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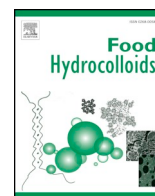
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Effect of stabilization method and freeze/thaw-aided precipitation on structural and functional properties of proteins recovered from brown seaweed (*Saccharina latissima*)



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ABSTRACT

Structural, functional and nutritional properties of protein recovered from brown seaweed, *S. latissima* with alkaline solubilization/isoelectric precipitation as a function of different post-harvest stabilization methods were studied. The latter included freezing at $-20\text{ }^{\circ}\text{C}/-80\text{ }^{\circ}\text{C}$, oven-drying, sun-drying, freeze-drying and ensilaging. Also, the efficacy of freeze/thaw-aided precipitation (F/T) in improving protein recovery of the process was evaluated. The freeze-dried, oven-dried, and $-20\text{ }^{\circ}\text{C}$ frozen seaweeds resulted in significantly higher protein yield than the $-80\text{ }^{\circ}\text{C}$ -frozen, sun-dried and ensiled biomasses. F/T increased protein precipitation and doubled total protein yield. Sun-drying and $-20\text{ }^{\circ}\text{C}$ -freezing caused extensive protein degradation as revealed by SDS-PAGE and HP-SEC, while oven-drying altered the seaweed protein structure with less α -helices. Functional properties of the seaweed proteins were remarkably affected by stabilization condition and F/T, but nutritional value of the proteins was only dependent on stabilization method. Thus, to efficiently recover seaweed proteins, its post-harvest stabilization condition must be carefully chosen based on the final application of the proteins.

1. Introduction

With new trends of vegetarian diets, there is a rise in the demand for vegetable protein sources, which to date mainly composes of legumes and cereals (Vilg & Undeland, 2017). These terrestrial protein sources, however, require arable land, fresh water and to a certain extent fertilizers, while an alternative sustainable protein source could be seaweed that grows in the sublittoral zone of the oceans without these demands (Kraan, 2013). One of the seaweed species that is easiest to cultivate and that quickly accumulates a large amount of biomass is the kelp *Saccharina latissima*. Unfortunately, protein content of kelp is relatively low (3–15% dry weight) (Fleurence, 1999), and consumption of whole brown seaweed plants may decrease the protein accessibility and digestibility due to the abundance of soluble fibers and polyphenols (Horie, Sugase, & Horie, 1995). To be used as a protein source for human, its protein should, therefore, be separated from non-protein components (Tamayo Tenorio, Kyriakopoulou, Suarez-Garcia, van den Berg, & van der Goot, 2018). If done using a multiple product biorefinery approach, it can possibly be economically interesting and even

compete with traditional plant-derived protein sources, such as soybeans (40% protein DW) (Vilg et al., 2015).

Freshly harvested seaweed has 70–90% moisture content and can deteriorate rapidly (Wong & Chikeung Cheung, 2001). Thus, it is crucial to stabilize and preserve the seaweed properly from the harvest-step until final usage or processing in large scale. Traditionally, fresh seaweeds harvested from the sea are dried before industrial processing. However, it has been reported that drying at high temperature can negatively affect the nutritional value of the brown seaweed (Chan, Cheung, & Ang, 1997). It has also been shown (Gupta, Cox, and Abu-Ghannam (2011) that a drying temperature already of $25\text{ }^{\circ}\text{C}$ reduced the total phenol and flavonoid content of brown seaweed by 49% and 51%, respectively, compared to fresh seaweed. On the other hand, when Wong and Chikeung Cheung (2001) evaluated the effects of oven- and freeze-drying on three *Sargassum* species, oven-drying significantly improved the protein extractability and the *in vitro* protein digestibility while protein from freeze-dried samples displayed significantly better physico-chemical properties. However, to the best of our knowledge, a comprehensive comparison of different stabilization techniques

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including freezing, drying and ensiling of seaweed and their impact on the yield of seaweed protein during an isolation process has not previously been systematically studied. Moreover, the relation between stabilization method and the functional properties of the proteins like solubility, emulsion capacity and oil/water absorption has not before been studied. The latter properties depend on the physicochemical and structural properties of the proteins and directly contribute to the taste, texture and consumer acceptance of the protein-containing food products (García-Vaquero, Lopez-Alonso, & Hayes, 2016).

Moreover, isolation of protein from brown seaweed is intricate due to a number of limiting barriers such as the polysaccharide-rich cell wall and abundance of polyphenols. Many of the reported processes to date are based on the fact that proteins from a homogenized sample have variable solubility in water depending on pH. By applying strong alkaline conditions, the algal protein gains a negative charge, increasing repulsion between protein molecules promoting the water interaction, i.e. increasing solubilization. Following a centrifugation step, protein can be then precipitated by drastically lowering the pH to the isoelectric point (pI) or by adding ammonium sulfate which however calls for an extra dialysis step. The latter resulted in protein yields of 6% for *Enteromorpha* (Kandasamy, Karuppiyah, & Rao, 2012), and 8% for *Kappaphycus* (Suresh Kumar, Ganesan, Selvaraj, & Subba Rao, 2014) while Vilg and Undeland (2017) achieved a protein yield of 16% for *S. latissima* and Harrysson et al. (2018) could obtain protein yields of 6.4% and 22.6% for *Ulva lactuca* and *Porphyra umbilicalis*, using isoelectric precipitation. The last studies illustrated how difficult it is to precipitate solubilized seaweed proteins, and only a fraction was recovered by the isoelectric precipitation. It has been reported that freeze/thawing of a protein solution can cause varying degrees of protein denaturation and precipitation depending on solute, electrolytes present, pH-shifting and freezing rate (Cao, Chen, Cui, & Foster, 2003). By using this concept, Hernández, Martínez, Hernández, and Urbano (1997) obtained protein concentrates of alfalfa press juice and, Xiong et al. (2017) successfully used freeze/thaw cycles for deproteinization of polysaccharides from *Cipangopaludina chinensis*. To date, this strategy has not been combined with classic pH-shift processing to increase protein recovery e.g. from seaweed.

Bearing these in mind, the present study aimed to evaluate the effect six different stabilization methods; freezing at $-20^{\circ}\text{C}/-80^{\circ}\text{C}$, oven-drying, sun-drying, freeze-drying and ensiling on the yield and quality of protein recovered from brown seaweed, *S. latissima*, using alkaline solubilization/isoelectric precipitation. Also, the efficacy of freeze/thaw-aided protein precipitation (F/T) in improving protein recovery of the process and its impact on nutritional and techno-functional properties of the proteins was evaluated.

2. Material and methods

2.1. Seaweed biomasses harvesting

Brown seaweed (*S. latissima*) seeded in the autumn of 2015 at Tjärnö, Sweden ($58^{\circ} 52' 31.931'' \text{N}$, $11^{\circ} 8' 47.434'' \text{E}$), was harvested in May 2016. The biomass was harvested by manually lifting the cultivation line, pulling the seaweed off and packing it in mesh bags. A part of the seaweed was hung on lines to dry outdoors for 10 days (day length of approximately 15–16 h), then transported in one big plastic bag before packing in smaller bags and stored at room temperature in darkness (called sun-dried biomass). The rest of the seaweed was then packed in plastic bags for the transport to Chalmers, Gothenburg, and stored at 4°C , overnight. The next day the seaweed was cut into smaller pieces (roughly $3 \times 3 \text{ cm}$) and used for each respective treatment:

Oven-drying: Biomass was dried in a heating cabinet at 40°C overnight, crushed by hand into small flakes, packed in Ziploc-bags and stored in darkness at room temperature.

Freezing: Biomass was frozen and stored in plastic bags at -20°C or -80°C .

Freeze-drying: Biomass frozen at -80°C was freeze-dried (FreeZone 6 L from LABCONCO, Kansas City, Missouri, USA) until constant weight, approximately 4 days of drying, vacuum-packed and stored at -80°C .

Ensilage: Approx. 10 kg of freshly harvested biomass was stored in buckets (modified to allow excess liquid runoff at the bottom and with a gas lock), with 20 ml added acid mix (formic acid/propionic acid, 65%/25% v/v) for 91 days, whereafter it was packed in Ziploc-bags frozen and stored at -80°C . At start and end of the ensiling process, pH was measured to be 4.42 and 4.32, respectively.

2.2. Protein isolation using the pH-shift method

Dry biomass (oven-, sun- and freeze-dried) was broken into small pieces and then milled using a coffee grinder (Rubicon 48068, 140W, Sweden) until a powder with a particle size $< 0.5 \text{ mm}$ was produced. Wet biomass (ensiled, -20°C and -80°C frozen) was minced using a food processor (KitchenAid 5KSM150) with a meat grinder attachment (Jupiter 478100) fitted with a 2 mm hole plate, packed in Ziploc-bags and stored at -80°C until used.

