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Assessment of the functional properties of protein extracted from the brown seaweed *Himanthalia elongata (Linnaeus)* S. F. Gray

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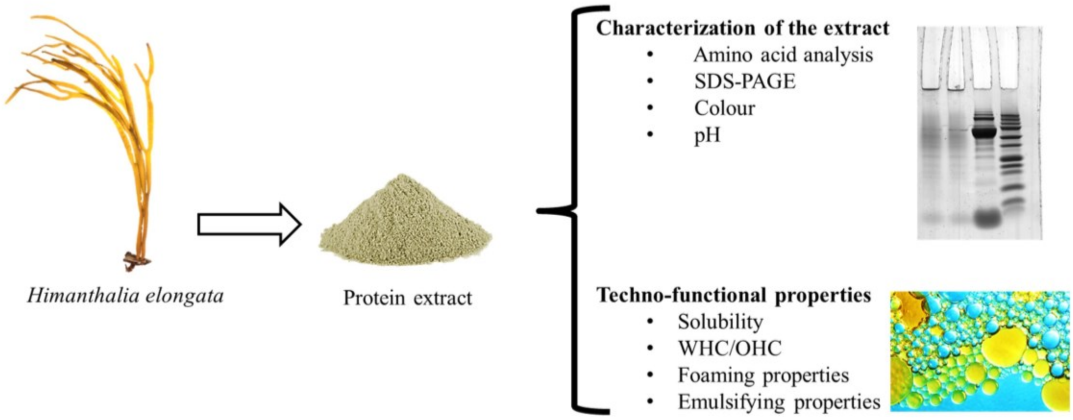

Abstract

A protein extract from the brown seaweed *Himanthalia elongata (Linnaeus)* S. F.Gray was prepared and its functional properties, colour and amino acid composition were assessed for its potential future use by the food industry. The total content of amino acids was determined as 54.02±0.46 g amino acids/ kg dry weight, with high levels of the essential amino acids lysine and methionine. SDS-PAGE showed 5 protein bands with molecular weights of 71.6, 53.7, 43.3, 36.4 and 27.1 kDa. The water holding capacity and oil holding capacity were determined as 10.27 ± 0.09 g H_2O / g and 8.1 ± 0.07 g oil / g respectively. Foaming activity and stability were higher at alkaline pH values. The emulsifying capacity and stability of the extract varied depending on the pH and oil used. These results demonstrate the potential use of *Himanthalia elongata* protein extract in the food industry.

Keywords: brown seaweed *Himanthalia elongata,* functional properties, amino acid profile, foaming properties, emulsifying properties.

1. Introduction

The World’s population is expected to reach 9.1 billion people within the next 40 years and it is predicted that food production will need to double in the next four decades. In this scenario protein supply will be critical for both human food and animal feed uses (Aiking, 2014).

At present, animal protein production for human consumption is inefficient, and on average the production of 1 kg of animal protein requires 6 kg of plant protein (Aiking, 2014). In terms of food sustainability, utilisation of less animal protein could be beneficial in terms of preventing the effects of climate change (Aiking, 2014). Plant proteins are cheaper to produce than animal proteins but lack essential amino acids. For example, lysine and tryptophan are deficient in cereals and methionine in legume crops (Ufaz & Galili, 2008). It is necessary therefore to find economically viable alternatives to both animal and terrestrial plant protein sources (Suresh Kumar, Ganesan, Selvaraj, & Subba Rao, 2014).

Protein contributes to the technofunctional properties of food products and can act as emulsifying agents, texture modifiers in addition to assisting with fat and water absorption and the whipping properties of foods (Ogunwolu, Henshaw, Mock, Santros, & Awonorin, 2009). These features all contribute to the taste, texture and consumer acceptance of food products (Ogunwolu et al., 2009). The functional properties of a protein concentrate depend on its physicochemical characteristics which include molecular weight, amino acid composition, net charge and surface hydrophobicity. The physicochemical characteristics of a protein extract often depend on the extraction conditions employed. For example, cowpea and pigeon pea protein isolates displayed differences in hydrophobicity, colour and enthalpies depending on the extraction technique (micellation technique versus isoelectric point precipitation) and the conditions employed (pH)(Mwasaru, Muhammad, Bakar, & Man, 1999).

Recently, demand for seaweed for human consumption has increased due to consumer demands for new and healthy "natural foodstuffs" produced in a sustainable manner. Seaweeds are known to be rich in minerals and certain vitamins, but they also can be a rich source of protein. The protein composition of seaweed and the primary sequences of the protein amino acids are different from those of land proteins and may be better suited for human consumption compared to other vegetable protein sources (Joel Fleurence, 1999). Most seaweeds also contain all the essential amino acids and brown macroalgae were reported to contain higher levels of the acidic amino acids aspartic and glutamic acid than red and green macroalgae (Joel Fleurence, 1999). *Himanthalia elongata* belongs to the brown macroalgae or Phaeophyta. It has a history of safe use and acceptability in cooking and was previously used to add a beefy or nutty-like flavour to dishes (Rhatigan, 2009). Indeed, previously, beef patty formulations produced using the seaweed *H. elongata* (40% inclusion) were rated the highest in terms of overall acceptability due to improvement in texture and mouth-feel without losing its sensory quality (Cox & Abu-Ghannam, 2013). However, only a few studies have been carried out detailing the functional properties of seaweed protein extracts (Kandasamy, Karuppiah, & Rao, 2012; Suresh Kumar et al., 2014).

