

# Seasonal variation in nitrogenous components and bioactivity of protein hydrolysates from *Porphyra dioica*

Julianne Stack<sup>1</sup> · Paul R. Tobin<sup>1</sup> · Anna Gietl<sup>2</sup> · Pádraigín A. Harnedy<sup>1</sup> · Dagmar B. Stengel<sup>2</sup> · Richard J. FitzGerald<sup>1</sup>

Received: 12 August 2016 / Revised and accepted: 15 January 2017 / Published online: 1 February 2017  
© Springer Science+Business Media Dordrecht 2017

**Abstract** The red macroalga *Porphyra dioica* has been harvested and consumed for centuries. Based on its nutritional composition, availability and consumer familiarity, significant potential exists to develop this species as a source of high-value functional food ingredients. Therefore, a detailed assessment of the natural variation in *P. dioica* nitrogenous components was performed to identify the optimal season for biomass harvesting with high bioactive peptide potential. Kjeldahl nitrogen analysis revealed that total nitrogen (TN) and protein nitrogen (PN) contents in *P. dioica* (expressed as (w/w) dry weight) from western Ireland ranged from 2.48 to 4.94% and 1.90 to 4.30%, respectively. Significant differences in protein contents were observed between samples collected in summer and winter months. Electrophoretic analysis also showed differences in the protein profiles of *P. dioica* collected at different times of the year. *P. dioica* protein extracts were hydrolysed with the food-grade proteolytic preparations, Alcalase 2.4 L and Flavourzyme 500 L, and significant seasonal differences were observed in in vitro bioactivity assays. The oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) values of the hydrolysates ranged from 229.5 to 1015.3 and 4.1 to 28.7  $\mu\text{mol}$  Trolox equivalent per gram of freeze-dried powder, respectively. The *P. dioica* hydrolysates also inhibited angiotensin-converting enzyme (ACE; half maximal inhibitory concentration,  $\text{IC}_{50}$ , 0.34 to 1.78  $\text{mg mL}^{-1}$ ) and dipeptidyl peptidase-IV

(DPP-IV;  $\text{IC}_{50}$ , 1.14 to 5.06  $\text{mg mL}^{-1}$ ). The results demonstrate the potential of *P. dioica* hydrolysates as health-enhancing food components or natural food preservatives due to their enzyme inhibitory and antioxidant activities.

**Keywords** Bioactive peptides · Enzymatic hydrolysis · Antioxidant · ACE · DPP-IV · Macroalgae

## Introduction

Macroalgae have a long history of use as animal feeds and human foods, particularly in South-East Asia and Japan, where seaweeds are an important commercial product. In 2008, approximately 21.6 billion sheets (~65,000 dry tonnes) of *Porphyra/Pyropia* were maricultured (Levine and Sahoo 2010). Although *Porphyra dioica* (and other ‘nori’ species), has been traditionally harvested and consumed, high-value applications of this marine species in Ireland appear to be limited. Some red macroalgae are reported to contain significant amounts of protein, i.e. up to 47% (w/w) dry weight (Fleurence 2004), and have been recognised as a promising source for the generation of protein-based biofunctional food ingredients (Harnedy and FitzGerald 2011). Due to its wide availability, nutritional composition and consumer familiarity, there may be a significant potential to develop *P. dioica* as a source of commercially valuable functional food ingredients. However, to realise this potential, a systematic assessment of the seasonal variation in the chemical and bioactive profile of *P. dioica* is required. This is due to the fact that the chemical composition of seaweeds including proteins, pigments and polysaccharides varies considerably depending on ambient environmental conditions such as water/air temperature, salinity, light and nutrient availability (Hafting et al. 2015).

✉ Richard J. FitzGerald  
dick.fitzgerald@ul.ie

<sup>1</sup> Proteins and Peptides Research Group, Department of Biological Sciences, University of Limerick, Limerick, Ireland

<sup>2</sup> Botany and Plant Science, School of Natural Sciences and Ryan Institute for Environmental, Marine and Energy Research, National University of Ireland Galway, Galway, Ireland

As well as providing amino acids and energy that are required for growth and maintenance, many food proteins contain, embedded within their primary sequences, peptide sequences capable of modifying particular physiological functions referred to as bioactive peptides (Nongonierma et al. 2016). These short amino acid sequences are inert within the sequence of the parent protein but can be released during gastrointestinal digestion, treatment with proteolytic preparations, food processing or fermentation (Pihlanto-Leppala 2000; Erdmann et al. 2008). Food-derived bioactive peptides may find applications in the management of a wide range of diseases by providing health benefits beyond their basic nutritional value (Harnedy and FitzGerald 2011). One such bioactivity relates to the ability of peptides to act as antioxidative agents.

Oxidative stress can damage macromolecules such as DNA, proteins and lipids, which may contribute to many illnesses such as diabetes and cancer, along with inflammatory, neurodegenerative and cardiovascular diseases. Antioxidants are thought to exert a positive effect on human health by protecting the body against damage by reactive oxygen species and free radicals (Ahn et al. 2004; Butterfield et al. 2006; Ngo et al. 2011), and consumer awareness of and demand for antioxidants from natural sources is increasing (Saha et al. 2015). Protein hydrolysates generated from sources such as egg and milk have been reported to inhibit lipid oxidation in various meat products (Pena-Ramos and Xiong 2003; Sakanaka and Tachibana 2006), and antioxidant protein hydrolysates have also been prepared from macro- and microalgae (Ko et al. 2012; Harnedy and FitzGerald 2013).

Type 2 diabetes mellitus causes significant morbidity and mortality and is characterised by both insulin deficiency and peripheral insulin resistance. The incretin hormones, glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), are rapidly cleaved and rendered metabolically inactive by the action of dipeptidyl peptidase-IV (DPP-IV; Deacon et al. 1995). Drug-based inhibition of this enzyme has therefore become a new approach for the treatment of type 2 diabetes mellitus. Interestingly, in this regard, natural DPP-IV inhibitory peptides have been generated following the hydrolysis of various food proteins such as those from milk (Nongonierma and FitzGerald 2013), egg (Van Amerongen et al. 2009) and the macroalga, *Palmaria palmata* (Harnedy and FitzGerald 2013).

High blood pressure is a major risk factor for the development of cardiovascular diseases (Kannel and Higgins 1990). The carboxydipeptidase, angiotensin-converting enzyme (ACE), has an important role in blood pressure regulation. ACE is involved in the transformation of angiotensin I to the powerful vasoconstrictor angiotensin II in the renin-angiotensin system, and the inactivation of the vasodilator bradykinin, in the kinin nitric oxide system (Ni et al. 2012). ACE inhibition is therefore considered to be a suitable

therapeutic approach in the management of hypertension. Although effective, there are several side-effects such as skin rashes and cough associated with the use of synthetic ACE inhibitors such as Captopril® (Atkinson and Robertson 1979). Consequently, there is substantial interest in identifying ACE inhibitors from natural sources for use in the management of hypertension. Several algal sources of ACE inhibitory peptides have been described in the literature (He et al. 2007; Cian et al. 2013; Harnedy and FitzGerald 2013).

The objective of this study was to identify the optimal season for harvesting of *P. dioica* biomass in order to subsequently yield high levels of peptide bioactivity. This study characterised the seasonal variability in nitrogenous components and the protein electrophoretic profiles of *P. dioica* collected from the west coast of Ireland. The effect of time of sample collection on the in vitro antioxidant, anti-diabetic (DPP-IV inhibitory activity) and cardioprotective potential (ACE inhibitory activity) of *P. dioica* protein hydrolysates was also investigated.

## Materials and methods

The proteolytic preparations, Alcalase 2.4 L and Flavourzyme 500 L, were from Novozymes A/S (Denmark). Tris-glycine-sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) buffer (10×) was obtained from National Diagnostics (USA). Trinitrobenzenesulphonic acid reagent was from Fisher Scientific (Ireland). Phenol solution (equilibrated with 10 mM Tris HCl, pH 8.0, 1 mM EDTA) and all other reagents were supplied by Sigma (Ireland).