A modified version of the pH-shift method used by Vilg and Undeland (2017) was performed on all biomasses in duplicate (Fig. 1) which is called classic pH-shift process. For each separate trial, approximately 3.5 g dry weight (DW) of wet or dry seaweed was weighed and added to a 600 ml beaker. Before homogenization all biomasses were adjusted to a moisture content of 88.5%, the highest measured moisture content of the wet biomasses, by adding cold de-ionized water (DI-water). The moisture-adjusted biomass was mixed with 6 parts (w/w) of cold DI-water and homogenized using a polytron (Ultra-turrax® T18 basic, IKA®) for 2 min at speed 4 (18000 rpm). The seaweed slurry/homogenate was kept on ice at all time.

The biomass was then osmoshocked for 15 min while kept on ice, before native pH was measured under stirring using a pH-meter (MeterLab® PHM210 standard pH meter). NaOH (1 M) was added to adjust the slurry to pH 12 and the slurry was then left to incubate under stirring on ice for 20 min. After incubation, samples for measurements of total protein were taken from the homogenate. Then, the slurry was centrifuged (Sorvall® RC-5C Plus) at $8500 \times g$, 4°C for 20 min. The resulting supernatant (S1) was separated from the pellet (P1) using a sieve ($\sim 0.5 \text{ mm}$) and weighed. Samples of S1 were taken for protein measurements before 1 M HCl was added to adjust S1 to pH 2 and left to incubate while stirring on ice for 20 min. In the classic pH-shift process (Fig. 1), S1 was centrifuged directly at $8500 \times g$, 4°C for 20 min. The resulting supernatant (S2A) was separated from the pellet (P2A) using a sieve ($\sim 0.5 \text{ mm}$) and weighed. The pellet (P2A), i.e. the protein, was collected and stored at -80°C .

In the pH-shift process with F/T, S1 was frozen overnight at -80°C immediately after adjusting it to pH 2, and thawed the next day under cold running water and centrifuged $8500 \times g$, 4°C for 20 min. Samples from the resulting supernatant (S2B) and pellet (P2B) were taken for protein measurements.

2.3. Measurement of protein solubility and yield

Protein solubilization yield, precipitation yield and total yield were studied by measuring protein content in the initial homogenate at target pH (H), first supernatant (S₁) and second supernatant (S₂). Calculation was done using equation number 1 and 2.

$$\text{Protein solubilization yield (\%)} = \frac{\text{Protein content of S1} \left(\frac{\text{mg}}{\text{ml}}\right) \times \text{volume S1}}{\text{Protein content of H} \left(\frac{\text{mg}}{\text{ml}}\right) \times \text{volume H}} \times 100 \quad (1)$$

$$\text{Protein precipitation yield (\%)} = \frac{(\text{Protein content of S1} (\frac{\text{mg}}{\text{ml}}) \times \text{volume S1}) - (\text{Protein content of S2} (\frac{\text{mg}}{\text{ml}}) \times \text{volume S2})}{\text{Protein content of S1} (\frac{\text{mg}}{\text{ml}}) \times \text{volume S1}} \times 100 \quad (2)$$

The protein yield of the entire pH-shift process was calculated using equation number 3.

$$\text{Total yield (\%)} = \frac{\text{Protein content of S1 (mg)} - \text{Protein content of S2 (mg)}}{\text{Protein content of H (mg)}} \times 100 \quad (3)$$

2.4. Characterization of recovered proteins

2.4.1. Moisture and protein analysis

The moisture content of each individual biomass was measured using an IR-scale (Precisa Moisture Balance HA 300). Approximately 0.5 g of biomass was used in each run and measurements were done in triplicate. The IR-scale was run at 80 °C. Protein content of the seaweed biomasses and their corresponding protein samples was measured using the Lowry method as modified by Markwell, Haas, Bieber, and Tolbert (1978).

2.4.2. Amino acid analysis

Amino acid profile of the seaweed biomasses and proteins was analyzed as explained previously by Abdollahi, Rezaei, Jafarpour, and Undeland (2018). Briefly, isolated protein and biomasses were freeze-dried and ground to fine powders. Approximately 30–50 mg of biomass powder and ~10–20 mg of protein powders was weighed into screw cap glass tubes. Four ml of 6 M HCl was added into each tube, air inside the tubes was replaced twice with nitrogen and samples were heated (with caps on) under a fume hood for 24 h at 110 °C using a heating block. Samples were loaded, automatically sampled and run in an LC/MS (Agilent 1100 HPLC, Waldbron, Germany) with a Phenomenex column (C18 (2) 250 μm × 4.6 μm × 3 μm), coupled to an Agilent 6120 quadrupole in the SIM positive mode (Agilent Technologies, Germany). Collected data were then compared against previously run amino acid standards.

2.4.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Finely ground powders of oven-dried, –20 °C frozen, sun-dried and freeze-dried biomasses and isolated proteins as most promising treatment based on protein yield were mixed with 1 M NaOH to create a solution containing ~3 mg protein/ml. The protein content of protein powders, dissolved in 1 M NaOH, and biomass samples were measured with the Markwell method (Markwell et al., 1978) and diluted to a final concentration of 2.1 mg protein/ml. The samples were mixed with 10X sample buffer and were then heated at 100 °C for 7 min, where after the samples were centrifuged for 5 min at 15000 g in room temperature. Ten μl of protein ladder (prestained dual color standard, 10–250 kDa, Bio-Rad, USA) and 15 μg of protein from each sample were loaded onto 4–20% precast mini linear gels (Bio-Rad, USA). Electrophoresis was conducted at a constant voltage of 205 V, using a Mini Protein II unit (Bio-Rad, USA). Gels were stained for 45 min with 0.02% (w/v) Coomassie Brilliant Blue R-250 in 50% (v/v) methanol and 7.5% (v/v) acetic acid, followed by a destaining for 70 min with 50% (v/v) methanol and 7.5% (v/v) acetic acid. Pictures of the gels were taken with Bio-Rad's Gel Doc 2000.

2.4.4. High-performance size-exclusion chromatography

Apparent molecular weight of the selected seaweed proteins was determined using high performance size-exclusion chromatography (HP-SEC) (Dionex HPLC, Dionex GmbH, Idstein, Germany) equipped with two serially connected Agilent columns: Agilent Bio SEC-5 (5 μm particle size, 150 Å pore size) and Agilent Bio SEC-5 (5 μm particle size and 300 Å pore size) and a UV-detector. The mobile phase was containing 100 mM of phosphate buffer (pH-7.5) with a flow of 0.85 ml/min and the results were monitored at an absorbance wavelength of 214 for 30 min. Samples were dissolved in the eluent to a final protein concentration of 1 mg/ml and centrifuged for 10 min at 10,000 × g followed by filtering the supernatant through a 0.45 μm pore size cellulose

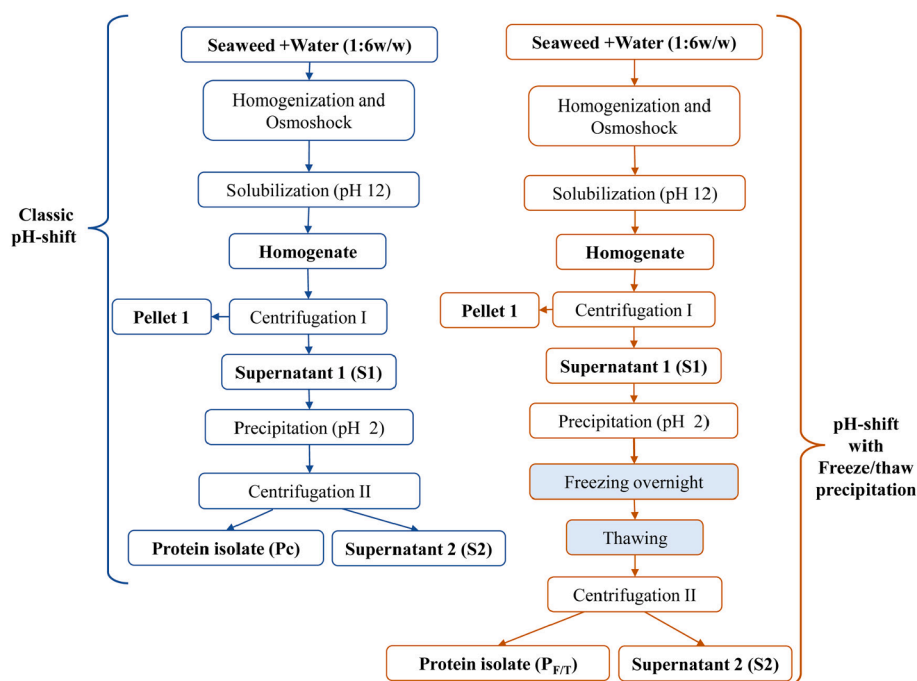


Fig. 1. Schematic overview of the classic pH-shift process applied to seaweed and the process run with the aid of freeze/thaw (F/T)-aided precipitation. Pc: protein isolated with classic pH-shift process. P_{F/T}: protein isolated using pH-shift processing with freeze-thaw aided precipitation.

