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Enrichment processes for the production of high-protein feed from the green seaweed *Ulva ohnoi*

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ABSTRACT

New sources of protein are required to supplement current animal- and plant-protein. Here, we quantify the quality and yield of four protein-enriched biomass products (PEB-I to PEB-IV) and a protein isolate (PI) from the commercially produced seaweed *Ulva ohnoi*. To decrease the content of components of the biomass that may be undesirable in feed, we have developed a multi-step biorefinery process to produce salt, sulfated polysaccharides (ulvan), and protein products. The content of protein increased from 22.2 ± 0.4% dry weight (dw) in unprocessed biomass to between 39.5 ± 1.9% in the PEB-IV and 45.5 ± 0.8% in the PI. The quality (mol % of essential amino acids [EAA]) of the protein products was similar to soybean meal, with 41.6 ± 0.1 and 43.4 ± 0.1 mol% EAA in PEB-I and the PI, respectively. The yield of PEB products varied from 16.3 ± 0.8 to 41.0 ± 0.8% of the unprocessed biomass, with PEB-I > PEB-II = PEB-III > PEB-IV. The yield of all PEB products was more than four-fold greater than the PI (4.4%). Conservatively, the biomass productivity of *U. ohnoi* is 70 t dw ha^{-1} year^{-1} resulting in a projected annual production (t dw ha^{-1} year^{-1}) of 24 t of salt, 4.3 t of ulvan, 29 t of PEB-I, or 3.2 t of PI using this biorefinery process. With nine-fold higher yield, and a protein product of similar quality to the PI, we recommend producing PEB-I by concentrating the protein through the extraction of salt and ulvan over the extraction of a PI for the development of food and feed products.

1. Introduction

Alternative and innovative sources of protein are required to supplement and replace both animal- and plant-protein in food and feed products. The global demand for meat from livestock (excluding fish) is projected to increase from approximately 313 million tonnes pa in 2014–2016 [1] to 455 million tonnes pa by 2050 [2], along with a projected increase in the global demand for plant protein (i.e., grains, legumes, and forage) for the production of livestock feed [3]. Notably, 85% of the world production of soybean is already being used for this purpose [4]. Seaweed is commonly promoted as a novel source of protein due to its typically high quality of protein, expressed as proportions of individual amino acids. However, the overall content of protein in seaweed as a percent of the whole biomass is typically low, compared with traditional protein sources such as soybean meal (45–49%) and fish meal (55–71%) [3], with a mean of 11.6% (range 2.98–26.8%) [5]. This is too low for whole, unprocessed seaweed to be suitable as a protein source in compound feed for monogastric livestock [6], with swine requiring feed containing 15–29% protein [3], higher trophic fish such as barramundi (*Lates calcarifer*) and Atlantic salmon (*Salmo salar*) requiring feed containing 42–55% protein [7,8], and lower trophic fish such as tilapia (*Oreochromis* spp.) and channel catfish (*Ictalurus punctatus*) requiring feed containing 30–40% protein [9]. Additionally, antinutritional compounds such as polysaccharides and phenolic compounds in seaweed can limit the digestibility of algal protein in the gut, particularly in carnivorous fish, further reducing the biologically available protein in the whole seaweed biomass [10,11]. Therefore, to develop seaweed as a protein source, it will be necessary to concentrate the protein, targeting a final product concentration of > 40%, while reducing the concentration of interfering compounds. There are two ways to do this, selectively extracting and isolating the protein fraction, or concentrating the protein by extracting non-protein components [12–14].

The green seaweed *Ulva ohnoi* (GenBank Accession number KF195501, strain JCU 1, [15]) is commercially cultured at a land-based aquaculture facility near Ayr, Queensland, Australia, with integrated production on farm primarily for bioremediation services. The productivity and biomass composition of *U. ohnoi* are well characterised

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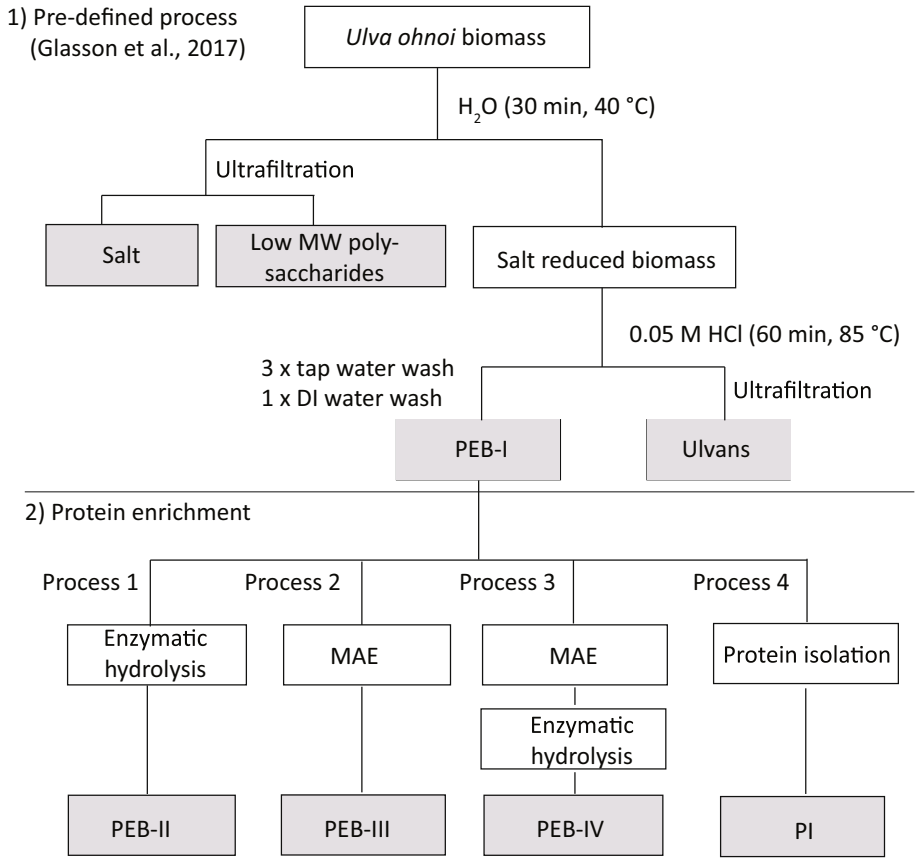

