A comparison of protocols for isolating and concentrating protein from the green seaweed *Ulva ohnoi*

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Abstract We compared protocols to isolate and concentrate protein from the green seaweed *Ulva ohnoi*. We quantified the effect of three factors on protein and essential amino acid yields and concentrations in protein isolates and residuals in a factorial experimental design. The three factors were starting material (as dry and milled or fresh and pulped), aqueous solvent-to-biomass ratio (20:1 or 5:1 *v/w*) and the incubation time in the aqueous solvent (incubated for 16 h at 30 °C or incubated for <1 min at ambient temperature). The protein isolation protocols increased the concentration of protein, total essential amino acids, methionine and lysine ~3 to 5-fold compared to whole *U. ohnoi* and were considerably more effective than the different protein concentrating combinations, which only increased protein and amino acid concentrations by 30–40 % in the residual biomass. The use of fresh and pulped biomass as the starting material, an incubation time of <1 min at ambient temperature and a low aqueous solution volume resulted in the highest protein isolate yield of 22 % of the protein found in seaweed. This study demonstrated that proteins from *U. ohnoi* were most effectively isolated by adopting protocols for terrestrial leaves compared to the protocols employed for seed crops as traditionally applied to seaweeds.

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Introduction

Seaweeds (marine macroalgae) have potential as a novel protein crop to reduce the pressure on traditional agricultural resources because they have high productivities (Bolton et al. 2009; Mata et al. 2010; Nielsen et al. 2012; Mata et al. 2016), and their culture does not require arable land or fresh water. The protein in seaweeds contains essential amino acids at proportions comparable to traditional protein sources, such as soybean meal and fishmeal (Angell et al. 2016). However, the essential amino acid content of seaweeds on a whole weight basis is low, resulting in a comparatively low "quantitative" protein resource even though it is a high "qualitative" protein resource. As an example, methionine, as a proportion of total amino acids, is approximately 50 % higher in seaweeds than in soybean meal, yet the concentration of methionine (concentration per unit weight) is more than three times lower in seaweeds (Angell et al. 2016). Consequently, seaweeds are only used in the livestock feed industry for their functional benefits based on a high mineral and fibre content (Dierick et al. 2009; Katayama et al. 2011; Evans and Critchley 2014). Utilising seaweeds as a protein resource in compound feeds of mono-gastric livestock will therefore require the processing of biomass to deliver a more concentrated form of this high-quality protein. Concentrating the protein content of plant material has traditionally been achieved directly by extracting and isolating the protein, or indirectly by extracting non-protein components to increase the protein content in the residual biomass (Berk 1992; Ju et al. 2001; Agboola et al. 2005; Tan et al. 2011).

In contrast, the isolation and concentration of protein from seaweeds are relatively unexplored and have focused on extraction protocols used for the dried and milled seed crops of soybean (Berk 1992), rice (Ju et al. 2001; Agboola et al. 2005) and canola (Tan et al. 2011). Yields of up to 48 % of total protein have been obtained for seaweeds using combined solvent (aqueous + alkaline) extractions (Fleurence et al. 1995; Wong and Cheung 2001a, b; Kandasamy et al. 2012; Kumar et al. 2014) of dried biomass. However, these yields are comparatively low because the efficiencies of extraction and isolation of protein from seaweeds are hindered by neutral polysaccharides and phenolic compounds that interact with the proteins and limit their solubility (Jordan and Vilter 1991; Fleurence et al. 1995; Wong and Cheung 2001b; Harnedy and FitzGerald 2011). Notably, seed crops and seaweeds are physiologically and biochemically distinct, with seeds having relatively low concentrations of insoluble polysaccharides and most of their protein is in the form of storage proteins. In contrast, leaves and seaweeds are physiologically and biochemically similar with high concentrations of insoluble polysaccharides and a diverse range of physiological proteins, many of which are associated with photosynthesis, including the enzyme RuBisCO, which represents up to 65 % of the total soluble protein in leaves (Ellis 1979; Spreitzer and Salvucci 2002). This suggests that protein extraction and isolation protocols for leaves should be suitable for seaweeds, but have not yet been examined. In these protocols, a mechanical protein extraction is applied to fresh biomass (Sinclair 2009; Bals and Dale 2011; Chiesa and Gnansounou 2011), making it an attractive protocol for the extraction and isolation of protein in fresh seaweeds.

An alternative process to improve the concentration of protein in seaweeds is the extraction of non-protein components. Indirectly enhancing the protein concentration through the extraction of non-protein components is a relatively simple process commonly employed for terrestrial crops (Berk 1992). In seaweeds, ash (external and internal salts and minerals) and soluble carbohydrates are the major non-protein material that could be relatively straightforward to remove. Ash represents between 20 and 50 % of the dry weight of seaweed (McDermid and Stuercke 2003; McDermid et al. 2007) and can be removed using freshwater rinsing and soaking (Neveux et al. 2014; Magnusson et al. 2016). Soluble carbohydrates represent between 6.5 and 38 % of dry weight of seaweeds (Maciel et al. 2008; Kraan 2012; Barros et al. 2013) and can be extracted using aqueous extraction at room temperature (Kolender and Matulewicz 2002; Maciel et al. 2008; Alves et al. 2013) and high temperatures (Yamamoto 1980; Barros et al. 2013), enzymatic digestion (BobinDubigeon et al. 1997; Melo et al. 2002) and acidic- (fucans) and alkaline-soluble extractions (Ray 2006).

Consequently, understanding the partitioning of protein and non-protein components in all fractions through multiple-step extraction processes is needed to develop the most suitable pathways for the concentration of proteins from seaweeds. Therefore, this study aims to quantify key procedural factors to optimise the isolation (through the extraction of the protein component) or concentration (through the extraction of the non-protein component) of protein for the commercially produced green seaweed *Ulva ohnoi*. To do this, three key factors that differ between seed extraction protocols and leaf extraction protocols were simultaneously examined for their efficacy in the isolation and concentration of protein using a factorial design.

Materials and methods

Sample preparation

The green seaweed *Ulva ohnoi* M. Hiroka and S. Shimada (Lawton et al. 2013) (GenBank accession numbers KFI195501 and KFI95536) was collected from commercial cultures at the MBD Energy aquaculture facility in Ayr, Queensland, Australia (19° 35′ 0″ S, 147° 24′ 0″ E) in August 2015 and held overnight in a recirculating system at the Marine and Aquaculture Research Facility Unit (MARFU), James Cook University, Queensland before harvesting for experiments. Separate collections were made for each experimental replicate ($n = 3$ collections over 3 weeks) as it was not logistically possible to perform all extractions simultaneously. Raw biomass was centrifuged to remove excess water and then oven dried (55 °C for 48 h) and milled (<1 mm) for processing using the 'dry and milled biomass protocol' (these samples were also used for biochemical analysis of ash, nitrogen and amino acids in the original material) or immediately processed using the 'fresh and pulped biomass protocol' (see below).

Experimental design

Three factors—'starting material', 'solvent-to-biomass ratio' and 'incubation time' for the initial aqueous extraction—were examined for both the isolation and concentration of protein using a factorial design. The starting materials were dry and milled biomass (generally applied to seeds—seed protocol) or fresh and pulped biomass (generally applied to leaves—leaf protocol). The solvent-to-biomass ratios in the initial aqueous extraction step were 20:1 (v/w) or 5:1. The incubation times were 16 h at 30 °C or <1 min at ambient temperature. The 5:1 aqueous solvent volume-to-biomass ratio was not practical for the dry and milled starting material as it formed a thick, dry paste that could not be centrifuged. Therefore, the 5:1 aqueous solvent volume-to-biomass ratio was not examined for the dry and milled starting material. The two starting materials required different processing protocols that are described below.

Dry and milled biomass protocol

The protocol, which is generally applied to seeds and previously to seaweeds, uses dried, milled biomass (DM) and is based on that described by Wong and Cheung (2001b) with slight modification. In brief, 20 g of dried, milled *U. ohnoi* was suspended in de-ionised water (20:1 *v/w*) and stirred overnight (16 h) at 30 °C or 15 s at ambient temperature (<1 min incubation time). Subsequently, for each treatment, the suspension was centrifuged at 3200× *g* for 30 min at 4 °C. The supernatant was then collected for protein precipitation (see below) and discarded after protein precipitation. The pellet was retained (referred to as the 'aqueous DM pellet') and re-suspended in de-ionised water and the pH adjusted to 12 using 1 M NaOH. The mixture was then stirred for 2 h (30 °C) before centrifugation as above. In contrast to Wong and Cheung (2001b), the reducing agent 2-mercaptoethanol was not used in the alkaline extraction step as it cannot be used for food-grade material (Turhan et al. 2003). The supernatant was then collected for protein precipitation (see below) and discarded after the precipitation of protein. The pellet was retained (referred to as the 'alkaline DM pellet') and dried in an oven at 55 °C, milled (<1 mm) and stored at −20 °C for ash, nitrogen and amino acid analysis (see below) (Fig. 1). The alkaline DM pellet is the alkaline DM total residual (Fig. 1).