Table 1

Protein content of differently preserved seaweed and final proteins and protein yield during different steps of pH-shift processing. Two types of processes were used: classic pH-shift process and pH-shift process with freeze/thaw-aided precipitation.

Treatment	pH-shift type	Solubilization Yield (%)	Precipitation Yield (%)	Total Yield (%)	Initial biomass protein content (%)	Final protein content (%)
Sun-dried	Classic	64.9 ± 8.52 ^b	11.5 ± 2.10 ^e	7.4 ± 0.38 ^f	6.1 ± 0.1 ^c	15.4 ± 0.4 ^f
	Freeze/thaw		18.6 ± 1.41 ^d	12.0 ± 0.67 ^d		20.3 ± 0.1 ^e
Oven-dried	Classic	77.2 ± 7.17 ^{ab}	13.0 ± 1.97 ^e	10.0 ± 0.59 ^e	8.0 ± 0.1 ^a	24.9 ± 0.2 ^c
	Freeze/thaw		26.1 ± 0.59 ^c	20.1 ± 1.41 ^b		40.5 ± 0.5 ^a
Frozen –20 °C	Classic	79.9 ± 9.10 ^{ab}	14.0 ± 1.73 ^e	11.1 ± 0.11 ^{de}	7.6 ± 0.1 ^b	22.0 ± 0.6 ^d
	Freeze/thaw		24.4 ± 2.60 ^c	19.3 ± 0.14 ^b		19.0 ± 0.8 ^e
Frozen –80 °C	Classic	65.7 ± 5.63 ^b	9.6 ± 1.52 ^c	6.3 ± 0.46 ^f	7.8 ± 0.4 ^{ab}	15.3 ± 0.8 ^f
	Freeze/thaw		25.3 ± 0.40 ^c	16.6 ± 1.16 ^c		26.2 ± 0.6 ^c
Ensiled	Classic	25.4 ± 4.46 ^c	30.0 ± 3.97 ^b	7.6 ± 0.34 ^f	6.6 ± 0.4 ^c	1.3 ± 0.1 ^h
	Freeze/thaw		42.5 ± 6.62 ^a	10.6 ± 0.22 ^e		2.0 ± 0.3 ^g
Freeze-dried	Classic	90.9 ± 0.28 ^a	12.3 ± 0.05 ^e	11.2 ± 0.01 ^e	8.2 ± 0.3 ^a	28.0 ± 0.9 ^c
	Freeze/thaw		29.1 ± 0.20 ^b	26.4 ± 0.10 ^a		37.6 ± 0.7 ^b

Different small letters in each column shows significant difference ($p < 0.05$).

acetate membrane (Agilent, USA). Ten μl of each sample was injected into system. A protein standard mix (0.1 kDa–670 kDa) was injected into the system and the retention time of the component against their logarithmic molecular weight was used as calibration curve. Average area of each peak from three independent injections was evaluated and presented as relative proportion of each protein peak.

2.4.5. Fourier transform infrared (FTIR) analysis of seaweed proteins

FTIR-spectra of the selected produced proteins were obtained by placing freeze-dried protein samples onto the crystal cell of a spectrophotometer (Nicolet 6700, Thermo Scientific, MA, USA) (Abdollahi & Undealnd, 2018). Scanned inverted wavenumbers ranged from 500 cm^{-1} to 4000 cm^{-1} , with data recorded once per 4 cm^{-1} . All spectra were recorded at room temperature ($25\text{ }^\circ\text{C}$) and 16 times scanning. To analyze secondary structure of proteins, the resolution of spectra was improved to separate overlapped peaks. To do so, the baseline of the FTIR spectra was initially corrected and subjected to Fourier self-deconvolution (FSD) using OriginLab software, version 94E (OriginLab Corporation, Northampton, MA, USA). Then, Gaussian function was used for multipeak fitting between 1600 and 1700 cm^{-1} and relative area of secondary structure components (α -helix, β -sheet, β -turn, and random coil) were determined by comparing to the total area of the secondary structure. Band assignment of the secondary structure elements was conducted based on previous literature (Kong & Yu, 2007).

2.4.6. pH-dependent solubility of dried seaweed proteins

Selected isolated proteins (20 mg) were weighed and added to 25 ml de-ionized water and was thoroughly vortexed. The 25 ml mixture was separated into aliquots of 5 ml in five 15 ml Falcon-tubes. Four of the tubes were adjusted to pH 5, 7, 9 and 11, respectively, using 1 M NaOH and controlling with a pH-meter (MeterLab[®] PHM210 standard pH meter). The fifth tube was left unaltered and referred to as “native pH” which was around 3. All tubes were incubated for 30 min, vortexed every 10 min and finally centrifuged for 10 min, at room temperature at $6000\times g$. Samples were taken from the resulting supernatants and analyzed for protein content with the Markwell method as described. Protein solubility of the different samples was calculated using equation (4).

$$\text{Solubility (\%)} = \frac{C_{\text{soluble proteins in supernatant}}}{C_{\text{protein initial suspension}}} \times 100 \quad (4)$$

where C indicates the protein concentration in $\mu\text{g/ml}$.

2.4.7. Emulsion activity index (EAI) and emulsion stability index (ESI)

At first, 30 mg of freeze-dried protein powder was mixed with 3 ml of de-ionized water in a 15 ml Falcon-tube, in triplicate. Two mixtures had their pH adjusted to 7 and 11, whilst the third was kept at its native pH. After pH-adjustment, all samples were vortexed at full speed for 1 min, where after 1 ml sunflower vegetable oil was added to each

sample followed by homogenization with a polytron (Ultra-turrax[®] T18 digital, IKA[®]) for 1 min at a speed of 20,000 rpm. Then, a 50 μl emulsion sample was taken from the bottom of the tube and mixed with 5 ml of 0.1% SDS-solution and used to measure and calculate the EAI and ESI were calculated as explained by Abdollahi and Undealnd (2018). Due to lack of sample in case protein from sun-dried biomass the EAI and ESI was just studied at pH 7 for this sample.

A small droplet of each emulsion was investigated by microscopy (Axiostar Plus, Carl Zeiss Microscopy, LLC, USA) using a 10x magnification (A-Plan 10x/0.25 Ph1 Var1, Carl Zeiss Microscopy, LLC, USA). Pictures of the investigated emulsions were taken with a microscope top mounted camera (Canon PowerShot G9, 12.1 Megapixels) with a 6x optical zoom lens.

2.4.8. Statistical analysis

To statistically determine significant differences within the resulting data from the six different stabilization treatments, one-way ANOVA (analysis of variance) was used with a significance level of 5% ($\alpha = 0.05$) together with Duncan *post-hoc* test for pairwise comparison. Unpaired Student's t-test was in some cases applied when investigating significance between two specific groups of data.

3. Results and discussion

3.1. Effect of stabilization method and F/T on protein yield during pH-shift processing

Protein solubilization yield, protein precipitation yield and total protein yield obtained during pH-shift processing of differently stabilized seaweed samples are summarized in Table 1. As can be seen, the stabilization methods clearly affected the protein solubilization yield of the studied samples after the first centrifugation step (Fig. 1), i.e., the amount of protein that could be extracted at pH 12. Freeze-dried, oven-dried, and $-20\text{ }^\circ\text{C}$ frozen biomasses achieved the highest protein solubilization yield (90.9%, 77.2%, and 79.9%, respectively) while the $-80\text{ }^\circ\text{C}$ frozen (65.7%), sun-dried (64.9%) and ensiled (25.4%) biomasses resulted in significantly lower values ($p < 0.05$). Solubilization capacity of proteins in the seaweed biomass is governed by the characteristics of the seaweed matrix such as amount of anionic or neutral polysaccharides, protein access, protein folding and interacting protein-binding compounds such as phenolics (Jordan & Vilter, 1991). Thus, it seems like different stabilization methods have affected solubilization capacity of the brown seaweed by changing molecular integrity or interactions in the seaweed cell wall.

