

# In vitro assessment of the cardioprotective, anti-diabetic and antioxidant potential of *Palmaria palmata* protein hydrolysates

Pádraigín A. Harnedy · Richard J. FitzGerald

Received: 26 October 2012 / Revised and accepted: 28 February 2013 / Published online: 13 April 2013  
© Springer Science+Business Media Dordrecht 2013

**Abstract** The contribution of protein fraction and proteolytic enzyme preparation to the in vitro cardioprotective, anti-diabetic and antioxidant activity of *Palmaria palmata* protein hydrolysates was investigated. Aqueous, alkaline and combined aqueous and alkaline *P. palmata* protein fractions were hydrolysed with the food-grade proteolytic preparations, Alcalase 2.4 L, Flavourzyme 500 L and Corolase PP. The hydrolysates had angiotensin converting enzyme (ACE) and dipeptidyl peptidase (DPP) IV inhibitory activity with IC<sub>50</sub> values in the range 0.19–0.78 and 1.65–4.60 mg mL<sup>-1</sup>, respectively. The oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) values ranged from 45.17 to 467.54 and from 1.06 to 21.59 μmol trolox equivalents/g, respectively. Furthermore, hydrolysates (1 mg mL<sup>-1</sup>) were shown to inhibit renin within the range 0–50 %. In general, Alcalase 2.4 L and Corolase PP hydrolysates of aqueous protein displayed the highest in vitro activity. The results indicate that protein fraction and enzyme preparation used have significant effects on in vitro biofunctional activity of the hydrolysates. This study demonstrates the potential of *P. palmata* protein hydrolysates as multifunctional functional food ingredients for the prevention/control of hypertension and type II diabetes.

**Keywords** *Palmaria palmata* · Protein hydrolysates · Bioactive peptides · Angiotensin converting enzyme · Renin · Dipeptidyl peptidase IV

## Introduction

Hypertension and type II diabetes are two of the leading public health problems in the industrialized world. These chronic diseases are interrelated and increasingly coexist in the aging population. Hypertension affects approximately 70 % of subjects with diabetes and the prevalence of hypertension is twice as high in diabetics than in those without the disease (Lago et al. 2007). Individuals suffering from either condition are strongly predisposed to atherosclerotic cardiovascular (CVD) and renal disease (Sowers 2004).

Peripheral blood pressure is controlled mainly by the renin–angiotensin system (RAS). Renin and ACE are two key enzymes in this system. RAS activity is initiated by cleavage of angiotensinogen to angiotensin (AT)-I by the acid proteinase, renin. In turn, the inactive decapeptide AT-I is converted to the vasoconstrictor, AT-II, by the carboxydipeptidase ACE (Meisel et al. 2006). Furthermore, ACE inactivates the vasodilator, bradykinin. Treatment of hypertension and heart failure has focused on manipulation of this pathway and in particular on the use of ACE inhibitors and angiotensin II (AT-II) receptor blockers (ARBs). However, because of incomplete protection provided by ACE inhibitors and ARBs, the combination of renin and ACE inhibitors/ARBs has recently emerged as a strategy for therapeutic treatment of hypertension (Li and Aluko 2010). By inhibiting renin, the production of AT-I is reduced, thus limiting the formation of AT-II generated by ACE and chymase in an ACE-independent pathway (Meisel et al. 2006).

Synthetic inhibitors of ACE, such as captopril, enalapril and lisinopril, have been used in the control of hypertension in humans (Norris et al. 2012). However, these drugs can cause adverse side effects, such as cough, allergic reactions,

P. A. Harnedy · R. J. FitzGerald (✉)  
Department of Life Sciences, University of Limerick,  
Limerick, Ireland  
e-mail: dick.fitzgerald@ul.ie

taste disturbances, and skin rashes (Wijsekara and Kim 2010). Therefore, an increased focus on identifying safer natural inhibitors in the prevention and treatment of hypertension is ongoing. To date, most research associated with the exploration of natural antihypertensive agents has been with ACE inhibitory peptides. A variety of ACE inhibitory peptides have been identified and characterised from numerous marine and non-marine protein sources. Some of these include macroalgae, fish, shellfish, dairy, animal, plant and egg sources. ACE inhibitory peptides have been isolated from macroalgae such as *Hizikia fusiformis*, *Undaria pinnatifida* and *Porphyra yezoensis* (Harnedy and FitzGerald 2011). However, limited information exists with regard to the inhibition of renin activity by protein hydrolysates. Protein hydrolysates with renin inhibitory activity include *Palmaria palmata*, flaxseed, hemp and pea protein hydrolysates (Fitzgerald et al. 2012; Udenigwe et al. 2009; Li and Aluko 2010; Girgih et al. 2011; Li et al. 2011). Flaxseed, hemp and pea protein hydrolysates were also shown to inhibit ACE in vitro. Furthermore, pea and hemp seed protein hydrolysates were shown to have antihypertensive activity in spontaneously hypertensive rats (SHR) and pea protein hydrolysates in human clinical trials (Li et al. 2011; Girgih et al. 2011). In this human trial, a 3-kDa freeze-dried permeate of the pea protein hydrolysate which showed weak renin and ACE inhibitory activities in vitro was reported to significantly reduce systolic blood pressure by 5–6 mmHg compared to the placebo, in normal to mildly hypertensive subjects.