In addition, a duplicate process was conducted and truncated after the initial aqueous extraction. This resulted in an aqueous supernatant and aqueous DM pellet. The aqueous DM pellet was dried in an oven at 55 °C, milled (<1 mm) and stored at −20 °C for ash, nitrogen and amino acid analysis (see below). This process was used to provide a quantitative compositional analysis of the seaweed biomass post-aqueous extraction (Fig. 1), which was not possible in the complete process as the aqueous DM pellet undergoes alkaline extraction (Fig. 1). The aqueous DM pellet is the aqueous DM total residual (Fig. 1).

Fresh and pulped biomass protocol

The protocol, which is generally applied to leaves, uses fresh, pulped biomass (FP) and is based on that described by Pirie (1969) with slight modification. In brief, 120 g (≈20 g dw) of freshly harvested *U. ohnoi* was pulped in de-ionised water at 20:1 (*v/w*), or a minimum amount of de-ionised water (5:1 *v/w*), using a stick blender (HB724 700W, Kenwood) and stirred overnight (16 h) at 30 °C, or for 15 s at ambient temperature (<1 min incubation time). Subsequently, for each treatment, the slurry was pressed and filtered through a 100 - μm mesh to provide a pressed cake (referred to as the 'aqueous pressed cake'), which was retained, and a suspension. For each treatment, the suspension was centrifuged as above.
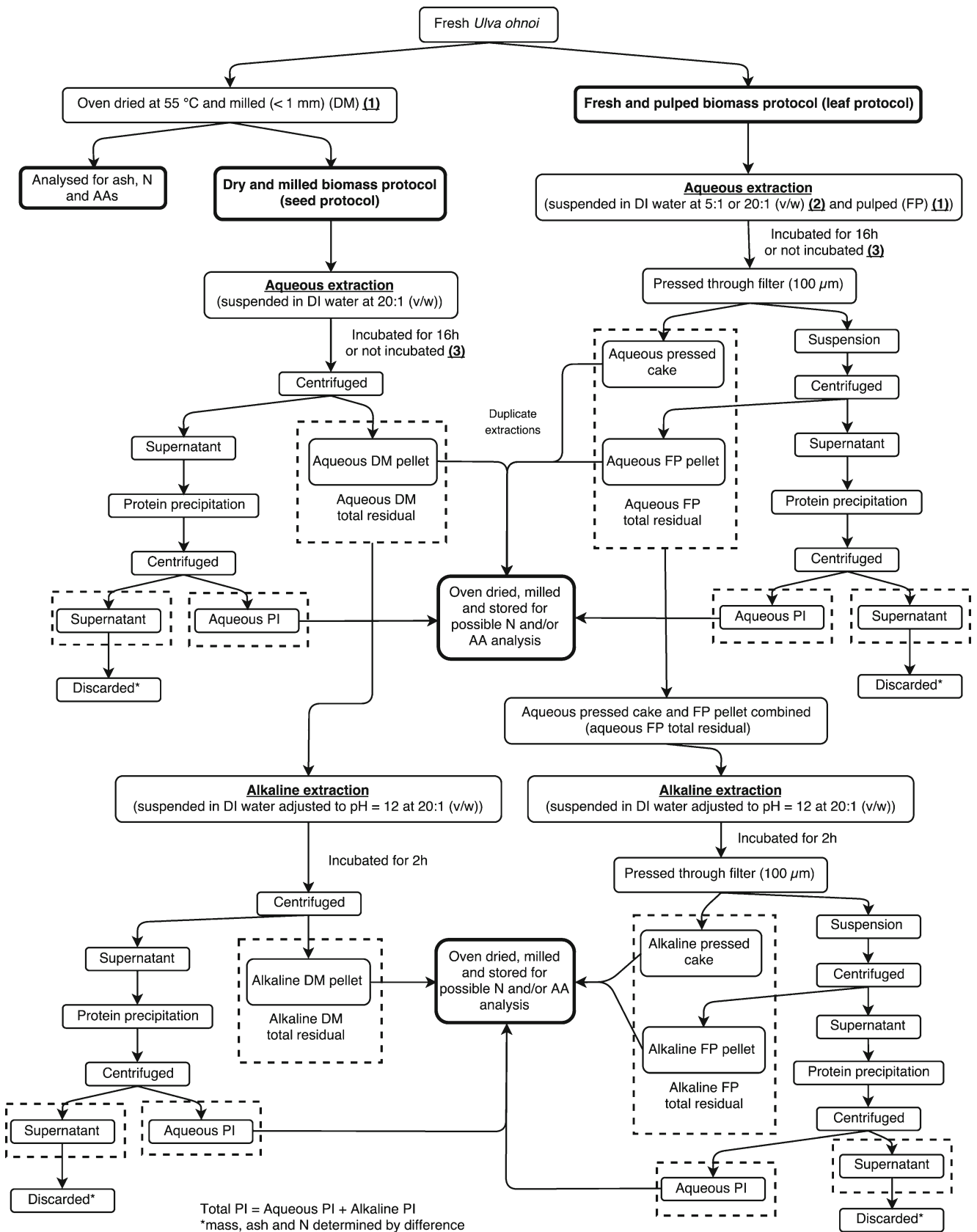
The supernatant was then collected for protein precipitation (see below) and discarded after the precipitation of protein.

The pellet was retained (referred to as the 'aqueous FP pellet'), combined with the aqueous pressed cake to give the aqueous FP total residual and re-suspended in de-ionised water and the pH adjusted to 12 using 1 M NaOH. The mixture was then stirred for 2 h (30 °C) before centrifugation as above. The slurry was then pressed and filtered through a 100 - μm mesh to provide a pressed cake (referred to as the 'alkaline pressed cake') and a suspension. The alkaline-pressed cake was dried in an oven at 55 °C, milled (<1 mm) and stored at −20 °C for ash, nitrogen and amino acid analysis (see below). The suspension was centrifuged, as above, and the supernatant was then collected for protein precipitation (see below) and discarded after the precipitation of protein. The pellet from the centrifugation was retained (hereafter referred to as the 'alkaline FP pellet') and dried in an oven at 55 °C, milled (<1 mm) and stored at −20 °C for ash, nitrogen and amino acid analysis (see below). Together, the alkaline-pressed cake and the alkaline FP pellet are the alkaline FP total residual (Fig. 1).

In addition, a duplicate process was conducted and truncated after the aqueous extraction. This produced an aqueous supernatant, an aqueous-pressed cake and an aqueous FP pellet. The aqueous pressed cake and aqueous FP pellet were dried in an oven at 55 °C, milled (<1 mm) and stored at −20 °C for ash, nitrogen and amino acid analysis (see below). This process was used to quantify the compositional analysis of the seaweed biomass post-aqueous extraction (Fig. 1), which was not possible in the complete process as the pressed cake and aqueous FP pellet undergo alkaline extraction (Fig. 1). Together, the aqueous pressed cake and the aqueous FP pellet are the aqueous FP total residual (Fig. 1).

Protein precipitation

Extracted protein was isolated from each supernatant by adjusting the pH to the isoelectric point (Ip) of the protein using HCl (10 % *v/v*). The Ip for each protein isolate was determined by subjecting the supernatant to incremental decreases in pH (using HCl) and determining turbidity (optical density at 750 nm) with a spectrophotometer (SPECTROstar Nano, BMG Labtech). The pH that gave the highest turbidity was taken as the Ip (Ju et al. 2001). This procedure was performed on the aqueous and alkaline extracted supernatants for both the dry and milled biomass and the fresh and pulped biomass. Each mixture containing the precipitated proteins was then centrifuged using the protocol described above. The precipitated proteins were oven dried at 55 °C, milled (<1 mm) and stored at −20 °C for nitrogen and amino acid analysis. The resulting dry protein powders from the aqueous and alkaline extractions are referred to as the 'aqueous' and 'alkaline' protein isolates (PIs), respectively.

Fresh *Ulva ohnoi*

Oven dried at 55 °C and milled (< 1 mm) (DM) **(1)**

Fresh and pulped biomass protocol (leaf protocol)

Analysed for ash, N and AAs

Dry and milled biomass protocol (seed protocol)

Aqueous extraction
(suspended in DI water at 5:1 or 20:1 (v/w) **(2)** and pulped (FP) **(1)**)

Incubated for 16h or not incubated **(3)**

Aqueous extraction
(suspended in DI water at 20:1 (v/w))

Incubated for 16h or not incubated **(3)**

Pressed through filter (100 μm)

Centrifuged

Duplicate extractions

Aqueous pressed cake

Suspension

Supernatant

Aqueous DM pellet

Aqueous DM total residual

Aqueous FP pellet

Aqueous FP total residual

Centrifuged

Protein precipitation

Supernatant

Centrifuged

Protein precipitation

Oven dried, milled and stored for possible N and/or AA analysis

Centrifuged

Supernatant

Aqueous PI

Aqueous PI

Supernatant

Discarded*

Discarded*

Aqueous pressed cake and FP pellet combined (aqueous FP total residual)

Alkaline extraction
(suspended in DI water adjusted to pH = 12 at 20:1 (v/w))

Alkaline extraction
(suspended in DI water adjusted to pH = 12 at 20:1 (v/w))

Incubated for 2h

Incubated for 2h

Centrifuged

Pressed through filter (100 μm)