When combining protein solubilization yields with protein precipitation yields, total protein yield is obtained (Table 1). Total protein yield obtained using the classic pH-shift process varied between 6.3 and 11.2% which was lower than the previously reported 16.01% achieved

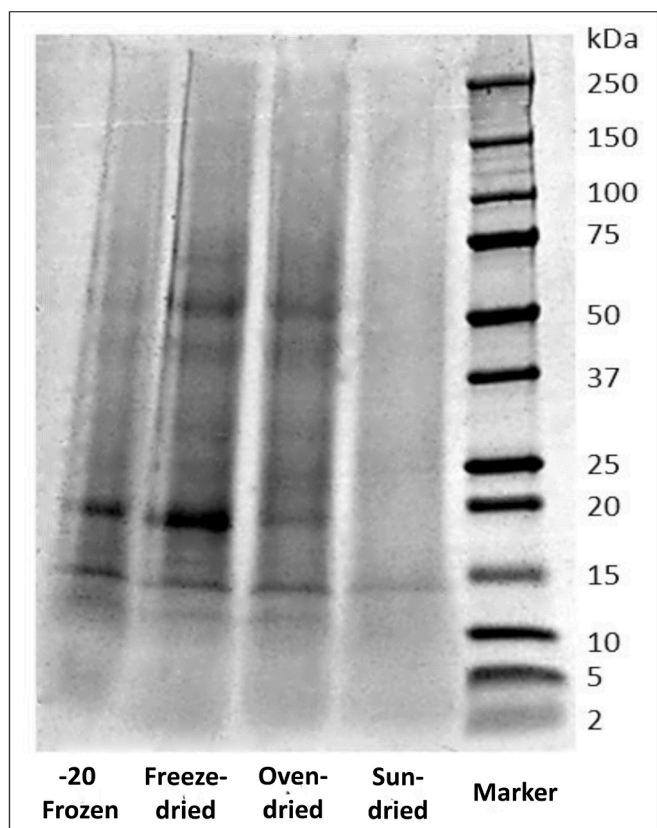


Fig. 2. Polypeptide pattern of differently preserved seaweed biomasses. 28.5 mg protein was loaded in each well.

by Vilg and Undeland (2017), using a very similar pH-shift process. However, their biomass was harvested in November whilst ours was harvested in May and as previous reports show, there is a crucial seasonal variation in the biomass composition of *S. latissima* (Vilg et al., 2015). Total protein yield obtained in this study was on the other hand higher than other previously reported protein yields from a pH-shift-like process; 5.71–6.48% for three green seaweed species (Kandasamy et al., 2012) and 7.81% for *K. alvarezii*, a species of red seaweed (Suresh Kumar et al., 2014).

Freeze-dried biomass resulted in the highest protein solubilization yield and total protein yield among the dehydration methods used in this study. This could be due to the unique conditions used in freeze-drying which can minimize the oxidative and thermal deterioration of seaweeds and preserve most of the original qualities of samples by preventing migration of fluids and solutes during drying (Robic, Sassi, & Lahaye, 2008). As shown in the polypeptide pattern of the biomasses (Fig. 2), freeze-drying had minimum destructive effect on *S. latissima* and most likely preserved its molecular integrity. This may also have minimized irreversible reactions of proteins with other cell wall components like polysaccharides and polyphenols which can prevent protein extractability in seaweeds. Similarly, Chan et al. (1997) and Robic et al. (2008) found the best nutritional quality and molecular integrity in freeze-dried *Sargassum hemiphyllum* and *Ulva*, respectively.

The lowest total protein yield was related to -80°C frozen and sundried biomasses. Sun-drying is usually used prior to colloid production (agar, carrageenan or alginate), from red and brown seaweeds, for economic reasons (Robic et al., 2008). However, sun-drying is strongly dependent on the weather and the length of the day and it is difficult to control drying rate and surrounding parameters (Chan et al., 1997). In this study no parameter other than the drying time was recorded. Chan et al. (1997) compared oven-drying, sun-drying, and freeze-drying and reported the lowest amount of ash, mineral, and total

vitamin C contents in the sun-dried seaweeds, which they explained with slower drying rate and long exposure time to air which increased the chance of leaching. Also, Young (1922) showed that sunlight can denature different kinds of proteins, which could explain why the proteins of the sun-dried biomass in this study only achieved a solubility of $\sim 60\%$ at alkaline pH. The possible denaturation of the proteins would irreversibly affect the folding of the protein and thereby reduce its solubility. The harsh effect of sun-drying on polypeptide pattern is also illustrated the SDS-PAGE results (Fig. 2).

Convection drying at 40°C (oven-drying) used in this study resulted in higher protein solubility and yield compared to sun-drying, but lower total yield than freeze-drying. The faster drying kinetic, with hot-air convection drying, was probably more efficient in inhibiting endogenous enzymatic breakdown of proteins (Robic et al., 2008) as reflected in the polypeptide pattern of oven-dried biomasses compared with the sun-dried biomass (Fig. 2). It has also been suggested (Wong & Chikeung Cheung, 2001) that the elevated temperature used in the oven-drying damage the seaweed cell walls more intensively than sun-drying which may facilitate protein extraction.

The seaweed biomass frozen at -20°C gave rise to similar protein solubility yield and total protein yield as oven-dried biomass (Table 1). Values were surprisingly lower for the -80°C frozen biomass, e.g. 14.2% lower extraction yield and 4.4% lower precipitation yield than the -20°C frozen biomass, implying that the function/folding of the proteins in the -80°C frozen biomass could be different. The larger ice crystals forming by slow freezing compared with fast freezing might under these circumstances cause favorable damages to structure cells better releasing protein from the seaweed matrix.

Seaweed samples stabilized by acid-aided ensilaging for 91 days resulted in minimum protein solubilization yield (25.4%) and total protein yield (7.6%). The process of ensilaging prevents microbial deterioration of biomasses by the low pH ($\sim 3\text{--}3.5$) but has a degrading effect on proteins (Ohshima & McDonald, 1978), by breaking proteins into peptides, amino acids and finally non-protein nitrogenous compounds like ammonia. In this study, the ensilaging process reduced the protein content of the seaweed by 37%, indeed changing the profile of proteins available for extraction. Ensilaging of seaweed has gained interest as a low energy stabilization method yielding feed for biorefinery (Cabrita, Maia, Sousa-Pinto, & Fonseca, 2017), but its negative side-effects on proteins should be considered and further studied if seaweed is considered as a new protein source.

To improve protein precipitation yield of seaweed protein, freeze/thawing of solubilized proteins was applied after adjustment to pH 2 as shown in Fig. 1. As can be seen in Table 1, when the F/T was applied, (see Fig. 1), the total protein yield was increased by a factor 1.6, 2.0, 1.7, 2.6, 1.4 and 2.4 compared to classic pH-shift processing for the sun-dried, oven-dried, -20°C frozen, -80°C frozen, ensiled and freeze-dried biomasses, respectively. Cryo-concentration of proteins, salts, buffers etc. during freezing of protein suspensions can cause varying degrees of protein denaturation and/or protein aggregation depending on solute, present electrolytes, pH-shifting and freezing rate (Cao et al., 2003; Chang et al., 1996). This aggregation and precipitation which is normally considered as a negative phenomenon could provide a tool for further recovering soluble proteins in classic pH-shift processing. Similarly, Hernández et al. (1997) obtained protein concentrates via freezing of alfalfa (a small flower plant used as livestock fodder) press juice. Also, Xiong et al. (2017) successfully used freeze/thaw cycles for deproteinization of polysaccharides from *Cipangopaludina chinensis*.

The protein content of the isolated proteins was significantly ($p < 0.05$) increased compared to the initial biomass in all cases, except for the ensiled biomass where it was significantly lower (Table 1). The proteins produced using the pH-shift method with F/T ($P_{F/T}$) had a significantly ($p < 0.05$) higher protein content than the proteins isolated using the classic pH-shift method (P_C) in all cases, except for the -20°C frozen biomass. The highest protein content was found in the proteins isolated from the freeze-dried and oven-dried biomasses

Table 2

Amino acid profiles (mg/100 g protein) of six types of differently preserved seaweed biomasses and their corresponding protein isolated with the classic pH-shift process (P_c) or that combined by freeze/thaw-aided precipitation (P_{F/T}). ▼ denotes a decrease, and ▲ an increase. Essential amino acids are shown bold.