## Sample preparation

Wild *Porphyra dioica* samples were collected at 6-week intervals from Spiddal, Co. Galway, Ireland (53° 14' 39" N, 9° 17' 55" W) over a 12-month period (July 2014 to July 2015). The seaweed was rinsed with sea water to remove sand and other particulate matter. The samples were freeze dried and subsequently ground with a Cyclotec Mill (1 mm screen, FOSS Tecator AB, Sweden). The freeze-dried, milled seaweed was then stored at room temperature in a light-protected, air-tight container. Analyses were carried out continuously during the sampling period in order to minimise the potential effects of long term storage on sample components.

## Determination of TN, NPN and PN

The TN content of the algal samples was quantified using a modification of the macro-Kjeldahl procedure described by Connolly et al. (2013) where 200 mg sample (in triplicate) was weighed out in N-free paper (Whatman, B-2 grade) and digested in 20 mL concentrated sulphuric acid with one

Kjeldahl catalyst tablet. Sodium caseinate of known protein content was used as a standard.

Milled, freeze-dried algal samples (~800 mg in triplicate) were resuspended in de-ionised water (dH<sub>2</sub>O; 1:20 (w/v)) and were stirred overnight at 4 °C prior to PN and NPN determination. The protein components were then precipitated by treatment with 72% (w/v) trichloroacetic acid (TCA, 12% (w/v) final concentration) following gentle stirring for 3 h at 4 °C. Samples were centrifuged for 15 min at 4 °C (4190×g, Hettich Universal 320R, Andreas Hettich GmbH & Co. KG, Germany). Kjeldahl nitrogen analysis of the pellet and supernatant was performed as described above to determine the PN and NPN contents, respectively, of *P. dioica*. The units of concentration for the nitrogenous components are expressed throughout in terms of (w/w) dry weight.

### Sodium dodecyl sulphate polyacrylamide gel electrophoresis

Algae were prepared for SDS-PAGE analysis using the method as described Wang et al. (2006). Protein concentration was measured using the Bradford assay (Bradford 1976) and 15.0 µg protein was loaded per lane using protein loading buffer (National Diagnostics, USA). SDS-PAGE analysis was carried out as described by Le Maux et al. (2016) using 12% (w/w) acrylamide gels stained with Coomassie Blue.

### Preparation of protein extracts

The procedure used to generate aqueous and alkaline-soluble protein extracts from milled freeze-dried *P. dioica* was as described by Harnedy and FitzGerald (2013). SDS-PAGE analysis of the extracted proteins was performed as described above. The combined aqueous and alkaline protein extracts were enriched by isoelectric precipitation by adjusting the pH with 1 N HCl to pH 4 as described by Harnedy and FitzGerald (2013).

### Determination of protein

The protein concentration of aqueous and alkaline-soluble extracts was determined in 96-well microplates by the Bensadoun and Weinstein (1976) modification of the Lowry et al. (1951) protein quantification protocol. The protein components of each sample (1 mL) were precipitated by adding 100 µL of 0.15% (w/v) sodium deoxycholate and 100 µL of 72% (w/v) TCA. The samples were incubated for 15 min on ice and then centrifuged for 10 min at 4 °C (21,250×g). Supernatant was removed. The protein pellets were resuspended with 1 mL of 2% (w/v) Na<sub>2</sub>CO<sub>3</sub> in 0.1 mol L<sup>-1</sup> NaOH. Combined reagent (1:1:100 2% (w/v) sodium tartrate 1% (w/v) CuSO<sub>4</sub>·5H<sub>2</sub>O/2% (w/v) Na<sub>2</sub>CO<sub>3</sub> in 0.1 mol L<sup>-1</sup> NaOH, 200 µL) was added to 20 µL samples and

standards in a 96-well microplate and incubated for 10 min. Folin Ciocalteu reagent (20 µL of 1 mol L<sup>-1</sup>) was then added. The plate was incubated for a further 30 min, and the absorbance was measured at 680 nm. All aqueous and alkaline protein extract samples were analysed in triplicate (*n* = 3) with reference to a bovine serum albumin protein standard curve (0–200 µg mL<sup>-1</sup>).

### Enzymatic hydrolysis of algal proteins

The macroalgal protein solutions (1.6% (w/v) in dH<sub>2</sub>O) were heated to 50 °C and adjusted to pH 7 using 0.5 M NaOH. The proteolytic preparations, Alcalase and Flavourzyme, were used at an enzyme/substrate (E/S) ratio of 1% (v/w) each. The reaction mixture was maintained at pH 7 using a pH-Stat (842 Titrand, Metrohm, Switzerland) during 4 h hydrolysis. The proteolytic enzymes were inactivated by heating the samples at 90 °C for 20 min. No-enzyme control samples were treated in the same manner. All samples were subsequently freeze dried.

### Monitoring the extent of hydrolysis and GP-HPLC

The extent of hydrolysis was assessed by the trinitrobenzenesulphonic acid method using the microplate format as described by Le Maux et al. (2016). All samples were analysed in triplicate (*n* = 3), and results were expressed as milligram of amino nitrogen released per milligram of freeze-dried powder (FDP). Gel permeation high-performance liquid chromatography (GP-HPLC) was performed as described by Spellman et al. (2005).

### Sample preparation for bioassays

Freeze-dried protein hydrolysate and unhydrolysed control samples were resuspended in the appropriate assay buffers at 30 mg mL<sup>-1</sup>. Undissolved material was removed by centrifugation for 5 min at room temperature (4190×g). Supernatants were diluted prior to bioassay as required.

### Bioactivity screening

All assays were carried out as independent triplicates assayed in triplicate using a microplate reader (BioTek Synergy HT, USA). The units of concentration for the bioactivity outputs were expressed throughout in terms of (w/w) dry weight of FDP.

### Antioxidant activity

The ferric-reducing antioxidant power (FRAP) and oxygen radical absorbance capacity (ORAC) activities of samples were determined using the methods described by Harnedy and FitzGerald (2013). The FRAP and ORAC activities were

expressed as micromoles of Trolox equivalents (TE) per gram of FDP.

#### DPP-IV inhibitory activity

DPP-IV inhibitory activity was determined by measuring the amount of free 7-amino-4-methyl-coumarin (AMC) liberated from the fluorogenic substrate Gly-Pro-AMC as described by Hamedy et al. (2015). The values were expressed as the mean  $IC_{50} \pm$  standard deviation ( $n = 3$ ). Diprotin A was used as a positive control ( $IC_{50}$  value,  $4.69 \mu\text{M}$ ).

#### ACE inhibitory activity

ACE inhibitory activity was determined using a fluorometric microtitre assay, as described by Connolly et al. (2014), using bovine lung ACE at  $8 \text{ mU mL}^{-1}$  activity. The values were expressed as the mean  $IC_{50} \pm$  standard deviation ( $n = 3$ ). Captopril was used as a positive control ( $IC_{50}$  value,  $25.14 \text{ nM}$ ).

#### Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 (GraphPad, USA). The results were analysed by one-way analysis of variance (ANOVA) at a significance level of

