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Enrichment of the protein content of the macroalgae *Saccharina latissima* and *Palmaria palmata*

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ABSTRACT

The large brown seaweeds (kelps) are potential sources of protein for animal feed. They have lower protein contents than most red and green algae, but due to potential for large-scale production, they may represent a significant future protein source. The impact of pH, temperature and polysaccharide-degrading enzymes on the solubility and extraction yields of protein from wet *Saccharina latissima* biomass was investigated. The protein solubility increased with increasing pH and reached maximum of 23% at pH 11, determined as total amino acids (TAA). The enzyme treatments increased the release of soluble compounds by 30–35%. The highest protein yield obtained was 19%, using a ratio of water to wet seaweed of 1:1 for extraction. Even if the yields can be increased by increasing the water amounts used for extraction, the majority of the protein would remain in the insoluble residue after separation. The strategy for production of a larger quantity of protein-enriched biomass was therefore to maintain the insoluble fraction as the product. A pilot scale production was carried out, also including the red algae *Palmaria palmata*. In total 750 kg *S. latissima* and 195 kg *P. palmata* were processed. The protein content in the product increased from 10 to 20% of dry weight (dw) for *S. latissima* and from 12 to 28% for *P. palmata*, with yields of 79 and 69%, respectively. The ash content was reduced from 44 to 26% and from 12 to 5% of dw, respectively, for the two species. The main protein loss was free amino acids, which constituted approximately 10% of TAA in the feedstocks. Less essential than non-essential amino acids were lost, thus, the essential amino acids were enriched in the product.

1. Introduction

Sustainable protein sources for animal feed and human food are needed to feed the growing global population. The demand for plant-based protein sources is therefore increasing, also reflected in the current trends towards reduced consumption of animal-based foods. In parallel, seaweed cultivation is an expanding industry, with the annual global production increasing from 10.6 to 32.4 mill. tonnes fresh weight from 2000 to 2018 [1]. The majority of the seaweed is produced in Asia. The European production of cultivated seaweed is only a few thousand tonnes [2] but is rapidly increasing, and the long Atlantic coast line, from Portugal to the Barents Sea, represents an enormous potential for cultivation and harvesting of seaweed. As a source of protein for high-volume applications, like animal feed, the big brown algae (kelps) are the most relevant, due to their high productivity. The brown algae have a relatively low protein content, typically 5–15% of dry weight (dw) [3,4]. However, due to potential for large-scale production, they nevertheless represent a significant future protein source. Due to the ease of cultivation, *Saccharina latissima* (sugar kelp) is so far the prominent cultivated species in Europe.

Protein sources used in animal feed contain at least 40% protein, for instance soybean meal 40–50% and soy protein concentrate and fish meal 60–70%. For application of brown-algal protein in animal feed, a product with a higher protein content than present in the biomass is

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therefore needed. Kelps also have a high ash content, which for *S. latissima* may exceed 40% of dry weight in spring [3] and a high iodine content (0.4–0.6% of dw) [5,6]. The contents of these components need to be reduced to allow high inclusion levels in food or feed, for palatability and safety, respectively.

Since feed protein has a low price, the processing should be simple and cheap, and preferably be part of a biorefinery where other, higher value products also are produced to increase the income. Most reports on protein extraction from macroalgae are on red algae, since these have a higher protein content than brown algae. The most frequently used method for protein isolation in laboratory scale is a two-step extraction, first by water, then by high pH, followed by isolation of the dissolved protein by precipitation, as described for green and red algae [7–9], and more recently for brown algae [10–12]. The protein extraction yield was 40–50% before the precipitation for *S. latissima* using the Lowry-method for protein quantification [12], and 56% based on total nitrogen for *Ascophyllum nodosum* without precipitation [11]. Isolation of the extracted protein by precipitation resulted in a purer product, but the yield was reduced to 16% [12]. The yields using water extraction at native pH were 15–20% for *A. esculenta* based on total nitrogen [13]. Low solubility of the brown-algal protein in water is the main limiting factor for the extraction yields. The solubility of *S. latissima* protein at native pH was 45–50%, but increased with increasing pH, to 100% at pH 12 [12]. Another challenge is the matrix polysaccharide alginate, which binds water and creates viscous solutions that limits the allowable solid concentrations during processing, imposing increasing volumes and costs for an industrial process.

The red algae *Palmaria palmata* (dulse) is reported to have a higher protein content and lower ash content than the kelps and have a lower content of indigestible compounds [14,15]. This species is therefore considered as more suited for food and feed applications than the kelps. Large-scale cultivation is, however, not yet established for *Palmaria*, and due to expected higher biomass costs, the species is likely to be more relevant as human food than a protein source for animal feed. Nevertheless, as the most abundant red algae in Northern Europe, *P. palmata* is a representative choice as a reference to brown algae for comparison of biomass properties and processing conditions.

Enzymes that break bonds between protein and other molecules, like polysaccharides and phlorotannins, may increase protein solubility. Polysaccharide degrading enzymes may additionally degrade cell walls and polysaccharide matrices and thus enhance the extraction yields. Treatment with xylanase and cellulase had significant effect on *P. palmata*, where the extracted soluble protein increased 4 to 11 times compared to water (neutral pH) and by 70% compared to the classical alkali extraction [8,16]. In these works, protein was determined in the extracts only, and no yields were calculated. Mæhre et al. [17] determined total amino acids in raw materials and extracts, and showed that the extraction yields increased from 29% by the alkali extraction to 70% using a pre-treatment with xylanase and cellulase. No reports on the use of enzyme treatment to increase extraction yields from brown algae seem to exist. Cellulase and alginate lyase have been used as pretreatment for fermentation to biofuels and other products [18–21], but the effect on protein release has not been reported.