The aim of the present study was to investigate the functional properties of protein extracts generated using food grade chemicals from the brown seaweed *H. elongata* commonly known as sea spaghetti. The solubility, water activity, water and oil holding capacity, emulsifying and foaming properties of the extracted protein were assessed. In addition the amino acid composition, colour and pH of the *H. elongata* protein extract were studied to assess the potential of this seaweed protein for use in the food industry.

2. Materials and method

2.1 Materials

Himanthalia elongata (Linnaeus) S. F. Gray provided by Porto-Muiños, Galicia, Spain was hand-harvested and collected at Muros, A Coruña, Galicia, Spain on the 20th of May 2013. Samples were freeze-dried, milled and vacuum preserved until further analysis. To avoid physicochemical modifications of the protein all reagents used during these experiments were food grade chemicals. Sunflower and olive oils from Musgrave House Ltd. (Cork, Ireland) and rapeseed, peanut and walnut oils from Lakeshore Foods Ltd. (Drogheda, Ireland) were purchased for use in this study.

2.2 Protein extraction and determination

Crude protein was extracted in triplicate according to the method of Galland-Irmouli et al. (1999). Briefly, 10 g of freeze-dried seaweed were suspended in 1 L of ultrapure water and ultra-sonicated for 1 hour using a Bransonic® 3510EMT (Branson Ultrasonic SA, Switzerland). This sample was left to stir overnight on a magnetic stirrer plate (IKA RCT basic safety control, Germany) at 4 °C. After 24 hours, the solution was centrifuged at 10000 x g for 1 hour and the supernatant decanted. The pellet fraction was re-suspended in 0.5 L of ultrapure water and subjected to a second extraction procedure as described above. Supernatants from both days were pooled together, and saturated to 80% with ammonium sulphate for 1 hour at 4 °C followed by centrifugation at 20000 x g for 1 h to precipitate the protein. The protein precipitates were diluted in a minimum volume of water and were subsequently dialyzed using Thermo Scientific™ SnakeSkin™ 3.5 kDa molecular weight cut off (MWCO) tubing (Fisher Scientific, New Hampshire State, USA) against ultrapure water at 4 °C overnight. Conductivity values were obtained for the water in dialysis tanks following incubation using a conductivity meter (Wissenschaftlich Technische Werkstätten, Germany). Dialyzed protein extracts were freeze-dried in an industrial scale freeze-drier FD 80 model

(Cuddon Engineering, New Zealand), vacuum sealed and stored at -20 °C until further analysis. The protein yield of this process was calculated as g protein extract / g seaweed on dry weight (DW). Nitrogen was analysed using a Leco Protein Analyser (Leco FP 628, Leco Corporation, USA). A factor of 6.25 was used to compute the protein value for the seaweed protein extract.

2.3 Total and free amino acid composition

For total and free amino acid composition analysis, *H. elongata* protein concentrate was hydrolysed in 6M HCl at 110°C for 23 hours following the method of Hill (1965). Samples were then deproteinized by mixing equal volumes of 24% (w/v) tri-chloroacetic acid (TCA) and sample, these were allowed to stand for 10 minutes before centrifuging at 14400 x g (Microcentaur, MSE, UK) for 10 minutes. Supernatants were removed and diluted with 0.2 M sodium citrate buffer, pH 2.2 to give approximately 250 nmol of each amino acid residue. Samples were then diluted 1 in 2 with the internal standard norleucine, to give a final concentration of 125 nm/mL. Amino acids were quantified using a Jeol JLC-500/V amino acid analyser (Jeol (UK) Ltd., Garden city, Herts, UK) fitted with a Jeol Na^+ high performance cation exchange column.

2.4. Polyacrylamide gel electrophoresis

Tris-Tricine-SDS-PAGE was performed using a Mini-PROTEAN® electrophoresis unit (Bio-Rad laboratories, USA) using Mini-PROTEAN® 10-20% Tris-Tricine Precast Gels (Bio-Rad laboratories, USA). Protein separation was performed according to the manufacturer's recommendations. Briefly, samples were diluted 1:1 in with loading buffer containing 200 mM Tris-HCl pH 6.8, 2% SDS, 40% glycerol, 0.04% Coomassie Blue G-250 and 350 mM DTT. Samples were heated at 55°C for 10 minutes and loaded in the precast gels in the electrophoresis unit in the presence of running buffer containing 100 mM Tris-base, 100 mM Tricine and 0.1% SDS. Running conditions were 30-35 mA for 3 hours and

15-20 mA for 2 hours. The Precision Plus Protein™ Dual Xtra Prestained Protein Standard (Bio-Rad laboratories, UK) was used as a molecular mass marker (250 - 2 kDa). After migration, protein bands were detected by Coomassie staining. Proteins were fixed in 40% methanol and 10% acetic acid for 40 minutes at low speed in an orbital shaker (VWR, USA). The gel was washed three times in ultrapure water for 5 minutes and then stained in Coomassie G-250 staining solution (Bio-Rad laboratories, USA) for 60 minutes in an orbital shaker. The gels were left overnight in ultrapure water with shaking and images were captured using a GS-800 densitometer (Bio-Rad laboratories, USA). All images were analyzed by Quantity One® software version 4.5.2 (Bio-Rad Laboratories, USA).