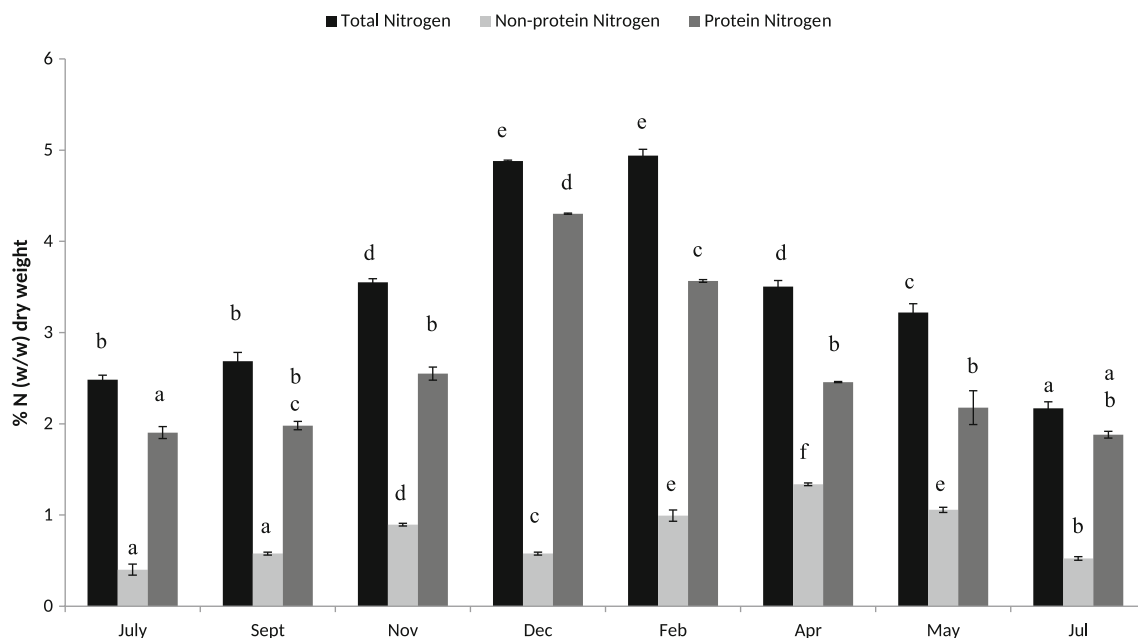
$p \leq 0.05$ . Where applicable, multiple comparisons were performed using Tukey's post hoc test.

## Results

### Quantification of nitrogenous components

Kjeldahl analysis of *P. dioica* samples revealed distinct seasonal variation of its nitrogenous components, with significant differences observed between samples collected in summer months and those collected in winter. The TN content was lowest during the summer months ( $2.48 \pm 0.05\%$  (w/w), July 2014); it increased significantly during the autumn ( $3.54 \pm 0.07\%$  (w/w), September 2014), peaking in the winter months ( $4.94 \pm 0.07\%$  (w/w)) and then decreased to  $2.17 \pm 0.07\%$  (w/w) in July 2015 (Fig. 1,  $p \leq 0.05$ ).

TCA was used to precipitate *P. dioica* proteinaceous material, which was then removed from the NPN-containing fraction by centrifugation. Kjeldahl analysis of these fractions revealed PN values ranging from  $1.88 \pm 0.04$  (July 2015) to  $4.30 \pm 0.06\%$  (w/w) (December 2014), and NPN values ranging from  $0.40 \pm 0.06$  (July 2014) to  $1.34 \pm 0.02\%$  (w/w) (April 2015). The *P. dioica* PN content exhibited a similar seasonal variation to that observed for TN, i.e. PN was lowest during the summer months, increased during autumn, peaking in the winter and then decreased during spring (Fig. 1). However,

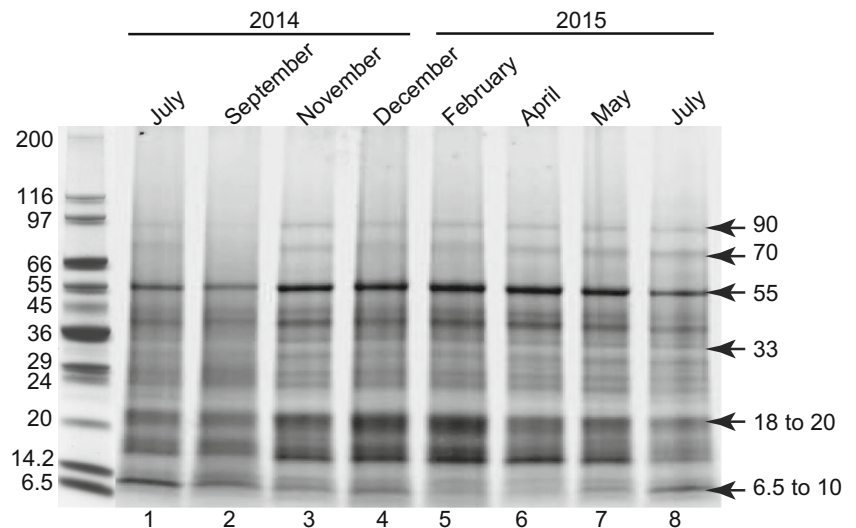


**Fig. 1** Total nitrogen (TN), non-protein nitrogen (NPN) and protein nitrogen (PN) contents of *Porphyra dioica* collected in July, September, November and December 2014 and February, April, May and July 2015. All results are reported as per cent nitrogen content of dry weight

*P. dioica*. Mean  $\pm$  SD ( $n = 3$ ). For analysis of each component (TN, PN or NPN), samples with different letters are significantly different at  $p \leq 0.05$



**Fig. 2** Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of proteins extracted from milled, freeze-dried *Porphyra dioica* collected at different times of the year. All lanes were loaded with 15 µg of protein



while *P. dioica* NPN content was also lowest during the summer months ( $0.40 \pm 0.06$  and  $0.52 \pm 0.02\%$  (w/w) in July 2014 and 2015, respectively), peak NPN contents were observed in spring ( $1.34 \pm 0.02\%$  (w/w) in April 2015,  $p \leq 0.05$ ).

**Protein characterisation and recovery**

Figure 2 shows the SDS-PAGE profiles of the total proteins extracted from milled freeze-dried *P. dioica*. Qualitative assessment of protein composition by SDS-PAGE showed different polypeptide profiles in samples collected at different times of the year. Some bands were common in all months, such as the 24 to 28 kDa, and 45 kDa bands, and appeared with regular intensity regardless of time of sample collection. However, some bands varied in intensity over the course of the year such as those at 6.5 to 10, 18–20, 33, 55, 70 and 90 kDa. Phycoerythrins purified from red macroalgae are composed of three subunits  $\alpha$ ,  $\beta$  and  $\gamma$ , which have apparent molecular masses of approximately 18, 20, and 30 to 33 kDa, respectively (Sun et al. 2004), which corresponded to some bands of increased intensity observed in winter and spring months (Fig. 2). The SDS-PAGE profiles of the July and September 2014 samples were less defined than those collected in other months.

In this study, aqueous and alkaline-soluble proteins were extracted from *P. dioica*. These fractions were then combined prior to enzymatic hydrolysis. The presence of polysaccharides and other algal compounds can limit proteolysis of algal proteins (Fleurence 2004), but protein enrichment by isoelectric precipitation minimises the inhibitory effect of other algal compounds on hydrolysis. Table 1 shows the seasonal variation in protein recovery from *P. dioica* samples collected at different times of the year. The protein recovery from *P. dioica* was lowest during the summer months ( $12.65 \pm 0.86$  mg protein g<sup>-1</sup> dry weight *P. dioica*, July 2014). It increased over

the winter months, peaking in December 2014 ( $47.98 \pm 0.8$  mg g<sup>-1</sup>), and then decreased again in summer ( $16.41 \pm 0.37$  mg g<sup>-1</sup>, July 2015). Significantly higher levels of total protein ( $32.94 \pm 1.11$  and  $28.27 \pm 0.79$  mg g<sup>-1</sup>) were recovered from the December 2014 and February 2015 *P. dioica* samples, respectively, compared with all other samples ( $p \leq 0.0001$ ). This is in agreement with the data in Fig. 1 showing that TN and PN contents were highest in these months.