Fig. 1. Flow chart of biorefinery process options 1–4 using enzymatic hydrolysis (process 1), microwave-assisted hydrolysis (MAE; process 2), a combination of these methods (process 3), or protein isolation (process 4) to generate high-concentration protein products. Shaded boxes represent potential or alternative product streams. MW, Molecular weight; DI, deionized water (milli-Q); PEB, protein-enriched biomass; PI, protein isolate.

under multiple growth conditions [15–20] and, with the supply of the biomass at a commercial scale, *U. ohnoi* is an ideal resource for the development of innovative algal products. In addition, *Ulva ohnoi* has previously been used as a model species to develop seaweed-specific protocols for the isolation or concentration of protein [14]. That study recommended protein isolation over protein concentration, with higher concentrations of protein in the isolate (230–260% higher than untreated biomass) compared with the concentrate (30–40% higher than untreated biomass). However, those protocols did not specifically target the removal of the soluble polysaccharides, the insoluble polysaccharides, or the lipid fractions to maximise enrichment of protein in the residual biomass, and yields of the protein isolate were < 5% of the untreated biomass, leaving scope for improvements. Moreover, since the water soluble sulfated polysaccharides (ulvans) present in species of *Ulva* are a high-value product in themselves, with unique gelling, bioactive, and functional properties [21,22], seaweeds from this genus are particularly suitable for the implementation of a cascading biorefinery with the step-wise fractionation and recovery of biomass components. In this process, a trade-off is expected between the yield and value of sequential product streams and the final concentration of protein in the protein product. The first two steps of this type of cascading biorefinery have been described previously [17,20], with the overall objective of producing 1) a seaweed salt; 2) high purity ulvans; and 3) a concentrated protein product. To date, this sequential production of a seaweed salt and high purity ulvan, has resulted in only a two-fold increase in the concentrated protein product (from 15 to 20% to 31–33%) [17,20]. However, this falls short of the target of > 40% protein in the final protein product for similarity to e.g. soybean meal. Notably, the extraction process of [20] is not optimised for the maximum removal of ulvans. At the low pH where ulvans are extracted, intractable polysaccharides (hemicellulosic glucuronans and xyloglycans) are insoluble and remain in the biomass, as does the insoluble fibre fraction (primarily cellulose), which together comprise up to 20% of the biomass of *Ulva* [16,23,24]. Further hydrolysis and extraction of these intractable polysaccharides has been demonstrated through digestion with enzymes [25–28], or by using water at high temperature and pressure during microwave assisted extraction (MAE) [23,29].

Therefore, in this study we will assess the quality and yield of protein products from the green seaweed *U. ohnoi* obtained at steps in a biorefinery process targeting the removal of non-protein components for separate product streams, and compare these to a protein isolate (PI). The final protein products are the protein-enriched biomass (PEB) obtained after removal of salt and ulvans (PEB-I), and the PEB after taking PEB-I through either enzymatic hydrolysis (PEB-II), microwave assisted extraction (MAE) (PEB-III), or a combination of MAE followed by enzymatic hydrolysis (PEB-IV) for the removal of insoluble carbohydrates. The initial steps of the removal of salt and ulvans using warm water and 0.05 M HCl at 85 °C, respectively, are pretreatment steps prior to enzymatic hydrolysis. An additional MAE step will also be used as further pretreatment for carbohydrate hydrolysis. The aim of this study is to define the process for the delivery of a high-quality, high-protein product from *U. ohnoi* for food and feed products, and to lay the foundation for a viable cascading biorefinery process for the production of high-value and commodity bio-products (salt, functional polysaccharides, and protein) from species of *Ulva*.

2. Materials and methods

2.1. Biomass

Biomass of *Ulva ohnoi* (Gen-bank accession number KF195501, strain JCU 1 [15]) was harvested from Pacific Reef Fisheries (19°29′S, 147°28′E; Ayr, Queensland, Australia), a land-based aquaculture facility where it is cultivated commercially. Collection of the biomass took place over multiple seven-day production cycles in October and November 2016. Harvested biomass was transported, chilled, to James

Cook University (Townsville, Queensland, Australia) where it was centrifuged (MW512; Fisher & Paykel Appliances Australia, Cleveland, Australia) to constant fresh weight (fw), split into 100 g fw portions, and stored in separate snap-lock bags at −20 °C until further processing. Sub-samples (50 g fw) of each harvest were dried (60 °C for 24 h) to determine the fw to dry weight (fw:dw) ratio and composition of the starting material as described in Section 2.4. Harvest-specific fw:dw ratios were used to calculate product yields as g of product per g dw biomass.

2.2. Protein-enriched biomass I

The PEB was produced by extracting salts and ulvan as described previously [17,20] (Fig. 1). Briefly, biomass (100 g fw portions) was submerged in 1 L of pre-heated milli-Q water (biomass:water ratio 1:10, 40 °C, 30 min) in 2 L beakers in temperature-controlled water baths (Grant JB Nova Unstirred Water Bath; LabGear Australia, Brisbane, Australia) to extract ash (salt) and low molecular weight polysaccharides from the biomass. Low molecular weight polysaccharides, from a known volume of the wash water, were recovered and purified by ultrafiltration and diafiltration with 5 volumes of milli-Q water (ÄKTA flux 6 fitted with a Xampler 10 kDa NMWC cartridge; GE Healthcare Australia, Parramatta, Australia), and subsequently freeze-dried [20]. The remaining wash water was evaporated to constant dw in an oven at 60 °C to quantify the amount of salt extracted. The remaining extracted biomass was heated in 1 L of 0.05 M hydrochloric acid (85 °C for 1 h) and stirred intermittently to selectively extract ulvans. The ulvan product in the aqueous phase was recovered by ultrafiltration and diafiltration as described above. The salt- and ulvan-extracted biomass was rinsed in 1.8 L of tap water three times, then rinsed in 1 L of milli-Q water once. The rinsed biomass was recovered by filtering over a 125-μm mesh and represents PEB-I. This process was repeated using 100 g fw portions of biomass from a single harvest to provide sufficient PEB-I for process development trials for protein enrichment to PEB-II and PEB-III as described in Section 2.3. Subsamples (n = 3) of intact starting biomass were freeze-dried for quantification of the content of nitrogen (% N), and the PEB-I biomass was freeze-dried in preparation for protein enrichment trials.