Dipeptidyl peptidase IV (DPP IV) rapidly metabolises glucagon-like peptide 1 (GLP-1) and glucose-independent insulinotropic polypeptide (GIP), two insulinotropic incretin hormones which enhance glucose-dependent insulin secretion and regulate postprandial blood glucose level (Green et al. 2004). These peptide hormones are inactivated by cleavage of the N-terminal X-Pro and X-Ala sequence by DPP IV. New approaches for development of anti-diabetic therapies are based on the development of incretin analogues and DPP IV inhibitors. To date numerous peptidomimetics, designed to mimic the N-terminal of incretin hormone dipeptides hydrolysed by DPP IV, have shown promising results in the treatment of type II diabetes. A limited number of peptides with DPP IV inhibitory activity have been isolated from food protein hydrolysates (Van Amerongen et al. 2009; Li-Chan et al. 2012; Uchida et al. 2011; Tulipano et al. 2011; Uenishi et al. 2012; Boots 2006; Nongonierma and FitzGerald 2013). Recently, in silico analysis of 34 animal and terrestrial plant proteins has identified a number of dietary proteins as potential precursors for generation of peptides with DPP IV inhibitory activity (Lacroix and Li-Chan 2012b). Thus far, there appears to be no information regarding the inhibition of DPP IV activity by macroalgal-protein derived peptides.

Reports have shown that some antihypertensive drugs, e.g., the ARB telmisartan, can also reduce the risk of type II diabetes (Kurtz and Pravenec 2004). It is believed that this risk reduction is associated with reduced AT II. AT II, the vasoconstrictory agent associated with high blood pressure, is believed to promote insulin resistance through its effects on insulin signalling pathways, oxidative stress, tissue blood flow sympathetic activity and adipogenesis. Furthermore, an increase in oxidative stress has been linked to both CVD and diabetes (Papaharalambus and Griendling 2007). With such a strong interrelationship between hypertension and type II diabetes, and the role of oxidative stress on the pathophysiology of these diseases, an opportunity exists to develop naturally derived agents for the management of these diseases.

Macroalgae, a popular food used in many oriental countries, have been identified as a potential source for generation of protein based biofunctional ingredients (Harnedy and FitzGerald 2011). These marine organisms, in particular the red species, are reported to contain significant levels of protein (Fleurence 2004). In the red macroalga, *P. palmata*, the protein content can be as high as 35 % (w/w) dry weight and the level and type of proteins can vary with season (Fleurence 2004). Mining of *P. palmata* proteins for peptides with multifunctional ACE, renin and DPP IV inhibitory activities provides an opportunity for development of a multi-action natural cardioprotective and anti-diabetic functional food ingredients. The objective of this study was to investigate the in vitro cardioprotective, anti-diabetic and antioxidant potential of hydrolysates generated from different *P. palmata* protein fractions.

## Material and methods

Abz-Gly-*p*-nitro-Phe-Pro-OH, Abz-Gly-OH-HCl, H-Gly-Pro-7-amino-4-methyl coumarin (AMC) and Diprotin A were from Bachem Feinchemikalien (Switzerland). 5-[(2 - Aminoethyl) amino] naphthalene-1-sulfonic acid (EDANs) was obtained from Anaspec (USA). Arg-Glu(EDANs)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dadcyl)-Arg, Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys (Boc)-OMe and recombinant human renin (EC 3.4.23.15) were from Cayman Chemicals (USA). Corolase<sup>®</sup> PP was provided by AB Enzymes (Germany), and Alcalase<sup>®</sup> 2.4 L and Flavourzyme<sup>®</sup> 500 L were obtained from Novozymes A/S (Denmark). ProtoGel<sup>®</sup>, ProtoGel<sup>®</sup> Resolving Buffer (4×), ProtoGel<sup>®</sup> Stacking Buffer (4×), Tris-Glycine-SDS PAGE Buffer (10×) and Protein Loading Buffer Blue (2×) were from National Diagnostics (USA). HPLC-grade water and acetonitrile was from VWR International (Ireland) and trinitrobenzenesulphonic acid (TNBS) reagent was from Fisher Scientific (Ireland). All other reagents were supplied by Sigma (Ireland).

## Sample preparation

Samples of *P. palmata* were collected at Black Head, Co. Clare, Ireland in October 2010. The seaweed was washed with clean seawater to remove sand and epiphytes prior to freeze-drying. The freeze-dried samples were pulverized with a Cyclotec™ Mill (1 mm screen, FOSS Tecator AB, Sweden) and subsequently stored at room temperature in an air tight container.

## Preparation of crude aqueous and alkaline soluble protein extracts

Crude aqueous and alkaline soluble protein extracts were prepared using the method described by Harnedy and FitzGerald (2013). In brief, 640 g of freeze-dried milled seaweed powder was suspended in de-ionised water (1:20 (w/v)) and gently stirred for 3 h at room temperature. The protein partitioning to the aqueous phase was removed following centrifugation at 4,190×g for 15 min at room temperature. The protein present in the supernatant was termed the aqueous protein extract. The pellet from the above was resuspended in 0.12 M NaOH (1:15 (w/v)) and gently stirred for 1 h at room temperature and the extract containing alkaline soluble proteins was removed following centrifugation as described above. The pellet from the above was subjected to a second alkaline extraction and both supernatants were combined. This material was termed the alkaline protein extract. The protein present in both the aqueous and alkaline extracts was collected by isoelectric precipitation at pH3.5 and 4.0, respectively. Following pH adjustment (pH3.5 and 4.0) samples were allowed to stand for 30 min at room temperature. The pellet obtained after centrifugation at 4,190×g for 15 min was resuspended in dH<sub>2</sub>O to a protein concentration of ~2 % (w/v). In order to determine if there were synergistic effects in the total protein complement, the water and alkaline extracted protein samples above were mixed in the ratio at which these protein fractions occur in *P. palmata* (42:58). This protein blend was termed the combined aqueous and alkaline protein extract.