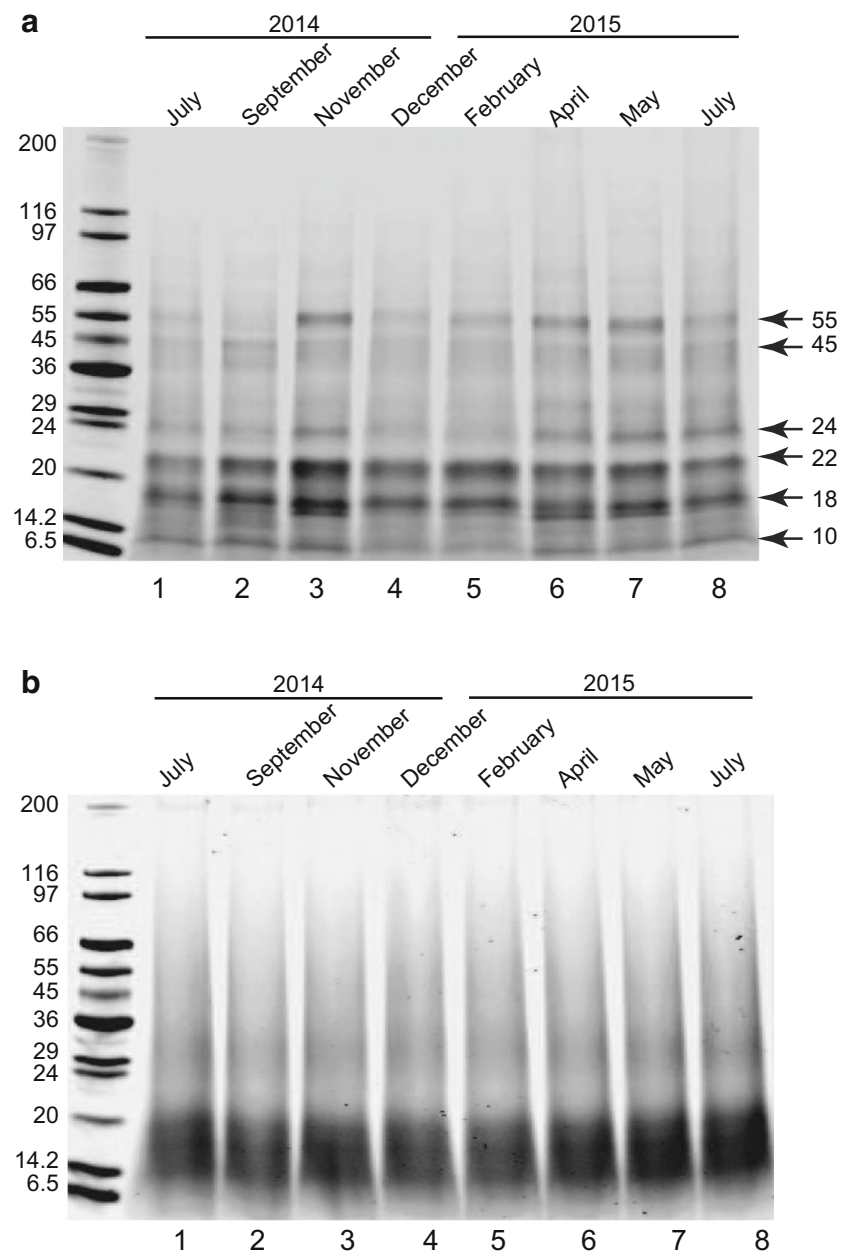
Figure 3 shows the SDS-PAGE profiles of the *P. dioica* aqueous and alkaline-soluble protein extracts. All the aqueous-soluble protein extracts have a number of shared protein bands (e.g. bands eluting at 22 and 45 kDa). However, significant variation in the intensity of several bands was also observed; e.g. bands at approximately 55 kDa were more intense in winter than in the July and September protein extracts. Additionally, bands at 10 and 24 kDa were less intense in samples collected in December and February. The SDS-PAGE profiles of the alkaline-soluble protein extracts were the same regardless of time of collection (Fig. 3b).

**Table 1** Concentration of aqueous, alkaline and total protein extracted from *Porphyra dioica*

Sample	Protein extracted (mg protein g <sup>-1</sup> dry weight)		
	Aqueous	Alkaline	Total
July 2014	3.96 ± 0.59 a	8.69 ± 1.45 a	12.65 ± 0.86 a
September 2014	3.66 ± 0.20 a	9.55 ± 0.49 a	13.21 ± 0.29 a
November 2014	14.47 ± 0.08 c	24.87 ± 0.39 c	39.34 ± 0.47 d
December 2014	15.04 ± 0.30 cd	32.94 ± 1.11 e	47.98 ± 0.80 f
February 2015	16.53 ± 0.22 de	28.27 ± 0.79 d	44.80 ± 0.57 e
April 2015	17.72 ± 0.90 e	19.32 ± 0.38 b	37.04 ± 1.28 d
May 2015	8.41 ± 0.05 b	11.60 ± 0.45 a	20.01 ± 0.50 c
July 2015	6.61 ± 0.64 b	9.80 ± 1.01 a	16.41 ± 0.37 b

Mean ± SD (n = 3). For each extract type (aqueous, alkaline or total), values with different lowercase letters are significantly different ( $p \leq 0.05$ )

**Fig. 3** Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of aqueous (a) and alkaline-soluble (b) proteins extracted from milled freeze-dried *Porphyra dioica* collected at different times of the year. All lanes were loaded with 15  $\mu\text{g}$  of protein



### Characterisation of the hydrolysates

Combined aqueous and alkaline protein extracts from *P. dioica* were hydrolysed using Alcalase and Flavourzyme for 4 h at 50 °C. An unhydrolysed control sample was also incubated for 4 h at 50 °C. Table 2 shows the changes in amino nitrogen concentration as a function of incubation time in freeze-dried enzyme-treated and unhydrolysed control (without enzyme) *P. dioica* protein extracts. The concentration of amino groups released was significantly higher in samples collected during the winter months. An increase of over 21 mg amino nitrogen  $\text{mg}^{-1}$  dry weight was observed after hydrolysis of the protein extracts from December, February and April compared with 1.74 and 10.54 mg amino

nitrogen  $\text{mg}^{-1}$  in July 2014 and 2015, respectively. There were no significant changes observed in the concentration of amino groups in the unhydrolysed control samples for all months.

GP-HPLC analysis (Fig. 4) was used to investigate the molecular weight distribution of the soluble proteinaceous constituents in the *P. dioica*, unhydrolysed control samples and associated hydrolysates. Unhydrolysed control samples from July and September 2014, and April, May and July 2015, all had significantly more soluble proteinaceous components >10 kDa than those from November and December 2014, or February 2015. Incubation with Alcalase and Flavourzyme modified the molecular masses of the soluble proteinaceous components of the samples. Apart from the July 2014 sample, higher amounts of low molecular weight

**Table 2** Concentration of amino nitrogen liberated in unhydrolysed *Porphyra dioica*, protein controls (control) and protein hydrolysates after 4 h hydrolysis with Alcalase and Flavourzyme (hydrolysate)

Sample	mg amino nitrogen mg <sup>-1</sup> FDP	
	Control	Hydrolysate
July 2014	0.83 ± 0.03 a	2.57 ± 0.14 b
September 2014	1.02 ± 0.05 a	11.28 ± 0.14 c
November 2014	0.91 ± 0.03 a	17.99 ± 0.16 d
December 2014	0.82 ± 0.09 a	21.95 ± 0.00 e
February 2015	0.59 ± 0.07 a	23.95 ± 0.42 f
April 2015	0.55 ± 0.06 a	23.03 ± 0.38 ef
May 2015	0.63 ± 0.06 a	19.28 ± 0.63 d
July 2015	0.54 ± 0.06 a	11.08 ± 0.55 c

Mean ± SD (n = 3). Data are expressed as milligrams amino nitrogen released per milligram of freeze-dried powder (FDP). Samples with different lowercase letters are significantly different (p ≤ 0.05)

peptides (<10 kDa) were seen in the protein hydrolysates compared with the unhydrolysed control. The majority of these peptides were <0.5 kDa.