2.5 Colour evaluation

The colour of the extracted protein powder generated from *H. elongata* was measured using an UltraScan PRO Spectrophotometer (HunterLab, Germany) with illuminant D_{65}, diffuse 8° observer angle and included automated specular component. White and black standards (HunterLab, Germany) were used during the calibration procedure previous all measurements. Readings are reported in the CIE L*, a*, and b* system, being L* (lightness), a* (redness/greenness), and b* (yellowness/blueness). The chroma (C*) and hue (h°) values were calculated using the following equations:

$$C^* = \sqrt{a^{*2}+b^{*2}}$$

$$h^o = \tan^{-1}\left(\frac{b^*}{a^*}\right) \times \frac{180}{\pi}$$

To compare the colours of this study with other colours we used the ΔE*ab that represents the distance between any two colours in CIELAB space defined by its three orthogonal coordinates L*, a* and b*. ΔE*ab was calculated as follows

$$\Delta E^*ab = \sqrt{(L_1^* - L_2^*)^2 + (a_1^* - a_2^*)^2 + (b_1^* - b_2^*)^2}$$

L_1^*, a_1^* and b_1^* are the colour attributes of one colour sample and L_2^*, a_2^* and b_2^* the colour attributes of another colour sample to compare. A result of ΔE*ab less than 2 is generally considered to be perceptually equivalent.

2.6 Determination of pH

Freeze-dried *H. elongata* protein was resuspended in distilled water at 1% w/v and the pH measured using a pH meter Orion, model 420A (Thermo Orion, Cambridgeshire, UK).

2.7 Water activity (a_w)

The water activity value of the extracted *H. elongata* protein (a_w) was measured using an AquaLab Water Activity System (Pullman, Wash., USA). Freeze-dried samples of protein crude extract of *H. elongata* were measured at 22±0.08 °C.

2.8 Water- (WHC) and Oil-holding capacity (OHC)

Water-holding capacity (WHC) and Oil-holding capacity (OHC) were determined using the method of Bencini (1986) with slight modifications. *H. elongata* protein extract samples (1 g) were mixed with 10 mL of distilled water or sunflower oil in a vortex mixer (Henry Troemner, USA) and then centrifuged at 2200 x g for 30 minutes. The supernatants obtained were decanted and the centrifuge tubes containing sediment were weighed. WHC and OHC were expressed as grams of water or sunflower oil held by 1 g of protein by using the following formula.

$$\text{WHC } / \text{ OHC } (\text{g } H_2O \text{ or sunflower oil/g protein concentrate}) = \frac{W_2 - W_1}{W_0} \times 100$$

Where; W_0 is the weight of the dry sample (g), W_1 the weight of the tube plus the dry sample (g) and W_2 weight of the tube plus the sediment (g).

2.9 Solubility (S)

The solubility (S) of the protein extract was determined by the method of Beuchat, Cherry, and Quinn (1975) with slight modifications. A dried protein extract powder dispersion was prepared in water at a concentration of 1% w/v and the pH was adjusted to pH 2-12 using 1 M HCl or NaOH. This mixture was shaken at room temperature for 45 minutes in a Multi Reax Vibrating Shaker (Heidolph, Germany) and centrifuged at 4000 g for 30 minutes. The amount of soluble protein was determined using the QuantiPro BCA Assay Kit (Sigma, St. Louis, USA). The percentage solubility (S (%)) of the protein extract at different pH conditions was calculated as follows.

$$\mathrm{S}\ (\%) = \frac{\text{Protein supernatant}}{\text{Protein full dispersion}} \times 100$$

2.10 Foaming capacity and stability

The foaming capacity (FC) of the extracted protein was determined according to the method described previously by Poole, West, and Walters (1984) with minor modifications. Protein suspensions were prepared at room temperature at a concentration of 1.5% w/v and the pH was adjusted using 0.1M NaOH or 0.1 M HCl to pH 2, 4, 6, 8 and 10, respectively. Protein suspensions were homogenised using a T 25 digital ULTRA-TURRAX® homogeniser (IKA®, Germany) at 10000 rpm for 1 minute and the volume of foam generated was measured in a graduated cylinder. FC was calculated as the volume of foam generated as a percentage of the initial volume of the solution using the formula:

$$\mathrm{FC}\ (\%) = \frac{\mathrm{V_F} - \mathrm{V_0}}{\mathrm{V_0}}\ \mathrm{X}\ 100$$

Where; V_0 is the initial volume of protein solution before homogenisation and V_F is the volume of foam generated after homogenisation.