Amino acid	Sun-dried Biomass (P _c /P _{F/T})	Oven-dried Biomass (P _c /P _{F/T})	Frozen-20°C Biomass (P _c /P _{F/T})	Frozen-80°C Biomass (P _c /P _{F/T})	Ensiled Biomass (P _c /P _{F/T})	Freeze-dried Biomass (P _c /P _{F/T})	FAO/WHO Adult (Infant)
Glycine	6.43 (7.69▲/5.84▼)	5.48 (7.47▲/5.54▲)	5.33(7.23▲/5.36▲)	5.30(7.18▲/5.56▲)	6.12(7.25▲/5.75▼)	5.58(7.42▲/5.20▼)	
Alanine	17.03 (14.74▼/10.06▼)	18.08(12.76▼/9.70▼)	18.84(15.05▼/10.57▼)	18.39(13.46▼/9.44▼)	16.87(15.40▼/9.30▼)	19.09(12.44▼/8.71▼)	
Serine	3.48 (3.66▲/4.30▲)	3.10(3.79▲/4.42▲)	3.05(3.74▲/4.29▲)	2.85(3.86▲/4.65▲)	3.24(3.84▲/4.70▲)	3.11(3.82▲/4.60▲)	
Proline	4.72 (4.55 ▼/4.48▼)	3.31(3.90▲/3.71▲)	3.33(3.82▲/3.61▲)	2.95(3.66▲/3.75▲)	3.59(4.55▲/4.49▲)	3.05(3.70▲/3.79▲)	
Valine	9.76 (9.76▼/8.61▼)	8.72(8.96▲/7.75▼)	8.72(8.98▲/7.98▼)	8.36(8.49▲/7.64▼)	8.54(9.92▲/8.54▲)	8.29(8.63▲/7.72▼)	3.9 (4.3)
Threonine	4.87 (5.43▲/6.83▲)	4.20(5.30▲/6.50▲)	4.13(5.46▲/6.57▲)	4.08(5.47▲/6.53▲)	4.58(5.63▲/7.11▲)	4.05(5.38▲/6.81▲)	2.3 (3.1)
Isoleucine	6.83 (7.03▲/6.22▼)	5.83(6.47▲/5.39▼)	5.78(6.83▲/5.39▼)	5.64(6.52▲/5.43▼)	6.09(7.22▲/6.86▲)	5.38(6.76▲/5.35▼)	3.0 (3.2)
Leucine	12.03 (12.23▲/11.24▼)	10.30(12.10▲/10.12▼)	10.19(12.28▲/9.97▼)	9.99(11.73▲/9.82▼)	10.72(12.20▲/10.123▲)	9.76(12.28▲/10.13▲)	5.9 (6.6)
Aspartic acid	3.93 (3.53▼/6.75▲)	4.44(5.31▲/8.17▲)	4.60(3.96▼/7.49▲)	4.64(5.23▲/8.05▲)	5.19(2.71▼/6.94▲)	4.94(5.65▲/8.41▲)	
Lysine	3.82 (4.71▲/6.71▲)	4.19(5.73▲/8.03▲)	4.30(5.74▲/7.69▲)	4.06(5.77▲/9.02▲)	4.06(5.25▲/6.67▲)	3.74(5.68▲/8.44▲)	4.5 (5.7)
Glutamic acid	13.36 (9.70▼/13.04▼)	18.74(10.51▼/14.45▼)	18.20(10.02▼/15.54▼)	19.94(11.14▼/13.85▼)	16.70(9.29▼/11.21▼)	20.11(10.64▼/13.15▼)	
Methionine	3.09 (3.08▼/3.13▲)	2.89(3.17▲/3.02▲)	2.72(3.24▲/2.83▲)	2.88(3.09▲/2.87▼)	2.76(3.41▲/3.25▲)	2.67(3.32▲/3.00▲)	1.6 (2.8)
Histidine	0.82 (0.98▲/0.71▼)	1.18(1.47▲/1.11▼)	1.15(1.37▲/1.03▼)	1.38(1.52▲/1.23▼)	1.26(1.39▲/1.04▼)	1.18(1.56▲/1.51▲)	1.5 (2.0)
Phenylalanine	6.05 (8.56▲/7.45▲)	5.60(7.26▲/6.21▲)	5.82(7.31▲/6.23▲)	5.66(7.11▲/6.14▲)	5.94(7.57▲/7.76▲)	5.14(6.41▲/6.10▲)	3.8 (5.2)
Arginine	2.58 (2.89▲/2.71▲)	2.61(3.70▲/3.55▲)	2.55(3.20▲/3.25▲)	2.56(3.71▲/3.66▲)	2.92(2.98▲/3.30▲)	2.61(4.13▲/4.20▲)	
Tyrosine	1.20 (1.45▲/1.91▲)	1.34(2.09▲/2.35▲)	1.28(1.78▲/2.20▲)	1.32(2.07▲/2.37▲)	1.43(1.40▼/1.85▲)	1.31(2.18▲/2.88▲)	
TEAA	47.27 (51.78▲/50.90▲)	42.91(50.46▲/48.12▲)	42.81(51.21▲/47.70▲)	42.05(49.70▲/48.67▲)	43.94(52.59▲/52.46▲)	40.21(50.02▲/49.06▲)	
TEAA/TA	0.47 (0.52▲/0.51▲)	0.43(0.50▲/0.48▲)	0.43(0.51▲/0.48▲)	0.42(0.50▲/0.49▲)	0.44(0.53▲/0.52▲)	0.40(0.50▲/0.49▲)	

TEAA: total essential amino acids; TAA: total amino acids.

TEAA: total essential amino acids; TAA: total amino acids.

(37.6% and 40.5% on freeze-dried powder basis, respectively). Kandasamy et al. (2012), using another version of the pH-shift process, produced protein concentrate from three green seaweed species that contained 33.4–60.4% protein on a DW basis. Similarly, Suresh Kumar et al. (2014), produced a protein sample from *K. alvarezii* by adding ammonium sulfate containing 62.3% protein (on DW basis). Harrysson et al. (2018) yielded protein isolates from *P. umbilicalis*, *U. lactuca*, and *S. latissima* with the pH-shift process, which contained 71.0, 51.2, and 40.7 protein on a DW-basis, respectively. The proteins produced from the freeze-dried and oven-dried biomasses in this study is therefore in the lower to mid-range of protein content for proteins produced from other seaweeds, but in accordance with other studies on *S. latissima*.

3.2. Amino acid profile of seaweed biomasses and extracted proteins

Amino acid (AA) composition for the biomasses and their respective proteins are shown in Table 2. Total amount of essential amino acids (TEAA) of initial seaweed biomasses was affected by the employed stabilization method. Sun-dried seaweed biomass had the highest TEAA (47 mg/100 g protein) and freeze-dried biomass had the lowest TEAA content (40 mg/100 g protein). However, almost all biomasses met the WHO/FAO adult and infant recommendations (WHO/FAO/UNU, 2007) for the EAA; valine, threonine, isoleucine, leucine, methionine, and phenylalanine and matches that of previous reports for *L. digitata* and *S. latissima* (Manns, Nielsen, Bruhn, Saake, & Meyer, 2017).

Regardless of the used stabilization method, the classic pH-shift process made the TEAA increase with ~5–10 g AA/100 g protein and when the pH-shift process was combined with F/T, the TEAA increased with ~4–9 g AA/100 g protein. Thus, both pH-shift process versions concentrated the EAA. However, both types of pH-shift processes reduced glutamic acid and alanine content in the final proteins compared to their counterpart biomasses. Comparing the EAA-profiles of the two different groups of obtained proteins to some common foodstuffs, like egg, beef and chicken, relative amounts of valine, isoleucine, leucine, methionine, phenylalanine and threonine is higher for the seaweed proteins. Our seaweed proteins also had higher relative content of valine, threonine, isoleucine, leucine, methionine, phenylalanine and TEAA than had beach pea proteins produced with pH-shift like processes (Chavan, McKenzie, & Shahidi, 2001). Further, they had a higher relative amount of all EAAs and TEAA than proteins from the brown seaweed *H. elongata* produced with ultrasound-aided solubilization and salting out precipitation (Garcia-Vaquero et al., 2016). To consider using these proteins as food they need to meet certain standards of EAA-contents; i.e. 45 g lysine/kg protein and 22 g methionine/kg proteins (Vareltzis & Undeland, 2012). All protein produced in this study meet these requirements. It can be calculated that an adult with 70 kg body weight needs to consume 191 g, 232 g, 300 g or 160 g of the dried protein produced of the oven-dried, -20 °C frozen, -80 °C frozen or freeze-dried biomasses with the classic pH-shift process, respectively, to cover the recommended daily intake of EAAs for adults, according to

the WHO and FAO (WHO/FAO/UNU, 2007). For proteins produced with the aid of F/T, the corresponding amounts are an intake of 156 g, 357 g, 218 or 124 g, respectively.

These results show that the choice of seaweed stabilization treatment and version of pH-shift process has a small impact on the AA-profile of the proteins, however, the choice has a great impact for the daily protein intake needed to cover the WHO/FAO recommendations.