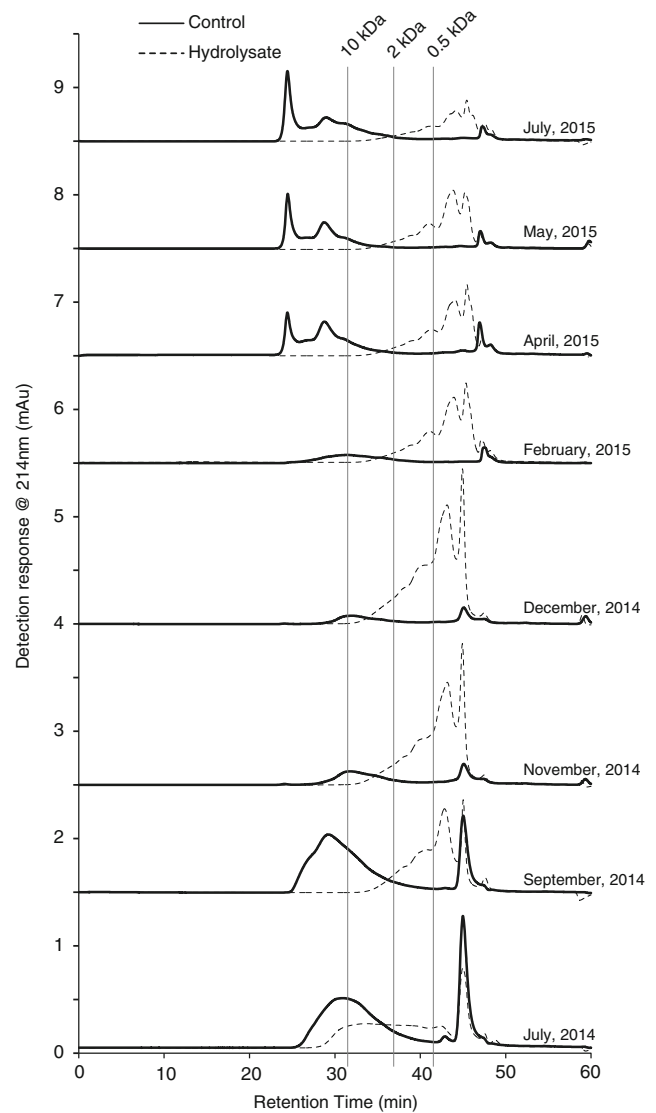
The time of harvest of samples used for generation of protein hydrolysates had a substantial effect on the molecular mass of the peptides generated (Fig. 4). The amount of low molecular weight peptides (<1 kDa) generated was greater in protein hydrolysates from samples collected in November and December 2014 (81.62 and 60.97% of soluble proteinaceous components, respectively), than those collected in July 2014 (28.37%). Interestingly, the unhydrolysed control samples collected in July 2014 contained as much low molecular weight components as the hydrolysate.

**In vitro assessment of biological activity**

The soluble proteinaceous components derived from *P. dioica* protein hydrolysates were assessed for in vitro antioxidant, ACE inhibitory and DPP-IV inhibitory activities.

*Antioxidant activity*

Seasonal variation was evident in the antioxidant activity of the *P. dioica* hydrolysates. As seen in Fig. 5, all the *P. dioica* protein hydrolysates, except those from July and September 2014, had significantly higher antioxidant activities (FRAP and ORAC) compared with the unhydrolysed controls (p ≤ 0.0001). This indicated that starting protein composition could affect the antioxidant activity of the peptides released. Additionally, the antioxidant potency of hydrolysates generated from winter and late spring protein extracts (November and December 2014 and February and April 2015) were significantly higher than those generated from summer and early

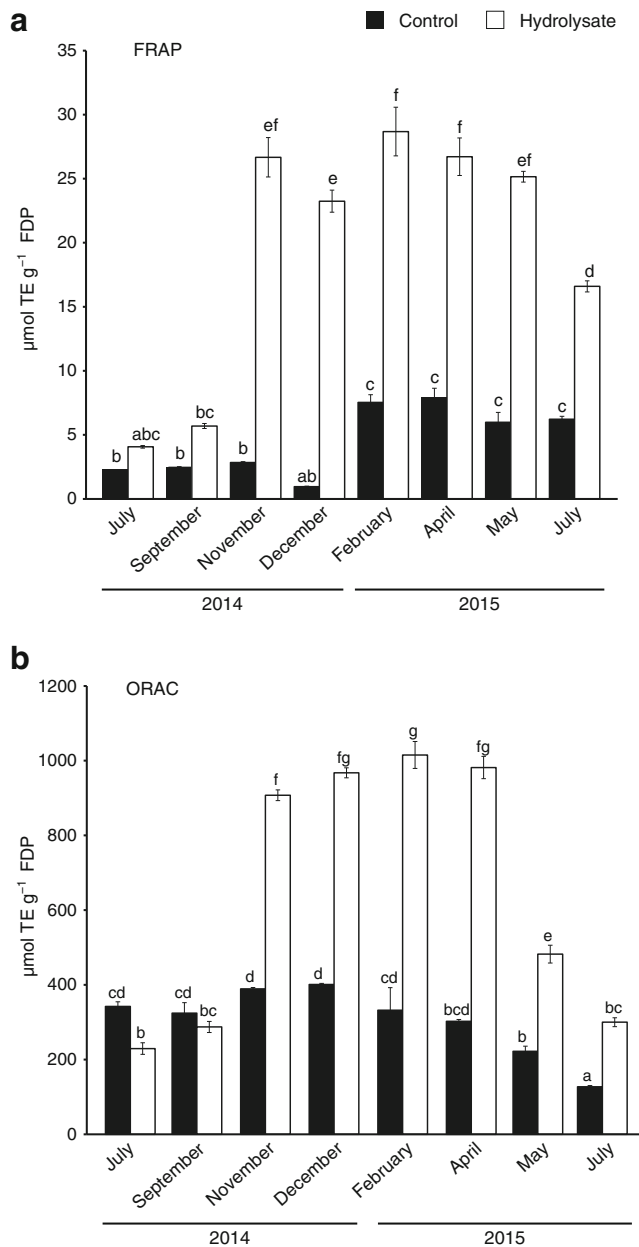


**Fig. 4** Gel permeation high-performance liquid chromatography (GP-HPLC) profiles showing the molecular mass distribution of the soluble proteinaceous components in unhydrolysed *Porphyra dioica* controls (control) and protein hydrolysates after 4 h hydrolysis with Alcalase and Flavourzyme (hydrolysate). Vertical lines indicate the retention times corresponding to 0.5, 2 and 10 kDa

autumn protein extracts (July and September 2014 and July 2015; Fig. 5, FRAP and ORAC, p ≤ 0.0001).

*DPP-IV inhibition*

Table 3 summarises the DPP-IV IC<sub>50</sub> values of the *P. dioica* protein hydrolysates and unhydrolysed control samples assessed in this study. Some seasonal variation was evident in the DPP-IV inhibitory activity of the *P. dioica* hydrolysates (Table 3). The DPP-IV IC<sub>50</sub> values of the hydrolysates generated from winter months (November 2014 to April 2015) were not statistically different from each other (1.14 ± 0.04 to 1.19 ± 0.21 mg mL<sup>-1</sup>; p > 0.05) but were more potent than



**Fig. 5** **a** Ferric reducing antioxidant power (FRAP) and **b** oxygen radical absorbance capacity (ORAC) of *Porphyra dioica*, unhydrolysed controls (control) and protein hydrolysates (hydrolysate). Values are expressed as mean  $\pm$  SD ( $n = 3$ ). For each assay, bars with different letters are significantly different at  $p \leq 0.05$ . Data are expressed as micromoles of Trolox equivalents (TE) per gram of freeze-dried powder (FDP;  $\mu\text{mol}$  of TE  $\text{g}^{-1}$ )

those generated from samples collected in peak summer (July 2014 and 2015,  $2.19 \pm 0.10$  and  $5.06 \pm 0.62$   $\text{mg mL}^{-1}$ , respectively;  $p \leq 0.05$ ). The DPP-IV inhibitory activity of the *P. dioica* protein hydrolysates generated from samples collected in April and May 2015 ( $1.14 \pm 0.04$  and  $1.98 \pm 0.01$   $\text{mg mL}^{-1}$ , respectively) were not significantly more potent than their unhydrolysed protein controls ( $1.78 \pm 0.21$  and  $2.00 \pm 0.01$   $\text{mg mL}^{-1}$ , respectively;  $p > 0.05$ ).

### ACE inhibition

Table 3 summarises the ACE  $\text{IC}_{50}$  values for the *P. dioica* protein hydrolysates and unhydrolysed control samples assessed in this study. With the exception of *P. dioica* collected in July 2014, all *P. dioica* protein hydrolysates had significantly more potent ACE inhibitory activity compared with the unhydrolysed protein controls ( $p \geq 0.05$ ). The time of harvest did not affect the ACE inhibitory ability of these hydrolysates ( $0.34 \pm 0.08$  to  $0.55 \pm 0.06$   $\text{mg mL}^{-1}$ ,  $p > 0.05$ ).

