expression of cDNA coding for a new allergen from the house dust mite, *Dermatophagoides farinae*: Homology with human heat shock cognate proteins in the heat shock protein 70 family. *The Journal of Biochemistry, 115*(3), 435–440.

Alston, J. M., Beddow, J. M., & Pardey, P. G. (2009). Agricultural research, productivity, and food prices in the long run. *Science, 325*(5945), 1209–1210.

An, S., Chen, L., Long, C., Liu, X., Xu, X., Lu, X., ... Lai, R. (2013). *Dermatophagoides farinae* allergens diversity identification by proteomics. *Molecular & Cellular Proteomics, 12*(7), 1818–1828.

Andersson, A., Rasool, O., Schmidt, M., Kodzius, R., Flückiger, S., Zargari, A., ... Scheynius, A. (2004). Cloning, expression and characterization of two new IgE-binding proteins from the yeast *Malassezia sympodialis* with sequence similarities to heat shock proteins and manganese superoxide dismutase. *The FEBS Journal, 271*(10), 1885–1894.

Barba, F. J., Grimi, N., & Vorobiev, E. (2015). New approaches for the use of non-conventional cell disruption technologies to extract potential food additives and nutraceuticals from microalgae. *Food Engineering Reviews, 7*(1), 45–62.

Barbarino, E., & Lourenço, S. O. (2005). An evaluation of methods for extraction and quantification of protein from marine macro-and microalgae. *Journal of Applied Phycology, 17*(5), 447–460.

Bauermeister, K., Wangorsch, A., Garoffo, L. P., Reuter, A., Conti, A., Taylor, S. L., ... Vieths, S. (2011). Generation of a comprehensive panel of crustacean allergens from the North Sea Shrimp *Crangon crangon*. *Molecular Immunology, 48*(15), 1983–1992.

Becker, E. W. (2007). Micro-algae as a source of protein. *Biotechnology Advances, 25*(2), 207–210.

Bernstein, I. L., & Safferman, R. (1973). Clinical sensitivity to green algae demonstrated by nasal challenge and in vitro tests of immediate hypersensitivity. *Journal of Allergy and Clinical Immunology, 51*(1), 22–28.

Bikker, P., van Krimpen, M. M., van Wikselaar, P., Houweling-Tan, B., Scaccia, N., van Hal, J. W., ... Lopez-Contreras, A. M. (2016). Biorefinery of the green seaweed *Ulva lactuca* to produce animal feed, chemicals and biofuels. *Journal of Applied Phycology, 28*(6), 3511–3525.

Bleakley, S., & Hayes, M. (2017). Algal proteins: Extraction, application, and challenges concerning production. *Foods, 6*(5), 33.

Bluhm, H., & Sack, M. (2009). Industrial-scale treatment of biological tissues with pulsed electric fields. *Electrotechnologies for extraction from food plants and biomaterials* (pp. 237–269). Springer.

Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. *Food Research International, 43*(2), 414–431.

Bruhn, A., Dahl, J., Nielsen, H. B., Nikolaisen, L., Rasmussen, M. B., Markager, S., ... Jensen, P. D. (2011). Bioenergy potential of Ulva lactuca: Biomass yield, methane production and combustion. *Bioresource Technology, 102*(3), 2595–2604.

Candas, D., & Li, J. J. (2014). MnSOD in oxidative stress response-potential regulation via mitochondrial protein influx. *Antioxidants & Redox Signaling, 20*(10), 1599–1617.

Chen, H., Yang, H., Wei, J., & Tao, A. (2014). In silico prediction of the T-cell and IgE-binding epitopes of Per a 6 and Bla g 6 allergens in cockroaches. *Molecular Medicine Reports, 10*(4), 2130–2136.

Crameri, R. (1998). Recombinant *Aspergillus fumigatus* allergens: From the nucleotide sequences to clinical applications. *International Archives of Allergy and Immunology, 115*(2), 99–114.

Cui, Y., Yu, L., Teng, F., Zhang, C., Wang, N., Yang, L., & Zhou, Y. (2016). Transcriptomic/proteomic identification of allergens in the mite *Tyrophagus putrescentiae*. *Allergy, 71*(11), 1635–1639.

De Coaña, Y. P., Parody, N., Fuertes, M.Á., Carnés, J., Roncarolo, D., Ariano, R., ... Alonso, C. (2010). Molecular cloning and characterization of Cup a 4, a new allergen from *Cupressus arizonica*. *Biochemical and Biophysical Research Communications, 401*(3), 451–457.