2.3. Protein-enriched biomass II - IV

The PEB-I was further enriched by solubilising any ulvans and insoluble carbohydrates that were intractable using three processes; 1) enzymatic hydrolysis of the remaining carbohydrates of PEB-I to produce PEB-II; 2) microwave-assisted extraction (MAE) of PEB-I to produce PEB-III; and 3) MAE of PEB-I, followed by enzymatic hydrolysis to produce PEB-IV (Fig. 2). Method development for the enzymatic hydrolysis (process 1) was performed by comparing the use of citrate buffer (50 mM, pH 5) or milli-Q water, with intact or milled (milled to 1 mm) PEB-I biomass in a factorial design (n = 3; Fig. 2). Enzymatic hydrolysis was performed in triplicate by suspending 2 g of PEB-I in 140 mL of either citrate buffer or milli-Q water (solid:liquid ratio of 1:70 w/v) and subjecting the suspensions to enzymatic hydrolysis with a commercial cellulase mixture (0.3 mL g^{-1} dw of PEB-I) (Viscozyme® L, Sigma Aldrich, Castle Hill, Australia) at 50 °C for 24 h with continuous stirring at 80 rpm (New Brunswick Scientific Innova® 44R incubator shaker; Eppendorf South Pacific, North Ryde, Australia) [27]. The solids were recovered and washed with one volume of milli-Q water before being freeze-dried, and this solid represents the PEB-II. Negative controls (biomass in citrate buffer, no enzyme added) and blanks (enzyme in citrate buffer, no biomass added) were also included. The concentration of solubilised carbohydrates in the hydrolysates was measured (see Section 2.4) to test the activity of the enzyme mixture and to account for carbohydrates originating from the enzyme mixture. The final method for enzymatic hydrolysis to produce PEB-II was chosen based on the highest content of N (% dw) quantified (see Section 2.4) in the PEB products.

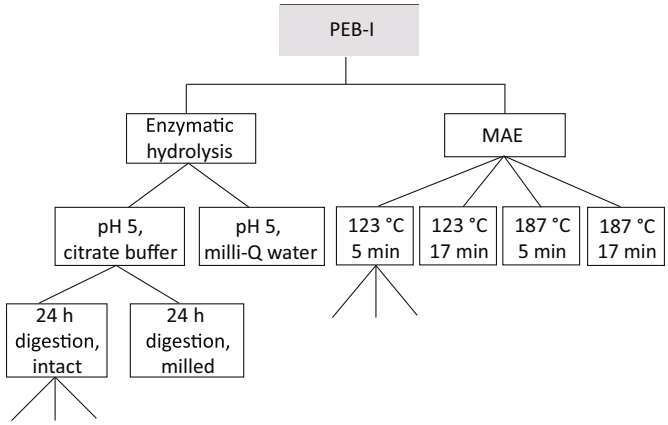

Fig. 2. Method development strategy to choose processing conditions for enzymatic hydrolysis (process 1) and microwave-assisted extraction (MAE; process 2). Experiments were performed on homogenised PEB-I from one harvest with procedural replication (enzymatic hydrolysis, n = 3; microwave, n = 4, technical replicates).

Method development for MAE (process 2) was performed by comparing extractions at 123 °C or 187 °C for 5 or 17 min (Fig. 2) at a biomass:water ratio of 1:34 w/v (n = 4). These settings were selected based on range finding experiments covering 101–209 °C and 1–21 min (results not shown). Biomass:water ratios of 1:10–1:34 were also tested during the range finding experiments and had no effect on the digestion efficiency, therefore the largest amount of water was chosen for ease of handling. Processing was performed with a focused microwave system (Speedwave Type SW-4; Berghof, Eningen, Germany) using 500.0 ± 0.1 mg dw of biomass (milled to 1 mm) samples immersed in milli-Q water in TFM™ PTFE microwave vessels. Samples were heated to the target temperatures within a 2-min ramp, held under autogenous pressure for the desired time, and then cooled to room temperature before depressurisation. The solid residue was recovered following rinsing twice with 10 mL of distilled water with centrifugation (3000 ×*g*, 25 °C, 20 min) between rinses. The supernatant was discarded while the solid residue was freeze-dried, and this dried material represents PEB-III. The method for MAE to produce PEB-III was chosen based on the highest content of N (% dw) quantified (see Section 2.4) in the PEB products.

Following method development, the effectiveness of the protein-enrichment processes and protein isolation were formally compared, with each process repeated in triplicate using biomass from separate harvests (n = 3, biological replicates). For process 3, PEB-I was first subjected to the selected method for MAE as a pre-treatment, followed by the selected method for enzymatic hydrolysis, and the dried material represents PEB-IV. For process 4, PEB-I biomass was immersed in 0.1 M NaOH in a liquid to biomass ratio of 20:1 v/w for 2 h at 30 °C under stirring at 800 rpm to extract the protein following the method described by Angell et al. [14]. Following centrifugation to pelletise and discard the solids, the supernatant was collected and the extracted protein was precipitated by acidification to pH 2.25 using 0.2 M HCl [14]. The precipitated protein was separated by centrifugation (3200 ×*g*, 4 °C, 30 min), the supernatant was discarded, and the pellet was freeze-dried. This pellet represents the PI.

2.4. Characterisation of unprocessed biomass and products

While quantifying and characterising the protein-enriched products were the main objectives of this research, the remaining product streams (salt, ulvan, and solubilised sugars in enzyme and microwave treatments aqueous phases) resulting from this cascading biorefinery model were also quantified to define the process as a whole. These