## Protein determination

Protein was determined by a modification of the Lowry protein method as described by Harnedy and FitzGerald (2013). All samples were analysed in triplicate ( $n=3$ ) with reference to a protein standard curve in the range 0–200 µg mL<sup>-1</sup> bovine serum albumin.

## Enzymatic hydrolysis of macroalgal proteins

Solutions (1.6 % (w/v) (aq) of the aqueous, alkaline and the combined aqueous and alkaline proteins were preheated to 50 °C and adjusted to pH7.0 with 2.0 M NaOH. Alcalase, Flavourzyme and Corolase PP were added at an

enzyme/substrate (E/S) ratio of 1 % (v/w) or (w/w). During hydrolysis (4 h) at 50 °C, the reaction mixture was maintained at pH7.0 using a pH-Stat (842 Titrando, Metrohm, Switzerland). The proteolytic enzymes were inactivated by heating at 90 °C for 20 min. Control protein samples, without added proteolytic preparation, were treated in the same manner. Hydrolysate samples were subsequently freeze-dried.

## Preparation of samples for bioassays

Freeze-dried protein hydrolysate samples were resuspended in assay buffer at a concentration of 50 mg mL<sup>-1</sup> for the DPP IV inhibition assay and stirred for 30 min at room temperature. Samples for all other assays were prepared in the respective assay buffers at 30 mg mL<sup>-1</sup>. Any insoluble material was removed by centrifugation at 4,190×g for 5 min at room temperature. The resultant supernatants were diluted prior to bioassays.

## Monitoring of extent of hydrolysis, SDS-PAGE and GPC-HPLC

During the course of hydrolysis the volume of base required to keep the reaction at pH7 (0.5 M NaOH) was monitored using a pH stat. Furthermore, the extent of hydrolysis was estimated by the TNBS method as described by Spellman et al. (2003) with some modifications. Briefly, 0.125 mL of standard/sample, prepared in 1 % (w/v) SDS, was added to 1.0 mL 0.2125 M sodium phosphate buffer pH8.2 and 1.0 mL 0.1 % (w/v) TNBS prepared in 0.2125 M sodium phosphate buffer pH8.2. Following mixing, the test tubes were incubated in the dark at 50 °C for 60 min. The reaction was stopped by the addition of 2.0 mL 0.1 N HCl and the absorbance was measured at 340 nm using an UVmini-1240 spectrophotometer (Shimadzu, USA). All samples were analysed in triplicate ( $n=3$ ) and the amino group content was determined with reference to a leucine standard curve in the range 0.0–2.0 mM. Results were expressed as mg amino groups released g<sup>-1</sup> protein. SDS PAGE was performed using a Mini Protean II electrophoresis system (Bio-Rad, USA) according to the method described by Harnedy and FitzGerald (2013). Gel permeation chromatography (GPC-HPLC) was performed as described by Spellman et al. (2005).

## Bioactivity screening

### *Angiotensin converting enzyme (ACE) inhibitory activity*

ACE inhibitory activity was measured fluorometrically based on the assay described by Sentandreu and Toldrá (2006) using 0.10M sodium borate buffer, pH8.3 containing 0.3 M NaCl and 4 mU mL<sup>-1</sup> ACE prepared from rabbit lung acetone powder. Rabbit lung acetone powder, 1:10 (w/v),

was gently stirred at 4 °C overnight in 0.10 M sodium borate buffer, pH 8.3 containing 5 % (v/v) glycerol. The suspension was centrifuged at 40,000×g for 30 min at 4 °C. The supernatant contained ACE activity. One unit (U) of ACE activity was defined as the amount of enzyme which hydrolyses 1 μmol Abz-Gly-Phe (NO<sub>2</sub>)-Pro min<sup>-1</sup> at 37 °C. Captopril (15 nM final concentration) was used as a reference ACE-inhibitory substance. The IC<sub>50</sub> values (inhibitory concentration that inhibits ACE activity by 50 %) reported as the average from three independent replicate assays (n=3).

#### Renin inhibitory activity

Renin inhibition activity was determined by quantifying the concentration of free EDANS liberated from the fluorogenic substrate Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dadcyl)-Arg (31.67 μM) in the absence and presence of macroalgal protein hydrolysates. In brief, 10 μL of each protein hydrolysate sample (final concentration 1 mg mL<sup>-1</sup>) was mixed with 160 μL assay buffer (0.05 M Tris-HCl buffer pH 8.0 containing 0.1 M NaCl) and 20 μL substrate. The reaction mixture was incubated at 37 °C for 5 min and the reaction was initiated by adding 10 μL of recombinant human renin (1.4 mU mL<sup>-1</sup>). The change in fluorescence was monitored over a 30 min period using a plate reader (BioTek Synergy HT, USA) at excitation and emission wavelengths of 360 and 460 nm, respectively. Sample was replaced with 10 μL of buffer in the uninhibited reactions. Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys (Boc)-OMe was used as a positive control. One unit of renin activity (U) was defined as the amount of enzyme which hydrolyses 1 μmol of Arg-Glu(EDANS)-Ile-His-Pro-Phe-His-Leu-Val-Ile-His-Thr-Lys(Dadcyl)-Arg min<sup>-1</sup> at 37 °C. The results were reported as % renin inhibition, where the activity of renin in the presence of protein or hydrolysate samples was expressed as a % activity in the absence of sample. The % inhibition reported for each hydrolysate sample was the mean of three independent replicates (n=3).

