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Different extraction procedures and analysis of protein from *Ulva* sp. in Brittany, France

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Abstract Seaweeds are well recognized as a potential protein source. The edible green seaweed, *Ulva* sp., is abundant in the Brittany Coast, France. This study examined the extraction of proteins and glycoproteins from this seaweed. Four different extraction procedures (*Procedure 1:* deionized water, DW; *Procedure 2:* lysis solution 1 (LS1) containing 8 M urea, 2% Tween 20, 2% Triton X-100, 30 mM dithiothreitol, and 1% polyvinylpyrolidine; *Procedure 3:* lysis solution 2 (LS2) containing 50 mM Tris-HCl buffer pH 8, 10 mM EDTA, 2 mM $Na_2S_2O_5$, and 1% Triton X-100; *Procedure 4:* 50 mM Tris-HCl buffer, pH 8) were applied to extract proteins from *Ulva* sp. in Brittany. The protein contents (%, dry basis) in the above extracts from *Procedures 1–4* were 4.36 ± 0.21, 11.88 ± 0.23, 10.34 ± 0.35, and 3.58 ± 0.48, respectively. Moreover, electrophoresis (SDS-PAGE) revealed that the protein profile varies with season. Three glycoprotein-rich fractions, namely, GP-1 (from *Procedure GP1*), GP-2-DA, and GP-2-DS (from *Procedure GP2*), were extracted from *Ulva* sp. GP-1 and GP-2-DA fractions have a higher protein content than neutral sugars, while GP-2-DS contains a higher amount of neutral sugars than proteins. Matrix-assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/MS) technique was applied to further proteomic analysis of each glycoprotein-rich fraction. GP-2-DS was hydrolyzed with protease enzyme to confirm the availability of proteins, and interestingly, the particular hydrolysate shows no original peaks in the MALDI-TOF/MS analysis. All three glycoprotein-rich fractions show no cytotoxicity in Vero cells at the tested concentration (500 mg dw mL^{-1}). Collectively, these results revealed that the extractable protein content and protein profile of *Ulva* sp. differ according to the extraction liquid system and the season. The utilization and value addition of proliferative *Ulva* sp. in Brittany as a protein source is promising but needs to consider the seasonal change of the protein profile.

Keywords *Ulva* sp. · Seaweeds · Seaweed proteins · Glycoproteins · Nutraceuticals

Introduction

Seaweeds have been recognized for their potential health benefits and applications in the food (Ito and Hori 1989; Mohamed et al. 2012), pharmaceutical (Cardozo et al. 2007; Wijesekara and Kim 2010), and cosmeceutical (Kijjoa and Sawangwong 2004; Kim 2014) industries. They are rich sources of bioactive compounds such as sulfated polysaccharides (Wijesekara et al. 2011a), phlorotannins (Wijesekara et al. 2010; Li et al. 2011), pigments (Pangestuti and Kim 2011), sterols (Wijesekara et al. 2011b), peptides, and proteins (Fleurence 1999; 2004; Kim and Wijesekara 2010; Samarakoon and Jeon 2012). Seaweed-derived proteins are an underexploited resource with potential applications in the food, pharma, cosmetic, feed, and other industries. The global importance of seaweeds as additional and sustainable protein source has been shown (Angell et al. 2016). Moreover, there has been an

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increased interest to extract proteins from seaweeds (Galland-Irmouli et al. 1999; Wong et al. 2006; Cordeiro et al. 2006; Nagai et al. 2008; Yotsukura et al. 2009; Kim et al. 2010; Harnedy and FitzGerald 2013). The protein content of seaweeds varies and depends on the species, season, and environmental growth conditions (Peinado et al. 2014). Generally, brown seaweeds have lesser protein content (7–16%, dry weight) than red (21%, dw) and green (10–26%, dw) seaweeds (Dawczynski et al. 2007; Fleurence 1999). Seaweed-derived proteins contain mainly glycoproteins, phycolectins, enzymes, phycoerythrins, and mycosporine-like amino acids. Among these, glycoproteins are hypothesized to have a key role in physiological function of seaweed cell walls. However, their extraction, purification, characterization, and the relationship between protein and sugar moieties need to be explored. Various liquid systems such as distilled water (Fleurence et al. 1995; Galland-Irmouli et al. 1999; Angell et al. 2017), buffers and alkaline solution 0.1 M NaOH (Fleurence et al. 1995; Kim et al. 2010), urea (Contreras et al. 2008), lysis solutions (Kim et al. 2010), and phenol-based extraction systems (Rice and Crowden 1987; Wong et al. 2006; Nagai et al. 2008; Contreras et al. 2008) have been introduced and applied to extract proteins from seaweeds.