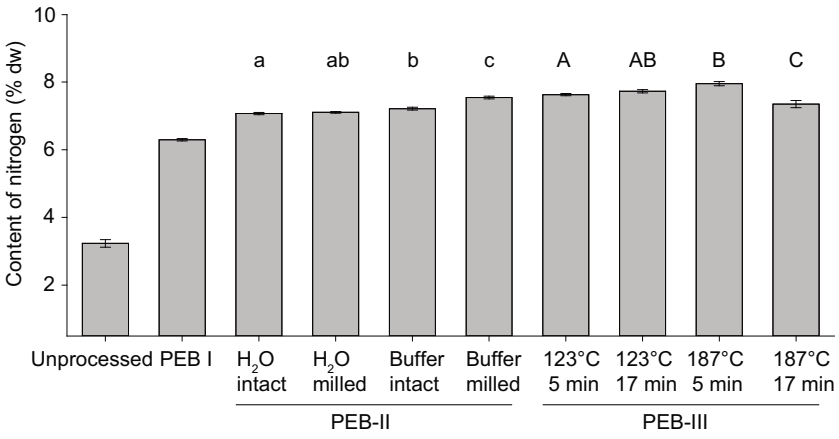

Fig. 3. Content of nitrogen (% dw) in unprocessed and processed biomass (PEB-I, PEB-II, and PEB-III) during method development. Letters above bars indicate significant groupings (Tukey's post hoc, $\alpha = 0.05$) within each process (PEB-II, lower case; PEB-III, upper case). Data presented as mean ± SE, n = 3 (technical replicates).

analyses were done on products resulting from the final processes only, not for those produced during method development. Yields were expressed as the percentage of the original unprocessed dry biomass (% yield), while composition was expressed as a percentage of product dry weight (% dw). Mineral composition (23 elements) was analysed as described in [17] in the unprocessed biomass, the salt, and all PEB products. The unprocessed biomass and all PEB products were characterised based on triplicate analyses (n = 3, from separate harvests) unless otherwise stated. Elemental analysis of % N was performed using GC-TCD after combustion in pure oxygen and was outsourced to OEA labs (http://www.oealabs.com, Callington, UK), and was performed on unprocessed biomass, washed biomass, PEB products, and the PI, as well as on PEB products produced during method development. Amino acids were analysed in the unprocessed biomass, all PEB products, and the PI at the Australian Proteome Analysis Facility, Macquarie University, Sydney, as described in [18]. The sum of the content of individual amino acids was then used as the measure of the content of protein, while the content of total essential amino acid was taken as the sum of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine. Cysteine is a minor components of the amino acids of *U. ohnoi* (0.36% of amino acids) [18] and was not quantified. The content of ash (inorganic salts) was quantified in the unprocessed biomass, the washed biomass, PEB products, and the PI by combustion in air at 550 °C for 6 h (SEM muffle furnace, LabTek, Brisbane, Australia). The content of moisture was quantified with a moisture analyser (MS-70; A&D, Toshima, Japan). The content of lipid was quantified gravimetrically following extraction using dichloromethane/methanol (2:1 v/v) as described in [30]. Fatty acids were extracted and quantified by GC–MS (7890 GC/5975C EI-MS, equipped with a DB-23 capillary column with a cyanopropyl stationary phase [60 m × 0.25 mm id × 0.15 μm]; Agilent Technologies Australia, Mulgrave, Australia) as described in [30]. The content of carbohydrate was calculated as 100 − Σ (ash + protein + lipid + moisture). The content of solubilised sugars (carbohydrates) was quantified in hydrolysate liquids using the phenol-sulfuric acid method with glucose as a standard [31].

2.5. Statistical analyses

Two-factor analysis of variance (ANOVA) was used to compare the effects of solvent (citrate buffer or water) and biomass form (intact or milled) (fixed factors) on the content of N (%) in PEB-II, and the effects of time and temperature (fixed factors) on the content of N (%) in PEB-III during method development. One-way ANOVA was used to compare the effects of the selected enzyme, MAE, and combined processes on the yield and composition (% of protein, ash, lipid, and carbohydrate) of PEB products. Data were log-transformed to improve homogeneity of variances if Levene's test for homogeneity of variances was significant. When ANOVA resulted in a significant difference in means ($\alpha = 0.05$), Tukey's HSD post-hoc tests were used to compare the means of the treatment groups. When there were significant interactions, the variance component (% variance explained, η^2) was calculated to interpret the relative importance of the significant terms in the model. All analyses were performed using Statistica Academic v. 13.3 (Tibco Software Inc.). The patterns in the quality of amino acids (average mol %) of unprocessed biomass, PEB products, and the PI were visualised using non-metric multidimensional scaling (nMDS; Primer 6.1.13). The Bray-Curtis similarity coefficient was used as a distance measure on square-root transformed data. In the same way, the quantity of specific amino acids as % dw in the algal samples were compared with the amino acid profiles of a typical soybean meal [32] and two types of white fish meal [33]. The mean of the content of amino acids from soybean meal samples collected over three collection times from seven soybean maturity zones was used as a representative number [32]. As cysteine was not analysed in the algal products in this study, the literature value of 0.36% of amino acids [18] was used for this comparison for unprocessed biomass, with the assumption of the retention or extraction of cysteine in the PEB products and PI being proportional to the quantified amino acids. All data is presented as mean ± standard error (SE).

3. Results and discussion

3.1. Method optimisation

The content of nitrogen (% N) increased from 3.2% in unprocessed biomass to 6.3% in PEB-I (salt-reduced and ulvan-extracted biomass; 94.7% increase; Fig. 3). The content of nitrogen increased by a further 12.3–19.8% in PEB-II (enzyme-digested biomass) compared with PEB-I, and there was an interactive effect of solvent (buffer vs. milli-Q) and biomass state (milled vs. intact) (ANOVA, $F_{1,11} = 26.3$, $p < 0.05$) on the content of nitrogen (% N) in PEB-II, with 57.6% of the variance explained by solvent, where the buffer was more effective for both intact and milled biomass, but the magnitude of change was significantly higher for milled biomass. Milled biomass hydrolysed in buffer yielded the highest content of nitrogen in PEB-II (7.5%) (Fig. 3), and therefore, this treatment was chosen for Process 1 for the formal comparison of processes. After accounting for carbohydrates originating from the enzyme cocktail (1.10 ± 0.03 mg glucose equivalents mL^{-1} in enzyme blanks), the addition of enzyme to biomass in buffer led to a 3-fold increase in solubilised carbohydrates in the hydrolysate compared to no addition of enzyme. The content of nitrogen increased by a further 16.7–26.4% in PEB-III (microwave-digested biomass) compared with PEB-I, and there was an interactive effect of treatment temperature and time (ANOVA, $F_{1,16} = 36.59$, $p < 0.05$), with 54.0% of the variance explained by the interaction, with shorter processing times being more effective than longer processing times at higher temperatures, but not

lower temperatures. Processing at 187 °C for 5 min yielded the highest content of nitrogen in PEB-III (7.95%) (Fig. 3), and therefore, this treatment was chosen for process 2 for the formal comparison of processes. Consequently, process 3 constituted microwave hydrolysis at 187 °C for 5 min (biomass: water ratio 1:34) followed by enzyme hydrolysis of milled biomass in citrate buffer to produce PEB-IV.