Foaming stability (FS) was expressed as the percentage of decrease of foam volume over time after 15, 30, 60, 90 and 120 minutes.

2.11 Emulsifying activity and emulsion stability

The emulsifying activity (EA) of the protein extracts was determined according to the methodology of Naczk, Diosady, and Rubin (1985) with slight modifications. The protein sample was diluted in Millipore ultrapure water (Millipore, Ireland) at a concentration of 1% w/v and the pH adjusted to between pH 2 to 10 using 0.1 M NaOH or 1 M HCl. The protein solution was homogenised for 30 seconds at 14000 rpm. To create an emulsion, commercial oils including sunflower, olive, rapeseed, peanut, and walnut oil were added to the aqueous phase containing the protein concentrate at oil: protein solution ratio of 3:2. The addition of oil was done in 2 steps. First half the volume of oil was added and the mixture was homogenized for 30 seconds at 14000 rpm and then the rest of the oil was added and the mixture homogenized for 90 seconds at the mentioned speed. The emulsion was placed in centrifuge tubes and centrifuged at 1100 x g for 5 minutes and the volume of the emulsion layer was measured. EA was calculated by the formula:

$$\mathrm{EA}\ (\%) = \frac{\mathrm{V_E}}{\mathrm{V_T}}\ \mathrm{X}\ 100$$

Being V_E the volume of the emulsion layer after centrifuging and V_T the all volume inside the tube.

To determine emulsion stability (ES), the previously prepared emulsions were heated at 85°C for 15 minutes, cooled at room temperature for 10 minutes and centrifuged again 1100 x g for 5 minutes. The ES was expressed as the % of EA remaining after centrifuging as follows:

$$\mathrm{ES}\ (\%) = \frac{\mathrm{V_{emulsion\ \ after\ \ heating}}}{\mathrm{V_{original\ \ emulsion}}}\ \mathrm{X}\ 100$$

2.12 Statistical analysis

In this study all measurements were carried out in triplicate. All statistical analyses were performed using SPSS for Windows v. 17.0. Normality of the data was tested with Kolmogorov-Smirnov and data were log-transformed before analysis when needed.

Differences in solubility and foaming capacity at different pHs were analyzed with one way ANOVA and post-hoc test HSD Tukey. Repeated measures general linear model was used to test differences in foaming stability with the effects of time, pH and pH*time included in the model and the differences analyzed using Games-Howell post-hoc test. The emulsion activity and stability were analyzed by multivariate general linear model, the effects of pH, oil and pH*oil interactions were contemplated in the model and post-hoc HSD Tukey tests were used to check the differences. In all cases, the criterion for statistical significance was $p < 0.05$.

3. Results and Discussion

3.1 Protein yield, total-free amino acid composition

The protein content of the obtained *H. elongata* protein extract was calculated as 63.38 ± 0.49 % using the LECO method described in section 2. The percentage yield obtained was 6.5 ± 0.7 % protein from the total seaweed extract when measured on a dry weight basis. This result is in agreement with previous studies which reported the level of protein extracted from this species of seaweed to be between 5-11% on a dry weight basis (Cofrades et al., 2010). The protein yield extracted could be improved by the addition of other non-food grade chemicals, such as 2-mercaptoethanol (Joël Fleurence, Le Coeur, Mabeau, Maurice, & Landrein, 1995). However, resultant protein would not have food grade status and would not be suitable for use in food products due to the toxic nature of 2-mercaptoethanol. Conductivity values obtained following dialysis of protein were 2.7 ± 0.03 (mS/cm) at 15.8 ± 0.09 °C and 11.9 ± 0.06 (µS/cm) at 15.8 ± 0.06 for dialysis water. A decrease in conductivity values was observed. This indicates that all salt was removed from the protein sample during the overnight dialysis.

The total and free amino acid composition of *H. elongata* protein extract (g/kg DW) is presented in Table 1. The total amino acid content of *H. elongata* was determined as 54.02 ± 0.46 g/kg DW (being the essential amino acids approximately 39.5% of the total amino acid composition), and the levels of the essential amino acids lysine (3.23 ± 0.05 g/kg DW) and methionine (1.96 ± 0.03 g/kg DW) were high as compared to other proteins from higher plants (Glew et al., 1997; Van Etten, Kwolek, Peters, & Barclay, 1967). In fact, cereals and legume crops are deficient in lysine and methionine respectively (Ufaz & Galili, 2008). Total amino acid content of *H. elongata* is in agreement with previous studies on *H. elongata* (22.4-61.4 g/kg DW) (Sánchez-Machado, López-Cervantes, López-Hernández, Paseiro-Losada, & Simal-Lozano, 2003) and in other brown seaweeds species (58.9-107.2 g/kg DW) (Mæhre, Malde, Eilertsen, & Elvevoll, 2014). Tris-Tricine gel analysis on the extract of *H. elongata* showed 5 protein bands with molecular weights values of 71.6, 53.7, 43.3, 36.4 and 27.1 kDa (Fig. 1).