The European seaweed industry is dominated by Norwegian, French, and Irish production, while Spain, Portugal, and UK are small producers and suppliers (Peinado et al. 2014). In France, the Brittany coast has been extensively explored for its rich seaweed biodiversity including *Ulva* sp. (Fleurence et al. 1994; Hardouin et al. 2014, 2016; Mabeau and Fleurence 1993).

Edible green seaweed *Ulva* sp. (sea salad) is well popular in Japan as "aonori" and used in Europe for soups and salad preparations. The nutritional value of proteins obtained from *Ulva pertusa* and *U. armoricana*, the digestibility of alkali-soluble proteins from *U. pertusa*, a comparison of different extractive procedures for proteins from *U. rigida* and *U. rotundata*, the use of enzyme-assisted extraction to yield antiviral and antioxidant fractions from *U. armoricana*, and isolation of four glycoproteins from *U. lactuca* have been previously reported (Abdel-fattah and Sary 1987; Denis et al. 2009; Fleurence et al. 1999; Fujiwara-Arasaki et al. 1984; Hardouin et al. 2016). However, characterization of glycoproteins available in *Ulva* sp. for sugar composition, electrophoresis pattern, and biological activities is not yet completely explored. Therefore, in the present study, four different liquid systems were applied to compare the extractable proteins and two different protocols were used to extract glycoprotein-rich fractions from *Ulva* sp. available in the Brittany coast, France. Matrix-assisted laser desorption ionization-time of flight/mass spectrometry (MALDI-TOF/MS) analysis was carried out to partially characterize the glycoprotein-rich fractions.

Materials and methods

Chemicals

Chemicals and reagents used in electrophoresis analysis were obtained from Sigma-Aldrich (USA). Ammonium sulfate, $(NH_4)_2SO_4$; ethylenediaminetetra acetic acid (EDTA); sodium thiosulfate; silver nitrate; trichloroacetic acid (TCA); Tris base; polyvinyl propelene (PVP); dithiothretol (DTT); and glycine were from Fisher Scientific, UK. All other chemicals and reagents were analytical grade chemicals or reagents. Deionized water (DW) was obtained from a Milli-Q water purification and filtration system with 18 MΩ.cm resistivity (Millipore, USA).

Seaweed *Ulva* sp.

Green proliferative seaweed *Ulva* sp. (Chlorophyta, Ulvales, Ulvaceae) was hand-picked in St. Gildas de Rhuys (47° 30′ 0″ N, 2° 49′ 60″ W), Brittany, France (from September 2015 to March 2016) and rinsed with fresh water to remove adherent sediments, macro-fauna, and epiphytes. A sample voucher (*Ulva* sp. GlycoGreen 09/2015) is kept at the author's laboratory for future reference. The seaweed was then freeze-dried, powdered, and kept at 4 °C until proteins and glycoproteins were extracted.

Extractable proteins from *Ulva* sp.

Protein was extracted using four different liquid systems (*Procedures 1–4*), namely, *Procedure 1:* deionized water (DW); *Procedure 2:* lysis solution 1 (LS1 containing 8 M urea, 2% Tween 20, 1% PVP, 30 mM DTT); *Procedure 3:* lysis solution 2 (LS2 containing 50 mM Tris-HCl buffer pH 8, 10 mM EDTA, 2 mM $Na_2S_2O_5$, and 1% Triton X-100); and *Procedure 4:* Tris-HCl buffer (50 mM, pH 8). The freeze-dried *Ulva* powder was extracted with each liquid system (1:20, *w*/*v*) in the *Procedures 1–4* as previously described (Kim et al. 2010). Briefly, each extraction mixture was extracted for overnight at the cold room followed by centrifugation (10,000×*g*, 5 min at 4 °C). Each yielded supernatant was dialysed (molecularporous membrane tubing, MWCO 6–8000; Spectrum Laboratories, Inc., Canada) overnight against DW and freeze-dried.

Extraction of glycoproteins from *Ulva* sp.

Glycoproteins were extracted according to two different procedures published previously (Go et al. 2009; Habeebullah 2015) with some modifications.