3.2. Biorefinery process

3.2.1. Salt

The yield of salt from the water wash was approximately 34% dw of the unprocessed biomass (Table 1), which was higher than previous results (23%) for the same species at identical conditions [17]. The amount of extractable salts could vary depending on the surface structure of the biomass, as the blades can be more or less 'crinkly' in appearance, with the crinkly blades potentially retaining more surface water during centrifugation. The content of ash (determined by combustion in air at 550 °C) in the unprocessed biomass here was approximately 22%, compared with 25% in [17], suggesting that more intracellular material, including minerals and low molecular weight ulvan, were extracted here. The composition of the salt also differed from previous studies using the same method [17], where the salt produced here had a lower Na:K ratio of 0.7 ± 0.1 (see Supplementary Table S1 for the detailed composition) compared with 2.6 in [17]. The main cations were similar to previous work, with Na, K, Mg, Ca, P, and S making up > 98% of the analysed elements, although proportions differed slightly, including a higher content of S in the current study. This difference may be ascribed to both the extraction of more intracellular components here (i.e. sulfated ulvans), and to the different cultivation conditions between the two studies, with the biomass used for the current work collected from a working aquaculture farm in 80 KL raceways using aquaculture waste water, while that of the earlier study was cultivated under controlled conditions with the addition of a comprehensive algal culture nutrient medium (MAF; Manutech Pty Ltd), containing both micro- and macro-minerals. The low Na:K ratio of 0.7 is noteworthy, as this is well within the range of the optimum intake ratio for humans [34–36], again highlighting the potential of the *Ulva* seaweed salt as a replacement for regular table salt (pure NaCl) in baked and processed goods as previously recommended by [17]. This concept of making a product from the mineral fraction of species of *Ulva* has been adapted to produce a mineral-rich liquid extract (referred to as MRLE or sap) by macerating fresh, unrinsed biomass of *U. fasciata* or *U. lactuca* in water and then separating the solid and liquid phases before continuing to further process the solid biomass residue in biorefinery models [37,38]. This sap constituted 26% and 14% of the starting material (dw), respectively, and is proposed as a liquid fertiliser similar to sap made from crushing *Kappaphycus alvarezii* and *Gracilaria edulis* [39]. Although no maceration is used here, a small fraction of low molecular weight ulvan polysaccharide is co-extracted with the salt, and made up 0.43 ± 0.001% of the dried salt product. Although the low MW ulvans in the salt wash are presented as a potential product stream in Fig. 1, the yields are too low to be a practical product to target for separation. However, ulvans elicit plant immune responses ([40], reviewed in [41]), and the effect of the salt wash product including the low MW ulvans on plant growth will be tested in future work.

3.2.2. Ulvan

The yield of ulvan from the 85 °C extraction was 6.1 ± 0.6% (Table 1), similar to that previously reported for this species using the same extraction conditions (8.2 ± 1.1%) [20], with biomass for the two studies collected from the same commercial growth system, six months apart. Extraction conditions impact both the yield and purity of the ulvan extract [20,41], hampering comparisons across the literature. While the yield achieved here is in the lower range, compared with reported yields of 2–27% [41], this extraction method results in a high purity extract [20].

Table 1
Yield of protein (as % of unprocessed biomass) and projected annual production of seaweed bioproducts based on biomass productivity of 70 t dw ha^{-1} $year^{-1}$ for *U. ohnoi*. Potential production is compared with published protein extraction yields (protein isolates, PI) from species of *Ulva* and land-based perennial grasses or grass/legume mixes, and is benchmarked against soybean meal. Data is presented as mean ± SE, n = 3 (biological replicates).

Sample ID	Yield (% of unprocessed biomass)	Potential production (t dw ha^{-1} $year^{-1}$)	Reference
Unprocessed biomass (% dw)	22.2 ± 0.4	70	This study
Salt	33.8 ± 1.0	23.7 ± 0.7	This study
Ulvan	6.1 ± 0.6	4.3 ± 0.4	This study
PEB-I	41.0 ± 0.8	28.7 ± 0.6	This study
PEB-II	23.6 ± 0.8	16.5 ± 0.5	This study
PEB-III	23.9 ± 0.9	16.7 ± 0.7	This study
PEB-IV	16.3 ± 0.8	11.4 ± 0.5	This study
PI	4.4 ± 0.3	3.2 ± 0.2	This study
Ulva lactuca PI	3–6	2.1–4.1	[49]
Ulva rigida PI	1.0–3.0	0.7–2.1	[50]
Ulva rotunda PI	1.4–3.6	1.0–2.5	[50]
Festulolium braunii PI	15–21	1.4–1.6	[52]
Festuca arundinacea PI	15–23	1.3–1.4	[52]
Grass/legume mix #36 PI	14–28	0.8–0.9	[52]
Soybean meal	45–53	1.0–1.1	[53,54]

3.2.3. Protein-enriched biomass and protein isolate

All PEB products and the PI had a significantly higher content (% dw) of nitrogen (ANOVA, $F_{5,12} = 62.3$, $p < 0.01$) and protein (sum of amino acids, % dw) ($F_{5,12} = 215.01$, $p < 0.01$) compared with the unprocessed starting material (Fig. 4). When comparing each PEB product to the previous extraction step (i.e., unprocessed biomass to PEB-I or PEB-I to PEB-II), PEB-I had the highest proportional increase in the content of nitrogen (81.3 ± 1.6% increase) compared with each of the other extraction steps (Processes 1–4; Fig. 4). Subsequently, the protein enrichment processes from 1 to 4 had smaller increases in the content of nitrogen, with the highest increase in PEB-III (process 3, 11.4 ± 1.4% increase), and the lowest increase in the PI step (process 4, 2.1 ± 0.5% increase). PEB-III had the highest content of nitrogen (9.3 ± 0.1% dw), which was double that of the starting material. The content of protein in PEB-I also had the highest proportional increase (87.2 ± 2.1%) compared with the preceding extraction step. This was followed by process 4, which resulted in a further increase in the content of protein of 9.8 ± 1.3%, while PEB-III and IV (from processes 2 and 3, respectively) had similar contents of protein compared with PEB-I (Fig. 4b). The PI had the highest content of protein at 45.5 ± 0.8% (Fig. 4b). The amino acid:nitrogen ratios (K_p) for each treatment were 4.79 (unprocessed biomass), 4.94 (PEB-I), 4.73 (PEB-II), 4.29 (PEB-III), 4.33 (PEB-IV), and 5.32 (PI), with lower amounts of non-protein N in PEB-I and the PI compared with unprocessed biomass, and higher amounts in PEB-III and PEB-IV. The K_p of the unprocessed biomass fell within previously reported values (90% of the data was between 4.42 and 5.83) for this species under experimental conditions, where the flux of nitrogen was manipulated [18], and was also close to the median value of green seaweeds in general (4.68) [5].