Supernatant

Alkaline DM pellet

Alkaline DM total residual

Oven dried, milled and stored for possible N and/or AA analysis

Alkaline pressed cake

Suspension

Protein precipitation

Centrifuged

Alkaline FP pellet

Alkaline FP total residual

Centrifuged

Supernatant

Supernatant

Aqueous PI

Protein precipitation

Discarded*

Centrifuged

Aqueous PI

Supernatant

Total PI = Aqueous PI + Alkaline PI
*mass, ash and N determined by difference

Discarded*

◄ **Fig. 1** An overview of the extraction protocols used for dry and fresh biomass and the factorial experimental design examined in this study. The factors and treatment levels were (*1*) the starting material as dry and milled biomass or fresh and pulped biomass, (*2*) an aqueous solvent-to-biomass ratio in the aqueous extraction step of 20:1 (*v/w*) or 5:1 and (*3*) an incubation time of 16 h at 30 °C or <1 min at ambient temperature. The recovered fractions of the biomass are highlighted by *broken line boxes*. Note: the mass, ash content and N content of the supernatants were determined by difference

Mass balance

All dried components from the aqueous and alkaline extractions for each treatment combination in the factorial experiment were weighed. These components were the aqueous and alkaline PIs and the aqueous and alkaline total residuals. The mass of all PIs, total residuals and supernatants are expressed as a percentage of the original biomass (mass yield) to give a mass balance of 100 %. The mass yield of the extracted material in the aqueous supernatant was determined by deducting the mass yield of the aqueous PI and the aqueous total residual from the original biomass. The mass yield of the extracted material in the alkaline supernatant was determined by deducting the mass yield of the alkaline PI and the alkaline total residual from mass yield of the aqueous total residual. The total PI for each treatment combination was calculated by the sum of the aqueous and alkaline PIs.

Ash analysis

The concentration of ash (% dw) in the original biomass and all aqueous and alkaline total residuals from each treatment combination was determined by incinerating samples at 550 °C for 6 h. The ash in all residuals is expressed as the percentage of the quantity of ash in the original biomass (% ash yield) and as a percentage of their dry weight (% dw). The difference between the ash yield in the original biomass and the aqueous and alkaline total residuals was used to determine the ash yield in the aqueous and alkaline supernatants, respectively. Due to insufficient sample amounts, the concentration of ash was not measured in any of the PIs and these were assumed to contain a negligible concentration of ash.

Nitrogen analysis

The concentration of nitrogen (% dw) in the original biomass and all dried components (aqueous and alkaline PIs and total residuals) for a selected sub-set of treatment combinations was analysed using an elemental analyser (OEA Laboratory Ltd., UK). Treatment combinations were selected based on a high potential to isolate or concentrate protein. The treatment combinations targeted for protein isolation were those with a relatively high mass yield in the total PI (combined aqueous and alkaline PIs). In contrast, treatment combinations targeted for

protein concentrates were those with a relatively high mass yields in the supernatant after protein precipitation (i.e. high non-protein mass extracted).

The concentration of N in all PIs, total residuals and supernatants are expressed as a percentage of the quantity of N in the original biomass (% N yield) to give a N balance of 100 % and also as a percentage of their dry weight (% dw). The N yield in the aqueous supernatant was determined by deducting the quantity of N in the aqueous PI and the aqueous total residual from the original biomass. The N yield in the alkaline supernatant was determined by deducting the N yield in the alkaline PI and the alkaline total residual from the N yield in the aqueous total residual. The amount of N extracted by the aqueous extraction was determined by deducting the N yield in the aqueous total residual from the original biomass. The amount of N extracted by the alkaline extraction was determined by deducting the N yield in the alkaline total residual from the N yield in the aqueous total residual. The total amount of N extracted (from both aqueous and alkaline extractions) was determined by deducting the N yield in the alkaline total residual from the N yield in the original biomass.

Protein and amino acid analysis

The concentration of amino acids (% dw) in the original biomass and selected PIs and aqueous residuals was analysed for a sub-set of treatment combinations based upon the selection criteria for N analysis (above). Amino acids were quantified after 24 h liquid hydrolysis in 6 M HCl at 110 °C using a Waters ACQUITY UPLC at the Australian Proteome Analysis Facility, Macquarie University, Sydney, using procedures based on the Waters AccQTag amino acid methodology (Cohen 2000; Bosch et al. 2006). The following amino acids were analysed: aspartic acid, asparagine, glutamic acid, glutamine, serine, histidine, glycine, threonine, alanine, arginine, valine, methionine, phenylalanine, isoleucine, leucine, lysine and proline. As asparagine is hydrolysed to aspartic acid, and glutamine to glutamic acid, during analysis, the sum of these amino acids was reported as asparagine/aspartic acid or glutamine/glutamic acid. The two remaining proteome amino acids, cysteine and tryptophan, were not analysed as they are minor constituents in *Ulva* spp. (Angell et al. 2012, 2014). The total amino acid (TAA) content was calculated based on the sum of the above amino acids and was used as the measure of protein. Total essential amino acid (TEAA) content was taken as the sum of arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine and valine. Protein, TEAA, methionine and lysine in the selected PIs and residuals are expressed as a percentage of the quantity of protein, TEAA, methionine and lysine in the

original biomass (% yield) and also as a percentage of their dry weight (% dw).

Data analysis

As the factorial experimental design was incomplete because the 5:1 aqueous solvent volume-to-biomass ratio was not examined for the dry and milled starting material, a series of univariate PERMANOVAs (PRIMER 6 & PERMANOVA+, PRIMER-E Ltd., UK) were used to analyse the effect of starting material (milled and dry or pulped and fresh), aqueous solvent-to-biomass ratio (20:1 or 5:1) and incubation time in aqueous solvent (16 h incubation time at 30 °C or <1 min incubation time at ambient temperature) on the mass yields of aqueous and alkaline PIs and total residual biomass (mass extracted), each as separate dependent variables. For all N and amino acid data, where only three factor combinations were analysed (DM.20.I (dry and milled starting material, 20:1 aqueous solvent-to-biomass ratio and 16 h incubation time at 30 °C in aqueous solvent), FP.20.I (fresh and pulped starting material, 20:1 aqueous solvent-to-biomass ratio and 16 h incubation time at 30 °C in aqueous solvent) and FP.5.NI (fresh and pulped starting material, 5:1 aqueous solvent-to-biomass ratio and <1 min incubation time at ambient temperature in aqueous solvent) (Table 1), the three treatment combinations were analysed as a single factor using PERMANOVAs.

All PERMANOVA analyses were conducted using Bray-Curtis dissimilarities on fourth root transformed data and 9999 unrestricted permutations of raw data. Tukey's multiple comparison was used to determine any differences between treatment combinations. The proportion of variation (%) of the total variation of the independent variable explained by a particular factor or factor interaction was calculated by eta-squared (% variance explained, η^2) = $MS_{factor}/MS_{total} \times 100$, where MS_{factor} and MS_{total} are the mean sum of squares of a particular factor and the total mean sum of squares, respectively (Anderson and Gorley 2007). For pairwise tests, Monte Carlo tests were also applied when the number of possible permutations was low. For these situations Monte Carlo P values (p (Monte Carlo)) were used to assess significance (Anderson and Gorley 2007).

Results

Protein precipitation

Turbidity in all protein solutions increased to a maximum with decreasing pH until a pH of approximately 2.25 (Supplementary Fig. S1). Therefore, a pH of 2.25 was taken as the isoelectric point for all the soluble protein fractions in this study.

Mass balance and ash

For both the aqueous and alkaline extraction steps, the mass of the starting material is divided into three components: (1) the total residual biomass (the DM pellet for dry and milled starting material and the pressed cake and FP pellet for the fresh and pulped starting material; see Fig. 1), (2) the protein isolate (PI) and (3) the supernatant after protein precipitation (supernatant) (Fig. 2). As described above, treatment combinations with potential as protein isolation protocols will have high mass yields in the total PI (combined aqueous and alkaline PIs) while treatment combinations with potential to produce protein concentrates will have high mass yields in the supernatant after precipitation (i.e. high non-protein mass extracted). On this basis, a subset of treatment combinations in the factorial experiment was selected for N and amino acid analysis (Fig. 2) (see below).