Procedure GP1: The freeze-dried *Ulva* powder was extracted with distilled water (DW) (1:10, *w*/*v*) at 50 °C for 6 h with

continuous stirring. The DW extract was collected by filtering through a nylon cloth and the above procedure was repeated for another two more times. All the three DW extractions were pooled and freeze-dried. The freeze-dried DW extract was then dissolved in 6 M urea solution (1:25, *w*/*v*, pH adjusted to 8.5 with $KHCO_{3(aq)}$) and kept overnight at 4 °C with continuous stirring. Then, the solution mixture was centrifuged (10,000×*g*, 10 min, 4 °C) and TCA (100%) was added to the supernatant to the final concentration of 5% (*v*/*w*). After overnight keeping at 4 °C, the mixture was centrifuged (10,000×*g*, 10 min, 4 °C) and the supernatant was discarded. The pellet was dissolved in DW (1:100, *w*/*v*), adjusted the pH to 7, and dialyzed against DW for 2 days (6–8 kDa dialysis membranes, 4 °C). After the dialysis, the pH was adjusted to 2 and centrifuged (10,000×*g*, 10 min, 4 °C). The pellet was washed with a mixture of absolute ethanol (EtOH) and diethyl ether (DE) (EtOH:DE = 3:1, 10 mL × 3 times), with EtOH (10 mL) and finally with DE (10 mL). Each washing step was followed by centrifugation (10,000×*g*, 10 min, 4 °C). The yielded pellet, glycoprotein isolate-1 (UvGP-1), was dried under a fume hood overnight, calculated (% of original sample weight, dry weight basis), and kept at −21 °C till further analysis (Fig. 1 is an outline of *Procedure 1*).

Procedure GP2: Freeze-dried *Ulva* sp. was soaked in DW (1:10, *w*/*v*) and continuously stirred overnight at 4 °C. The water extract was then filtered through a cotton cloth, and two volumes of ethyl alcohol (absolute; 1:2, *v*/*v*) were added to the filtrate to precipitate polysaccharides (overnight at 4 °C). The upper layer was collected by filtration through a nylon cloth and the precipitated polysaccharides were discarded. Then, $(NH_4)_2SO_4$ salt was added to 50% saturation to the collected filtrate and kept overnight at 4 °C. The precipitated protein-conjugated salt was separated from the liquid medium by centrifugation (10,000×*g* for 10 min at 4 °C) and dissolved in DW followed by dialysis (6–8 kDa membranes, 4 °C, 2 days) against DW. After the dialysis, two glycoprotein isolate fractions were yielded: the dialysis aggregates (UvGP-2-DA) settled at the bottom of the dialysis tube, and the dialysis soluble (UvGP-2-DS) isolate fractions. Both UvGP-2-DS and UvGP-2-DA were freeze-dried; the yields were calculated (% of original sample weight, dry weight basis) and kept at −21 °C till further analysis (Fig. 2 is an outline of *Procedure 2*).

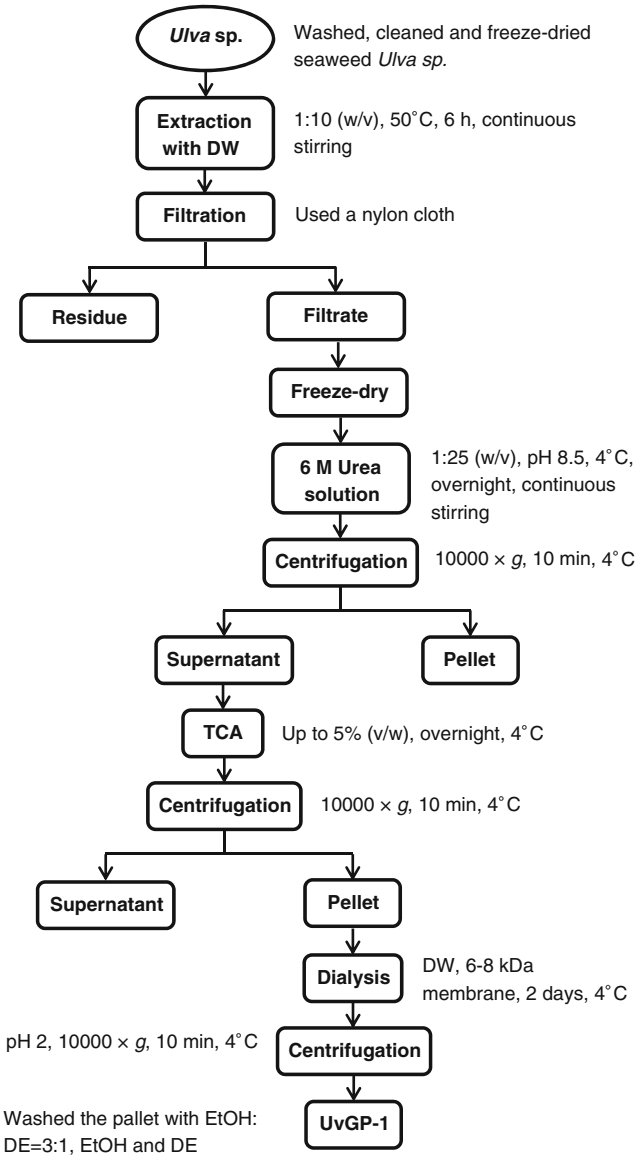

Fig. 1 Glycoprotein extraction from *Ulva* sp.: *Procedure GP1* (Go et al. 2009)