In addition to changes in the quantity of protein, the quality of the protein (as molar ratios of individual amino acids) was also affected by the protein-enrichment and PI processes (Fig. 5a). The PI had the highest proportion of essential amino acids (43.4 ± 0.1 mol% compared with 39.7 ± 0.1 mol% in unprocessed biomass, and 41.6 ± 0.1 in PEB-II; supplementary Table S2), and was distinct from the other treatments in the nMDS plot. PEB-III and PEB-IV were similar in their composition of amino acids, and had the highest molar proportions of valine, phenylalanine, and isoleucine (see supplementary Table S2 for

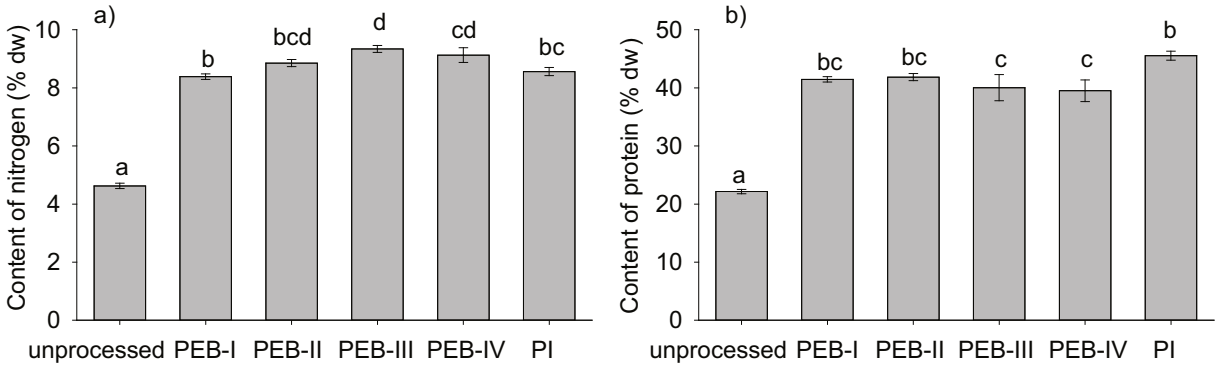

Fig. 4. Content (% dw) of nitrogen (a) and protein (sum of amino acids) (b) in unprocessed and processed biomass (corrected for content of moisture). Common letters above bars represent non-significant groupings (Tukey's HSD, $\alpha > 0.05$). Data presented as mean ± SE, n = 3 (biological replicates).

full amino acid profiles). The PEB products, and in particular the PI, were all similar to soybean meal in terms of content of amino acids (averaged data extracted from [32]) and white fish meal made from heads and viscera, or heads alone, of cod [33] (Fig. 5b). Notably, the unprocessed biomass of *U. ohnoi* was distinct from the other treatments with a much lower content of all amino acids (Fig. 5b). Compared with soybean meal, the PI had higher content of Met (1.0 vs. 0.8% dw), but a lower estimated content of Cys (0.2 vs. 0.9% dw), two amino acids that are typically limiting in plant-based proteins. Importantly, since 66% of the protein in the formulated feed used in the Norwegian salmon industry is plant-based and mainly sourced from soy [8], the similar contents of amino acids in soybean meal (45–53%, [3,32]) and the algal products here (39.5–45.5%) suggest that the PEB products and PI may be suitable as a partial fish meal replacement in some feed formulations, especially in herbivorous species where the content of carbohydrate (fibre) pose less of an issue.

The content of ash was significantly reduced in all PEB products and the PI (1.7 ± 0.3–5.0 ± 0.1% dw) compared with unprocessed biomass (22.3 ± 0.5% dw; ANOVA, $F_{5,12} = 1207.4$, $p < 0.01$; Fig. 6). This reduction was the result of the water wash prior to the extraction of ulvan, as evident from the yield of salt (Section 3.2.1), and there were only minor changes with each consecutive extraction step in the biorefinery process. While the proportions of cations varied between treatments, sulfur remained dominant, making up between 39.7 ± 1.3% (unprocessed biomass) and 64.7 ± 1.6% (PEB-III) of the analysed minerals (Supplementary Table S3). The sulfur, calcium, potassium, magnesium, and sodium combined, represented between 79.4 ± 3.1–96.0 ± 0.4% of the analysed minerals. Excess dietary sulfur can cause cerebrocortical necrosis in ruminants due to the production of hydrogen sulfide during fermentation within the rumen, and a dietary limit of 3.5 g S kg^{-1} in the feed is established for cattle, while a limit of 4 g S kg^{-1} in the feed is established for swine and poultry to limit osmotic diarrhea [42]. Considering these limitations, inclusion of the unprocessed biomass should be limited to < 7.5% in cattle or 8.6% in swine and poultry, while PEB-I could be included at 21.4% and 24.5% of feed, respectively. More sulfur was removed in PEB-III and PEB-IV, and these could be included at 49.0 and 52.2% in cattle, and 56.0 and 59.6% in swine and poultry, respectively, without exceeding the limits for sulfur. Similar considerations need to take place for any other elements that have specified feed intake limits.