De Vouge, M. W., Thaker, A. J., Zhang, L., Muradia, G., Rode, H., & Vijay, H. M. (1998). Molecular cloning of IgE–binding fragments of *Alternaria alternata* allergens. *International Archives of Allergy and Immunology, 116*(4), 261–268.

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

Fleurence, J. (2004). Seaweed proteins. *Proteins in Food Processing,* 197–213.

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.

Godfray, H. C. J. J. R., Beddington, I. R., Crute, L., Haddad, D., Lawrence, J. F., & Muir, C. (2010). Food security: The challenge of feeding 9 billion people. *Science (New York, N.Y.), 327*(5967), 812–818.

Grabarek, Z., Tao, T., & Gergely, J. (1992). Molecular mechanism of troponin-C function. *Journal of Muscle Research & Cell Motility, 13*(4), 383–393.

Gruehn, S., Suphioglu, C., O'Hehir, R. E., & Volkmann, D. (2003). Molecular cloning and characterization of hazel pollen protein (70 kD) as a luminal binding protein (BiP): A novel cross-reactive plant allergen. *International Archives of Allergy and Immunology, 131*(2), 91–100.

Hindley, J., Wünschmann, S., Satinover, S. M., Woodfolk, J. A., Chew, F. T., Chapman, M. D., & Pomés, A. (2006). Bla g 6: A troponin C allergen from *Blattella germanica* with IgE binding calcium dependence. *Journal of Allergy and Clinical Immunology, 117*(6), 1389–1395.

Inc, T. F. S. (2012). ImmunoCAP Specific IgE. Retrieved November 23, 2017, from http://www.phadia.com/en-GB/5/Products/ImmunoCAP-Assays/1/.

Ivanciuc, O., Schein, C. H., & Braun, W. (2003). SDAP: Database and computational tools for allergenic proteins. *Nucleic Acids Research, 31*(1), 359–362.

Jeong, K. Y., Kim, C., Un, S., Yi, M., Lee, I.-Y., Park, J. W., ... Yong, T.-S. (2010). Allergenicity of recombinant troponin C from *Tyrophagus putrescentiae*. *International*