Determination of extractable protein content

For protein quantification, the freeze-dried protein extracts (Ex. 1–4 from *Procedures 1–4*) were dissolved in DW (1 mg mL^{-1}) and the protein content was determined by the bicinchonic acid colorimetric method (BCA assay) in a microplate according to the manufacturer's guidelines (Thermo Scientific, USA). Similarly, *Ulva* and its extracted glycoprotein fractions (UvGP-1, UvGP-2-DS, and UvGP-2-DA) were hydrolyzed in 0.1 M HCl for 3 h at 100 °C and kept in ice to cool for 10 min, followed by neutralization with the same volume of 0.1 M NaOH. The upper layer was collected for the quantification of protein content by BCA assay. Briefly, 25 μL from the supernatant of the extract was mixed with 200 μL of BCA assay reagent mixture. The microplate was incubated at 37 °C for 30 min and the absorbance was measured at 540 nm (Multiskan, Thermo Scientific). Bovine serum albumin (BSA) was used to develop the standard curve (0–500 μg mL^{-1}).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

For the extractable proteins, freeze-dried *Ulva* (0.1 g) was extracted with Tris-HCl buffer (50 mM, pH 8) (1:10, *w*/*v*)

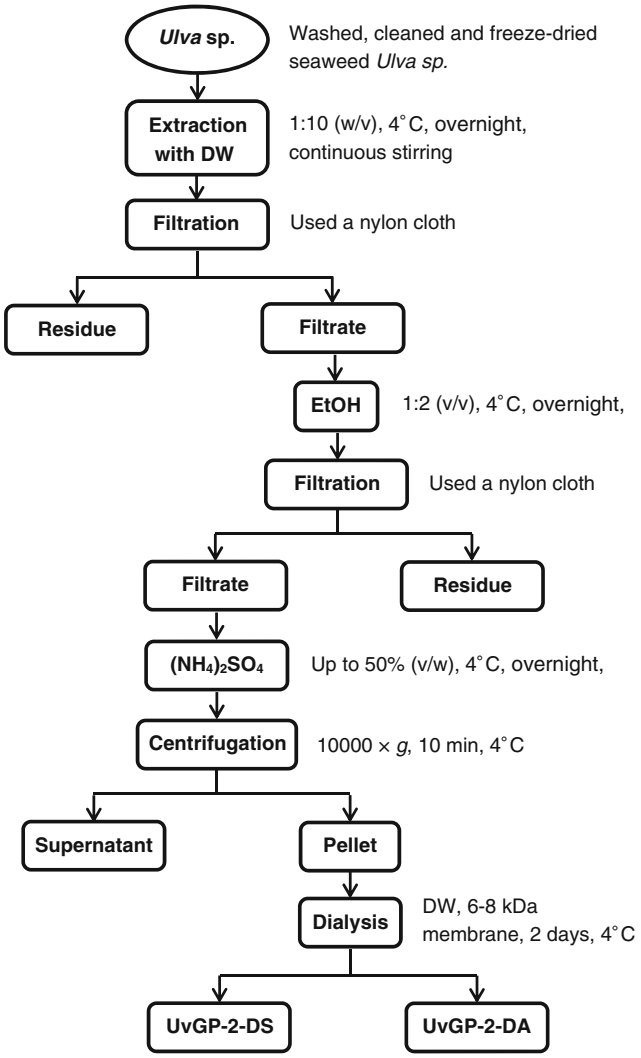

Fig. 2 Glycoprotein extraction from *Ulva* sp.: the *Procedure GP2* (Habeebullah 2015)

overnight at 4 °C and the upper layer collected for electrophoresis analysis. Of the *Ulva* protein extract, 200 μL was carefully collected from the upper layer and mixed with 800 μL of acetone. The mixture was kept at room temperature for 2 h and then centrifuged at 10000×*g* for 10 min. The resulting protein pellet (~ 1 mg) was dissolved in 12 μL of loading buffer (10% of 50 mM Tris-HCl buffer, pH 6.8, 40% of 10% sodium dodecyl sulfate solution (*w*/*v*), 20% of glycerol, 25% of 2-mercaptoethanol, and 5 of 1% bromophenol blue solution) and preheated at 90 °C for 2 min prior to the SDS-PAGE analysis. For the extracted glycoprotein fractions; each fraction was dissolved in loading buffer at 2 mg mL^{-1} prior to SDS-PAGE loading.