Protein-enrichment processes significantly decreased the proportion of carbohydrates (ANOVA, $F_{5,12} = 45.48$, $p < 0.0.1$) from 44.8% in the unprocessed biomass to a low of 40.8% in PEB-III and PEB-IV, with the PI having a higher proportion of carbohydrates at 50.9% (not corrected for moisture) (Fig. 6). Alkali-soluble glucuronans and xyloglycans were likely co-extracted and then co-precipitated with the protein in the protein isolation step, resulting in a high proportion of carbohydrates remaining in the PI. This could be mitigated by using chromatography, or treatment with more targeted enzymes such as glucuronidase followed by ultrafiltration or dialysis, as further clean-up steps prior to precipitation or spray-drying of the protein isolates at scale. Although there were significant reductions in the content of carbohydrates, these were typically < 10% and none of processes 1–4 were effective at removing the more intractable carbohydrates from the

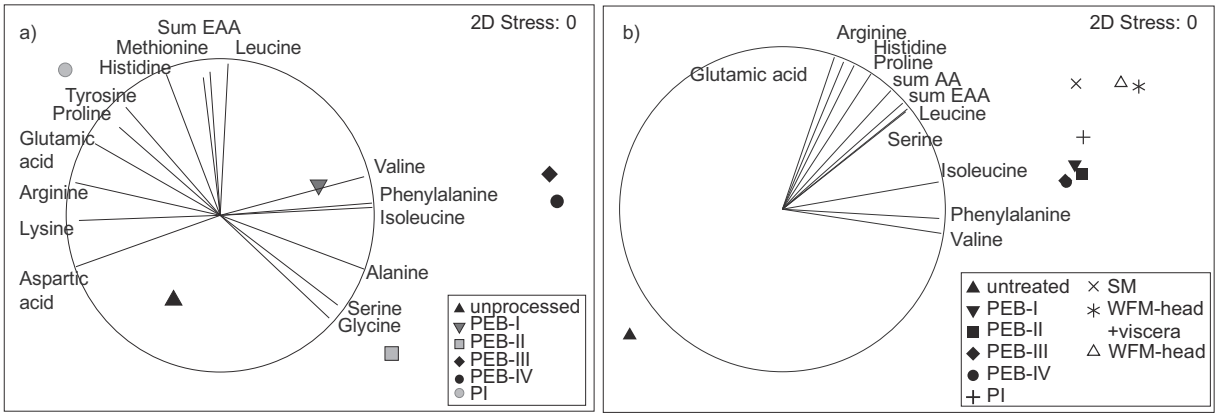

Fig. 5. The nMDS scaling ordinations of the a) quality of amino acids in *U. ohnoi* as average mol % (n = 3 biological replicates) in untreated biomass, protein-enriched biomass (PEB-I, PEB-II, PEB-III, and PEB-IV), and protein isolate (PI), and b) quantity of amino acids as % dw in unprocessed biomass, protein-enriched biomass, and protein isolate, compared with an average soybean meal (SM) [32], and white fish meal (WFM) made from fish heads and viscera, or heads alone [33]. Lines represent vector loadings of the specific amino acids (Pearson's correlation, a) R > 0.8, b) R > 0.9), with the size and direction of each vector representing the relative abundance of that amino acid in that region of the plot. EAA, essential amino acids; AA, amino acids.

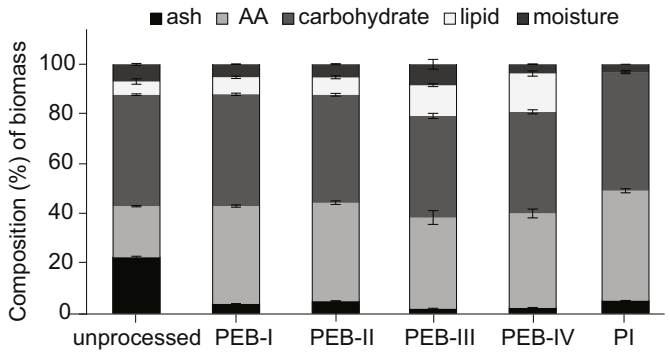

Fig. 6. Proximate composition (% dw) of untreated and protein-enriched biomass (PEB-I, PEB-II, PEB-III, and PEB-IV), and protein isolate (PI). Data presented as mean ± SE, n = 3 (biological replicates).

PEB products. During the industrial saccharification of lignocellulosic biomass (i.e., energy crops, forestry residues, corn stover, and bagasse), a pretreatment step of acid, alkali, or hydrothermal processing is used to solubilise the hemicellulose and lignin that hold the cellulose fibrils (bundles of cellulose strains) together, to render lignocellulosic feedstock more accessible for enzymatic hydrolysis [43,44]. While pretreatments often result in increased yields of hydrolysed sugars of enzyme-treated seaweeds [45–47], the different carbohydrate structure (lack of lignin) of seaweed biomass compared to terrestrial biomass suggests that, in some cases, pretreatment can be bypassed completely [28,48]. One aim of this study was to determine whether the warm acid extraction step (80 °C) of the ulvan, with or without a microwave treatment, would be a sufficient pretreatment to facilitate enzymatic hydrolysis. However, this was not the case and harsher conditions would be required for the pre-treatment of *U. ohnoi*. A previous biorefinery process designed around *U. lactuca* used a pre-treatment of 150 °C for 10 min before enzymatic digestion for 24 h at 50 °C, which released approximately 60% of the carbohydrates as monosaccharides and yielded a protein-enriched residue with a concentration of 40% protein and 16% ash [27]. Based on in vitro models of digestion, this protein-enriched residue was suggested as a potential feed for swine with a 90% organic matter digestibility, and a potential feed for cattle, with a rumen fermentation similar to alfalfa [27]. Here, after the freshwater rinse and acid extraction of ulvan polysaccharides (approximately 14% of total polysaccharides in the unprocessed biomass), PEB-I had a concentration of protein of 39.3% (not corrected for moisture content), and only 3.7% ash (minerals). Based on the comparative quality of PEB-I here and the protein-enriched residue of [27], it may not be necessary to further manipulate the composition of the *U. ohnoi* biomass beyond the extraction of salt and ulvan to make it suitable as a feed for monogastric, and potentially ruminant livestock, noting the limitations for inclusion rates based on the content of sulfur and consideration of other elements.