3.3. Effect of stabilization method and F/T on the structural properties of seaweed proteins

3.3.1. Polypeptide pattern and molecular weight of proteins

The polypeptide pattern of the biomasses, (Fig. 2), varied depending on their stabilization method. The freeze-dried and oven-dried biomasses displayed the highest number of bands, three in the range of ~37–75 kDa and four at ~10–15 kDa. There were also two slightly more intensive band around 50 kDa and 20 kDa. The -20°C frozen biomass displayed less intensity for the higher molecular weight (HMW) bands, however, the lower molecular weight (LMW) bands were very similar to those of the freeze-dried and oven-dried biomasses. The sun-dried biomass only displayed one band at ~15 kDa indicating severe protein degradation. These results show that the choice of stabilization treatment remarkably influenced the polypeptide pattern of the initial biomass and resulted in high degree of protein degradation in sun-dried and -20°C frozen biomasses. To the best of our knowledge, no previous polypeptide analysis on *S. latissima* biomass has been reported. However, Kim et al. (2011) performed proteome analysis of *Saccharina japonica*, a close relative to *S. latissima* revealing the following identity of specific bands; 57 kDa: 6-phosphogluconate dehydrogenase, 51 kDa: trypanothione reductase and ATP synthase subunit beta (chloroplastic), 41 kDa: actin-1, 40 kDa: elongation factor Tu, 39 kDa: glyceraldehyde-3-phosphate dehydrogenase, 37 kDa: phosphoglycerate kinase. The 51, 41, 40 and 39 kDa polypeptides could be the same as in our seaweed biomasses.

HP-SEC was also used to evaluate the molecular weight of extracted proteins as a function of both stabilization method and F/T. Chromatographic results and relative molecular weight profile of proteins based on the percentage area of peaks are shown in Fig. 3. All protein extracts had a peak eluted before the largest protein in the standard mix (Thyroglobulin bovine 670 kDa) which might be related to soluble protein aggregates. Kadam, Álvarez, Tiwari, and O'Donnell (2017) also reported on such a peak related to supramolecular aggregated proteins in Irish brown seaweed (*Ascophyllum nodosum*). This early HMW peak has also been reported in terrestrial plant-based protein extracts, from e.g. legume and faba bean (Yang, Liu, Zeng, & Chen, 2018) which is not detectable by SDS-PAGE. Highest proportional area of this peak was observed in proteins from freeze-dried and oven-dried biomasses and the lowest amount was found in proteins from the -20°C frozen biomass. This may reflect that higher degree of protein degradation occurred in biomasses which were frozen at -20°C and in the sundried biomass. F/T slightly increased the proportional area of this early peak which may imply its positive effect on precipitation of HMW protein aggregates. The second peak, which was the dominant peak in all proteins, was assigned to 28 kDa proteins with its highest proportional area in proteins from -20°C frozen and sundried biomasses. The relative percentage of this peak also slightly increased in proteins after the freeze-thaw cycle, except for in the protein isolated from -20°C frozen seaweed. Harnedy and FitzGerald (2013) also showed that alkali-soluble proteins from red seaweed *Palmaria palmata* had a low average molecular weight, but water solubilized proteins from the same species had a molecular weight that ranged from 55–14 kD. However, Kadam et al. (2017) reported a maximum molecular weight for protein isolated from Irish brown seaweed (*A. nodosum*) of 3.8 kDa when first using acid and then alkaline extraction. Garcia-Vaquero et al. (2016), also found several bands in the range of ~27–75 kDa in proteins isolated with a pH-shift like process from the

brown seaweed *H. elongata*. However, they aided their protein precipitation using ammonium sulfate which could facilitate the recovery of larger peptides. Also, as visible in Fig. 2., LMW peaks with 5 kDa, 0.3 kDa and < 0.1 kDa were detectable in all protein extracts while their lowest percentage was obtained in proteins isolated from freeze-dried seaweed. Our results thus show that the choice of stabilization method affects the polypeptide pattern of the *S. latissima* biomass and molecular weight distribution of the corresponding isolated proteins.

3.3.2. Secondary structure of seaweed proteins

The amide I absorption zone between 1600 and 1700 cm^{-1} can be useful to evaluate the secondary structure of proteins as it is the sum of overlapping component bands: α -helix, β -sheet and β -turn and random coils as shown in Fig. 4a by curve fitting of Amide I bands of different proteins (Carbonaro & Nucara, 2010). As further summarized in Fig. 4b, seaweed proteins like most other plant proteins showed higher proportion of β -sheet than other secondary structure elements. The results also showed that molecular secondary architecture of seaweed proteins was quite sensitive to stabilization method. The oven-dried seaweed protein showed the lowest proportion of α -helix structure (13%) which was mainly transformed to random coil structure. Sun-dried protein showed the highest proportion of α -helix structure (24%) compared to the other proteins, while proteins from freeze-dried and -20°C frozen biomasses showed some degree of alteration in secondary structure as revealed by relative reduction in α -helix structure. It has been widely reported previously that changes in the secondary structure of most of proteins are reflected by the loss of α -helix which is mainly responsible to maintain protein native structure. The latter is mainly stabilized by hydrogen bonds between the carbonyl oxygen ($-\text{CO}-$) and amino hydrogen ($-\text{NH}-$) of the polypeptide chain (Sun, Huang, Hu, Xiong, & Zhao, 2014; Timilsena, Adhikari, Barrow, & Adhikari, 2016). It has been reported that intense mechanical forces (Chen, Zhou, Xu, Zhou, & Liu, 2017) and long-term exposure to non-ambient temperatures like heating (Sun et al., 2014) oven-drying, freeze-drying (Timilsena et al., 2016) and freezing (Harnkarnsujarit, Kawai, & Suzuki, 2015) can cause reduction of α -helix and increase in unordered secondary structure of proteins. The elevated and reduced temperature applied during oven-drying and freezing as well as freeze-drying have possibly caused unfolding of proteins and affected protein conformation by disrupting hydrogen bonds and hydrophobic interactions within the α -helix, thereby inducing a more unordered structure. In accordance with this, the ambient temperature used during sun-drying has caused minimum side effect on protein conformation. F/T also caused changes in the secondary structure of all seaweed proteins causing transformation of organized native α -helix structure to unordered structures; the intensity of the transformation depended on the stabilization condition that the proteins had previously experienced. Highest proportional changes in secondary structural components as a function of F/T occurred in sun-dried biomass reflecting higher degree of protein unfolding and denaturation. Complex changes in the liquid environment surrounding proteins took place in the supernatant during freeze-thawing, causing varying degree of protein aggregation and denaturation which caused disturbing and weakening of hydrogen bonds and further reduction of native α -helical structure in all seaweed proteins.

3.4. Effect of stabilization method and F/T on the functional properties of seaweed proteins

3.4.1. pH-dependent solubility of seaweed proteins

The pH-dependent solubility of both types of protein as a function of stabilization method is shown in Fig. 5a and b. All proteins isolated using the classic pH-shift method showed a similar and very high solubility (80–100%) in the neutral and alkaline pH range. However, in the acid range, the samples of the sun-dried biomass displayed moderately high solubility whilst the remaining proteins greatly lost protein solubility in the span pH 7–pH 3, reaching down to ~10–20%. Garcia-