SDS-PAGE was performed using a Mini Protean II electrophoresis system (Bio-Rad, USA) with 5% stacking gel and 10% separation gel (Laemmli 1970). The molecular weights of bands of the protein fractions were compared by reference to the relative migration of standard proteins applied: bovine serum albumin (66 kDa) and casein (22 kDa) proteins. The gels were stained with $AgNO_3$ (0.1 g of $AgNO_3$ with 37 μL of formaldehyde in 100 mL of DW). The stained gels were revealed with Na_2CO_3 solution (2.5 g of Na_2CO_3 in DW with 25 μL of formaldehyde). Further staining was stopped by 50% EDTA solution.

Neutral sugar content

Neutral sugar content of the raw *Ulva* and its glycoprotein fractions were assessed according to Dubois method with slight modifications (Hardouin et al. 2014, 2016). Briefly, the samples were mixed with 75% phenol solution and conc. H_2SO_4 acid was added. After stirring for 10 s and incubation at 20 and 30 °C (10 min each), the absorbance was measured at 490 nm. Anhydrous D-glucose was used to obtain the standard curve.

Matrix-assisted laser desorption ionization-time of flight/mass spectrometry analysis Each glycoprotein-rich fraction (GP-1, GP-2-DA, and GP-2-DS) and the protease (enzyme Protamex, Novozyme, Denmark) hydrolysate of GP-2-DS and the enzyme used were subjected to MALDI-TOF/MS analysis. 2,5-Dihydroxybenzoic acid (DHB) was used as the matrix.

Cell culture

African green monkey kidney cells (Vero, ATCC CCL-81) were grown in Eagle's minimum essential medium (MEM, Eurobio) supplemented with 8% fetal calf serum (FCS, Eurobio) and 1% of antibiotics PCS (10,000 IU mL^{-1} penicillin, 25,000 IU mL^{-1} colimycin, 10 mg mL^{-1} streptomycin; Sigma-Aldrich).

Cytotoxicity assays based upon cell viability

Cytotoxicity of the yielded glycoprotein fractions from *Ulva* was tested on cultured Vero cells, as previously described (Hardouin et al. 2014, 2016). Briefly, cytotoxicity was evaluated by incubating Vero cell suspensions (3.5×10^5 cells mL^{-1}) with different concentrations of glycoproteins ranging from 1 to 200 μg mL^{-1} diluted in supplemented Eagle's MEM, in the wells of a microplate. Cytotoxicity was tested by cell viability after 72 h incubation (37 °C, 5% CO_2) using the neutral red dye method. Optical density (OD) was measured at 540 nm. The 50% cytotoxic concentration (CC_{50}) was defined as the concentration that reduced the OD of treated cells to 50% of that of untreated cells. CC_{50} values were expressed as the percentage of cytotoxicity:

$$(\%C) : \left[(ODc)_C - (ODc)_{MOCK} / (ODc)_C\right] \times 100$$

where $(ODc)_C$ and $(ODc)_{MOCK}$ are the OD values of the untreated cells and treated cells, respectively (Langlois et al. 1986).

Statistical analysis

All biochemical tests were done in triplicates (n = 3) and cytotoxicity was evaluated in quadruplicates (n = 4). The data are expressed as mean ± standard deviation. Duncan's multiple range test was performed for the mean comparison at 5% significance level ($p \leq 0.05$).

Results

Extractable protein content

The extractable protein content (%, dw) varies according to the liquid system used (Fig. 3). LS1 yielded the highest extractable proteins (11.88 ± 0.23) followed by LS2 (10.34 ± 0.35). The DW (4.36 ± 0.21) and Tris-HCl (3.58 ± 0.48) extractions showed no significant difference. The use of organic liquid systems with protein extraction enhancers increases the extractable proteins, but in the case of biorefinery of this proliferative *Ulva* sp. for food and feed industry, these organic systems are limiting factors. A variety of extraction systems have been introduced to isolate proteins from seaweeds. However, the yield and purity of proteins vary according to the seaweed species and the composition of cell wall polysaccharides, which interfere with extraction and purification. Moreover, the seasonal variations of the protein content of seaweeds need to be fully studied for sustainable industrial application.