Aqueous PI mass yields ranged from 0.25 ± 0.03 to 2.18 ± 0.02 % and alkaline PI mass yields ranged from 2.11 ± 0.18 to 4.56 ± 0.24 % (Fig. 3a). For aqueous PIs, aqueous extraction incubation time (Pseudo-$F_{1,17}$ = 110.74, $p < 0.001$) and aqueous solvent volume-to-biomass ratio (Pseudo-$F_{1,17}$ = 17.01, $p < 0.001$) had significant effects on mass yields, explaining 83 and 13 % of the variance, respectively. Treatments with an incubation time of <1 min (mean = 1.54 ± 0.06 %) had higher aqueous PI mass yields compared to those that were incubated for 16 h at 30 °C (mean = 0.34 ± 0.01 %). A lower aqueous solvent volume-to-biomass ratio also resulted in a higher aqueous PI mass yield (mean = 1.33 ± 0.38 %) compared to a higher ratio (mean = 0.75 ± 0.16 %). The highest overall aqueous PI mass yield resulted when fresh and pulped biomass was used with a 5:1 aqueous solvent volume-to-biomass ratio and a <1 min

Table 1 The combination of treatments that were analysed for N and amino acids

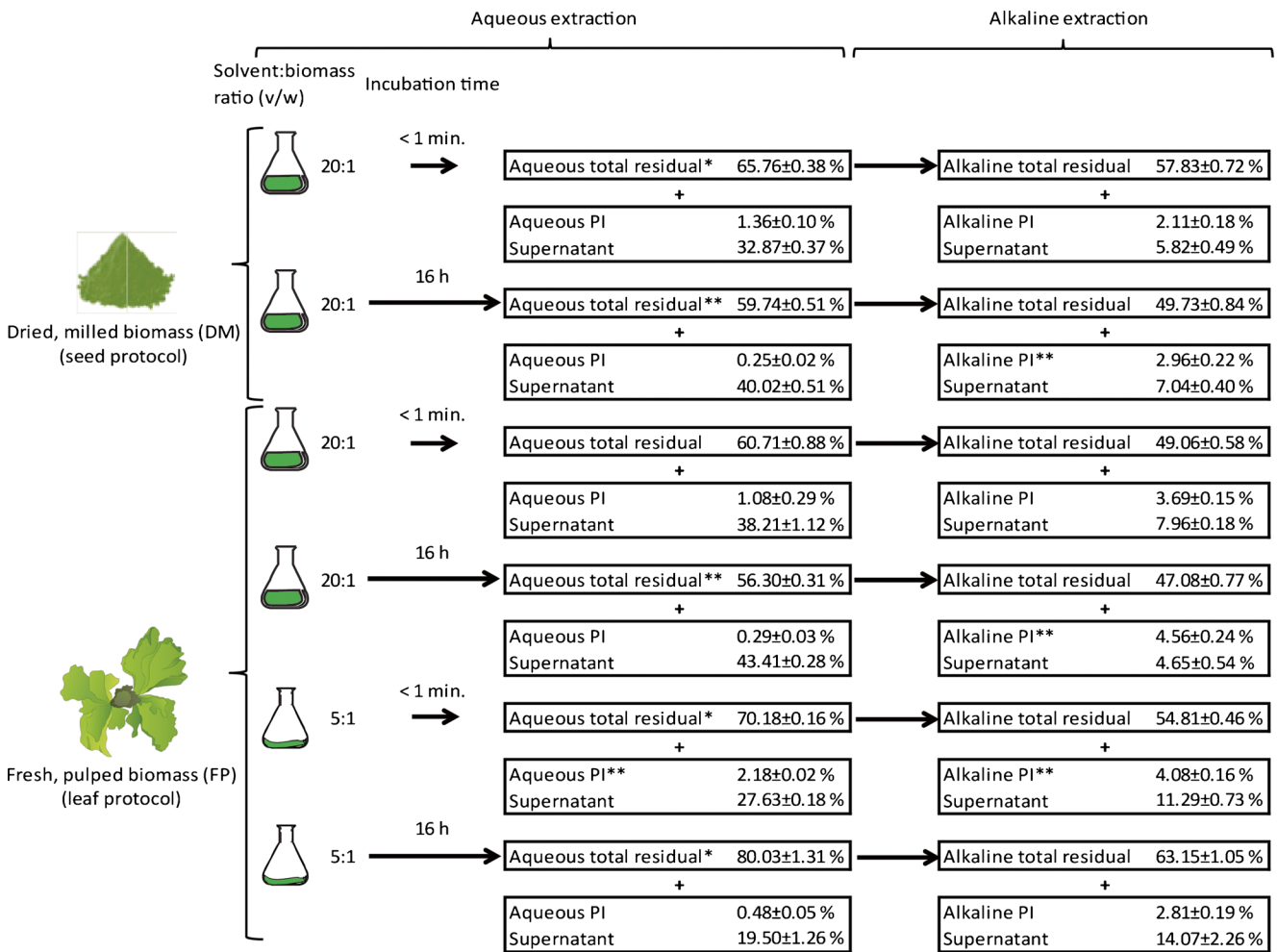| ID	Starting material	Aqueous solvent-to-biomass ratio (v/w)	Incubation time of aqueous extraction
DM.20.I	Dry and milled (DM)	20:1 (20)	16 h (I)
FP.20.I	Fresh and pulped (FP)	20:1 (20)	16 h (I)
FP.5.NI	Fresh and pulped (FP)	5:1 (5)	Not incubated (<1 min) (NI)

Aqueous extraction   Alkaline extraction

Solvent:biomass ratio (v/w)   Incubation time

Dried, milled biomass (DM) (seed protocol)

20:1 — < 1 min.
Aqueous total residual*	65.76±0.38 %	→	Alkaline total residual	57.83±0.72 %
+			+	
Aqueous PI	1.36±0.10 %		Alkaline PI	2.11±0.18 %
Supernatant	32.87±0.37 %		Supernatant	5.82±0.49 %

20:1 — 16 h
Aqueous total residual**	59.74±0.51 %	→	Alkaline total residual	49.73±0.84 %
+			+	
Aqueous PI	0.25±0.02 %		Alkaline PI**	2.96±0.22 %
Supernatant	40.02±0.51 %		Supernatant	7.04±0.40 %

Fresh, pulped biomass (FP) (leaf protocol)

20:1 — < 1 min.
Aqueous total residual	60.71±0.88 %	→	Alkaline total residual	49.06±0.58 %
+			+	
Aqueous PI	1.08±0.29 %		Alkaline PI	3.69±0.15 %
Supernatant	38.21±1.12 %		Supernatant	7.96±0.18 %

20:1 — 16 h
Aqueous total residual**	56.30±0.31 %	→	Alkaline total residual	47.08±0.77 %
+			+	
Aqueous PI	0.29±0.03 %		Alkaline PI**	4.56±0.24 %
Supernatant	43.41±0.28 %		Supernatant	4.65±0.54 %

5:1 — < 1 min.
Aqueous total residual*	70.18±0.16 %	→	Alkaline total residual	54.81±0.46 %
+			+	
Aqueous PI**	2.18±0.02 %		Alkaline PI**	4.08±0.16 %
Supernatant	27.63±0.18 %		Supernatant	11.29±0.73 %

5:1 — 16 h
Aqueous total residual*	80.03±1.31 %	→	Alkaline total residual	63.15±1.05 %
+			+	
Aqueous PI	0.48±0.05 %		Alkaline PI	2.81±0.19 %
Supernatant	19.50±1.26 %		Supernatant	14.07±2.26 %

Fig. 2 Mass balance (as % of original seaweed biomass) of all treatment combinations in the factorial experimental design (Fig. 1). The mass of the starting material is divided into three components after each extraction; the total residual biomass (the DM pellet for dry and milled starting material and the pressed cake and FP pellet for the fresh and pulped starting material; see Fig. 1), the protein isolate (PI) or the residual supernatant after protein precipitation (supernatant). *Asterisk:* analysed for N. *Double asterisk*: analysed for N and amino acids. Note: the 5:1 aqueous solvent volume-to-biomass ratio was not practical for the dry and milled starting material as it formed a thick, dry paste that could not be centrifuged. Therefore, the 5:1 aqueous solvent volume-to-biomass ratio was not examined for dry and milled starting material

incubation time at ambient temperature (2.18 ± 0.02 %, Fig. 2). The aqueous PI of this treatment was therefore analysed for N and amino acids.

For alkaline PIs, starting material (Pseudo-$F_{1,17}$ = 83.19, $p < 0.001$) and the interaction between the aqueous solvent volume-to-biomass ratio and aqueous extraction incubation time (Pseudo-$F_{1,17}$ = 24.59, $p < 0.001$) had significant effects on PI mass yields, with these factors explaining 69 and 20 % of the variance, respectively. Alkaline PI mass yields were higher when fresh and pulped biomass was used (mean = 3.78 ± 0.21 %) compared to dry and milled biomass (mean = 2.54 ± 0.23 %). When a high aqueous solvent volume-to-biomass ratio was used, the result was higher alkaline PI mass yields when the aqueous extraction was incubated for 16 h at 30 °C (mean = 3.76 ± 0.39 %) compared to when it was not (mean = 2.90 ± 0.37 %) ($t = 5.28$, $p < 0.001$). However, when a low aqueous solvent volume-