3.2 Colour evaluation

The colour of the protein extracted from *H. elongata* was determined by measuring the lightness (L*), redness (a*), and yellowness (b*) values. L* represents the degree of lightness, being the value 100 white and the value 0 black. Redness is represented by +a* and -a∗ values reflect greenness. Yellowness is represented by +b*, while -b* indicates blueness (Aziah & Komathi, 2009). The value chroma (C*) represents the degree of departure from grey toward pure chromatic colour and the value hue (h°) represents the quality by which we distinguish one colour from another as red, yellow, green, blue or purple.

The colour values of *H. elongata* protein concentrate were determined as L* 59.36±0.04, a* 0.47±0.05, b* 16.26±0.11, C* 16.26±0.11 and h° 88.34±0.18. Colour is one of the most important appearance attributes of food since it influences consumer's acceptance (Jiménez-Aguilar et al., 2011). To our knowledge there are no studies on the colour of seaweed protein extracts and the colour of *H. elongata* protein concentrate from this study was perceptually different to other vegetable and seaweed powders available in the literature, as well as to dairy protein powders commonly used by the food industry (see Table 2). Determination of colour in food products could be useful due to the association between the colour and the taste and flavour perception in humans (Spence, Levitan, Shankar, & Zampini, 2010). In this sense it is demonstrated that the people's judgments of flavour identity are often affected by the changing of a food or colour (Spence et al., 2010).

3.3 pH and water activity (a_w)

The pH of *H. elongata* protein dispersions was determined as 3.99±0.02 and the a_w of the freeze dried protein powder was 0.47±0.01 measured at temperature 22.07±0.08 °C. Both physicochemical characteristics are important for the storage of the product before further use of these proteins by industry and its later applications. Foods with higher aw show rapid deterioration due to biological and chemical changes since aw influences microbial growth, lipid oxidation, non-enzymatic and enzymatic activities in foods (Sablani, Kasapis, & Rahman, 2007). The a_w of the protein powder of *H. elongata* is comparable to other dried food products such as sliced almonds (0.476 at 20 °C), wheat flour (0.523 at 25 °C) and starch (0.56 at 25 °C) (Schmidt & Fontana, 2008).

3.4 Water- (WHC) and Oil-holding capacity (OHC)

Interactions of water and oil with proteins are important for the food industry because of their effects on the flavour and texture of foods (Suresh Kumar et al., 2014). Intrinsic factors of the proteins such as the amino acid composition, protein conformation, surface polarity / hydrophobicity can affect their WHC and OHC (Yu, Ahmedna, & Goktepe, 2007).

The WHC represents the ability of a protein to associate with water under water limiting conditions. The WHC of the protein concentrate from *H. elongata* was determined as 10.27±0.09 g water / g protein concentrate. This value was higher than previous studies on seaweed extracts generated from species including *Kappaphycus alvarezii*, which had a WHC value of 2.22±0.04 g water / g protein (Suresh Kumar et al., 2014) and 4.67±0.04 g water / g Kapparazii powder^{TM} (Ramli, Daik, Yarmo, & Ajdari, 2014). Few studies have been conducted on seaweeds, normally on full seaweed powder, and our results were similar to WHC values described for *Hypnea japonica* seaweed powder (11.8-14 g water / g), *Hypnea charoides* seaweed powder (10.9-12.4 g water / g) and *Ulva lactula* seaweed powder (8.68-9.71 g water / g) (Wong & Cheung, 2000) and lower than the reported WHC of *Undaria pinnatifida* seaweed powder (19-44 g water / g) (Suzuki, Ohsugi, Yoshie, Shirai, & Hirano, 1996). A high WHC value for a protein helps to maintain freshness and moist mouth feel of baked foods and is associated with reduced moisture loss in packed bakery goods (Chandi & Sogi, 2007). Protein ingredients with excessively high WHC may dehydrate other formula components (Zayas, 1997c). High values of WHC are desirable in viscous food products such as sausages, custards, dough and baked products because these values help to hold water without dissolution of protein, providing thickening and viscosity (Seena & Sridhar, 2005).

The OHC is another important property of food ingredients used in formulated food. High OHC are desirable for flavour retention and improving the palatability of products (Tiwari, Tiwari, Mohan, & Alagusundaram, 2008). The OHC of *H. elongata* protein concentrate in this study was 8.1±0.07 g oil / g. This compared favourably to the OHC value obtained for *Kappaphycus alvarezii* protein (1.29±0.20 g oil / g) (Suresh Kumar et al., 2014) or Kapparazii powder^{TM} (5.11±0.36 g oil / g) (Ramli et al., 2014) and it was similar to other products such as rice bran protein concentrates (3.74-9.18 g oil / g) (Chandi & Sogi, 2007). The ability of protein to bind fat is very important in uses including meat replacers and extenders (Ogunwolu et al., 2009) and it is a requisite for the formulation of foods such as sausages, cake batters, mayonnaise and salad dressings (Chandi & Sogi, 2007).

The WHC and OHC values obtained for *H. elongata* protein concentrate extracted in this study demonstrates that it could be a useful protein extract for multiple food applications such as in improving the texture and palatability of different food formulations.