The protein isolation protocols used in laboratory-scale result in low yields due to the low protein solubility and a considerable loss in the precipitation steps. They also apply large amounts of water, which must be removed by centrifugation or filtration. Furthermore, most studies have used dried biomass. Due to the high water content of the biomass (80–90%), drying is energy-demanding and expensive. For industrial applications, processing should therefore be carried out on wet biomass, to omit two drying steps. Thus, the present work has had two aims, first, to obtain more knowledge about the solubility of the *S. latissima* protein, as well as extraction yields from wet biomass, and secondly, to use the data as basis for development of a simple and scalable process for production of a protein-enriched product from the seaweed biomass, using a minimum amount of water. As part of the work, this process was demonstrated in a larger scale, also including *P. palmata* as a reference. Wet biomass, 750 kg *S. latissima* and 195 kg *P. palmata*, were processed for production of a protein-enriched ingredient for evaluation in animal feeding trials. Due to low extraction yields, the chosen strategy for the large-scale processing was to maintain the un-solubilised part of the biomass as the product, since the major part of the protein remained in this fraction. A protein-enriched product was obtained by removal of non-proteinous, soluble components, such as salts (ash), while maintaining as much as possible of the protein in a non-soluble state.

2. Materials and methods

2.1. Raw materials

Cultivated *S. latissima* was harvested at the coast of Trøndelag, Norway in June 2015 and May 2016, and wild *P. palmata* was collected in Bodø, Norway at several dates in the period December–March 2015–2016. The *Palmaria* biomass was cleaned of epiphytes and associated species, then briefly spray-rinsed with freshwater to remove sand and surface salt, and drained of surface water, before vacuum-packing and freezing at −20 °C until processing. *Saccharina* was cultivated on ropes and cleaning was not needed. This biomass was drained for seawater and stored in plastic bags at −20 °C until processing. The *Palmaria* batches were combined before processing and analyses (Table 1). For *Saccharina*, the batch from 2015 was used for all laboratory scale experiments, while the batch from 2016 was used for the large-scale processing. For small-scale laboratory tests, portions of 3–5 kg wet biomass were milled frozen in a hammer mill with 10 mm sieve (Schutte Mini Mill, Buffalo, NY, USA). The milled biomass was split in smaller portions that were thawed before each experiment. For pilot-scale processing, milling was an integrated part of the process (Section 2.3).

2.2. Protein solubility and extraction in laboratory scale

For tests of suitable dw-concentrations in the extraction experiments, 2, 5 and 8% dw were obtained by adding 32, 20 and 10 mL deionised water to 8, 20 and 30 g thawed, milled *Saccharina* biomass (water to biomass ratios 4:1, 1:1 and 1:3, respectively) in 50 mL plastic centrifuge tubes. In the standard protocol for tests of other parameters, 5% dw was used, obtained by adding 20 mL deionised water to 20 g *Saccharina* and 30 g water to 10 g *Palmaria* (1:1 and 3:1), respectively. pH was adjusted when required using NaOH or HCl. When enzymes were used, these were added after pH-adjustment. The total mass was always ~40 g, including pH-adjustment and enzyme additions. The seaweed suspensions were incubated on a rotary mixer (Labinco LD76, Labinco BV, Breda, The Netherlands). Incubation times and temperatures are described for the respective experiments. After incubation, the tubes were centrifuged (3500 ×*g*, 15 min, ambient temp.). The wet mass of pellets and supernatants were recorded before dw-determinations and freeze-drying for analyses of N and total amino acids.

Enzymes used were Alginate lyase (Sigma A1603) ("Aly-1"), 0.5 mg/g dw seaweed; Alginate lyase AL951, provided by CEVA, France ("Aly-2"), 0.2 mg/g dw seaweed; Cellulase Cellic CTec2® (Sigma SAE0020), 2

Table 1
Composition of raw materials.

Species	Harvesting date	Dry weight (dw) [% of ww]	Ash [% of dw]	Total aa [% of dw]	Total N [% of dw]
S. latissima	June 2015	10.5 ± 0.5	43.8 ± 0.1	11.3 ± 0.3	1.98 ± 0.02
S. latissima	May 2016	9.7 ± 0.8	44.6 ± 0.6	10.1 ± 0.8	1.97 ± 0.04
P. palmata	December 2015 – March 2016	20.8 ± 1.7	10.5 ± 0.1	11.6 ± 0.2	2.95 ± 0.03

μL/g dw seaweed; Xylanase (Sigma X2629) 12.5 mg/g dw seaweed. The enzyme concentrations were selected based on previous studies, eg. alginate lyase from Sigma [18,20,21], cellulase [20,21], and xylanase [22], and recommendations from CEVA for their alginate lyase. When single enzymes were used, pH and temperatures were for Aly-1: pH 6.5, 40 °C; Aly-2: pH 6.5, 30 °C; Cellic CTec2: pH 5.5, 50 °C; Xylanase: pH 5.0, 24 °C, with incubation time 8 h. When alginate lyases and the cellulase were used simultaneously, the pH was 5.8, and the temperature was 30 or 40 °C for 5 h, before increasing to 50 °C for 3 additional hours. When they were used sequentially, initial pH and temperature were as for the single enzymes. Before the 2nd enzyme was added after 5 h, pH and temperature were adjusted to 5.3 and 50 °C when the cellulase was added, and to 6.2 and 30 or 40 °C when the alginate lyases was added, before further incubation for 3 h. The incubation times were based on pre-tests using 3, 5, 8, and 12–16 h (over night) incubations. The changes after 5 h were not significant for the cellulase, xylanase and Aly-1, while for Aly-2, the reaction continued with decreasing rates at least until 8 h.

Released aqueous phase from the seaweed, denoted "seaweed water", was calculated as the supernatant mass minus added water and presented as the %-fraction of the water in the unprocessed biomass. Soluble protein (total amino acids, TAA) and N was calculated using the concentration of TAA and N in the supernatant multiplied by the total amount of water in supernatant and pellet (i.e. assuming an equal distribution of the soluble compounds in all water), and presented as the %-fraction of total N and TAA in the sample.