to-biomass ratio was used, higher alkaline PI mass yields resulted when the aqueous extraction was incubated for <1 min at ambient temperature (mean = 4.08 ± 0.16 %) compared to when it was incubated for 16 h at 30 °C (mean = 2.81 ± 0.19 %) ($t = 3.611$, p (Monte Carlo) = 0.0233). The highest alkaline PI mass yields resulted when fresh and pulped biomass was used and either incubated for 16 h at 30 °C at a high aqueous solvent volume-to-biomass ratio (mass yield = 4.56 ± 0.24 %) or incubated for <1 min at ambient temperature at a low aqueous solvent volume-to-biomass ratio (4.08 ± 0.16 %). The highest alkaline PI mass yield when dry and milled biomass was used resulted when the aqueous extraction was incubated for 16 h at 30 °C with a high aqueous solvent volume-to-biomass ratio (2.96 ± 0.22 %). Therefore, the alkaline PIs of these three treatment combinations were analysed for N and amino acids (see Table 1 and Figs. 3 and 4).
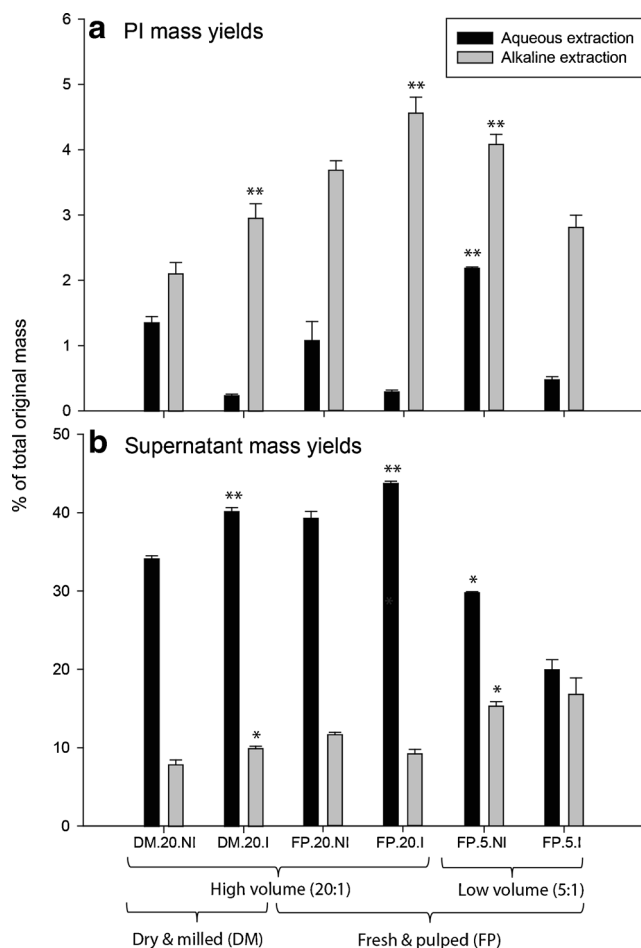
Fig. 3 The proportion of the original biomass in the protein isolates (PIs) (**a**) and the supernatants (**b**) (±SE) for aqueous and alkaline extractions for the six treatment combinations examined in the factorial design (see Figs. 1 and 3). *Single asterisk* and *double asterisks* indicate PIs (**a**) and total residuals (**b**) that were analysed for total N or total N and amino acids, respectively. *I* = incubated for 16 h at 30 °C and *NI* = no incubation (<1 min at ambient temperature)

Aqueous supernatants yielded between 19.50 ± 1.26 and 43.41 ± 0.28 % of the original biomass (Fig. 3b). In contrast, the alkaline supernatant yield was much lower at 4.65 ± 0.54 to 17.07 ± 2.26 % of the original biomass. For the aqueous supernatant mass yields, there was a significant effect of starting material (Pseudo-$F_{1,17} = 15.22$, $p = 0.003$) and a significant interaction effect between aqueous solvent volume-to-biomass ratio and incubation time (Pseudo-$F_{1,17} = 65.58$, $p < 0.001$); however, aqueous solvent volume-to-biomass ratio explained most of the variance (80 %). The highest mass yield in the aqueous supernatant occurred when an aqueous solvent volume-to-biomass ratio of 20:1 was used compared to a ratio of 5:1 for treatments incubated for 16 h at 30 °C ($t = 15.15$, $p < 0.001$, mean $= 41.71 \pm 0.80$ and 19.50 ± 1.26 % for 20:1 and 5:1, respectively) and <1 min at ambient temperature ($t = 12.17$, p < 0.001, mean $= 35.54 \pm 1.30$ and 27.63 ± 0.18 % for 20:1 and 5:1, respectively); however, this difference was greater when the aqueous solvent was

incubated for 16 h at 30 °C compared to when it was incubated for <1 min at ambient temperature (Pseudo-$F_{1,17} = 65.58$, $p < 0.001$). In contrast, higher mass yields in the alkaline supernatant resulted when the lower aqueous solvent volume-to-biomass ratio was used (Pseudo-$F_{1,17} = 50.03$, $p < 0.001$, 61 % of variance explained). The proportion of the mass yielded in the aqueous supernatant that consisted of ash was between 43.80 ± 2.53 and 62.77 ± 3.43 %, with supernatant mass yields from extractions that had an aqueous solvent volume-to-biomass ratio of 5:1 (mean $= 59.80 \pm 2.23$ %) having a higher proportion of ash compared to those that had a ratio of 20:1 (mean $= 48.80 \pm 1.23$ %) (Pseudo-$F_{1,17} = 14.21$, $p < 0.001$). However, aqueous supernatants from treatments with an aqueous solvent volume-to-biomass ratio of 20:1 yielded significantly more ash from the original biomass (mean $= 65.99 \pm 2.27$ %) compared to those with a ratio of 5:1 (mean $= 48.86 \pm 2.82$ %) (Pseudo-$F_{1,17} = 193.45$, $p < 0.001$). The solvent volume explained most of the variance (68 %) in the proportion of total ash removed from the original biomass. The highest supernatant mass and ash yields were in the aqueous supernatant when the biomass was incubated for 16 h at 30 °C in a solvent volume-to-biomass ratio of 20:1 for both types of staring material (supernatant mass yield $= 40.02 \pm 0.51$ and 43.41 ± 0.28 %, ash yield $= 61.47 \pm 3.27$ and 75.17 ± 1.26 % for dry and milled and fresh and pulped, respectively). Therefore, these treatment combinations were assessed for protein concentration efficiency by analysing N and amino acids in the aqueous total residual (Fig. 2 and 3b).

The concentration of ash in all total residuals was lower than the original biomass (28.50 ± 0.52 %) and ranged from 12.55 ± 0.41 to 20.43 ± 0.33 % for aqueous total residuals and from 16.81 ± 0.20 to 24.09 ± 0.08 % for alkaline total residuals (Fig. S2). For aqueous total residuals, aqueous solvent volume-to-biomass ratio (Pseudo-$F_{1,17} = 60.12$, $p < 0.001$) and starting material (Pseudo-$F_{1,17} = 44.67$, <0.001) had significant effects on the concentration of ash, explaining 53 and 39 % of the variance, respectively. There was also a significant interaction between starting material and incubation time (Pseudo-$F_{1,17} = 5.80$, $p = 0.033$), but overall, this was not an important effect as it explained only 5 % of the variance. A higher aqueous solvent volume-to-biomass ratio resulted in a lower concentration of ash in the aqueous total residual (mean $= 15.88 \pm 0.88$ %) compared to a lower ratio (mean $= 19.34 \pm 0.54$ %). The concentration of ash in the aqueous total residual was also lower when fresh and pulped material was used (mean $= 16.35 \pm 0.98$ %) compared to dry and milled biomass (mean $= 18.40 \pm 0.73$ %), with this effect greater when the aqueous solvent was incubated for 16 h at 30 °C. Therefore, the aqueous total residual with the lowest concentration of ash resulted when fresh and pulped biomass was used with a 20:1 aqueous solvent volume-to-biomass ratio with an incubation time of 16 h at 30 °C (12.55 ± 0.41 %).

Fig. 4 The distribution of N from the original seaweed biomass (**a**) and concentration of N in recovered components (±SE) (**b**). N from the original biomass is divided among the residual biomass (the pellet for dry and milled starting material (DM pellet) and the pressed cake and pellet (FP pellet) for the fresh and pulped starting material), protein isolate (PI) and residual supernatant after protein precipitation (supernatant). *I* = Incubated for 16 h at 30 °C and *NI* = no incubation (<1 min at ambient temperature). Note: the PIs for aqueous extractions for DM.20.I and FP.20.I did not yield sufficient mass for N analysis and were assumed to contribute a negligible amount of N to the N yield balance. *Dashed line* represents the concentration of N in the original biomass
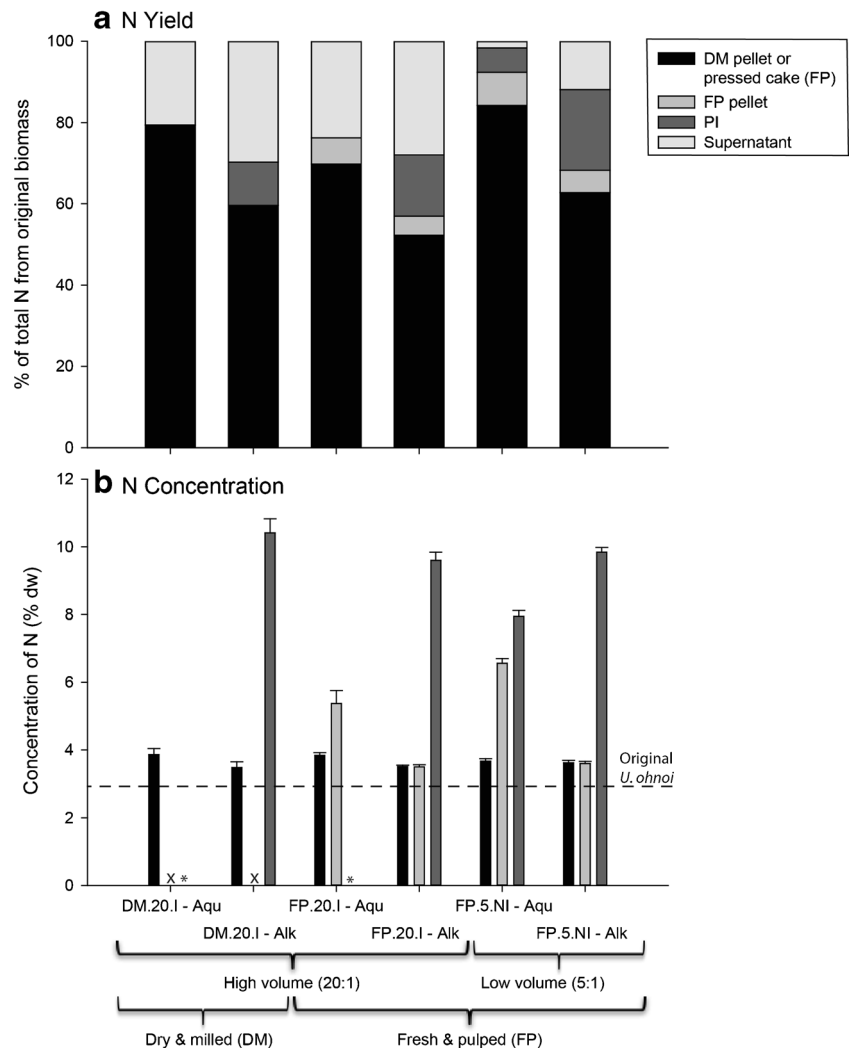
These treatment effects on the concentration of ash in the aqueous total residual were carried through the alkaline extraction, and the same treatment combination resulted in the lowest concentration of ash in the alkaline total residual (Supplementary Fig. S2).