3.5 Solubility

Protein solubility is one of the most important parameters of a food ingredient because it influences other functional properties such as foaming and emulsifying properties. High nitrogen solubility is required for protein concentrates to be used as functional ingredients in many foods including beverages, dressings, coffee whiteners, whipped toppings and confections (Chandi & Sogi, 2007).

The pH had a significant influence effect on the solubility of *H. elongata* protein. Minimum solubility was observed at pHs 2 (25±1.1 %) and 4 (22.5±0.5 %), increasing with the pH (45.3±0.9 %, 65.1±0.8 %, 87.3±3 % at pH 6, 8 and 10 respectively) until reaching its highest values at pH 12 (96.15±0.15 %). Similar solubility values for proteins extracted from seaweeds and legumes have been reported in other studies (Chau, Cheung, & Wong, 1997; Suresh Kumar et al., 2014). The minimum solubility at pH 4 could be explained to the fact that the proteins have their isoelectric point at this pH and at more acidic or alkaline pHs the protein will acquire a net positive or negative charge respectively, which favours the repulsion of molecules and thereby increases the solubility of the protein (Seena & Sridhar, 2005).

3.6 Foaming capacity and stability

Proteins in foams contribute to the uniform distribution of fine air cells in the structure of the foods, improving its smoothness, lightness and allowing the volatilization of flavours that enhances the palatability of the food products (Zayas, 1997b).

The FC of *H. elongata* protein concentrate was influenced by the pH of the solution. The lowest FC was observed at pH 2 (6.98±0.16 %) and increased significantly until reaching its highest levels at pHs 6, 8 and 10 that did not show statistically significant differences (64.44±2.22, 55.56±6.92 and 71.52±4.81 %). The FC of *H. elongata* protein was higher than previous studies on seaweed protein extracts that showed maximum FC of approximately 54% at pHs4 and 2 for *Kappaphycus alvarezii* (Suresh Kumar et al., 2014) and *Enteromorpha* sp. (Kandasamy et al., 2012) respectively. The lowest FC at pH 2 and 4 could be attributed to the protein behaviour at its isoelectric point (Ragab, Babiker, & Eltinay, 2004). The higher FC at pHs 6, 8 and 10 of the proteins of *H. elongata* could be due to increased net charges on the proteins that weakened the hydrophobic interactions, increasing in this way the flexibility of the protein. This fact allows proteins to diffuse more rapidly to the air-water interface and encapsulate air particles, enhancing foam formation (Aluko & Yada, 1995).

The FS was significantly affected by time ($F_5=864.66$, $p<0.001$), pH ($F_4=8649.94$, $p<0.001$) and the interaction between both factors time*pH ($F_{20}=37.27$, $p<0.001$). So differences in FS were analyzed at each time for the different pHs (Fig. 2). The protein concentrate of *H. elongata* showed low foaming stability at pHs 2 and 4, being statistically significantly different to the rest of the groups after the 30th minute. At pH 10 the protein showed statistically significant better FS when compared to other pHs. After 30 minutes the FS decreased and showed no statistical differences to FS at pHs 6 and 8 at the 90th minute. This behaviour was similar to previous reports on cowpea (Ragab et al., 2004) and sesame seed (Khalid, Babiker, & Tinay, 2003) protein concentrates.

3.7 Emulsifying activity and stability

Emulsifying properties are one of the most important properties in the manufacturing of formulated foods (Zayas, 1997a) and good emulsifying properties are desired to make milk like beverages and meat analogs (Tiwari et al., 2008).

The EA of the protein concentrate of *H. elongata* at 5 different pH (ranged 2-10) and 5 different oils (sunflower, olive, rapeseed, peanut and walnut oils) is shown in Fig. 2. There were statistically significant differences in EA between the oils (F_4=9.19, p<0.001), pH (F_4=88.86, p<0.001) and the interaction oil*pH (F_{16}=4.92, p<0.001), so the data was analyzed at every pH taking into account the type of oil used. In general the lowest EA was appreciated at pH 4 for all oils used (68.8-82.9%), with no statistically significant differences between them. Differences in EA between oils were demonstrated at pH 2. Protein suspended in peanut oil showed the highest EA (99.7 %) and rapeseed oil the lowest EA (80%) at pH 2. Protein suspended in walnut oil and olive oil showed the highest (95.7 %) and the lowest (81.3%) EA values at pH 6, respectively. At pH 8 and 10 there were no statistical significant differences between oils and the EA was high (90.7-100%). The high EA found in *H. elongata* protein concentrate are in good agreement with previous studies with *Kappaphycus alvarezii* protein that showed high EA in different oils (75.68- 99.67 %) (Suresh Kumar et al., 2014). The V-shaped pattern of the EA profile for *H. elongata* protein concentrate (Fig. 2) might be due to variations in the hydrophilic-lipophilic balance of the proteins at different pH value within the range given (Sathe, Deshpande, & Salunkhe, 1982). These changes are similar to those described for different legume proteins (Chau et al., 1997). Previous studies suggested that the EA of some products depends on the nature and the concentration of the protein present in them (Dickinson, Galazka, & Anderson, 1991).