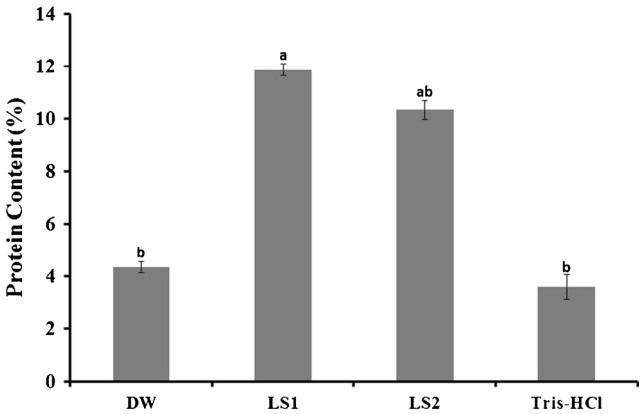

Fig. 3 Extractable protein content (%, dw) of different extraction liquid systems; DW, LS1, LS2, and Tris-HCl buffer. Data are shown as mean ± SD and means with the same letter are not significantly different at $p \leq 0.05$

SDS-PAGE of *Ulva* sp. proteins

An electrophoresis technique, sodium dodesylsulfate polyacrylamide gel electrophoresis (1-D or 2-D) was used to identify and determine the molecular weight of dominant protein bands in the extracted seaweed proteins. Here, the Tris-HCl buffer-extracted proteins from *Ulva* sp. were subjected to electrophoretic separation and the resulting protein profile changes according to the season (Fig. 4). Interestingly, it has clearly expressed the sharp protein bands during the early winter months (October and November, 2015) than September 2015 and March 2016. Moreover, some protein bands are only expressed during the early winter months.

Yield, protein, neutral sugar contents, and cytotoxicity of glycoprotein-rich fractions

In this study, two previously published procedures were used and *Procedure 1* yielded glycoprotein fraction, GP-1 (yield; 0.54%), and *Procedure 2* yielded two glycoprotein fractions namely, GP-2-DA, which aggregates at the bottom of the dialysis tube (yield is 0.52%, dry weight basis) and the soluble fraction GP-2-DS (yield; 1.98%). Figure 5 presents protein and neutral sugar contents of the three glycoprotein-rich fractions along with the original freeze-dried seaweed sample *Ulva* sp. (U). GP-2-DA has the highest protein content (33.42 ± 1.73%), followed by GP-1 (24.99 ± 0.95%) and GP-2-DS (22.68 ± 1.74%). GP-2-DS has the highest neutral sugar content (28.58 ± 1.69%), followed by GP-2-DA (18.25 ± 1.7%) and

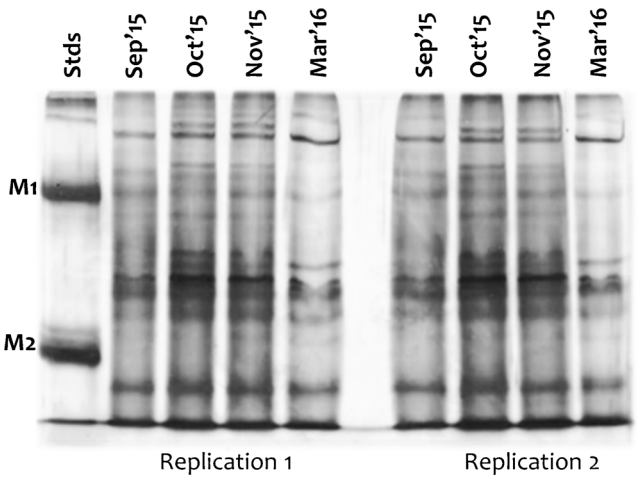

Fig. 4 SDS-PAGE protein profile of *Ulva* sp. in Brittany, France. Electrophoresis was performed using a Mini Protean II electrophoresis system (Bio-Rad, USA) with 5% stacking gel and 10% separation gel. The molecular weights of bands of the protein fractions were compared by reference to the relative migration of standard proteins applied; bovine serum albumin (66 kDa) and casein (22 kDa). The gels were stained with $AgNO_3$ solution

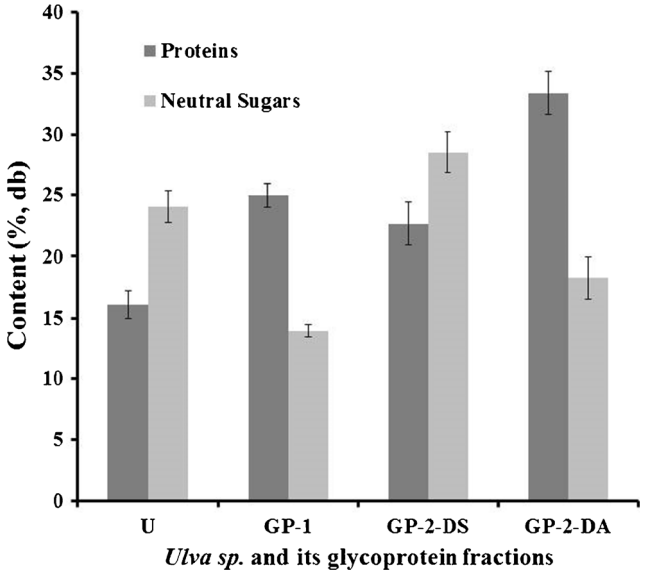

Fig. 5 Extractable protein and neutral sugar contents (%, dw) of yielded three glycoprotein-rich fractions; GP-1, GP-2-DS, and GP-2-DA (mean ± SD)

GP-1 (13.96 ± 0.47%). The freeze-dried *Ulva* sp. contains 16.08 ± 1.14% of proteins and 24.09 ± 1.27% of neutral sugar.