Archives of Allergy and Immunology, 151(3), 207–213.
Kuehn, A., Hilger, C., Lehners-Weber, C., Codreanu-Morel, F., Morisset, M., Metz-Favre, C., ... Hentges, F. (2013). Identification of enolases and aldolases as important fish allergens in cod, salmon and tuna: Component resolved diagnosis using parvalbumin and the new allergens. *Clinical and Experimental Allergy: Journal of the British Society for Allergy and Clinical Immunology, 43*(7), 811–822.
Ledesma, A., Villalba, M., & Rodríguez, R. (2000). Cloning, expression and characterization of a novel four EF-hand Ca2+-binding protein from olive pollen with allergenic activity. *FEBS Letters, 466*(1), 192–196.
Levitan, S., Sher, N., Brekhman, V., Ziv, T., Lubzens, E., & Lotan, T. (2015). The making of an embryo in a basal metazoan: Proteomic analysis in the sea anemone *Nematostella vectensis*. *Proteomics, 15*(23–24), 4096–4104.
Lu, I.-F., Sung, M.-S., & Lee, T.-M. (2006). Salinity stress and hydrogen peroxide regulation of antioxidant defense system in *Ulva fasciata*. *Marine Biology, 150*(1), 1–15.
Miao, Z., & Gaynor, J. J. (1993). Molecular cloning, characterization and expression of Mn-superoxide dismutase from the rubber tree (Hevea brasiliensis). *Plant Molecular Biology, 23*(2), 267–277.
Nishikawa, M., Takemoto, S., & Takakura, Y. (2008). Heat shock protein derivatives for delivery of antigens to antigen presenting cells. *International Journal of Pharmaceutics, 354*(1), 23–27.
Parniakov, O., Apicella, E., Koubaa, M., Barba, F. J., Grimi, N., Lebovka, N., ... Vorobiev, E. (2015). Ultrasound-assisted green solvent extraction of high-added value compounds from microalgae *Nannochloropsis* spp. *Bioresource Technology, 198*, 262–267.
Parniakov, O., Barba, F. J., Grimi, N., Marchal, L., Jubeau, S., Lebovka, N., & Vorobiev, E. (2015). Pulsed electric field and pH assisted selective extraction of intracellular components from microalgae *Nannochloropsis*. *Algal Research, 8*, 128–134.
Polikovsky, M., Fernand, F., Sack, M., Frey, W., Müller, G., & Golberg, A. (2016). Towards marine biorefineries: Selective proteins extractions from marine macroalgae *Ulva* with pulsed electric fields. *Innovative Food Science & Emerging Technologies, 37*, 194–200.
Postigo, I., Gutiérrez-Rodríguez, A., Fernández, J., Guisantes, J. A., Suñén, E., & Martínez, J. (2011). Diagnostic value of Alt a 1, fungal enolase and manganese-dependent superoxide dismutase in the component-resolved diagnosis of allergy to pleosporaceae. *Clinical & Experimental Allergy, 41*(3), 443–451.
Radauer, C., Bublin, M., Wagner, S., Mari, A., & Breiteneder, H. (2008). Allergens are distributed into few protein families and possess a restricted number of biochemical functions. *Journal of Allergy and Clinical Immunology, 121*(4), 847–852.
Rihs, H., Chen, Z., Rozynek, P., & Cremer, R. (2001). Allergenicity of rHev b 10 (manganese-superoxide dismutase). *Allergy, 56*(1), 85–86.
RStudio Team (2015). RStudio: Integrated development for R. RStudio, Inc., Boston, MA, from http://www.rstudio.com.
Shen, H., Au, L., Lin, W., Liaw, S., Tsai, J., & Han, S. (1997). Molecular cloning and expression of a *Penicillium citrinum* allergen with sequence homology and antigenic crossreactivity to a hsp 70 human heat shock protein. *Clinical & Experimental Allergy, 27*(6), 682–690.
Stice, C. (2014). WhooPea: Plant Sources Are Changing the Protein Landscape. Retrieved from https://www.giiresearch.com/report/lux322725-whoopea-plant-sources-are-changing-protein.html.
Stice, C., & Basu, A. (2015). The great protein shift: Re-thinking one of the fundamental building blocks of the human diet. Retrieved May 7, 2015, from http://quarterly.luxresearchinc.com/quarterly/?p=139.
Subasinghe, R., Soto, D., & Jia, J. (2009). Global aquaculture and its role in sustainable development. *Reviews in Aquaculture, 1*(1), 2–9.
Thomas, K., Herouet-Guicheney, C., Ladics, G., Bannon, G., Cockburn, A., Crevel, R., ... Vieths, S. (2007). Evaluating the effect of food processing on the potential human allergenicity of novel proteins: International workshop report. *Food and Chemical Toxicology, 45*(7), 1116–1122.
Tilman, D. (1999). Global environmental impacts of agricultural expansion: The need for sustainable and efficient practices. *Proceedings of the National Academy of Sciences, 96*(11), 5995–6000.
Tinghino, R., Barletta, B., Palumbo, S., Afferni, C., Iacovacci, P., Mari, A., ... Pini, C. (1998). Molecular characterization of a cross-reactive *Juniperus oxycedrus* pollen allergen, Jun o 2: A novel calcium-binding allergen. *Journal of Allergy and Clinical Immunology, 101*(6), 772–777.
Van Krimpen, M. M., Bikker, P., Van der Meer, I. M., Van der Peet-Schwering, C. M. C., & Vereijken, J. M. (2013). *Cultivation, processing and nutritional aspects for pigs and poultry of European protein sources as alternatives for imported soybean products.* Wageningen UR Livestock Research.
Wagner, S., Sowka, S., Mayer, C., Crameri, R., Focke, M., Kurup, V. P., ... Breiteneder, H. (2001). Identification of a *Hevea brasiliensis* latex manganese superoxide dismutase (Hev b 10) as a cross-reactive allergen. *International Archives of Allergy and Immunology, 125*(2), 120–127.
Weichel, M., Glaser, A. G., Ballmer-Weber, B. K., Schmid-Grendelmeier, P., & Crameri, R. (2006). Wheat and maize thioredoxins: A novel cross-reactive cereal allergen family related to baker's asthma. *Journal of Allergy and Clinical Immunology, 117*(3), 676–681.
Wu, L., Zhao, W., Yang, R., & Chen, X. (2014). Effects of pulsed electric fields processing on stability of egg white proteins. *Journal of Food Engineering, 139*, 13–18.