2.3. Large scale processing

The seaweed biomass was processed in a pilot-scale facility containing a coarse grinder (Monster, Stette AS, Skodje, Norway), a fine grinder (Ultra 2 MEW 623, Mado GmbH, Schwarzwald, Germany), a scraped surface heat-exchanger (Votator 2, Waukesha Cherry-Burrell, Delavan, Wisconsin, USA), a 1000 L stirred tank, and a continuous tricanter centrifuge (Z23–3, Flottweg, Vilsbiburg, Germany). Frozen batches (20 kg) of seaweed biomass were moved to a cold room (4 °C) one day before start of the processing, to soften the biomass before milling. For *S. latissima* (2016) 750 kg was processed, divided in two batches. For each batch of 375 kg, 150 L tap water was added after milling and transfer to the tank. To reduce the risk for microbial growth during incubation, the slurry was heated by recirculation via the heat exchanger (70 °C) and back to the tank until 65 °C was reached (~25 min), followed by a holding time at 65 °C for 10 min. The remaining part of the water and approximately 20 kg ice was added to cool the biomass since the tank had no cooling system. The total time above 50 °C was less than 45 min. The pH was adjusted to 6.2–6.3 by addition of H_2SO_4, and alginate lyase (Aly-2) was added (0.3 g/kg dw seaweed). The total amount of added water per batch summed up to 558 kg (water to biomass ratio 1.5:1), resulting in a final dw of 3.9%. The biomass was incubated for 15 h at 28–32 °C with continuous stirring. The milled *P. palmata* biomass (195 kg) was added ~550 L tap water (to 4.9% dw), and pH was adjusted to 4.5–5 by H_2SO_4. Due to a shorter incubation time and lower pH, no heat-treatment was included. Incubation with xylanase (Sigma X2629, 5.4 g/kg dw seaweed) was performed at pH 4.5–5, 30 °C for 5 h. For practical reasons and caused by reduced accuracies due to the handling of the large volumes, some of the incubation conditions (time, temperature, or enzyme concentration) deviated from laboratory-scale. Based on the pre-tests (Section 2.2) and the lab-scale data (Section 3.3), these deviations are not expected to have had significant effect on the results.

After incubation, the biomass slurries were separated in a tricanter (2822 ×g, 3.3 min retention time). The liquid phases were discarded, while the sediment phases (353 and 75 kg respectively) were frozen at −20 °C for later drying (not a part of this work). Samples for analyses (5–10 mL) were collected at different stages of the process (after milling, after enzyme treatment, and from the solid and liquid phases after separation), frozen at −20 °C and freeze-dried.

2.4. Analytical methods

2.4.1. Dry weight, ash and nitrogen

The dry weight of raw materials and samples was determined gravimetrically after drying at 105 °C until constant weight (typically 24 h). Ash content was determined after heating dry samples at 600 °C for 12 h. Total nitrogen (N) was determined using a CHNS-O elemental combustion system (Costech Instruments ECS 4010, Valencia, USA). The measurements were performed using 3–4 replicates.

2.4.2. Amino acids and protein

Freeze-dried, ground samples were hydrolysed in 6 M HCl containing 0.4% mercaptoethanol for 24 h at 110 °C. The samples were filtered, pH was adjusted to 2.2, and the samples were further diluted with citrate buffer (pH 2.2) for the HPLC analysis. The acid hydrolysis converts glutamine and asparagine to glutamic and aspartic acid, respectively, and tryptophan is partly degraded. The hydrolysed samples were analysed as described by Forbord et al. [3]. The total amino acids are presented as the sum of amino acids, not corrected for water added during hydrolysis. In laboratory-scale experiments with *P. palmata*, protein was determined using the "Lowry-assay" in microwell format [23]. For analysis of free amino acids in the supernatants, the samples were treated with 10% sulphosalicylic acid to precipitate proteins before analysis using pre-column *o*-phthaldialdehyde derivatization and reverse-phase high-performance liquid chromatography [24,25]. The analyses were performed using 2–3 replicates.

2.4.3. Iodine

Iodine in raw materials and the products after large-scale processing were determined according to Roleda et al. [6].

2.5. Statistics

To determine significant differences between means, Student's unpaired two-tailed *t*-test, assuming equal variances was performed for experiments performed in triplicates, using GraphPad Prism (version 9.2.0 for Windows, GraphPad Software, San Diego, California USA.

3. Results

3.1. Biomass composition

The protein content on dry weight basis determined as TAA was similar (10–11% of dw) in the two batches of *S. latissima* and the combined *P. palmata* batches, while the N content was ~50% higher in *Palmaria* (Table 1). However, due to the higher dry weight, the amino acid content of the fresh biomass was twice as high for *Palmaria* as for *Saccharina*, and the N-content three-fold higher. The ash-content was 44% of dw in *S. latissima*, four-fold higher than in *P. palmata*. We have used TAA as a measure for the protein content. By the additional determination of total N, more information about the nature of the N-containing compounds of the biomass was obtained. For instance, the higher N-content indicates a considerably higher content of non-protein N in the *Palmaria* batch than in the two *Saccharina* batches.

3.2. Impact of pH and temperature on S. latissima protein solubilities and extraction yields

Since the volume of added water will affect the extraction yields, three dry-weight concentrations (2, 5 and 8%) were compared at native pH (6.5–7) to select suitable conditions for the studies. Only total N was analysed in this experiment. The solubility was 30–35% at all conditions, when the yields, as expected, increased with increasing amounts of added water, from 18 to 26%. At the lowest dw-concentration, i.e.

when most water was added, the biomass absorbed water (Fig. 1), probably due to osmotic effects. In this case, the volume of supernatant after centrifugation was smaller than the volume of added water. A dw-concentration of 5% was selected for the further work, corresponding to a 1:1 ratio of added water to wet biomass. This relatively low water amount was selected for comparison of extraction conditions, since changes in water-binding abilities and texture will be more easily detected than at lower biomass concentrations.