#### Dipeptidyl peptidase (DPP) IV inhibitory activity

DPP IV inhibitory activity was determined by measuring free AMC (7-amino-4-methyl-coumarin) liberated from the fluorogenic substrate Gly-Pro-AMC. In brief, 10 μL of protein hydrolysate sample was mixed with 30 μL 0.02 M Tris-HCl buffer, pH 8.0 containing 0.10 M NaCl and 1 mM EDTA, and 50 μL, 200 μM H-Gly-Pro-AMC. Following 5 min incubation at 37 °C the reaction was initiated by addition of 10 μL DPP IV (8 mU mL<sup>-1</sup>). The change in fluorescence was monitored over a 30-min period using a plate reader at excitation and emission wavelengths of 360 and 460 nm, respectively. One unit (U) of DPP IV activity was defined as the amount of enzyme which hydrolyses 1 μmol H-Gly-Pro-

AMC min<sup>-1</sup> at 37 °C. Diprotin A (5 μM final concentration) was used as a reference DPP IV-inhibitory substance. The IC<sub>50</sub> value reported for each hydrolysate sample was the mean value from three independent replicate assays (n=3).

#### Ferric reducing antioxidant power (FRAP) activity

The FRAP of hydrolysate samples was determined using the method described by Kelman et al. (2012) with some modifications. Freshly prepared FRAP reagent (150 μL; 0.3 M acetate buffer [pH 3.6], 0.01 M 2,4,6-tripyridyl-s-triazine [TPTZ], 0.02 M FeCl<sub>3</sub>·6H<sub>2</sub>O 10:1:1) heated to 37 °C was pipetted into microtitre plate wells and the absorbance was read at 590 nm using a plate reader. Test sample (20 μL), Trolox (standard) and MeOH (blank) was then added and the absorbance (590 nm) was read after 30 min incubation at 37 °C. The FRAP of each hydrolysate was expressed as μmol of Trolox equivalents per gram of freeze-dried powder (μmol of TE g<sup>-1</sup> dw). All assays were performed in triplicate (n=3).

#### Oxygen radical absorbance capacity (ORAC) assay

The ORAC assay was also performed using a plate reader. All reagents were prepared in 0.075 M sodium phosphate buffer (pH 7.0). Each sample or standard (50 μL) was mixed with 50 μL 0.78 μM fluorescein and pre-incubated at 37 °C for 15 min. The reaction was initiated by the addition of 25 μL 0.221 M 2,2'-Azobis(2-methylpropionamide) dihydrochloride (AAPH) solution. The fluorescence (excitation: 458 nm, emission: 520 nm) was recorded every 5 min over 2 h. The ORAC value was calculated and expressed as μmol of Trolox equivalents per gram of freeze-dried powder (μmol of TE g<sup>-1</sup> dw). All assays were performed in triplicate (n=3).

#### Statistical analysis

The results were analysed by one-way analysis of variance (ANOVA) at a significance level of *p*=0.05. Where applicable, multiple comparisons were performed using Tukey's and Games-Howell post-hoc tests. All analysis was performed using SPSS (SPSS, version 18, IBM Inc., USA)

## Results and discussion

#### Enzymatic hydrolysis of *P. palmata* protein fractions

The type of bioactive peptides generated from a particular protein is dependent on two factors: (a) the primary sequence of the protein substrate and (b) the specificity of the enzyme(s) used. With the exception of three studies



which involve the hydrolysis of protein rich fractions from *P. palmata*, *Undaria pinnatifida* and *Porphyra columbina*, the generation of protein hydrolysates from macroalgal proteins has involved direct hydrolysis of aqueous suspensions of macroalgal cells with food-grade proteolytic enzymes (Cian et al. 2012; Sato et al. 2002b; Fitzgerald et al. 2012). In the present study different protein fractions were extracted from *P. palmata* prior to hydrolysis. These were the aqueous, alkaline and a combination of aqueous and alkaline protein extracts. Many reports have shown that the presence of algal fibre (both soluble and insoluble), phenolic compounds, trypsin inhibitors along with protein glycosylation all have an inhibitory effect on the digestibility of algal proteins (Fleurence 2004). Extraction of proteins prior to hydrolysis therefore helps to remove some of these inhibitory agents. Furthermore, extraction of different protein fractions rather than extraction of total protein can act as a pre-enrichment step if peptides with promising biofunctional activity are derived from particular protein fractions.

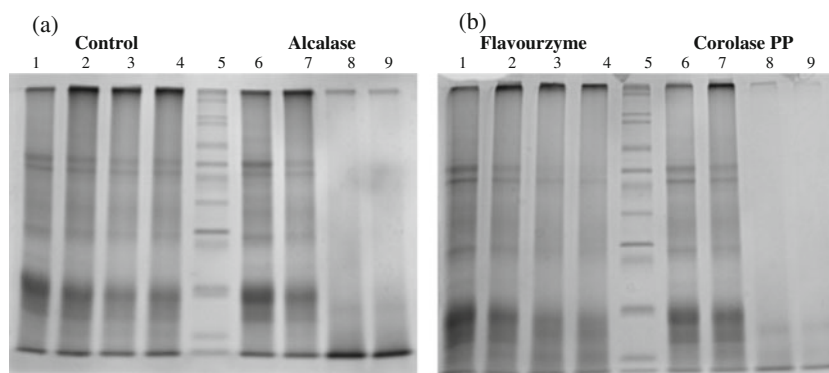
Food-grade proteolytic preparations are routinely used in the generation of protein hydrolysates for human consumption. These preparations can arise from plant, animal or microbial sources. Alcalase, a proteolytic preparation derived from *Bacillus licheniformis* mainly contains subtilisin endoproteinase and a minor glutamyl endopeptidase activity (Kalyankar et al. 2013). Corolase PP, derived from porcine pancreas, contains the intestinal proteinase activities trypsin, chymotrypsin and elastase (Mullally et al. 1994). Flavourzyme, derived from *Aspergillus oryzae*, contains both endoproteinase and exopeptidase activity (Smyth and FitzGerald 1998). Given their broad specificities, hydrolysis with these enzyme preparations leads to the generation of hydrolysates with different peptide profiles.