Cytotoxicity assay has revealed that three glycoprotein-rich fractions show no cytotoxicity in Vero cells at the tested concentration (up to 500 mg mL^{-1}, in DW).

MALDI-TOF/MS analysis of isolated glycoproteins

The MALDI-TOF/MS spectrum (Fig. 6) clearly indicates that the glycoprotein fractions consist of proteins. The protease-hydrolyzed GP-2-DS hydrolysate (GP-2-DS + E) does not show original bands as presented in the original fraction GP-2-DS. This was further compared with the enzyme (E) spectrum and the both spectrums show almost similar bands. To the best of our knowledge, this is the first report on MALDI-TOF/MS analysis of seaweed-derived (from *Ulva* sp.) glycoproteins to ensure the presence of protein part.

Discussion

The extraction of proteins from plant tissues is complicated since they contain interference constituents such as polysaccharides (lignin, cellulose, hemicelluloses) and phenolic compounds (Cremer and Van de Walle 1985; Granier 1988; Saravanan and Rose 2004). Hence, a phenol-based extraction procedure for proteins from plant tissues has been employed to overcome this issue (Saravanan and Rose 2004; Wang et al. 2006; Faurobert et al. 2007). Similarly, the main constraint in seaweed protein extraction is the presence of cell wall mucilage including anionic or neutral polysaccharides (Ito and Hori 1989; Fleurence et al. 1995; Nagai et al. 2008). Another constraint is due to the low extraction of proteins, poor resolution in gel electrophoresis, and insufficient extract quantities for further analysis. Until now, most of seaweed protein extractions were performed to compare the efficacy of liquid system used to yield high protein extracts from seaweeds (Fleurence et al. 1995; Kim et al. 2010). Among the various extraction liquid systems, phenol-based extraction has been proved to be the most efficient (Wong et al. 2006; Nagai et al. 2008; Contreras et al. 2008). In this study, application

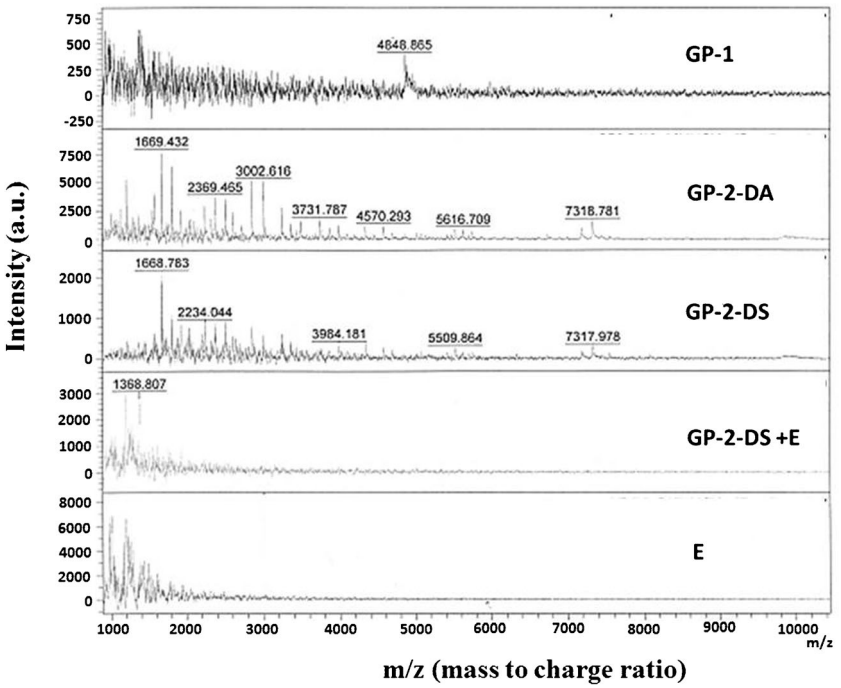

Fig. 6 MALDI-TOF/MS spectrum of three glycoprotein-rich fractions (GP-1, GP-2-DA, and GP-2-DS), the protease hydrolysate (GP-2-DS + E) and the enzyme (E)

of lysis solution systems yielded higher extractable proteins compared to water and buffer. However, the recent trend is for green extraction conditions, specifically in the food and pharmaceutical industries. Hence, the water or buffer extraction of proteins is of much interest and recommended. Moreover, it has been shown that the addition of distilled water (1:10, *w/v*) and holding for a few hours results in an osmotic shock and facilitates protein extraction from seaweeds (Fleurence et al. 1995; Barbarino and Lourenço 2005).