In total five independent experiments were run, including 4–10 different conditions in each, some as screening without replicates, and some with replicates. The results from four of the experiments are presented, including the screening test of biomass concentrations presented above, and effects of pH, temperature and enzyme treatments, presented below. Additionally, an experiment to identify suitable incubation times for the enzyme treatments was performed. In general, the standard deviations between replicate samples from one experiment were small, typically ±5% for N and < ±2% for TAA, while the variation between the same condition in independent experiments was higher, up to +/− 8–10% for both. The reason for this is most likely small sample amounts and heterogeneity of the biomass, which will affect the amounts of protein and water, both used for the calculations. Therefore, only clear differences and trends are discussed.

The solubility of N-containing compounds and total amino acids increased with increasing pH, to maximum values of 23% for TAA and 39% for total N at pH 11 (Fig. 2a). For the large-scale processing, a heating to ~65 °C was included to reduce the risk for bacterial growth during processing. This temperature could in theory either increase the protein solubility, or reduce the solubility due to denaturation. A test where samples were incubated for 1–4 h at 65 °C was therefore performed. The high temperature had no significant effect on TAA or N solubility or yields, neither positive nor negative (Fig. 2). The solubility of the total dry matter calculated in the same way was 10% higher after incubation at 65 °C than at room temp (63–65% vs 57–61%). Based on all data, the solubility of the protein as TAA was approximately 20% at native pH. The solubility of total N was higher, on average approximately 35%, probably due to the presence of soluble, non-proteinous N-containing compounds. Free amino acids were analysed in some of the experiments and were 100% soluble.

The solubility represents the theoretical maximum extraction yield at the actual condition. The real yield will be lower, as it also will be determined by the amount of water used for extraction and the separation efficiency between undissolved solids and the liquid phase. The water-binding ability of alginate, the dominating polysaccharide in *Saccharina* harvested in May–June, decreases with decreasing pH. As a measure for changes in the water-binding ability and texture that may affect the separation efficiencies, we calculated the amount of aqueous phase from the seaweed ("seaweed water") that was released to the supernatant after centrifugation, and also plotted the pellet masses. In accordance with decreasing water binding capacity, the amount of seaweed water released during centrifugation at standard conditions increased with decreasing pH, and the pellet masses decreased (Fig. 2b). However, the increasing solubility with increasing pH more than compensated for the reduced liquid release, resulting in increasing protein yields with increasing pH (Fig. 2c). The temperature had no significant effect on pellet masses and liquid release. The highest yield of solubilised protein and other N-containing compounds in the supernatant was obtained at pH 11 (Fig. 2c) and was 16 and 29% for TAA and N, respectively.

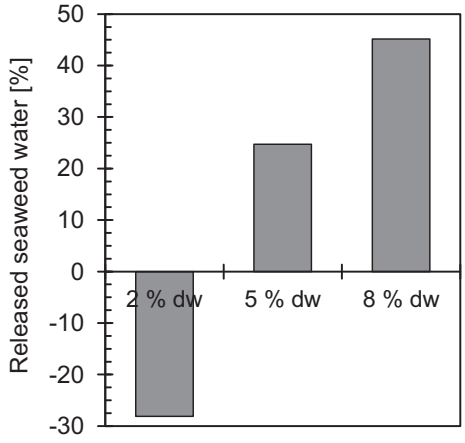

Fig. 1. Seaweed aqueous phase ("seaweed water") released by centrifugation at three biomass concentrations, presented as % of the biomass water content. The biomass concentrations correspond to approx. 19, 48 and 76% ww (water to biomass ratios 4:1, 1:1 and 1:3).

3.3. Enzyme treatment for increased extraction yields

As an attempt to increase the extraction yields, biomass samples were treated with alginate and cellulose-degrading enzymes to disrupt the cell walls and the alginate matrix. The enzymes were used individually and combined. Due to different pH optima for the cellulase (5–5.5) and the alginate lyases (6–7), the enzymes were tested simultaneously at intermediate conditions and sequentially at their optimum conditions. Since pH affects the protein solubility, a control without enzyme, incubated at pH 5.8, was included. The cellulase and the two alginate lyases all improved the separation efficiencies, shown as significantly decreased pellet mass, compared to the control without enzyme (Fig. 3a). However, combinations of cellulase and alginate lyase, either simultaneously, or sequentially with the alginate lyase first, had a far more pronounced effect. Combinations were the cellulase was used first, were less efficient. The combined alginate lyase and cellulase treatment increased the dry-weight yields in the supernatants by 30–35% (Fig. 3b). The trends were less clear for the TAA and N-yields, with no significant differences between one or two enzymes. The TAA-yields only partly correlated with the solubilities (Supplementary data, Fig. S1), but in general, the highest TAA solubilities (24%) and yields (20%) were obtained for alginate lyase, alone and in some of the combinations with cellulase. The more pronounced effects of the enzymes on the dry weight yields than on the protein yields can be explained by the far higher solubility of total dry weight than of the protein, since only the soluble components will be extracted. The added enzymes will constitute a part of the protein in the soluble phase. Based on calculations assuming 50–80% protein in the enzyme preparations, maximum 5% of the measured TAA could come from the enzymes.

3.4. Composition of the protein fraction

Free amino acids constituted approximately 10% of the total amino acids in the raw materials. After extraction at pH 3.1, free amino acids constituted 60% of total amino acids in the supernatant. This fraction decreased to 40% at pH 11, due to the higher amounts of protein solubilised with increasing pH. In the supernatants after enzyme treatment and heating to 65 °C, the fraction of free amino acids was 40–44%. Alanine, aspartic acid and glutamic acid were the dominating free amino acids (Fig. 4a). While the free amino acids were enriched in the supernatant, the fraction of the typical protein amino acids, including all the essential, decreased (Fig. 4b), and these were thus enriched in the pellet. Taurine constituted 2.9 mg/g dw in the raw materials and 2.5% of TAA, but was not determined in the analysis of free amino acids and is not included in the graphs.