Table 1 shows that the concentration of amino groups released as determined by the TNBS assay during hydrolysis of all three *P. palmata* protein fractions was higher when Alcalase and Corolase PP were used compared to Flavourzyme. An increase of  $16.04 \pm 0.12$  and  $34.78 \pm 0.81$  mg amino group  $\text{g}^{-1}$  protein was observed during the hydrolysis of the aqueous protein fraction with Alcalase and Corolase PP, respectively, compared to  $6.71 \pm 0.29$  mg amino group  $\text{g}^{-1}$  protein with the aqueous protein hydrolysate generated with Flavourzyme. An increase of  $6.43 \pm 0.17$ ,  $16.59 \pm 0.37$  and  $4.05 \pm 0.06$  mg amino group  $\text{g}^{-1}$  protein was observed in the alkaline protein fraction when hydrolysed with Alcalase, Corolase PP and Flavourzyme, respectively. An increase of  $8.81 \pm 0.20$ ,  $21.8 \pm 0.58$  and  $4.54 \pm 0.04$  mg amino group  $\text{g}^{-1}$  protein was observed with the combined aqueous and alkaline proteins hydrolysates generated with Alcalase, Corolase PP and Flavourzyme, respectively. Furthermore, no significant changes were observed in the pH and the amino group concentration in the control samples during 4 h incubation. These results concur with previous studies which have shown that *P. palmata* does not possess endogenous proteolytic activity (Harnedy and FitzGerald 2013). Therefore, the increase in amino group concentration during hydrolysis is attributable to the proteolytic action of Alcalase and Corolase PP, and to a lesser extent Flavourzyme on the *P. palmata* protein samples. This was further confirmed by SDS-PAGE and GPC analysis (Figs. 1 and 2). SDS-PAGE was used to visualise the total protein profiles of the unhydrolysed substrates and their degradation during incubation with the proteolytic enzyme preparations. GPC analysis was used to access the molecular weight distribution of soluble proteinaceous components in the unhydrolysed protein fractions and associated hydrolysates.

**Table 1** Concentration of amino groups liberated during the hydrolysis of *Palmaria palmata* aqueous, alkaline and a mixture of aqueous and alkaline protein fractions

Protein fraction	Hydrolysis time (min)	(mg amino group $\text{g}^{-1}$ protein)			
		Control	Alcalase	Flavourzyme	Corolase PP
Aqueous	0	$8.15 \pm 0.55^a$	$8.34 \pm 0.33^a$	$8.28 \pm 0.40^a$	$8.51 \pm 0.20^a$
	120	$8.13 \pm 0.95^a$	$21.54 \pm 0.20^b$	$12.72 \pm 1.46^b$	$36.11 \pm 1.32^b$
	240	$7.94 \pm 0.38^a$	$24.38 \pm 0.21^c$	$14.99 \pm 0.11^c$	$43.29 \pm 1.01^c$
Alkaline	0	$6.56 \pm 0.04^a$	$6.51 \pm 0.17^a$	$6.65 \pm 0.25^a$	$6.23 \pm 0.02^a$
	120	$6.31 \pm 0.44^a$	$11.52 \pm 0.01^b$	$9.67 \pm 0.09^b$	$21.04 \pm 0.52^b$
	240	$6.54 \pm 0.07^a$	$12.94 \pm 0.00^c$	$10.70 \pm 0.19^c$	$22.82 \pm 0.39^c$
Aqueous and alkaline	0	$6.38 \pm 0.17^a$	$6.46 \pm 0.30^a$	$6.59 \pm 0.2^a$	$7.15 \pm 0.36^a$
	120	$6.20 \pm 0.18^a$	$13.34 \pm 0.17^b$	$10.17 \pm 0.02^b$	$25.44 \pm 0.18^b$
	240	$6.22 \pm 0.38^a$	$15.27 \pm 0.50^c$	$11.13 \pm 0.24^c$	$28.95 \pm 0.94^c$

Mean  $\pm$  SD ( $n=3$ ). When comparing values for each protein fraction hydrolysed with different enzymes different letters at 0, 120 and 240 min are significantly different at  $p < 0.05$



**Fig. 1** Sodium dodecyl sulphate polyacrylamide gel electrophoresis profiles of *Palmaria palmata* combined aqueous and alkaline proteins and combined aqueous and alkaline protein hydrolysates. Here, 20  $\mu\text{g}$  of all samples was loaded onto the gel. *Lanes 1 and 6*: aqueous proteins; *lanes 2 and 7*: 0 min hydrolysate samples; *lanes 3 and 8*: 120 min

hydrolysate samples; *lanes 4 and 9*: 240 min hydrolysate samples; *lane 5*: molecular weight standards. **a** Undigested control and Alcalase digested sample profiles; **b** Flavourzyme and Corolase PP digested sample profiles

The SDS-PAGE profiles show that the combined aqueous and alkaline protein fractions were significantly hydrolysed by Alcalase and Corolase PP even after 2 h incubation (Fig. 1a [lane 8] and b [lane 8]). However, the same protein fraction was not extensively degraded when incubated with Flavourzyme (Fig. 1b, lanes 3 and 4). This trend was also seen in the SDS profiles with the individual aqueous and alkaline protein digests (data not shown). Furthermore, GPC analyses showed that the quantity of low molecular weight peptides <10 kDa generated was significantly higher in protein hydrolysates generated with Alcalase and Corolase PP than in the samples incubated with Flavourzyme (Fig. 2). Furthermore, a majority of these peptides were  $\leq 2$  kDa. Low molecular mass peptides are generally more readily absorbed across the gastrointestinal tract (Meisel et al. 2006). Therefore, if bioactive, these <2 kDa *P. palmata* peptides could potentially reach their target site in a bioavailable format.