The ES was also statistically affected by the oil (F_4=13.85, p<0.001), pH (F_4=4.93, p<0.05) and the interaction of oil*pH (F_{16}=4.83, p<0.001), so the data was analyzed at pH values 2-10 taking into account the type of oil used (Fig. 3). In general rapeseed and walnut oils showed the worst ES at all pH values analysed. ES was more effective in sunflower, olive and peanut oils at pH values 6 and 8. The ES could be affected by several factors such as pH, droplet size, net charge, interfacial tension, viscosity and protein conformation (Hung & Zayas, 1991). In this case, the high ES after heating at 85°C for 15 minutes might be attributed to the dissociation of some proteins, resulting in the formation of subunits with more hydrophobic groups and thus stronger interactions with the lipid phase (Chau et al., 1997).

The functional properties of *H. elongata* protein concentrate suggest that this novel protein could be suitable for its use in the formulation of a wide variety of food products. More studies are needed on the sensory attributes of the final food product after the incorporation of the novel protein source (Lin, Huff, & Hsieh, 2002). The presence of contaminants, anti-nutritional factors and allergens in novel proteins should also be evaluated (Garcia-Vaquero & Hayes, 2016).

4. Conclusions

Presently, there is high demand from food manufacturers for protein alternatives to dairy and meat sources which ideally will be cheaper, with similar nutritional quality and also with excellent functional properties. Seaweeds have a long history of use in food products, however, it is still an underutilised source of protein. The WHC and OHC of the protein extract generated from *H. elongata* in this study along with its foaming and emulsifying properties suggest that it could be suitable for its use in the formulation of a wide variety of food products such as sausages, breads, and cakes as well as soups and salad dressing.

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Table 1. Total and free amino acid composition (g / kg DW) of *Himanthalia elongata* protein concentrate.

Essential amino acids	**Total amino acids (g/kg DW)**	**Free amino acids (g/kg DW)**
Threonine	3.25±0.04	0.12±0.00
Valine	4.28±0.18	
Methionine	1.96±0.03	
Isoleucine	2.28±0.07	
Leucine	2.05±0.07	
Phenylalanine	2.28±0.08	
Lysine	3.23±0.05	
Histidine	2.01±0.11	0.09±0.00
Non-essential amino acids		
Aspartic acid	5.94±0.04	
Serine	2.77±0.07	
Glutamic acid	7.52±0.05	0.32±0.01
Proline	2.55±0.25	
Glycine	2.98±0.03	
Alanine	3.32±0.13	0.11±0.01
Cysteine	3.14±0.16	0.13±0.03
Tyrosine	1.41±0.06	
Arginine	3.05±0.01	
Total amino acids	54.02±0.46	0.73±0.02

Table 2. Comparison of the colour of the protein concentrate from *Himanthalia elongata* and vegetable powders, seaweed products and common dairy proteins used by the food industry. At ΔE*ab < 2, the two colours compared are perceptually equivalent.

Sample	**Source**	**L***	**a***	**b***	**ΔE*ab**	**Reference**
Protein concentrate	*Himanthalia elongata*	59.36	0.47	16.26		Present study
Bakery powder	Pumpkin	32.98±1.35	4.70±1.16	23.26±1.03	27.62	Pongjanta, Naulbunrang, Kawngdang, Manon, and Thepjaikat (2006)
Protein powder	Soybean	97.16±0.06	0.03±0.01	2.68±0.10	40.17	Mwasaru, Muhammad, Bakar, and Man (1999)
Protein powder	Pigeon pea	82.27±0.34	−0.77±0.24	6.77±0.38	26.42	Mwasaru et al. (1999)
Protein powder	Cowpea	76.14±1	3.15±0.25	4.40±0.20	20.72	Mwasaru et al. (1999)
Kapparazii powder ™	*Kappaphycus alvarezii*	89.51±0.02	-1.27±0.03	5.49±0.02	32.06	Ramli, Daik, Yarmo, and Ajdari (2014)
TA150	*Eucheuma cottonii*	82.69±0.23	2.10±0.01	17.16±0.15	23.4	Chan, Mirhosseini, Taip, Ling, and Tan (2013)
Seakem CM611	*Chondrus crispus*	88.87±0.13	1.60±0.05	11.08±0.13	29.98	Chan et al. (2013)
Gelcarin GP812	*Chondrus crispus*	83.84±0.68	2.13±0.02	13.13±0.27	24.74	Chan et al. (2013)
Gelcarin GP911 NF	*Chondrus crispus*	83.63±0.29	2.18±0.09	12.37±0.51	24.64	Chan et al. (2013)
Grindsted ® CL220	Red seaweeds	87.51±0.42	0.27±0.01	11.91±0.57	28.48	Chan et al. (2013)
Calcium caseinate	Dairy product	91.78	-2.09	9.42	33.23	Krupa-Kozak, Bączek, and Rosell (2013)
Sodium caseinate	Dairy product	93.70	-2.46	6.77	35.75	Krupa-Kozak et al. (2013)
Whey proteins isolate	Dairy product	91.09	-2.32	10.86	32.31	Krupa-Kozak et al. (2013)

Lightness (L*), redness (a*), yellowness (b*), Chroma (C*) and distance between two colours (ΔE*ab).