### Discussion

A thorough investigation of the natural variation in *P. dioica* nitrogenous components was required in order to identify the optimal season for biomass harvesting with high bioactive peptide potential. *P. dioica* protein content ranged from  $9.40 \pm 0.23$  to  $21.52 \pm 0.04\%$  (w/w) when calculated from experimentally obtained % PN values after using a NTP conversion factor of 5.00 (Angell et al. 2016). These are similar to protein contents previously reported for *Porphyra* spp. (Smith et al. 2010). As was the case with the seasonal trend observed for TN and PN content, *P. dioica* protein content was lowest during the summer months and highest in winter, with a greater than twofold difference being observed. This increase may be due to higher levels of phycobiliproteins such as phycoerythrin. Phycobiliproteins are light harvesting antennae-protein pigments found in red algae, cyanobacteria and others, and can represent up to 50% of the total protein content of algal cells (Glazer 1994). Phycoerythrin is thought to have a nitrogen-storage function. As seawater nitrogen is generally plentiful during the late autumn and winter months, phycoerythrin levels can increase at this time (Martinez and Rico 2002).

Other studies have also reported that the concentration and composition of proteins in macroalgae vary considerably with season and also with fluctuations in nutrient levels, in particular nitrogen (Harnedy and FitzGerald 2011). The protein contents of *Gracilaria cervicornis* and *Sargassum vulgare* were reported to be higher during spring than in summer and autumn, and these protein levels were positively correlated with nitrogen level and negatively with water temperature and salinity (Marinho-Soriano et al. 2006). Wong and Cheung (2000) reported a similar pattern of seasonal variation in protein contents for *Pterocladia capillacea*, *Ulva lactuca* and *Jania rubens*, while Rouxel et al. (2001) reported that the protein content of *Porphyra umbilicalis* decreased from  $20.6 \pm 1.5\%$  in February to  $12.4 \pm 1.0\%$  in August 1997.

The data reported in this study suggests that not only the protein content (Fig. 1; Table 1) but also the protein composition (Figs. 2 and 3) of *P. dioica* varies with time of collection. Electrophoretic analysis showed different polypeptide profiles in samples collected at different times of the year, with some



**Table 3** Dipeptidyl peptidase (DPP) IV and angiotensin converting enzyme (ACE) inhibitory activities of *Porphyra dioica*, unhydrolysed controls (control) and protein hydrolysates (hydrolysate)

Sample	DPP-IV IC <sub>50</sub> (mg mL <sup>-1</sup> )		ACE IC <sub>50</sub> (mg mL <sup>-1</sup> )	
	Control	Hydrolysate	Control	Hydrolysate
July 2014	3.63 ± 0.35 a	5.06 ± 0.62 b	0.90 ± 0.14 a	1.78 ± 0.06 bc
September 2014	7.21 ± 0.11 c	1.64 ± 0.19 de	1.39 ± 0.08 bd	0.51 ± 0.06 e
November 2014	2.06 ± 0.02 d	1.17 ± 0.07 e	1.62 ± 0.07 bc	0.38 ± 0.03 e
December 2014	2.46 ± 0.41 d	1.19 ± 0.21 e	1.56 ± 0.04 bc	0.42 ± 0.02 e
February 2015	4.14 ± 0.10 ab	1.17 ± 0.09 e	1.12 ± 0.29 a	0.34 ± 0.08 e
April 2015	1.78 ± 0.21 de	1.14 ± 0.04 e	1.41 ± 0.21 bd	0.43 ± 0.06 e
May 2015	2.00 ± 0.01 d	1.98 ± 0.01 de	1.85 ± 0.25 c	0.28 ± 0.12 e
July 2015	4.71 ± 0.15 ab	2.19 ± 0.10 d	1.04 ± 0.35 ad	0.55 ± 0.06 e

Mean ± SD ( $n = 3$ ). For each assay, samples with different lowercase letters are significantly different ( $p \leq 0.05$ )  $IC_{50}$  inhibitory concentration that inhibits enzyme activity by 50%

bands being common throughout the seasons while others varied in intensity over the course of the year. To our knowledge, this is the first report of the seasonal variation in total protein composition and recovery from *P. dioica*. Additionally, the SDS-PAGE profiles of the July and September 2014 samples were less defined than those collected in other months. This may reflect a period of rapid growth during the summer months of a particularly warm Irish summer (Climate Annual, Ireland, 2014 (2015)) or could be due to the high levels of algal carbohydrates present. The total carbohydrate content of the *P. dioica* samples in this study peaked in July 2014 at  $57.48 \pm 1.76\%$ , with the lowest values observed in December 2014 ( $26.21 \pm 1.84\%$ ; data not shown). This variation is consistent with previous reports of seasonal variation in total carbohydrate content of *Porphyra* spp. (Karsten et al. 1999).

Previous studies have demonstrated that the proteolytic preparations, Alcalase and Flavourzyme, can generate bioactive peptides from macroalgae (Harnedy and FitzGerald 2013). Alcalase is derived from *Bacillus licheniformis*. Its main activity is a subtilisin endoproteinase, but it also has a minor glutamyl endopeptidase activity (Kalyankar et al. 2013). Flavourzyme, which is derived from *Aspergillus oryzae*, has both exopeptidase and endoproteinase activities (Smyth and FitzGerald 1998). Enzymatic hydrolysis with these preparations led to a greater increase of amino nitrogen in the samples collected in December, February and April than those collected in July 2014 and 2015. These differences may be due to the differences in protein composition between samples collected at different times of year as shown in Figs. 2 and 3. There were no significant changes observed in the concentration of amino nitrogen in the unhydrolysed control samples for all months. Therefore, the observed increase in amino group concentration in the hydrolysed samples can be attributed to the proteolytic actions of Alcalase and Flavourzyme.

The predominantly phototrophic life of algae means they are exposed to high levels of oxidative stress. To our knowledge, this study is the first description of *P. dioica* protein hydrolysates possessing antioxidant activity with maximal FRAP and ORAC activities of  $29.76 \pm 0.48$  and  $1015.25 \pm 36.08 \mu\text{mol TE g}^{-1}$ , respectively, being observed for the February 2015 hydrolysate. These values are comparable with those reported for *P. palmata* (Harnedy and FitzGerald 2013; Beaulieu et al. 2016), *Solieria chordalis*, *Saccharina longicruris*, *U. lactuca* (Bondu et al. 2015) and other marine protein hydrolysates (Khantaphant et al. 2011; Cian et al. 2012). Seasonal variation in the antioxidant activity of *P. dioica* protein hydrolysates was observed in this study, with hydrolysates generated from winter and late spring samples having significantly more potent antioxidant activities than those generated from summer and early autumn samples. This may be due to accumulation of phycobiliprotein such as phycoerythrin (Martinez and Rico 2002), which is known to possess antioxidant abilities (Yabuta et al. 2010), during this time period. Trinitrobenzenesulphonic acid analysis of the *P. dioica* Alcalase and Flavourzyme hydrolysates showed that samples from winter and late spring months had a higher concentration of amino nitrogen released than those from summer months. This indicates a greater extent of hydrolysis in these samples (Table 2). This agrees with previous reports that peptides exhibiting antioxidant activity generally have low molecular masses (Rajapakse et al. 2005). As peptides of low molecular mass may be more easily absorbed across the gastrointestinal tract (Meisel et al. 2006), these hydrolysates are potentially interesting for investigation as functional food ingredients. They may also find application as food preservatives to prolong food quality by combating lipid peroxidation. The use of different proteolytic preparations or combinations of the same could lead to the generation of *P. dioica* hydrolysates with more potent antioxidant activities as has

been reported by other studies (He et al. 2007; Harnedy and FitzGerald 2013). It is also possible that enhanced beneficial activities could be observed if multiple *P. dioica* protein hydrolysates were combined.