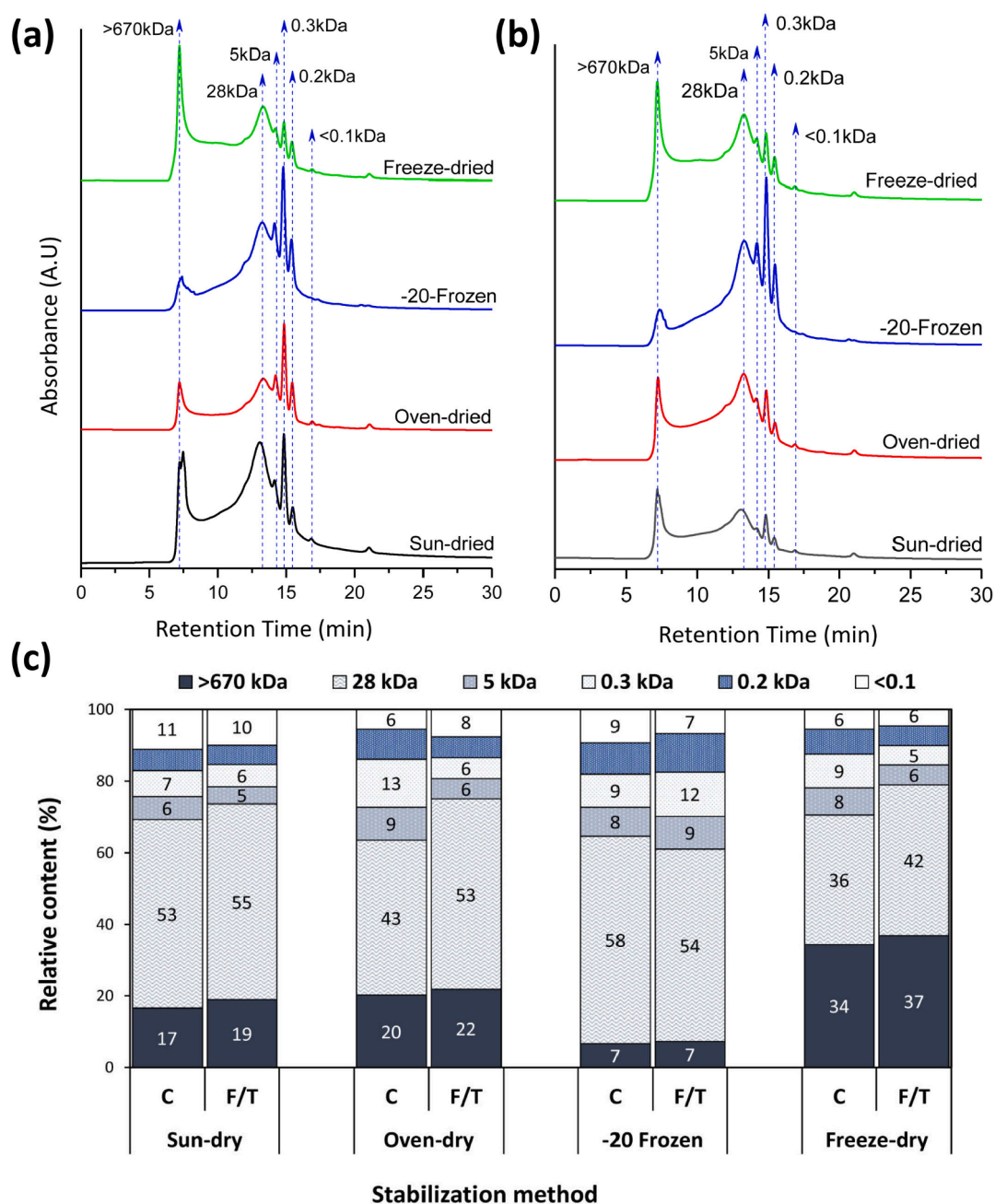


Fig. 3. HP-SEC chromatogram showing the profile of proteins extracted from differently preserved seaweeds by the classic pH-shift process (a), with a freeze/thaw-aided precipitation (b) and their relative percentage of total peak area (c). Arrows point at the molecular weight size of main peaks. C: classic pH-shift process and F/T: pH-shift process with freeze/thaw-aided precipitation.

Vaquero et al. (2016) found high solubility (65–96%) for protein isolated from the brown seaweed *H. elongata* at pH 8–12, but Suresh Kumar et al. (2014) reported maximum solubility of 58% at pH 12 for a protein concentrate from the red algae, *K. alvarezii*. Both studies mentioned above found minimum solubility of the isolated seaweed proteins at pH 4, which was assumed the isoelectric point of the proteins. However, we found minimum solubility of our proteins at pH 3. pH-dependent-solubility of proteins in water depends on several factors including surface characteristics of their amino acids, molecular weight and conformational status and, not least, ionic strength (Timilsena et al., 2016). Via interaction between e.g. chloride ions and the proteins, salt can also change the proteins' isoelectric point. Both mentioned studies had dialyzed their protein extract due to the use of ammonium sulfate-induced precipitation, which was not done here. In

addition to salt content, denaturation and hydrolysis of protein can alter its solubility, i.e. smaller peptides usually have higher solubility (Culbertson, 2005). The later may explain high water solubility of protein from sun-dried seaweeds.

For the proteins produced using the pH-shift method with F/T originating from the oven-dried and freeze-dried biomasses, the solubility pattern is very like that of their proteins isolated using the classic pH-shift method, see Fig. 5b. However, the pattern of the proteins produced using the pH-shift method with F/T originating from -20 °C frozen biomass followed that of the sun-dried biomass, i.e. moderately high protein solubility in the acid range. To conclude, the choice of preservation method influenced the pH-dependent solubility of both types of produced proteins.

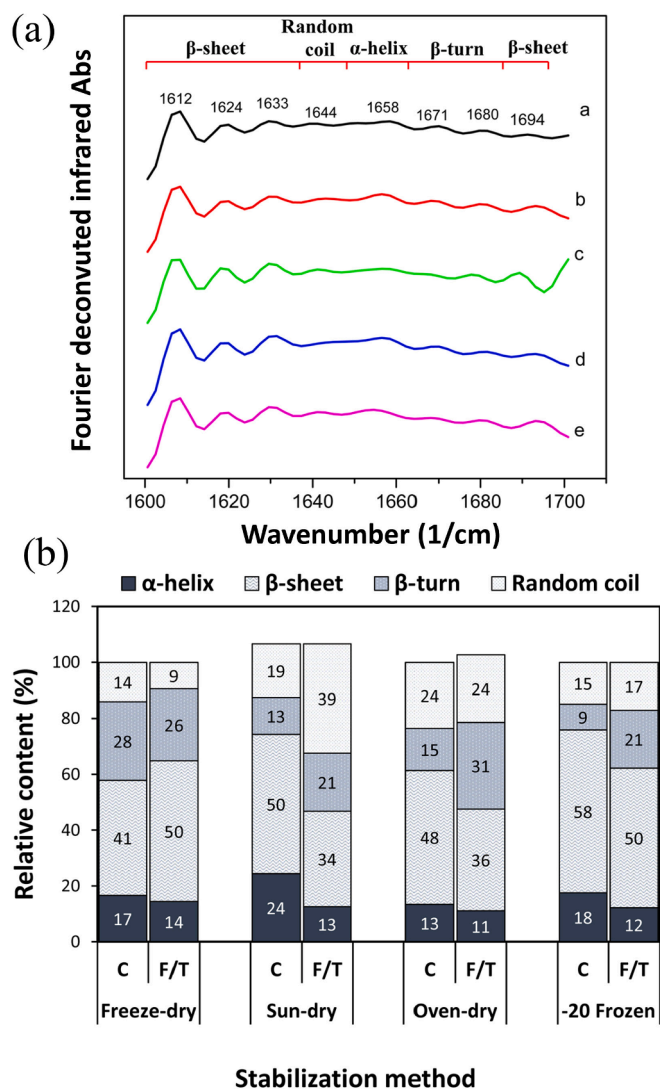


Fig. 4. Representative FTIR second derivative spectrum (a), and relative content of the secondary structural features (b) of protein isolated from differently preserved seaweeds by classic pH-shift process a: sun-dried seaweed protein, b: oven-dried seaweed protein, c: 20 °C frozen seaweed protein, d: freeze-dried seaweed protein and e: freeze-dried seaweed protein recovered by freeze-thaw (F/T) aided pH-shift process. C: classic pH-shift process and F/T: pH-shift process with freeze/thaw-aided precipitation.

3.4.2. Emulsion capacity of seaweed proteins

Both groups of protein produced from the sun-dried and -20 °C frozen biomasses displayed relatively high EAI values (~80–110 m²/g) at the studied pH-values (pH 2, 7 and 11) (Fig. 6a and b). Proteins produced from the oven-dried and freeze-dried biomasses displayed a relatively low EAI at pH 2 (~10–30 m²/g), somewhat higher at pH 7 (~50–70 m²/g) and reaching their maximum at pH 11 (~60–90 m²/g). To have a good emulsifying effect, the protein needs to be soluble at the pH of the aqueous part of the oil/water-mixture to reach the lipid interface. Preferably it should have a relatively high surface hydrophobicity to interact with the lipid phase (Shevkani, Singh, Kaur, & Rana, 2015). The protein solubility of proteins isolated using the classic pH-shift method and proteins isolated using the pH-shift method with F/T originating from the sun-dried and -20 °C frozen biomasses were high throughout the pH range 3–11, except for the proteins isolated using the classic pH-shift method of the -20 °C frozen biomass at pH ~3, which then correlates well to their EAI-patterns. The low protein solubility in the acid range and the high solubility in the neutral/alkaline range obtained for both types of protein from oven-dried and