3.5. Impact of enzyme treatment on protein extraction yields from *P. palmata*

A limited number of laboratory-scale experiments were carried out with the red algae *P. palmata*. The experiments were performed in a

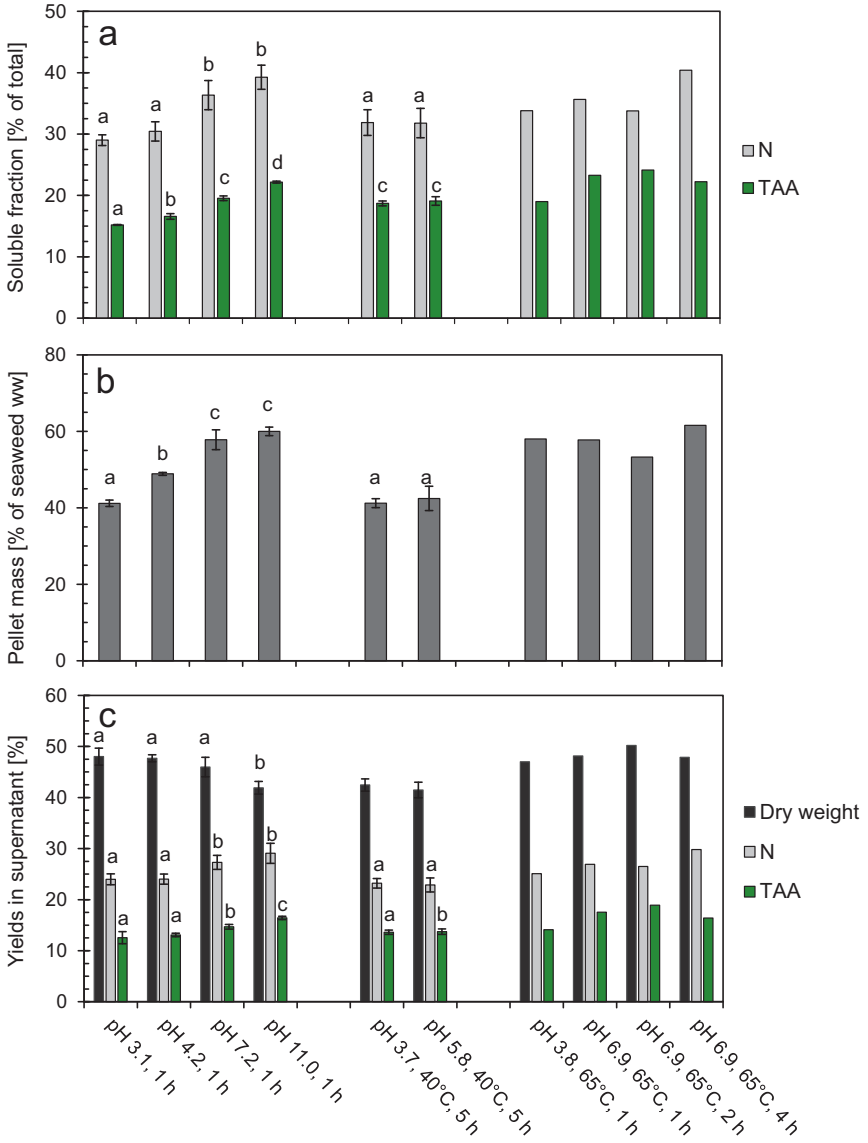

Fig. 2. Effect of pH at room temperature, and temperature at different pHs, on protein solubility and extraction yields. a) Solubility of N and total amino acids (TAA); b) Pellet mass after centrifugation at standard conditions; c) Yields of dry weight, nitrogen (N) and TAA in the supernatant after centrifugation.
The samples incubated at 65 °C were further incubated at 30 °C over night (15–17 h). Error bars: average with SD for 3 replicates. No replicates at 65 °C. Letters indicate significant different values.

similar way as for *Saccharina* using ~5% dry weight during extraction, obtained by addition of water in the ratio 3:1. The pellet mass after centrifugation was only 27%, compared to ~50% for *Saccharina*, probably an effect of the lack of alginate and a higher content of soluble components in *Palmaria*. Treatment with a xylanase reduced the pellet mass from 27 to 12% of the seaweed mass (Supplementary Data Fig. S2), compared to the reduction from 50% to 20–22% for *S. latissima* after treatment with alginate lyase and cellulase. The *Palmaria*-experiments were carried out in an early phase of the work, when protein was determined using the Lowry-assay. The determined protein solubility and yields are therefore not directly comparable to the results for *Saccharina*, but provides an indication of the effect of the enzyme treatment. At native pH, approximately 40% of the protein was soluble, and the yield in the supernatant was 32%. The xylanase treatment increased the protein yield in the supernatant from 32% without enzyme, to 46% after 2 h and 49% after 22 h incubation with enzyme.

3.6. Large-scale processing for protein enrichment

The protein extraction yields from *Saccharina* using a 1:1 dilution with water and native pH was 15–20% as TAA and 25–30% as N. The yields in the supernatant can be increased by increasing the extraction (water) volumes, but can never exceed the solubility, which was maximum 22–24% and 35% for TAA and N, respectively, at the experimental conditions applied. Thus, the majority of the protein would remain in the insoluble fraction after solid-liquid separation. These yields were obtained using a laboratory bucket centrifuge, which gave a packed pellet. For the large scale processing, which utilised a continuous tricanter centrifuge, more liquid would remain in the sediment phase and the extraction yields would be even lower. Furthermore, the extracted protein would need isolation from a diluted solution of several thousand litres. We therefore decided to maintain the sediment fraction as product, rather than the extracted protein. Although higher extraction yields, the same strategy was chosen for *Palmaria*, due to the large water volumes needed for an efficient extraction.