The *P. dioica* DPP-IV IC<sub>50</sub> values reported in this study, which ranged from 1.14 ± 0.04 to 5.06 ± 0.62 mg mL<sup>-1</sup>, are comparable if not more potent than previously published food-derived DPP-IV inhibitory protein hydrolysates. Hydrolysates generated from Alaska pollock skin-derived collagen, *P. palmata* and rice bran had IC<sub>50</sub> values in the range of 0.80–2.59, 1.65–4.60 and 2.3 ± 0.1 mg mL<sup>-1</sup>, respectively (Hatanaka et al. 2012; Harnedy and FitzGerald 2013; Guo et al. 2015). However, direct comparison of the potency of DPP-IV inhibitory activities of multiple protein hydrolysates is made difficult by the use of different assays and assay conditions and when IC<sub>50</sub> values for positive controls such as Diprotin A may not be reported. There was some seasonal variation evident in the *P. dioica* DPP-IV IC<sub>50</sub> values, indicating that seasonal variation in total protein composition and recovery from *P. dioica* affects the DPP-IV inhibitory ability of peptides released after enzymatic hydrolysis. Consumption of seaweed has been previously reported to influence glycaemic control in humans (Paradis et al. 2011). The DPP-IV inhibitory activities of the April and May 2015 show that *P. dioica* protein hydrolysates were not more potent than their unhydrolysed protein controls. This suggests that whole *P. dioica* along with its extracts may possess health benefits such as anti-diabetic activity.

There have been numerous reports of macroalgal protein hydrolysates and peptides having ACE inhibitory activity in vitro (Suetsuna 1998; He et al. 2007; Cian et al. 2013; Harnedy and FitzGerald 2013). The ACE IC<sub>50</sub> values obtained for the *P. dioica* hydrolysates in this study ranged from 0.28 to 1.78 mg mL<sup>-1</sup>, indicating that their potency is similar, if not higher, than previously published *P. palmata*, *Polysiphonia urceolata* and *Porphyra yezoensis* (0.19–0.78, <1 and 1.52–3.21 mg mL<sup>-1</sup>, respectively) protein hydrolysates (Suetsuna 1998; He et al. 2007; Harnedy and FitzGerald 2013). These data show that the time of harvest did not affect the ACE inhibitory ability of these hydrolysates (0.34 ± 0.08 to 0.55 ± 0.06 mg mL<sup>-1</sup>, *p* > 0.05). This may indicate that the proteinaceous compounds responsible for the ability of *P. dioica* hydrolysates to inhibit ACE activity may be constantly expressed throughout the year, which was not the case for antioxidant activity. This is in contrast to Harnedy et al. (2014) who reported significantly more potent ACE inhibitory activities in *P. palmata* hydrolysates collected in July when compared with those collected in April or October. Interestingly, hydrolysates from *P. palmata* and *P. urceolata* displayed different degrees of ACE inhibitory activity depending on the proteolytic preparation used (He et al. 2007; Harnedy and FitzGerald 2013). Future studies using different proteolytic preparations could therefore be used to

improve the potency of the *P. dioica* ACE and DPP-IV inhibition.

In conclusion, this study revealed significant differences in protein content and composition between samples collected in summer and winter months. This work also reported for the first time that Alcalase and Flavourzyme protein hydrolysates generated from *P. dioica* had antioxidant, ACE-inhibitory and DPP-IV inhibitory activities comparable with or more potent than other published marine protein hydrolysates. Furthermore, winter samples yielded protein hydrolysates with the most potent antioxidant and DPP-IV inhibitory activities. The results herein highlight the importance of understanding seasonal effects on the protein composition of *P. dioica* as time of collection has implications for the ensuing generation of peptides with specific biological activities by enzymatic hydrolysis. As most commercial *Porphyra* spp. crops are not specifically grown for a high bioactive content but as a commodity (Hafting et al. 2015), this new knowledge could be useful for the future exploitation of these macroalgae as sustainable sources of proteinaceous biofunctional food components. Peptide identification studies are necessary to identify the bioactive peptides generated from *P. dioica* collected at different times of the year, as well as the verification of their physiological effects in in vivo studies.

**Acknowledgements** The authors acknowledge Alice Nongonierma and Alan Connolly for assistance with GP-HPLC analysis. This work was supported under the National Development Plan 2007–2013, through the Marine Functional Food Research Initiative, and the Food Institutional Research Measure, administered by the Department of Agriculture, Food, and the Marine, Ireland under grant issue 13/F/536.

## References

- Ahn C-B, Jeon Y-J, Kang D-S, Shin T-S, Jung B-M (2004) Free radical scavenging activity of enzymatic extracts from a brown seaweed *Scytosiphon lomentaria* by electron spin resonance spectrometry. *Food Res Int* 37:253–258
- Angell AR, Mata L, de Nys R, Paul NA (2016) The protein content of seaweeds: a universal nitrogen-to-protein conversion factor of five. *J Appl Phycol* 28:511–524
- Atkinson AB, Robertson JI (1979) Captopril in the treatment of clinical hypertension and cardiac failure. *Lancet* 2:836–839
- Beaulieu L, Sirois M, Tamineaux É (2016) Evaluation of the in vitro biological activity of protein hydrolysates of the edible red alga, *Palmaria palmata* (dulse) harvested from the Gaspé coast and cultivated in tanks. *J Appl Phycol* 28:3101–3115
- Bensadoun A, Weinstein D (1976) Assay of proteins in the presence of interfering materials. *Anal Biochem* 70:241–250
- Bondu S, Bonnet C, Gaubert J, Deslandes É, Turgeon SL, Beaulieu L (2015) Bioassay-guided fractionation approach for determination of protein precursors of proteolytic bioactive metabolites from macroalgae. *J Appl Phycol* 27:2059–2074
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254