The proportion of lipid was significantly higher in PEB-III and PEB-IV (12.3 ± 0.5 and 15.4 ± 1.0%, respectively) compared with the unprocessed biomass (5.3 ± 1.1%), PEB-I (6.8 ± 0.5%), and PEB-II (7.0 ± 0.6%), while no lipids were extracted into the PI (ANOVA, $F_{5,12} = 87.33$, $p < 0.01$; Fig. 6). The content of fatty acids in unprocessed biomass and PEB products followed the same pattern as the total lipids, while the composition of fatty acids (as % of the sum of all fatty acids) differed between PEB products (Table S4), where lower proportions of polyunsaturated fatty acids (PUFAs) were present in PEB-III and PEB-IV. This was expected due to the oxidation of double bonds during the high temperature treatment of the microwave. Although the content of fatty acids in the unprocessed biomass was similar to a previous study [16], the composition differed, and the main fatty acids here were palmitic (C16:0), oleic (C18:1), palmitoleic (C16:1), behenic (C22:0), and alphalinolenic (C18:3) acids, which together made up 90–94% of the fatty acids in all PEB products and the unprocessed biomass, with no C18:4 detected. Unsaturated fatty acids made up 28–32% of the fatty acids (Table S4), which is also substantially lower than the approximately 60% previously reported for species of *Ulva* [16,30,37]. Previous biorefinery models using species of *Ulva* have removed the lipid fraction as a separate product [37], and while the fatty acid profile of the current harvests of *U. ohnoi* were more saturated than previously reported for *U. ohnoi*, it is preferable to retain the lipids in the protein-enriched biomass to maintain a source of fatty acids in the feed and to minimise processing, since the yield of fatty acids is typically < 3% of the dry biomass [16,30,37].

3.3. Yield and productivity

The yield of PEB products varied from 16.3 ± 0.8 to 41.0 ± 0.8% of the unprocessed biomass with PEB-I > PEB-II = PEB-III > PEB-IV (Table 1). The lower yield of PI (4.4 ± 0.3%), was similar to that reported for *U. ohnoi* using alkaline extraction (4.1%) [14], but without the biorefinery steps of removing salt and ulvans. Similar yields of protein isolates from other species of *Ulva* have also been reported using both mechanical and chemical extraction methods. Mechanical blending, osmotic shock, enzymatic digestion, or the use of pulsed electric fields for cell disruption to release water soluble proteins from *U. lactuca* yielded between 3 and 6% PI (as g PI produced per 100 g biomass) [49], while osmotic shock alone or followed by alkaline extraction yielded 1.0–1.4 and 3.0–3.6% PI from *U. rigida* and *U. rotundata*, respectively [50].

Based on production data collected over two years from Pacific Reef Fisheries, where the seaweed for this study was collected, the biomass productivity of *U. ohnoi* is conservatively 70 t dw ha^{-1} $year^{-1}$, which equates to 20 g dw m^{-2} day^{-1} for 350 days of production. This biomass productivity, combined with the yield of the PEB products (as % of unprocessed biomass), results in a potential annual production of 24 t dw ha^{-1} $year^{-1}$ of salt, 4.3 t dw ha^{-1} $year^{-1}$ of ulvan, and between 11 (PEB-IV) and 29 (PEB-I) t dw ha^{-1} $year^{-1}$ of PEB products, or 3.2 t dw ha^{-1} $year^{-1}$ of PI (Table 1). Similarly, this biomass productivity can be used in conjunction with previously reported yields of PI from *U. lactuca* [49] and from *U. rigida* and *U. rotundata* [50], to estimate the potential annual production of PI, which is then 0.7–4.1 t dw ha^{-1} $year^{-1}$ (Table 1). Since biomass productivities vary with geographical region and climate, a comparative estimate of potential annual productivities of protein products from *Ulva* cultivated in land-based systems in temperate regions can be calculated by multiplying the values in Table 1 by a factor of 0.64, which assumes a biomass production potential of 45 t dw ha^{-1} $year^{-1}$ following [51], and identical yields of PEB products and PI. Perennial grasses also have the potential for use as innovative protein feedstock under a biorefinery model, where the soluble fraction of protein is recovered to provide feed for monogastric livestock, while the residual fibrous biomass can be used as feed for ruminants or further processed [52]. The maximum estimated productivity of true protein of the grasses *Festulolium braunii* and *Fastuca arundincea*, and a grass legume mix, was between 0.8 and 1.6 t dw ha^{-1} $year^{-1}$, similar to PIs from species of *Ulva*.

Given that the majority of the soybeans produced globally are used for feed, the productivity of soybean protein is a useful comparison for bench-marking purposes. The average annual productivity of soybeans is approximately 2.6 ton ha^{-1} by [53], with a protein content of 45–53% dw [54]. Assuming that all soybeans are used for the production of protein rich soybean meal, and that there is a 100% yield of protein into the meal (as % of protein in unprocessed soybeans), this results in a potential productivity of approximately 1.1 t dw ha^{-1} $year^{-1}$ (Table 1), only a quarter of the potential productivity of the PI from *Ulva*. It is worth highlighting that if the PI from *U. ohnoi* was further purified to increase the concentration of amino acids for specific feeds, even a decrease in yield (and therefore projected productivity) by a further 50% would still result in a higher

potential productivity than soybean meal. However, as the yield and, therefore, the annual productivity of PEB-I from *U. ohnoi* is nine-fold greater than that of the PI, with a similar content and quality of protein, it is recommended that a biorefinery process consisting of the removal of salt and ulvan to concentrate the protein in the residual biomass is used over the extraction of a PI for the production of feed products.

4. Conclusion

The minimised process of extracting salt and ulvan produces a high yield of protein-enriched biomass (as PEB-I) with a content of protein of over 40% dw and 44 mol% of essential amino acids. This two-step biorefinery enrichment process is recommended over more complex enrichment processes, which lack significant further increases in the concentration of protein, and over the protein isolation process, which has a product yield nine times lower and of similar quality. Importantly, this study establishes PEB-I as a suitable product for testing as a protein source in animal feed models (i.e., ruminant, swine, poultry, and aquaculture animals). While the land-based production of seaweed, particularly of *Ulva*, is an emerging industry mainly associated with the bioremediation of nutrient-rich effluents from aquaculture or used as a feed input in the production of sea urchins and abalone [55,56], the development of biorefinery processes such as this one, with defined products of salt, ulvan (polysaccharides), and protein-enriched biomass, provides improved avenues for product development.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.algal.2019.101555.

Declaration of Competing Interest

No conflicts, informed consent, human or animal rights applicable.

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