S. latissima, 750 kg wet weight, and *P. palmata*, 195 kg wet weight, were processed. The biomass dry weight during processing was ~4 and ~ 5% for *Saccharina* and *Palmaria*, respectively. The alginate lyase Aly2 (alone) and the xylanase were used to facilitate solid-liquid separation. This would increase the protein loss to the supernatant, but since more of the polysaccharides will be solubilised and removed, the protein concentration of the sediment will increase. Total amino acids and nitrogen were determined in the liquid phase ('supernatant') and the sediment phase, enabling a complete material balance (Table 2). For

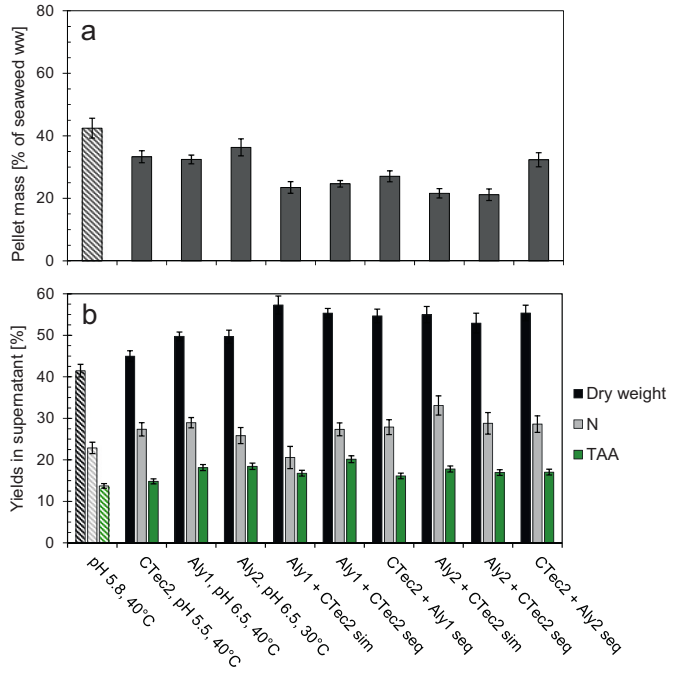

Fig. 3. Effect of treatment with alginate lyases and cellulase on pellet mass and extraction yields.
a) Pellet mass after centrifugation at standard conditions; b) Yields of dry weight, nitrogen (N) and total amino acids (TAA) in the supernatant after centrifugation. Hatched bars: Control without enzyme.
Conditions when the alginate lyases and cellulase were used simultaneously: pH 5.8, temperature 30 or 40 °C for 5 h, before increasing to 50 °C for 3 additional hours. Used sequentially: Initial pH and temperature as for the single enzymes. Adjustment after 5 h before the 2nd enzyme was added to pH 5.3 and 50 °C when the cellulase was added, and to 6.2 and 30 or 40 °C when the alginate lyases were added, before further incubation for 3 h. Error bars: Average of two replicates with variation range.

Saccharina, 63% of the N and 90% of TAA remained in the sediment after centrifugation. The values for *Palmaria* were lower, 72 and 69% respectively, in agreement with the higher solubility of the *Palmaria* protein obtained in the laboratory-scale experiments. The amino acids that were lost to the liquid phase, were mainly alanine, glutamic acid and aspartic acid for *Saccharina*, and glutamic and aspartic acid for *Palmaria* (Supplementary Data, Fig. S3 and S4). In addition to the analysed compounds, the removed, soluble components would include mannitol for *Saccharina* and soluble polysaccharides for *Palmaria*. Approx. 15% of the essential amino acids were lost for *Saccharina* and 25% for *Palmaria*, compared to the loss of 31 and 41% of TAA. The essential amino acids were thus enriched in the sediments, by 9% for *Saccharina* and 18% for *Palmaria*. The protein content as TAA increased from 10.1% of dw in the raw material to 19.6% in the product (sediment) for *S. latissima*, and from 11.6 to 27.7% of dw for *P. palmata* (Fig. 5). For *Saccharina*, mainly ash was removed, resulting in an increased content not only of protein, but also other organic compounds, which includes alginate, cellulose, and membrane lipids. For *Palmaria*, the content of other organic compounds was reduced, since the major part of the polysaccharides are soluble. The iodine of the *S. latissima* biomass was reduced by 80%, from 6 to 1 g/kg. The iodine content was reduced relatively more than the ash content, indicating that iodine was looser bound than the salts in general.

4. Discussion

The amino acid composition of the two species applied in the work was the same as reported in previous studies of seaweed harvested in Norway, and the total content of amino acids were similar to the values reported for biomass harvested at the same time of the year [3,14,26–28]. We had expected a higher protein content in *P. palmata* than in *S. latissima*, but TAA-content of 10% in *Palmaria* biomass harvested in the autumn has been reported previously [27]. A batch harvested in May at the same location as the one used in our study, contained 19% TAA [26], similarly to the value for spring harvesting reported by Gaillard et al. [27]. Free amino acids constituted approx. 10% of the total amino acids in unprocessed biomass. The levels were similar to those reported for *A. esculenta* and *P. palmata*, 10.1, and 9.2% of TAA, respectively [29]. The dominating free amino acid in *S. latissima* was alanine, followed by glutamic and aspartic acids, also in accordance with the data for *A. esculenta* [29].

We analysed both total amino acids and total N. Total N is reported as the actual, measured value, without conversion to protein, since the conversion factor will change during processing and be different for the supernatants and the pellets/sediments. Considering the raw materials, the factor TAA/N was 5.1 and 5.7 for the two *Saccharina* batches, but only 3.9 for *P. palmata*. For the batch harvested in May at the same location, the ratio was 4.7 [26]. The low ratio in the winter-batch used in the present work is probably caused by a high content of nitrate, which has its maximum from November to March [30]. Considering the products from the pilot-scale processing, the ratio was 6.3 for *Saccharina*, but still only 3.8 for *Palmaria*. This shows that for *Saccharina*, relatively more non-protein N than protein was removed to the soluble phase, while for *Palmaria*, protein and other N-containing compounds were removed in the same ratio.