Nitrogen balance

The aqueous solvent extracted between 7.65 ± 1.05 and 23.77 ± 0.21 % of the total nitrogen from the original biomass (N yield, Fig. 4a). Significantly, more N was extracted by the aqueous extraction for treatment combinations FP.20.I (23.77 ± 0.21 %, $t = 8.41$, p (Monte Carlo) = 0.001) and DM.20.I (20.58 ± 3.19 %, $t = 5.03$, p (Monte Carlo) = 0.007) compared to treatment combination FP.5.NI (7.65 ± 1.05 %). However, the aqueous PI mass yields for these treatment combinations were extremely low (<0.29 % of original biomass (Fig. 2)), indicating that almost no N was likely isolated for the FP.20.I or the DM.20.I treatment combinations (insufficient

material meant that N analysis was not possible for these PIs). In contrast, the FP.5.NI treatment combination, which extracted the least amount of N from the original biomass during the aqueous extraction (7.65 ± 1.05 %), recovered the most N in the aqueous PI (5.98 %, Fig. 4a).

The alkaline solvent extracted similar amounts of the total N from the original biomass for all three treatment combinations (proportion of original N extracted (N yield) = 19.27 ± 0.27, 19.82 ± 1.75 and 24.09 ± 0.83 % for FP.20.I, DP.20.I, and FP.5.NI, respectively) and all yielded alkaline PIs (Fig. 4a). Of this alkaline extracted N, treatment combination FP.20.I recovered proportionally more N in the alkaline PI (78.19 ± 2.67 % of alkaline extracted N recovered in alkaline PI) compared to treatment combinations FP.5.NI (57.70 ± 3.95 %, $t = 4.04$, p (Monte Carlo) = 0.015) and DM.20.I (54.13 ± 4.62 %, $t = 3.91$, p (Monte Carlo) = 0.019), which recovered similar proportions. Treatment combinations FP.5.NI and FP.20.NI recovered similar amounts of the total N from the original biomass in the alkaline PI (13.83 ± 0.47 and 15.08 ± 0.73 %, respectively), but only

FP.20.NI had a significantly higher alkaline PI N yield compared to DM.20.I (10.66 ± 1.05 %, $t = 3.33$, p (Monte Carlo) = 0.033).

Combined, the aqueous and alkaline solvents extracted less than half of the total N from the original biomass, leaving most of the N in the final residual biomass (i.e. the alkaline DM pellet for DM.20.I or the combined alkaline press cake and alkaline FP pellet for FP.20.I and FP.5.NI treatment combinations) (Fig. 4a). The FP.5.NI treatment combination extracted significantly less N in total (31.74 ± 0.25 % of total N in the original biomass remaining in total residual) compared to FP.20.I (43.05 ± 0.69 %, $t = 14.32$, p (Monte Carlo) < 0.001) and DM.20.I (40.40 ± 2.35 %, $t = 3.40$, p (Monte Carlo) = 0.029), which extracted similar amounts of N. However, the FP.5.NI treatment combination recovered the most extracted N in the total PI (62.41 ± 0.25 % of total extracted N) compared to FP.20.I (35.01 ± 0.69 %, $t = 16.99$, p (Monte Carlo) < 0.001) and DM.20.I (26.76 ± 2.35 %, $t = 6.06$, p (Monte Carlo) = 0.004) (Fig. 4a). The proportion of total N lost to the total residual supernatant and not recovered in either the total residual biomass or total PI was lower for the FP.5.NI treatment combination (11.93 ± 0.13 %) compared to the FP.20.I and DM.20.I treatment combinations (27.96 ± 0.21 and 29.75 ± 3.19 %, respectively).

The concentration of nitrogen in PIs and residual biomass

The concentration of N (% dw) in all recovered components (DM pellets, FP pressed cakes, FP pellets and PIs) was higher compared to the original seaweed biomass for aqueous and alkaline extractions across all three treatment combinations that were analysed (Fig. 4b). The aqueous PI from the FP.5.NI treatment combination had a N concentration of 7.94 ± 0.17 % dw and was 173.79 ± 8.25 % higher than the original biomass (2.90 ± 0.07 %). The concentration of N in the alkaline PIs of all three treatment combinations was significantly higher than the aqueous PI from the FP.5.NI treatment combination (PERMANOVA pair-wise comparisons, $p < 0.05$) and ranged from 9.60 ± 0.23 % dw to 10.40 ± 0.41 % dw (230.78 ± 1.77 to 258.43 ± 7.99 % higher than original biomass).

The concentration of N in the residual DM pellets and pressed cakes ranged from 3.48 ± 0.17 to 3.86 ± 0.17 % dw and were 19.78 ± 3.57 to 32.96 ± 4.55 % higher than the original biomass, with all DM pellets and pressed cakes for all treatments having a similar concentration of N for both aqueous and alkaline extractions (Fig. 4b). Further, with the exception of the FP.20.I treatment combination ($t = 3.85$, p (Monte Carlo) = 0.018), there was no difference in the concentration of N between aqueous DM pellets or pressed cakes and alkaline DM pellets or pressed cakes. The concentration of N in the FP.20.I and FP.5.NI aqueous FP pellets (5.36 ± 0.38 and 6.55 ± 0.13 % dw, respectively) was 85.20 ± 14.97 and 125.97 ± 1.82 % higher, respectively, than

the original biomass and significantly higher than the alkaline FP pellets (3.50 ± 0.06 and 3.60 ± 0.06 % dw for FP.20.I and FP.5.NI, respectively) (Pseudo-$F_{1,11} = 161.73$, $p < 0.001$) (Fig. 4b).

Protein isolation

The protein yields (determined by TAA) of PIs ranged from 12.28 ± 1.32 to 21.57 ± 0.57 % for the three treatment combinations, with the alkaline PI providing all of the yield for treatment combinations FP.20.I and DM.20.I and most of the yield for FP.5.NI (71 %) (Table 2). The protein yield of the alkaline PIs for all three treatment combinations were similar; however, FP.5.NI had a significantly higher total yield due to the addition of the aqueous PI, which was not obtained for the other treatment combinations. This pattern was the same for TEAA yield. Methionine yields in the alkaline PI were similar across the three treatment combinations; however, the alkaline PI of FP.5.NI was significantly higher than that of DM.20.I (Table 2). In total, FP.5.NI had the highest methionine yield due the addition of the aqueous PI. Lysine yields in the alkaline PI were significantly higher for the FP.20.I treatment compared to the DM.20.I and FP.5.NI treatments, which were similar. However, due to the addition of the aqueous PI, treatment FP.5.NI had the highest lysine yield in total (Table 2).

The concentration of protein (TAA), TEAA, methionine and lysine in all PIs were substantially higher compared to the original biomass for all three treatment combinations (Table 2). The alkaline PIs for the three treatments had similar concentrations of protein, and TEAA, however, the DM.20.I alkaline PI had significantly higher concentrations of methionine compared to the FP.5.NI alkaline PI and significantly higher concentrations of lysine compared to both FP.20.I and FP.5.NI alkaline PIs (Table 2). The aqueous PI of treatment combination FP.5.NI had significantly lower concentrations of protein, TEAA, methionine and lysine compared to all alkaline PIs. As a result, the total PI for the FP.5.NI treatment combination had significantly lower concentrations of protein, TEAA, methionine and lysine compared to the DM.20.I treatment combination and significantly lower concentrations of lysine compared to the FP.20.I treatment combination (Table 2).