#### In vitro assessment of biological activity

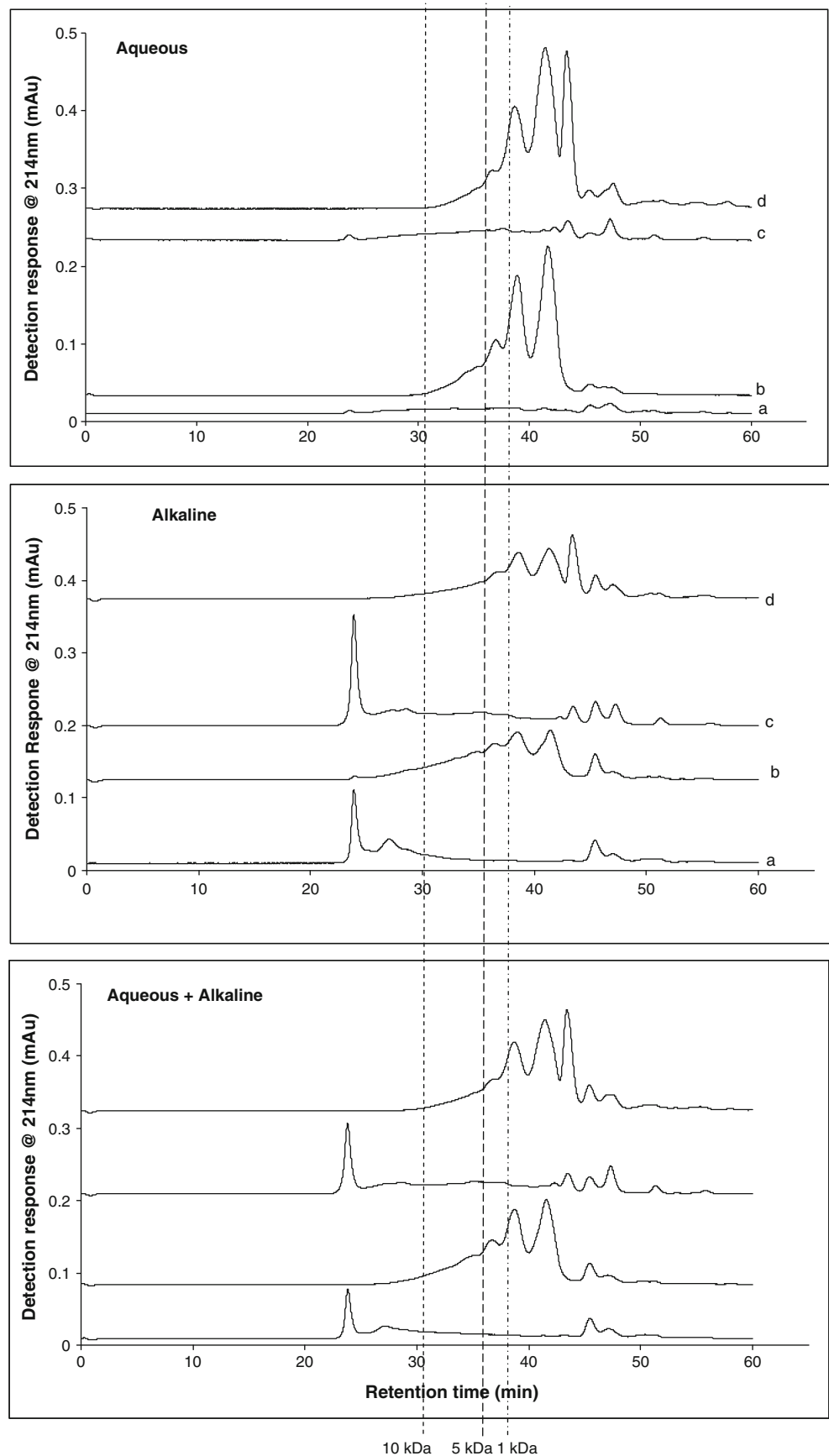
The soluble proteinaceous components derived from *P. palmata* protein hydrolysates were assessed for ACE, renin and DPP IV inhibitory and antioxidant activity *in vitro*.

#### ACE inhibitory activity

As already outlined, ACE is a carboxydipeptidase activity involved in the generation of vasoconstrictory peptides that lead to the elevation of blood pressure. Several macroalgal protein hydrolysates and peptides have been shown to exhibit ACE inhibitory activity *in vitro* (Cian et al. 2012; He et al. 2007; Sato et al. 2002a, b; Cha et al. 2006; Lee et al. 2005; Saito and Hagino 2005; Suetsuna 1998a, b; Suetsuna and Nakano 2000). However, to date it would appear that no reports are available on the ACE inhibitory activity of *P. palmata* hydrolysates.