The low solubility of brown algal protein hampers extraction. In our studies, the solubility of the *S. latissima* protein was in the range 15 to 24% based on total amino acids, while the solubility of total N was 29–40%. This is lower than reported by Vilg and Undeland [12], who found a solubility of 45–50% at native pH, increasing to 100% at pH 12. The difference is most likely related to the biomass harvesting time. Their biomass was harvested in November, when the dw is around 20% due to a high content of laminaran, with a lower ash and protein content than in spring (eg [4]). However, the trends were similar, with increasing solubility with increasing pH and no significant effect of temperature. Wijers et al. [31] did not determine the solubility, but their N-extraction-yields from *S. latissima* varied from 30% at native pH to ~45% at pH 11–12, using biomass harvested in June and 3% dw during extraction, in agreement with our results. A likely reason for the general low solubility of seaweed protein is a high fraction of membrane proteins compared to other protein sources used in food and feed, which mainly are seeds, like soybeans and other legumes. The even lower solubility of protein from brown algae than for red algae may be due to cross-links with polyphenols, which occur in considerably higher concentrations in brown than in red algae [32]. We have used the total water in the biomass for calculation of the protein solubility. However, due to the presence of alginate in *Saccharina*, parts of the water will be bound in the gel and not available to dissolve protein. Since relatively small amounts of water were used for determination of the solubilities, the actual solubility could be higher. This can be verified by use of lower biomass concentrations.

Polysaccharide-degrading enzymes were tested for increased extraction yields. In theory, this should not affect protein solubility, unless breaking bonds to other molecules, like phlorotannins. The increased solubilities observed when using alginate lyases are probably due to release of proteins trapped by the alginate matrix, but an increasing amount of free water for protein solubilisation when the gel is disrupted, can also have contributed. The enzyme treatment reduced the pellet volumes significantly and increased the release of soluble compounds. Pre-treatment of brown algae using polysaccharide-degrading enzymes have been used for fermentation. The effect on protein has not been assessed, but alginate lyase in combination with cellulase had more effect on the rheology than cellulase alone [21], in agreement with our results, and indicates that the alginate lyase improves the access for

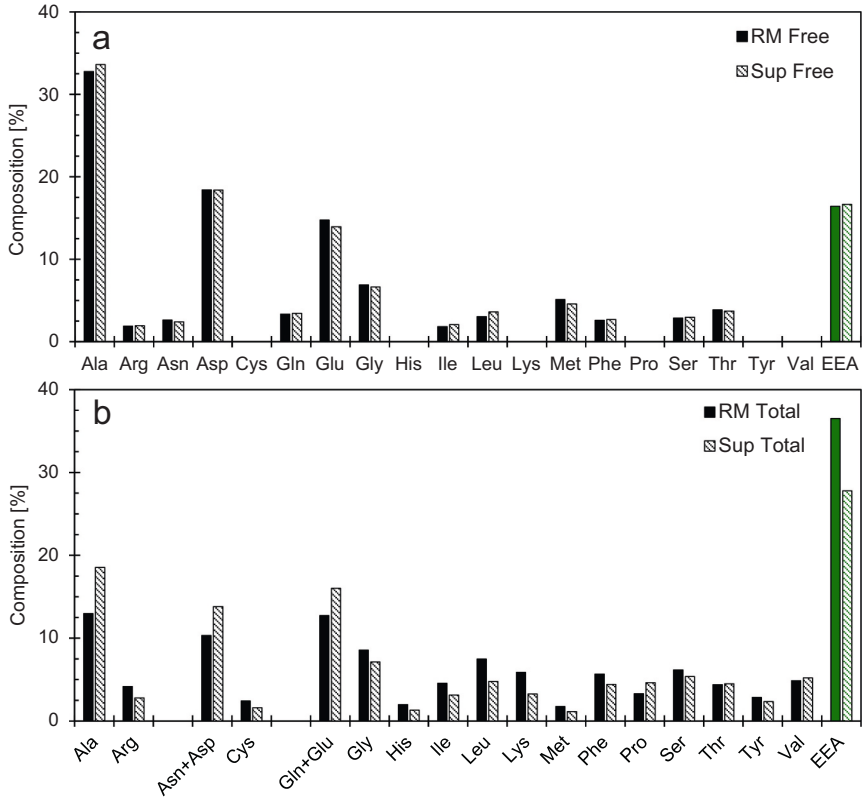

Fig. 4. Free (a) and total (b) amino acids in raw materials (RM) and supernatant (Sup). The supernatant data are average values for the treatment at 65 °C at pH 6.9 and samples from the enzyme treatment, in total 7 samples. Total amino acids were 110 mg/g dw in RM and 39 mg/g dw in Sup, excluding taurine, and free amino acids were 11 and 17 mg/g dw, respectively. EEA: Sum of essential amino acids.

Table 2
Material balances for recovered solids, ash and protein (as N and total amino acids) after separation. The masses of liquid phase and sediment include added water.