Protein concentration

The aqueous total residual yielded between 73.9 and 89.6 % of the protein, TEAA, methionine and lysine from the original biomass, with no difference between the two treatment combinations analysed (DM.20.I and FP.20.I, Table 3). For fresh and pulped biomass, where the total residual was divided into a pressed cake and suspended material, the pressed cake yielded most of the protein, TEAA, methionine and lysine (Table 3).

Table 2 The yield (% of original) and concentration (% dw) of protein (TAA), total essential amino acids (TEAA), methionine (Met) and lysine (Lys) for protein isolates obtained for the three treatment combinations analysed in this study as well as those for other studies. The concentration of protein, TEAA, Met and Lys are also presented for the original biomass *Ulva ohnoi* from this study

	Protein		TEAA		Met		Lys	
	% Yield	% dw	% Yield	% dw	% Yield	% dw	% Yield	% dw
Original *Ulva ohnoi*	NA	13.58 ± 0.42a	NA	6.33 ± 0.21a	NA	0.23 ± <0.01a	NA	0.84 ± 0.03a
PIs (this study)								
DM.20.I (Alk only)	12.28 ± 1.32a	56.04 ± 2.35b	12.97 ± 1.4a	27.56 ± 1.16b	15.2 ± 1.15a	1.18 ± 0.03b	11.22 ± 0.86a	3.17 ± 0.01b
FP20.I (Alk only)	17.13 ± 1.39ab	50.87 ± 2.39bc	18.17 ± 1.48ab	25.13 ± 1.21bc	21.22 ± 1.98abc	1.07 ± 0.07bc	15.17 ± 0.83b	2.78 ± 0.07c
FP.5.NI (Aq + Alk)	21.57 ± 0.57b	47.43 ± 0.78c	22.61 ± 0.59b	23.16 ± 0.39c	27.6 ± 0.63b	1.03 ± 0.01c	18.92 ± 0.23c	2.54 ± 0.04d
Aq	6.28 ± 0.07c	39.07 ± 0.32d	6.59 ± 0.08c	19.08 ± 0.16d	8.14 ± 0.29d	0.86 ± 0.03d	6.22 ± 0.15d	2.38 ± 0.03e
Alk	15.29 ± 0.64a	50.85 ± 0.87b	16.03 ± 0.67a	24.83 ± 0.43b	19.47 ± 0.85c	1.1 ± 0.01b	12.71 ± 0.35a	2.61 ± 0.05cd
Other studies								
Ulva lactuca[a]	36.4*	73.9	NA	29.83	NA	0.47	NA	3.54
Hypnea charoides[a]	46.3*	78.7	NA	27.92	NA	1.35	NA	3.26
Hypnea japonica[a]	45.4*	78.7	NA	31.54	NA	1.67	NA	3.79
Sargassum hemiphyllum[b]	9.5*	81.2	NA	31.96	NA	1.11	NA	4.48
Sargassum hemiphyllum[b]	7.8*	70.6	NA	28.20	NA	0.86	NA	3.85
Sargassum henslowianum[b]	33.1*	69.2	NA	31.50	NA	0.70	NA	3.82
Sargassum henslowianum[b]	27.0*	71.2	NA	27.99	NA	0.63	NA	4.08
Sargassum patens[b]	48.0*	81.2	NA	33.51	NA	0.79	NA	4.88
Sargassum patens[b]	37.8*	70.6	NA	27.68	NA	0.71	NA	4.25

Common lowercase letters within variables (columns) indicate no significant difference (PERMANOVA pairwise comparisons, p (Monte Carlo) > 0.05)

Aq aqueous PI, *Alk* alkaline PI, * Crude protein (N*6.25), *NA* not applicable

[a] Wong and Cheung (2001b)　[b] Wong and Cheung (2001a)

Table 3 The yield (% of original material) and concentration (% dw) of protein (TAA), total essential amino acids (TEAA), methionine (Met), lysine (Lys) and ash in the residual biomass of the aqueous extraction step for DM.20.I and FP.20.I (see Table 1). The concentration of protein, TEAA, Met, Lys and ash are also shown for the starting material (*Ulva ohnoi*). Note: the aqueous total residual for FP.20.I consisted of the aqueous pressed cake and aqueous FP pellet. These are reported separately and as a total residual

	Protein		TEAA		Met		Lys		Ash	
	% Yield	% dw	% Yield	% dw	% Yield	% dw	% Yield	% dw	% Yield	% dw
Ulva ohnoi	NA	13.58 ± 0.42a	NA	6.33 ± 0.21a	NA	0.23 ± <0.01a	NA	0.84 ± 0.03a	NA	28.50 ± 0.52a
Aqueous residual biomass										
DM.20.I (DM pellet)	81.34 ± 2.47a	18.49 ± 0.62b	83.55 ± 2.48a	8.85 ± 0.29b	84.83 ± 2.99a	0.33 ± 0.02b	79.5 ± 1.87a	1.12 ± 0.03bc	38.53 ± 3.27a	18.38 ± 1.54b
FP.20.I (total)	80.48 ± 0.46a	19.65 ± 0.21b	83.49 ± 0.54a	9.52 ± 0.11b	89.6 ± 2.61a	0.38 ± 0.01c	73.98 ± 0.75a	1.11 ± 0.01b	24.83 ± 1.26b	12.55 ± 0.41c
Pressed cake	73.35 ± 0.32b	18.86 ± 0.27b	75.9 ± 0.33b	9.09 ± 0.13b	80.39 ± 2.69a	0.35 ± 0.02bc	67.99 ± 0.63b	1.08 ± 0.01c	23.48 ± 1.21b	12.65 ± 0.44c
FP pellet	7.13 ± 0.22c	27.72 ± 0.22c	7.59 ± 0.25c	13.75 ± 0.08c	9.21 ± 0.41b	0.61 ± 0.02d	6.00 ± 0.17c	1.44 ± 0.01d	1.35 ± 0.05c	11.05[a]d

Common lowercase letters within variables (columns) indicate no significant difference (PERMANOVA pairwise comparisons, p (Monte Carlo) > 0.05)

NA not applicable

[a] Pooled sample due to insufficient sample mass

The aqueous total residual from both the DM.20.I and FP.20.I treatments had significantly higher concentrations of protein, TEAA, methionine and lysine compared to the original seaweed biomass. Concentrations of protein, TEAA, methionine and lysine were 36.17 ± 4.15, 39.88 ± 4.25, 42.03 ± 5.23 and 33.12 ± 3.62 % higher for the DM.20.I treatment, respectively, and 44.70 ± 1.21, 50.46 ± 1.46, 63.75 ± 3.41 and 32.24 ± 2.06 % higher for the FP.20.I treatment, respectively, compared to the original seaweed biomass. The total residuals for the DM.20.I and FP.20.I treatments had similar concentrations of protein, TEAA and lysine; however, FP.20.I had a significantly higher concentration of methionine compared to DM.20.I (Table 3). The pressed cake portion of the FP.20.I aqueous total residual was similar in protein, TEAA, methionine and lysine concentration compared to the aqueous total residual of the DM.20.I treatment. However, the aqueous FP pellet portion of the FP.20.I aqueous total residual had significantly higher concentrations of protein, TEAA, methionine and lysine (Table 3). Concentrations of protein, TEAA, methionine and lysine were 104.30 ± 5.14, 117.59 ± 5.21, 163.77 ± 5.23 and 71.84 ± 3.76 % higher in the aqueous FP pellet compared to the original seaweed biomass.

Discussion

Yields of protein isolates from the green seaweed *U. ohnoi* were higher when protocols for the extraction of leaves were used as opposed to seeds. This demonstrates, for the first time, an approach to isolating protein inspired by the physiological and morphological similarities between seaweed and leaves. Importantly, the quality of the isolate produced was similar for both protocols for concentrations of protein, total essential amino acids and methionine. This supports the use of protocols for the isolation of protein from leaves for processing seaweed biomass.

The total protein isolate (PI) for each extraction included an aqueous-solvent extracted PI and an alkaline-solvent extracted PI. For the aqueous PI, an incubation time of <1 min at ambient temperature resulted in significantly higher mass yields than a 16 h incubation at 30 °C. This effect of incubation time was consistent across both types of starting material (dry and milled vs. fresh and pulped) and both aqueous solvent to biomass ratios (20:1 and 5:1). Lower PI mass yields when aqueous extractions were incubated for 16 h at 30 °C may be due to heat-related denaturing of proteins over time. Indeed, long incubation times (16 h) at low temperatures (4 and 22 °C) resulted in no difference in aqueous protein isolate yields for the red seaweed *Palmaria palmata* compared to shorter incubation times (4 and 7 h) (Harnedy and FitzGerald 2013). Similarly, for leaves, aqueous solvent extractions are either processed immediately after pulping or incubated at low

temperatures to prevent proteins from deteriorating (Fernández et al. 1999; Chiesa and Gnansounou 2011). However, more N was extracted from those treatment combinations that were incubated for 16 h at 30 °C (DM.20.I and FP.20.I) compared to those that were not (FP.5.NI treatment combination), demonstrating that the lower PI mass yields relating to incubation time were a result of lower protein precipitation rates rather than lower protein extraction rates. This suggests that if the extracted proteins were denatured, this did not reduce their solubility, but rather their rate of precipitation. Previous studies on the extraction of protein from seaweeds have also used long incubation times at above ambient temperatures (Fleurence et al. 1995; Wong and Cheung 2001a, b). However, these studies precipitated proteins using ammonium sulphate, rather than acid, and did not distinguish between extracted and isolated protein, making it difficult to elucidate any denaturing effects on protein precipitation. For the alkaline PI, the use of fresh and pulped biomass resulted in significantly higher mass yields compared to when dry and milled biomass was used. This effect has been established for leaves (Bals and Dale 2011; Chiesa and Gnansounou 2011) and suggests that the proteins extracted from *U. ohnoi* are similar in nature to those in leaves and are likely denatured during the drying process. However, as with the aqueous PI, differences in N yields in the alkaline PI stemmed from different protein precipitation yields of extracted N rather than differences in the amount of total N extracted by the alkaline solvent, suggesting that any denaturing of proteins limits precipitation, rather than extraction.