Table 2 summarises the  $\text{IC}_{50}$  values for each of the protein hydrolysates and associated control samples analysed in this study. In agreement with literature reports,  $\text{IC}_{50}$  values of 15 and 177 nM were obtained for the synthetic ACE inhibitory drugs captopril and enalapril, respectively (Norris et al. 2012). Unhydrolysed protein control samples and all protein hydrolysates generated with Flavourzyme show significantly lower ACE inhibitory activity ( $>2 \text{ mg mL}^{-1}$ ) than equivalent protein hydrolysates generated with Alcalase and Corolase PP (Table 2). With the exception of the combined aqueous and alkaline protein hydrolysates significantly higher ACE inhibitory activity was seen with Alcalase generated hydrolysates compared to those generated with Corolase PP. Furthermore, in relation to hydrolysates generated with Alcalase and Corolase PP, significantly higher ACE inhibitory activity was observed with hydrolysates generated from aqueous and combined aqueous and alkaline protein extracts compared to equivalent hydrolysates generated from the alkaline protein extracts. ACE  $\text{IC}_{50}$  values of 0.086/0.099, 0.107, 0.219, and 0.070  $\text{mg mL}^{-1}$  have been reported for *Undaria pinnatifida* hydrolysates generated with Protease S, pepsin, Proleather FG-F and Protease N, respectively (Sato et al. 2002a, b). Furthermore, ACE  $\text{IC}_{50}$  values in the range 1.52–3.21  $\text{mg mL}^{-1}$  were reported with *Porphyra yezoensis* hydrolysates (Suetsuna 1998a). The ACE  $\text{IC}_{50}$  values obtained for all hydrolysates generated with Alcalase, and the aqueous and combined aqueous and alkaline hydrolysates generated with Corolase PP ranged from 0.19 to 0.41  $\text{mg mL}^{-1}$ . This would indicate that the potency of some of the *P. palmata* protein hydrolysates generated in this study have similar if not higher ACE inhibitory activity than *Undaria pinnatifida* and *Porphyra yezoensis* protein hydrolysates. However, direct comparison of ACE inhibitory potencies of different protein hydrolysates is difficult when different assays and assay conditions are used and when  $\text{IC}_{50}$  values for positive controls such as captopril and enalapril are not reported (Murray et al. 2004).

**Fig. 2** Gel permeation HPLC profiles showing the molecular mass distribution of the soluble proteinaceous components in *Palmaria palmata* protein hydrolysates taken at 240 min: **a** undigested control, **b** digested with Alcalase, **c** digested with Flavourzyme and **d** digested with Corolase PP. *Dashed lines* show the retention times corresponding to 1, 5 and 10 kDa



**Table 2** Angiotensin-converting enzyme (ACE) and dipeptidyl peptidase (DPP) IV inhibitory activity of *Palmaria palmata* protein hydrolysates

	Protein substrate	Proteolytic activity			
		Control IC <sub>50</sub> (mg mL <sup>-1</sup> )	Alcalase	Flavourzyme	Corolase PP
ACE	Aqueous	>2.00	0.19±0.03 <sup>a</sup>	>2.00	0.33±0.04 <sup>bc</sup>
	Alkaline	>2.00	0.41±0.07 <sup>c</sup>	>2.00	0.78±0.05 <sup>d</sup>
	Aqueous and Alkaline	>2.00	0.28±0.05 <sup>ab</sup>	>2.00	0.34±0.04 <sup>bc</sup>
DPP IV	Aqueous	>5.00	2.52±0.05 <sup>e</sup>	>5.00	1.65±0.12 <sup>a</sup>
	Alkaline	>5.00	>5.00	4.60±0.09 <sup>f</sup>	3.16±0.07 <sup>b</sup>
	Aqueous and Alkaline	>5.00	4.24±0.02 <sup>e</sup>	>5.00	2.26±0.09 <sup>d</sup>

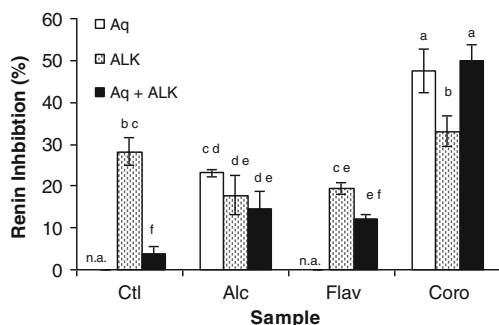
Mean±SD ( $n=3$ ). IC<sub>50</sub> with different letters for each of the activities are significantly different at  $p<0.05$

IC<sub>50</sub> inhibitory concentration that inhibits enzyme activity by 50 %

### Renin inhibitory activity

The protein substrate and the type of enzyme used significantly affected the renin inhibitory potential of the macroalgal protein hydrolysates (Fig. 3). No renin inhibitory activity was observed with the aqueous protein control sample and the aqueous protein hydrolysate generated with Flavourzyme. A significant increase in renin inhibitory activity was observed with the hydrolysate generated with Corolase PP (47.62±5.16 %) compared to that generated with Alcalase (23.10±0.91 %). This was in good agreement with the renin inhibition reported for *P. palmata* aqueous protein hydrolysates generated with papain (41.89±3.22 at 1 mg mL<sup>-1</sup>) (Fitzgerald et al. 2012).