- Butterfield DA, Abdul HM, Opii W, Newman SF, Joshi G, Ansari MA, Sultana R (2006) Pin1 in Alzheimer's disease. *J Neurochem* 98:1697–1706
- Cian RE, Alaiz M, Vioque J, Drago SR (2013) Enzyme proteolysis enhanced extraction of ACE inhibitory and antioxidant compounds (peptides and polyphenols) from *Porphyra columbina* residual cake. *J Appl Phycol* 25:1197–1206
- Cian RE, Martinez-Augustin O, Drago SR (2012) Bioactive properties of peptides obtained by enzymatic hydrolysis from protein byproducts of *Porphyra columbina*. *Food Res Int* 49:364–372
- Climate Annual, Ireland, 2014 (2015) *Met Éireann*. <http://www.met.ie/climate/MonthlyWeather/clim-2014-ann.pdf>. Accessed 6/7/2016
- Connolly A, Piggott CO, FitzGerald RJ (2013) Characterisation of protein-rich isolates and antioxidative phenolic extracts from pale and black brewers' spent grain. *International J Food Sci Technol* 48:1670–1681
- Connolly A, Piggott CO, FitzGerald RJ (2014) *In vitro* alpha-glucosidase, angiotensin converting enzyme and dipeptidyl peptidase-IV inhibitory properties of brewers' spent grain protein hydrolysates. *Food Res Int* 56:100–107
- Deacon CF, Johnsen AH, Holst JJ (1995) Degradation of glucagon-like peptide-1 by human plasma *in vitro* yields an N-terminally truncated peptide that is a major endogenous metabolite *in vivo*. *J Clin Endocr Metab* 80:952–957
- Erdmann K, Cheung BW, Schroder H (2008) The possible roles of food-derived bioactive peptides in reducing the risk of cardiovascular disease. *J Nutr Biochem* 19:643–654
- Fleurence J (2004) Seaweed proteins. In: Yada RY (ed) *Proteins in food processing*. Woodhead Publishing Limited, Cambridge, UK, pp 197–213
- Glazer AN (1994) Phycobiliproteins—a family of valuable, widely used fluorophores. *J Appl Phycol* 6:105–112
- Guo L, Harnedy PA, Zhang L, Li B, Zhang Z, Hou H, Zhao X, FitzGerald RJ (2015) *In vitro* assessment of the multifunctional bioactive potential of Alaska Pollock skin collagen following simulated gastrointestinal digestion. *J Sci Food Agric* 95:1514–1520
- Hafting JT, Craigie JS, Stengel DB, Loureiro RR, Buschmann AH, Yarish C, Edwards MD, Critchley AT (2015) Prospects and challenges for industrial production of seaweed bioactives. *J Phycol* 51:821–837
- Harnedy PA, FitzGerald RJ (2011) Bioactive proteins, peptides, and amino acids from macroalgae. *J Phycol* 47:218–232
- Harnedy PA, FitzGerald RJ (2013) *In vitro* assessment of the cardioprotective, anti-diabetic and antioxidant potential of *Palmaria palmata* protein hydrolysates. *J Appl Phycol* 25:1793–1803
- Harnedy PA, O'Keeffe MB, FitzGerald RJ (2015) Purification and identification of dipeptidyl peptidase (DPP) IV inhibitory peptides from the macroalga *Palmaria palmata*. *Food Chem* 172:400–406
- Harnedy PA, Soler-Vila A, Edwards MD, FitzGerald RJ (2014) The effect of time and origin of harvest on the *in vitro* biological activity of *Palmaria palmata* protein hydrolysates. *Food Res Int* 62:746–752
- Hatanaka T, Inoue Y, Arima J, Kumagai Y, Usuki H, Kawakami K, Kimura M, Mukaiharu T (2012) Production of dipeptidyl peptidase IV inhibitory peptides from defatted rice bran. *Food Chem* 134:797–802
- He HL, Chen XL, Wu H, Sun CY, Zhang YZ, Zhou BC (2007) High throughput and rapid screening of marine protein hydrolysates enriched in peptides with angiotensin-I-converting enzyme inhibitory activity by capillary electrophoresis. *Bioresour Technol* 98:3499–3505
- Kalyankar P, Zhu Y, O'Keeffe M, O'Cuinn G, FitzGerald RJ (2013) Substrate specificity of glutamyl endopeptidase (GE): hydrolysis studies with a bovine alpha-casein preparation. *Food Chem* 136:501–512
- Kannel WB, Higgins M (1990) Smoking and hypertension as predictors of cardiovascular risk in population studies. *J Hypertens* 8:S3–S8
- Karsten U, West JA, Zuccarello GC, Nixdorf O, Barrow KD, King RJ (1999) Low molecular weight carbohydrate patterns in the Bangiophyceae (Rhodophyta). *J Phycol* 35:967–976
- Khantaphant S, Benjakul S, Ghomi MR (2011) The effects of pretreatments on antioxidative activities of protein hydrolysate from the muscle of brownstripe red snapper (*Lutjanus vitta*). *LWT-Food Sci Technol* 44:1139–1148
- Ko SC, Kim D, Jeon YJ (2012) Protective effect of a novel antioxidative peptide purified from a marine *Chlorella ellipsoidea* protein against free radical-induced oxidative stress. *Food Chem Toxicol* 50:2294–2302
- Le Maux S, Nongonierna AB, Barre C, FitzGerald RJ (2016) Enzymatic generation of whey protein hydrolysates under pH-controlled and non pH-controlled conditions: impact on physicochemical and bioactive properties. *Food Chem* 199:246–251
- Levine IA, Sahoo D (2010) *Porphyra*: harvesting gold from the sea. IK International Pvt Ltd
- Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193:265–275
- Marinho-Soriano E, Fonseca PC, Carneiro MA, Moreira WS (2006) Seasonal variation in the chemical composition of two tropical seaweeds. *Bioresour Technol* 97:2402–2406
- Martinez B, Rico JM (2002) Seasonal variation of P content and major N pools in *Palmaria palmata* (Rhodophyta). *J Phycol* 38:1082–1089
- Meisel H, Walsh D, Murray B, FitzGerald R, Mine Y, Shahidi F (2006) ACE inhibitory peptides. In: *Nutraceutical proteins and peptides in health and disease*. CRC Press, Boca Raton, pp 269–315
- Ngo DH, Wijesekara I, Vo TS, Ta QV, Kim SK (2011) Marine food-derived functional ingredients as potential antioxidants in the food industry: an overview. *Food Res Int* 44:523–529
- Ni H, Li L, Liu G, Hu SQ (2012) Inhibition mechanism and model of an angiotensin I-converting enzyme (ACE)-inhibitory hexapeptide from yeast (*Saccharomyces cerevisiae*). *PLoS One* 7:e37077
- Nongonierna AB, FitzGerald RJ (2013) Dipeptidyl peptidase IV inhibitory and antioxidative properties of milk protein-derived dipeptides and hydrolysates. *Peptides* 39:157–163
- Nongonierna AB, O'Keeffe MB, FitzGerald RJ (2016) Milk protein hydrolysates and bioactive peptides. In: *Advanced dairy chemistry*. Springer, Berlin, pp 417–482
- Paradis ME, Couture P, Lamarche B (2011) A randomised crossover placebo-controlled trial investigating the effect of brown seaweed (*Ascophyllum nodosum* and *Fucus vesiculosus*) on postchallenge plasma glucose and insulin levels in men and women. *Appl Physiol Nutr Metab* 36:913–919
- Pena-Ramos EA, Xiong YL (2003) Whey and soy protein hydrolysates inhibit lipid oxidation in cooked pork patties. *Meat Sci* 64:259–263
- Pihlanto-Leppala A (2000) Bioactive peptides derived from bovine whey proteins: opioid and ACE-inhibitory peptides. *Trends Food Sci Tech* 11:347–356
- Rajapakse N, Mendis E, Byun HG, Kim SK (2005) Purification and *in vitro* antioxidative effects of giant squid muscle peptides on free radical-mediated oxidative systems. *J Nutr Biochem* 16:562–569
- Rouxel C, Daniel A, Jerome M, Etienne M, Fleurence J (2001) Species identification by SDS-PAGE of red algae used as seafood or a food ingredient. *Food Chem* 74:349–353
- Saha SK, McHugh E, Murray P, Walsh DJ (2015) Microalgae as a source of nutraceuticals. In: *Phycotoxins: chemistry and biochemistry*, Wiley, NY, 2nd edn. pp 255–291
- Sakanaka S, Tachibana Y (2006) Active oxygen scavenging activity of egg-yolk protein hydrolysates and their effects on lipid oxidation in beef and tuna homogenates. *Food Chem* 95:243–249
- Smith JL, Summers G, Wong R (2010) Nutrient and heavy metal content of edible seaweeds in New Zealand. *N Z J Crop Hort* 38:19–28
- Smyth M, FitzGerald RJ (1998) Relationship between some characteristics of WPC hydrolysates and the enzyme complement in commercially available proteinase preparations. *Int Dairy J* 8:819–827

- Spellman D, Kenny P, O’Cuinn G, FitzGerald RJ (2005) Aggregation properties of whey protein hydrolysates generated with *Bacillus licheniformis* proteinase activities. *J Agric Food Chem* 53:1258–1265
- Suetsuna K (1998) Purification and identification of angiotensin I-converting enzyme inhibitors from the red alga *Porphyra yezoensis*. *J Mar Biotechnol* 6:163–167
- Sun L, Wang S, Gong X, Chen L (2004) A rod-linker-contained R-phycoerythrin complex from the intact phycobilisome of the marine red alga *Polysiphonia urceolata*. *J Photochem Photobiol B* 76:1–11
- Van Amerongen A, Beelen-Thomissen M, Van Zeeland-Wolbers L, Van Gilst W, Buikema J, Nelissen J (2009) Egg protein hydrolysates. PCT Patent Application WO/2009/128713
- Wang W, Vignani R, Scali M, Cresti M (2006) A universal and rapid protocol for protein extraction from recalcitrant plant tissues for proteomic analysis. *Electrophoresis* 27:2782–2786
- Wong KH, Cheung PCK (2000) Nutritional evaluation of some subtropical red and green seaweeds: part I—proximate composition, amino acid profiles and some physico-chemical properties. *Food Chem* 71:475–482
- Yabuta Y, Fujimura H, Kwak CS, Enomoto T, Watanabe F (2010) Antioxidant activity of the phycoerythrobilin compound formed from a dried Korean purple laver (*Porphyra* sp.) during in vitro digestion. *Food Sci Technol Res* 16:347–352