	Mass [kg]			Recovered [%]		
	Raw material	Liquid phase	Sediment	Liquid phase	Sediment	Total
S. latissima						
Wet weight	757	1527	353	–	–	–
Dry weight	73	38.3	28.9	52.4	39.6	92.0
Ash	32.6	24.2	7.9	74.2	24.2	98.4
N	1.4	0.44	0.90	30.9	62.8	93.7
Total amino acids	7.2	1.7	**5.7**	23.5	79.4	102.9
P. palmata						
Wet weight	195	662	**75**	–	–	–
Dry weight	37	15.5	9.8	42.2	26.7	68.9
Ash	3.4	2.8	0.47	82.7	13.8	96.5
N	1.0	0.2	0.72	18.7	71.8	90.5
Total amino acids	3.9	0.7	2.7	18.6	69.3	87.9

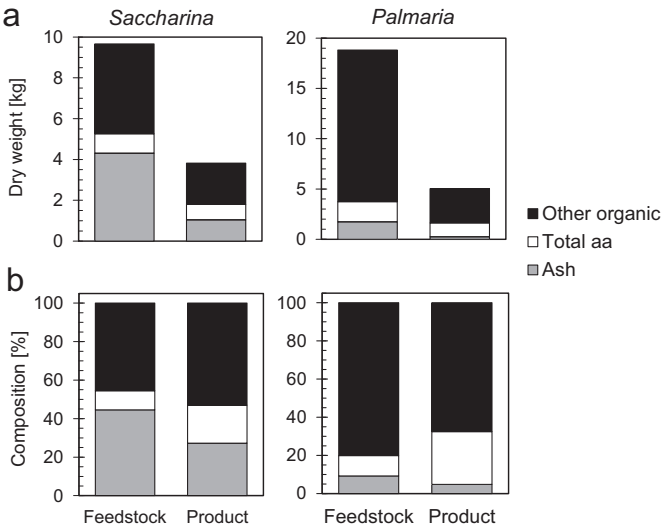

Fig. 5. Composition of feedstocks and products (sediments). a): Presented as total masses and b): as % of dry weight. "Other organic" is calculated as difference between dry weight and (ash + TAA).

the cellulase. It is likely that a large part of the soluble protein in the seaweed biomass are enzymes, including proteases. It can therefore not be excluded that endogenous proteases have contributed to solubilisation of the protein during the relatively long incubation periods used for the enzyme-treatments. However, since the heat-treatment of the biomass at 65 °C did not reduce the yields of solubilised protein, a possible contribution has not been significant. Only one concentration was used for each of the enzymes. The optimum concentrations with respect to effects and costs therefore need to be determined.

The extraction yields will be a function of the solubility, the released seaweed water, and the amount of water added for extraction. By using these as input variables in a simple calculation model (Supplementary

data, Table S1), assuming 100% solubility, 50% of the seawater released and water addition in a ratio 1:1 to the seaweed wet weight, the yield in the supernatant will be 76%. The yield for a compound with 20% solubility will be 15%. Increasing the released seaweed water to 80% will increase the yields to 90% for the completely soluble compound, but only to 18% for a 20% soluble compound. A pellet mass of 11%, as for *Palmaria*, 3:1 added water, and 40% solubility will give a yield of 40%. The extraction yields can be increased by using more water for extraction. The calculated yields assuming 10:1 added water and 80% seaweed water released are 98 and 20% for a 100% and a 20% soluble compound, respectively. This clearly illustrates that more extensive methods for solubilisation of the protein is needed for high-yield extraction of the brown-algal protein. The experimental values obtained are well in agreement with these theoretical calculations, also those obtained in pilot scale.

We used wet biomass, as a more industrially relevant raw material than dried biomass. The extraction yields might be higher from dried biomass due to disruption of cell walls and increased porosity caused by the drying, as well as easier milling to smaller particle sizes. Abdollahi et al. [33] showed considerably higher protein extraction yields from freeze-dried than from frozen, wet biomass. However, in a preliminary experiment using freeze-dried *Saccharina*, we obtained extraction yields of 9% at pH 7 and 17% at pH 11 based on TAA, and 17 and 28% based on N, i.e. in the same range as for wet biomass, using the same dw-concentrations (data not shown).

Due to the low extraction yields, the chosen strategy for production of a larger quantity of protein-enriched biomass was to maintain the protein-rich insoluble fraction as the main product. Removal of undesired compounds rather than extraction of the protein is a known technology used for other plant proteins, for instance production of soy protein concentrate. For the green macroalgae *Ulva ohnoi* the protein content, as TAA, was increased from 22 to 40–45% by this strategy, but with a yield of only 40% [34]. We recovered 79% of the *Saccharina* protein (as TAA) in the sediment, and the protein content of the sediment product was 20% of dw. The corresponding values for *Palmaria* were 69 and 28%, respectively. From a nutritional point of view, it is important that the essential amino acids were enriched in the product. Protein was not analysed in the pellets in the small-scale laboratory tests, but calculated as the difference between the raw materials and the supernatant, TAA in the pellets from *Saccharina* varied between ~20% of dw without enzyme treatment, and up to 35% after treatment with alginate lyase and cellulase. The higher yields and lower protein content in the sediment in large-scale production can be explained by a less efficient removal of soluble compounds, mainly salts, from the sediment phase due to the use of a continuous centrifuge. For *Palmaria*, the higher solubility of the 'non-protein' resulted in removal of a larger part of these components, and a higher increase in the protein content of the sediment.

Optimization of the process should involve identification of the optimum trade-off between the protein content of the product, yields and processing costs. The process can be combined with isolation of valuable compounds from the liquid phase, which easily can be recovered by membrane filtration. This would primarily include the polysaccharide fucoidan, but lost, soluble protein and peptides can also be recovered. For a further increase of the protein content of the sediment fraction of *Saccharina,* removal of the insoluble polysaccharides, alginate, and cellulose will be needed. This will require enzymatic degradation. Xylanases and cellulases are part of commercial enzyme blends for biomass conversions, for instance biofuel production, while no industrial alginate lyases are available. Since alginate is a valuable molecule, a chemical extraction could be considered, but this will imply a more extensive and expensive process for subsequent isolation of the protein. For application as food or feed ingredient, the digestibility needs further investigations. The insoluble fraction is likely to be less digestible than extracted and solubilised protein and further processing may be needed.

CRediT authorship contribution statement

IMA: Conceptualization, Methodology, Investigation, Supervision, Data Curation, Writing - original draft, Writing - review & editing. ISS: Methodology, Investigation, Data Curation, Writing - original draft, Writing - review & editing. BT: Methodology, Investigation, Writing - review & editing. MYR: Resources, Investigation, Writing - review & editing. RS: Methodology, Investigation, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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