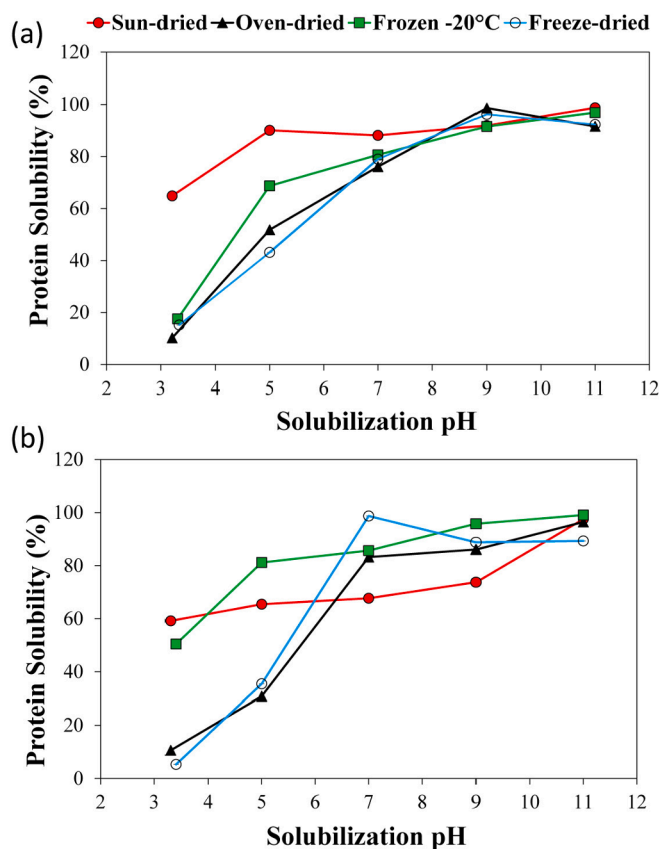


Fig. 5. Protein solubility curves of proteins produced from a selection of the differently stabilized seaweeds by the classic pH-shift process (a) and the pH-shift process with freeze/thaw-aided precipitation (b) as a function of pH.

freeze-dried biomasses also correlates well to their EAI-patterns. However, proteins isolated from the oven-dried and freeze-dried biomasses showed considerably higher ESI compared to proteins from the sun-dried and -20 °C frozen biomasses (see Fig. 2 ad 3). Similarly, Garcia-Vaquero et al. (2016) and Suresh Kumar et al. (2014) reported high emulsion activity for proteins isolated from the brown seaweed *H. elongata* and the red algae *K. alvarezii* although they used different analysis methods. EAI-values for the proteins of our study were higher than those reported for proteins of hake (*Merluccius capensis*) (2.03 m²/g), egg white (5.18 m²/g), soy (7.39 m²/g) and pea (7.79 m²/g), all measured at pH 7 (Tomé, Pires, Batista, Sousa, & Raymundo, 2014). A freeze-thawing cycle had no effect on the EAI of proteins isolated from the sun-dried and -20 °C frozen biomasses while it reduced EAI at pH 7 and 11 of proteins isolated from the oven-dried and freeze-dried biomasses. Freeze-thawing however improved the proteins' ESI (Fig. 6c and d).

In addition to the EAI/ESI measurements, the created emulsions were investigated microscopically, see Fig. 7. Emulsions prepared from proteins isolated using the classic pH-shift method with F/T of the sun-dried and of the -20 °C frozen seaweed showed small droplets of oils at native pH, however, with less effective dispersion than at higher pH. This correlates well to their solubility as well as their EAI-values at this pH. Looking at images for both types of proteins from the oven-dried and freeze-dried biomasses, these produced poor to no emulsion-systems at pH 2. However, at all studied pHs, proteins isolated using the classic pH-shift method from the -20 °C frozen and the sun-dried seaweeds depicted more effective dispersion of oil, yielding emulsion with smaller oil droplets than proteins from oven-dried and freeze-dried seaweeds. Similarly, Zhao et al. (2015) showed that soy proteins with

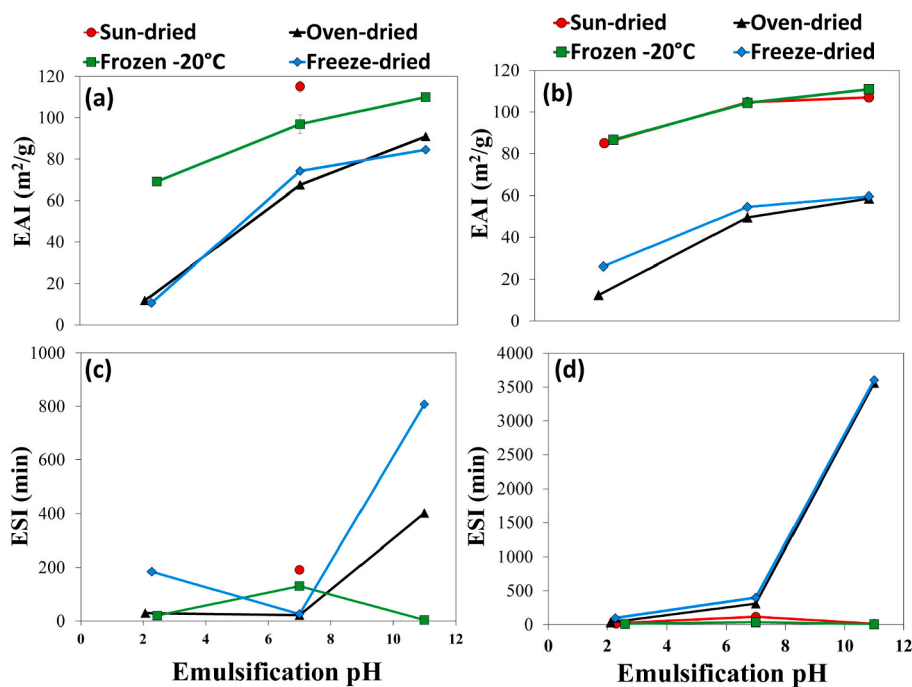


Fig. 6. Emulsion activity index (EAI) and emulsion stability index (ESI) of proteins produced from a selection of the differently stabilized seaweed biomasses by classic pH-shift process (a and c) and pH-shift process with freeze/thaw-aided (F/T) precipitation (b and d) as a function of pH.

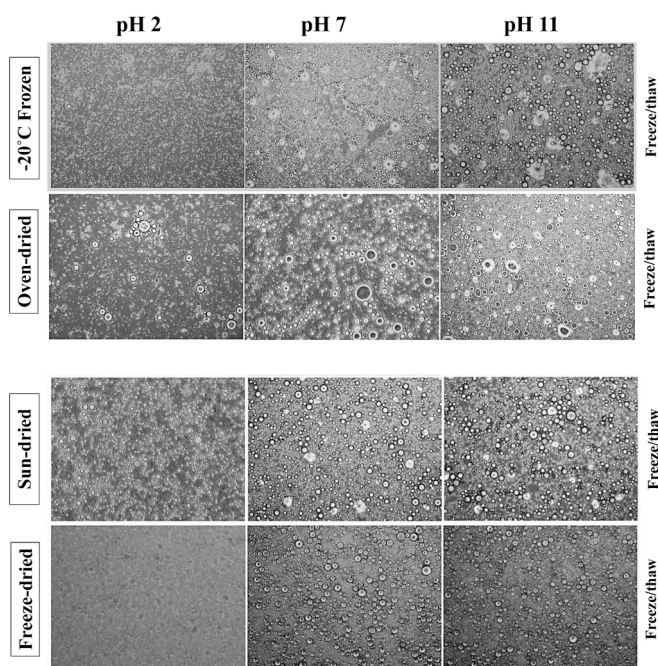


Fig. 7. Representative microscopic picture of emulsions formed by proteins recovered from a selection of the differently stabilized seaweeds by the pH-shift process with freeze/thaw-aided (F/T) precipitation as a function of pH.

higher emulsion capacity produced smaller oil droplets with finer dispersion. A high protein solubility and EAI around pH 7 means that our seaweed proteins could be applicable in a lot of foods requiring emulsification in that pH range. However, more work is needed to optimize the conditions for each protein since the stability (ESI) of the emulsions varied vastly over the whole pH range.

4. Conclusions

The post-harvest stabilization method greatly affected protein solubilization and precipitation yield of brown seaweed, *S. latissima* when subjected to alkaline solubilization/isoelectric precipitation, i.e., pH-shift processing. The highest protein solubilization yield was given by freeze-dried and oven-dried biomasses, while the ensiled biomass gave the lowest yield. Total protein yield did not exceed 11.2% with the classic pH-shift process. Interestingly, freeze/thawing of the first supernatant of the process after adjusting it to pH 2 could increase protein precipitation and thereby the total protein yield up to 2-fold in most of the biomasses. Functional properties and quality of the seaweed proteins were also remarkably affected by both stabilization method and application of a freeze-thaw cycle. Stabilization with sun-drying and -20 °C freezing caused a high degree of protein degradation. Protein isolated from all biomasses had high solubility at neutral and alkaline pH's while the protein from sun-dried biomass had high solubility also at acidic pH's. Proteins recovered from sun-dried and -20 °C frozen biomasses had considerably higher emulsion activity than the proteins recovered from freeze-dried and oven-dried biomasses, while in case of emulsion stability it was reverse. Regardless of stabilization condition and incorporation of a freeze-thaw cycle, all proteins met the daily intake recommendations for total essential amino acids and for lysine alone. In conclusion, to recover seaweed proteins efficiently, its post-harvest stabilization condition must be carefully chosen based on the final application of the proteins as well as economic and environmental aspects.

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