Overall, the PI protein yields obtained in this study (12.28–21.57 %) were moderate compared to other studies on seaweeds, which range from 7.8 to 48.0 % (Fleurence et al. 1995; Wong and Cheung 2001a, b;) and lower than those reported for other species of *Ulva* (26.8–36.4 %) (Fleurence et al. 1995; Wong and Cheung 2001b) (Table 2). These three studies applied methodologies that used dry and milled biomass, had high aqueous solvent to biomass ratios and a 16 h incubation time for the aqueous extraction. However, the key differences between these studies and this one were that extractions were repeated 5–6 times (as opposed to once here), the reducing agent 2-mercaptoethanol was used during the alkaline extraction to increase protein solubility by breaking disulphide linkages (not used here) and proteins were precipitated using ammonium sulphate (precipitated using acid here). While any or all of these may have been responsible for the higher PI protein yields, repeating extraction protocols 5–6 times and the use of 2-mercaptoethanol are unlikely to be transferred to larger scales for food or feed production because repeating extraction protocols on the same biomass would yield diminishing returns and the use of 2-mercaptoethanol is not permissible for the extraction of proteins for human or livestock consumption (Turhan et al. 2003). Alternative food grade reducing agents (cysteine-hydrochloride-monohydrate

and *N*-acetyl-*l*-cysteine) have been successfully used to improve protein isolation yields from seaweeds (Harnedy and FitzGerald 2013) and could be incorporated into protein isolation protocols at scale. Consequently, protein isolation yields may be increased beyond those reported here if a food grade reducing agent is used in conjunction with the use of fresh and pulped biomass.

As PI protein yields for seaweeds (up to 48 %) are considerably lower than those that are routinely achieved for terrestrial seed crops (>75 %) (Berk 1992; Ju et al. 2001), there is considerable scope to improve the PI protein yields of seaweeds. Further increases in PI protein yields will result from increasing protein precipitation efficiencies (i.e. increasing the proportion of extracted protein that is precipitated and isolated) and/or increasing protein extraction efficiencies (i.e. the total protein removed from original biomass). As this study is the first to quantify protein precipitation yields for a seaweed by quantifying the N and protein in original biomass, the total residual biomass and the PI, it is difficult to elucidate which parameters examined in other studies affect protein precipitation rates. However, there is evidence to suggest that further improvements in extraction efficiencies of fresh biomass protocols can be achieved. For example, novel cell disruption techniques, such as enzyme preparations, microwave, and ultrasound, have improved the extraction efficiencies of protein and other cellular-bound components in dry seaweed biomass (Harnedy and FitzGerald 2013; Kadam et al. 2013) and fresh terrestrial leaves (Barba et al. 2015; Šic Žlabur et al. 2016). These could provide similar improvements for fresh seaweed biomass. Indeed, Le Guillard et al. (2016) extracted 54 % of protein (as TAA) from the red seaweed *Grateloupia turuturu* using a combination of enzymes and ultrasound on fresh biomass. Alternatively, other methodologies that are based on completely different principles to traditional solvent extractions could be explored for seaweeds, such as hydrothermal liquefaction (Yu et al. 2011; Jazrawi et al. 2015;).

The concentrations of protein in the total PIs in this study (47.43–56.04 % dw) were approximately 20–40 % lower than those of other studies on seaweeds (range from 69.2–81.2 % dw as TAA), which included a PI from *Ulva lactuca* (73.9 % dw) (Table 2). Despite this lower concentration of protein, PIs from this study had similar concentrations of total essential amino acids (1–30 % lower here) (Table 2). Moreover, they had 20–90 % more methionine than PIs obtained from *Sargassum* spp. (Wong and Cheung 2001a) and 120–150 % more methionine than the PI obtained from *U. lactuca* (Wong and Cheung 2001b) (Table 2). In contrast, the concentration of lysine was lower compared to other studies (3–50 % lower here) (Table 2). These differences in the concentration of protein, total essential amino acids, methionine and lysine may be associated with the use of the reducing agent 2-mercaptoethanol, which is used to improve protein solubility by breaking di-sulphur bonds, or the use of ammonium

sulphate for protein precipitation, as these were the two major differences between the extraction protocols used in this study and by Wong and Cheung (2001b). Irrespective, the concentration of protein, total essential amino acids, methionine and lysine in the PIs in this study were 250–400 % higher than the original seaweed biomass.

Protein in the original seaweed biomass can be concentrated in the total residual biomass after aqueous and alkaline extractions if more non-protein material is removed relative to protein. The aqueous extraction removed considerably more non-PI mass (supernatant mass yield) than the alkaline extraction for all treatment combinations. For the aqueous extraction, a higher aqueous solvent volume-to-biomass ratio (20:1) and a 16 h incubation time extracted the most non-PI mass for both types of starting material. Most of this non-PI mass was ash (~50 %) and non-precipitated protein (~30 %). The remaining extracted material (~20 %) represented approximately 10 % of the original biomass and was likely soluble fibre, which constitutes 11.3–12.6 % dw in *U. ohnoi* (Magnusson et al. 2016; Mata et al. 2016). The co-extraction of protein with ash and soluble fibre during the aqueous extraction step resulted in only a moderate (30–40 %) increase in the concentration of protein and essential amino acids in the aqueous total residual relative to the original biomass (Table 3). Correspondingly, the concentration of protein, total essential amino acids, methionine and lysine in the aqueous total residuals were 55–70 % less than the concentrations in the PIs. These findings demonstrate that protein isolation protocols are more suited to seaweeds with low concentrations of protein, such as *U. ohnoi*, compared to protein concentration protocols. However, more promising results have recently been reported for *U. lactuca* when dry and milled seaweed was incubated in an aqueous solution at high temperatures and subjected to enzymatic hydrolysis of carbohydrates (Bikker et al. 2016).

One novel outcome for the concentration of protein resulted from the analysis of the aqueous FP pellet portion of the total residual for the leaf protocol treatments, which was high in N, protein and essential amino acids. Any protein-rich residual components could be combined with aqueous PIs to improve yields, without detracting from the quality of the PI. Alternatively, as there was little difference in the concentration of N and only a small increase in the concentration of ash between the aqueous and alkaline total residuals, the aqueous total residual could likely undergo an alkaline extraction step without a significant reduction in the concentration of protein or ash in the final residual biomass. This would allow for co-production of an alkaline PI along with a protein-concentrated residual biomass (alkaline total residual). However, as the yields for protein isolation improve, it follows that the concentration of protein and essential amino acids in the final alkaline residual biomass will decrease. A similar process has been proposed for terrestrial leaves (Sinclair 2009), where

relatively low protein extraction yields ensure that enough protein is retained in the residual to be utilised as a feed. However, this is a new concept for seaweeds and there are clearly many stepwise protocols to consider when processing seaweed biomass, with a caveat that these should be viable at scale.

Both PIs and total residuals had higher concentrations of protein, TEAA, methionine and lysine compared to the original seaweed biomass; however, only the PIs are suitable as protein sources for mono-gastric livestock. This is because PIs had concentrations of protein, TEAA, methionine and lysine that were 5–70 % higher than soybean meal, but total residuals had concentrations that were 45–60 % lower than soybean meal (Angell et al. 2016). Instead, the total residual biomass after either aqueous or alkaline protein extractions may be suitable as a feed for ruminant livestock due to their reduced ash (salt) content (Masters et al. 2007).

In conclusion, the protein isolation protocols in this study increased the concentration of protein, total essential amino acids, methionine and lysine ~3 to 5-fold compared to whole *U. ohnoi* and were considerably more effective than the different protein concentrating combinations, which only increased protein and amino acid concentrations by 30–40 %. The use of fresh and pulped biomass as the starting material, an incubation time of <1 min at ambient temperature and a low aqueous solution volume resulted in the highest protein isolate yield of 22 % of the protein found in seaweed. Consequently, this study has demonstrated that proteins from the green seaweed *U. ohnoi* were most effectively isolated by adopting methodologies for terrestrial leaves. However, the best protein isolate yields are lower than other studies for seaweeds, and together, they are considerably lower than those for terrestrial seed crops, both of which infer that the physiology and morphology of the seaweeds will need to be considered on a case-by-case scenario. Regardless of species-specific responses to protein isolate protocols, this study highlights the need to develop cell disruption and extraction techniques to improve protein yields to deliver on the paradigm of using seaweeds as an alternative protein crop.

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