The protein content of seaweeds varies and depends on the species, season, and environmental growth conditions (Harnedy and FitzGerald 2011; Peinado et al. 2014). For example, the protein content of *Palmaria palmata* (Rhodophyta) is higher in the winter-spring period than in the summer-early autumn period (Galland-Irmouli et al. 1999). In this study, some protein bands were more clearly expressed during the winter months than in other seasons. Seaweeds may contain non-protein nitrogen (N, such as free nitrates, pigments, nucleic acids), resulting in an overestimation of their protein content (Angell et al. 2016) such as when using the general N-to-protein conversion factor of 6.25 in the common Kjeldahl procedure). Therefore, N-to-protein conversion factors of 5.38, 4.59, and 5.13 for brown, red, and green seaweeds, respectively, have been suggested (Lourenco et al. 2002; Makkar et al. 2016) and species-specific conversion factors have been determined for some seaweeds (Biancarosa et al. 2017). Shuuluka et al. (2013) proposed a factor of 4.45 for *Ulva* spp.

Glycoproteins are available in all living organisms with the possible exception of bacteria. To date, some glycoproteins have been isolated from seaweeds (Hwang et al. 2008; Go et al. 2009; Go et al. 2010; Rafiquzzaman et al. 2013; Choi et al. 2014; Habeebullah 2015; Rafiquzzaman et al. 2015a, 2015b). However, their structures and functional roles in seaweeds are yet to be widely investigated. In the present study, two procedures were used to yield glycoprotein-rich fractions. To the best of our knowledge, this is the first study on glycoprotein extraction of *Ulva* sp. from Brittany, France. We followed two different procedures previously used to extract glycoproteins from brown seaweeds but slightly modified both procedures for green seaweed. Moreover, MALDI-TOF/MS analysis was performed in this research to analyze the glycoprotein-rich fractions for the first time. It has been previously published that seaweed-derived glycoproteins have health beneficial bioactivities such as preventing liver cells, promotes IEC-6 cell proliferation, anti-inflammatory, antioxidant, and anti-Alzheimer's activities. Interestingly, cytotoxicity assay revealed that all three glycoprotein-rich fractions show no cytotoxicity in Vero cells. Glycoproteins are embedded in the seaweed cell wall matrix and believed to be incorporated in seaweed adhesion to a stratum.

Another key type of the reported seaweed-derived proteins are the arabinogalactan proteins (AGPs) and one of our further studies is to search for these yielded glycoprotein-rich fractions for the presence of AGPs. These AGPs and hydroxyproline-rich glycoproteins (HRGP) are found in the cell wall of green seaweeds (Domozych et al. 2012). The cell wall of green seaweed, *Codium fragile*, contains low amounts of HRGP (Estevez et al. 2009) and AGPs (Fernández et al. 2010). Moreover, AGPs have been strongly implicated in terrestrial plant developmental process but their roles are as yet poorly known (Popper 2011). There are two moieties in AGPs and the carbohydrate component accounts around 90% (dry weight basis) and mostly contains arabinose and galactose residues. The protein component is approximately 10% (dry weight basis) and rich in hydroxyproline residues (Fincher et al. 1983).

Although the structures and activity relationships of seaweed-derived proteins are yet to be explored extensively, it has shown that some seaweeds contain proteins comparable with terrestrial protein sources like soybean and other leguminous plants (Norziah and Ching 2000; Cian et al. 2015). Moreover, seaweed proteins contain all essential amino acids (Galland-Irmouli et al. 1999; Dawczynski et al. 2007). Interestingly, after extracting the agar from the red seaweed, *Gracilaria fisheri*, the by-product is rich in proteins, which contain high amounts of essential amino acids (Laohakunjit et al. 2014). According to our preliminary analysis, the yielded GP-1 and GP-2-DA fractions have richer amino acid composition than GP-2-DS (data not shown). To date, a large number of different seaweeds have been explored for their protein quantity and quality (Harnedy and FitzGerald 2011), but their value and the applications in the food industry are yet to be promoted.

Collectively, these results suggested that *Ulva* sp. from the Brittany coast, France, is rich in proteins and glycoproteins and could be utilize as a potential protein source. However, the extractability and availability of different protein bands vary according to the extraction medium and season. Further characterization and purification of the extracted glycoprotein-rich fractions are in progress.

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