Fig. 1. Tris-Tricine SDS-PAGE of the protein extract from *Himanthalia elongata*. (a) a molecular mass marker (250 - 2 kDa), (b) *Himanthalia elongata* protein extract.

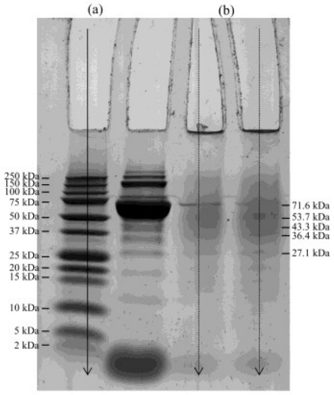
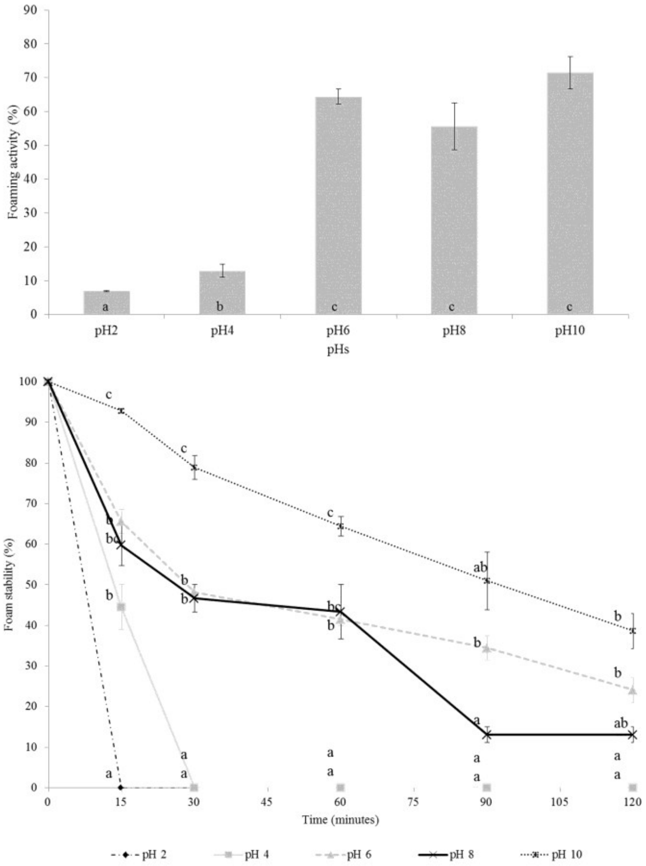
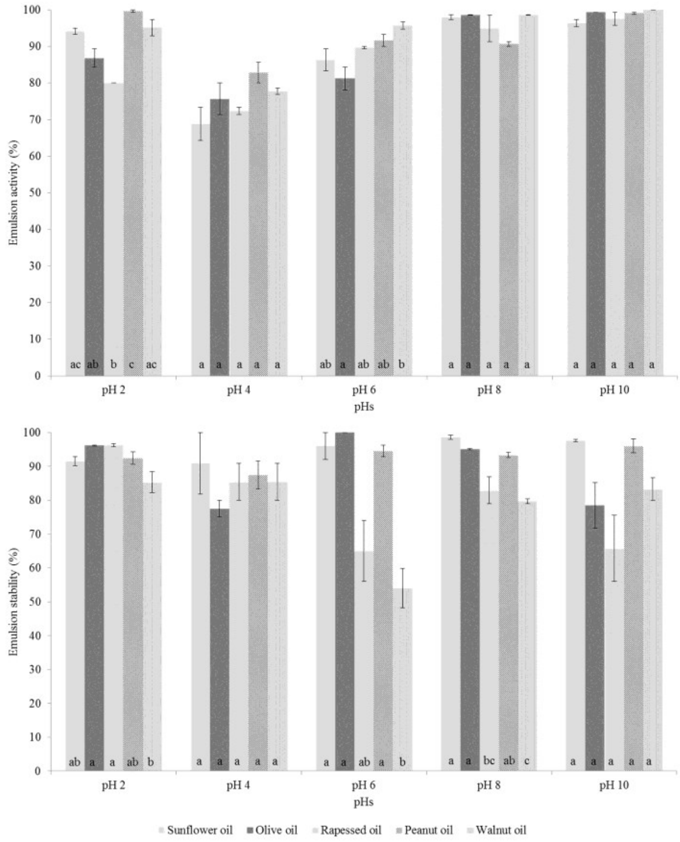


Fig. 2. Foam capacity and stability (%) of *Himanthalia elongata* protein concentrates at different pHs. Results are expressed as mean ± standard error of the mean (SEM).



Different letters indicate statistically significant differences ($p<0.05$) in FC and FS between the different pHs.

Fig. 3. Emulsifying activity and stability (%) of *Himanthalia elongata* protein concentrates at different pHs and oils.



Different letters indicate statistically significant differences ($p<0.05$) in EA and ES between the different oils at the same pH.

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