No significant difference was observed between the renin inhibitory potency of the alkaline protein control sample (28.18±3.22 %) and the alkaline protein hydrolysate generated with Corolase PP (33.09±3.57 %). Furthermore, lower renin inhibition was observed for alkaline hydrolysates generated with Alcalase and Flavourzyme with 17.72±4.68 % and 19.48±1.48 % inhibition recorded, respectively. It



**Fig. 3** The renin inhibitory activity of *Palmaria palmata* protein hydrolysates. The concentration of protein hydrolysate used in all cases was 1 mg mL<sup>-1</sup>. All results are reported as % inhibition. Mean±SD ( $n=3$ ). n.a. no inhibition detected. Aq, Alk and Aq+Alk stand for aqueous, alkaline and combined aqueous and alkaline protein fractions. Ctl, Alc, Flav and Coro represent Control (unhydrolysed), Alcalase, Flavourzyme and Corolase PP hydrolysates. Bars with different letters are significantly different at  $p<0.05$

would appear that compounds with renin inhibitory activity in the control sample maybe degraded during hydrolysis with Alcalase and Flavourzyme. With the combined aqueous and alkaline protein hydrolysates no significant difference in renin inhibitory activity was observed between the control protein sample and the Flavourzyme generated hydrolysate. A significant increase in renin inhibitory activity was observed with hydrolysates generated with Corolase PP (49.94±3.97 %) compared to those generated with Alcalase (14.58±4.23 %). In contrast to results obtained for ACE inhibition, all *P. palmata* protein hydrolysates generated with Corolase PP show significantly higher renin inhibition activity than those generated with Alcalase. Furthermore, higher potency was observed with aqueous protein-derived Corolase PP hydrolysates than equivalent alkaline protein-derived samples. Pea protein hydrolysates generated with thermolysin were reported to inhibit renin by 17 % at 1 mg mL<sup>-1</sup> (Li et al. 2011). This would indicate that some of the hydrolysates generated in this study are more potent than the pea protein hydrolysate. Renin IC<sub>50</sub> values of 1.5–2.8 and 0.81 mg mL<sup>-1</sup> have been reported for flaxseed and hemp seed protein hydrolysates, respectively (Girgih et al. 2011; Udenigwe et al. 2009). IC<sub>50</sub> values were not determined herein but it would appear that the macroalgal protein hydrolysates generated in this study are as potent if not more potent than flaxseed and hemp seed protein hydrolysates. Again direct comparison of renin inhibitory activity of different protein hydrolysates is difficult when different assay conditions are employed (e.g., units of enzyme used in assay).

### DPP IV-inhibitory activity

Control of DPP IV activity is an insulin regulatory strategy for the treatment of type II diabetes. To date, a number of peptidic components similar in structure to the incretin hormones, GIP and GLP-1, have shown promising results as DPP IV inhibitory components. A limited number of these components are food protein-derived peptides (Nongonierma and FitzGerald 2013; Van Amerongen et al.



2009; Li-Chan et al. 2012; Uchida et al. 2011; Tulipano et al. 2011; Uenishi et al. 2012; Boots 2006). However, to the best of our knowledge, there appears to be no information in the literature in relation to the inhibition of DPP IV by macroalgal protein hydrolysates. In this study, *P. palmata* protein hydrolysates generated with Alcalase and Corolase PP, in general, showed highest DPP IV inhibitory activity (Table 2). With aqueous protein hydrolysates, both the control protein sample and the protein hydrolysate generated with Flavourzyme had  $IC_{50}$  values  $>5$  mg mL<sup>-1</sup>. A significant increase in DPP IV potency ( $IC_{50}$  value:  $1.65 \pm 0.12$  mg mL<sup>-1</sup>) was observed with the aqueous protein hydrolysate generated with Corolase PP compared to that generated with Alcalase ( $2.52 \pm 0.05$  mg mL<sup>-1</sup>).  $IC_{50}$  values  $>5$  mg mL<sup>-1</sup> were observed with the alkaline protein control sample and the alkaline protein hydrolysate generated with Alcalase.  $IC_{50}$  values of  $4.60 \pm 0.09$  and  $3.16 \pm 0.07$  mg mL<sup>-1</sup> were recorded with hydrolysates generated with Flavourzyme and Corolase PP, respectively. A similar trend was seen with the combined aqueous and alkaline protein hydrolysates as was seen with the aqueous protein hydrolysates. Both the control protein sample and the protein hydrolysate generated with Flavourzyme had  $IC_{50}$  values  $>5$  mg mL<sup>-1</sup>. An  $IC_{50}$  value of  $4.24 \pm 0.02$  mg mL<sup>-1</sup> was observed for the Alcalase hydrolysate while a significant increase in DPP IV potency ( $IC_{50}$  value:  $2.26 \pm 0.09$  mg mL<sup>-1</sup>) was observed with the combined aqueous and alkaline protein hydrolysate generated with Corolase PP. As observed with the results on renin inhibitory activity, all protein hydrolysates generated with Corolase PP showed significantly higher DPP IV activity than those generated with Alcalase and Flavourzyme. Furthermore, for hydrolysates generated with Alcalase and Corolase PP, significantly higher activity was observed with hydrolysates generated from aqueous soluble protein compared to equivalent hydrolysates derived from alkaline soluble proteins.  $IC_{50}$  values in the range 0.4–1.5 and 0.6–0.875 mg mL<sup>-1</sup> were observed with lysozyme hydrolysates generated with Newlase F, Umamizyme, Promod 278, pepsin and Alcalase, and sodium caseinate hydrolysates generated with hydrolysed bromelain, papain and Scintillase CS150L, respectively (Van Amerongen et al. 2009; Boots 2006). Casein hydrolysates generated with Newlase F, Umamizyme, Promod 278 and Alcalase exhibited DPP IV  $IC_{50}$  values in the range 2.7–5.0 mg mL<sup>-1</sup> (Van Amerongen et al. 2009). This would indicate that the potency of macroalgal protein hydrolysates generated in this study were similar or slightly lower than the potency reported for lysozyme hydrolysates, and for sodium caseinate hydrolysates generated with bromelain, papain and Scintillase CS150L. Furthermore, the potency was similar, or higher, than the potency of casein protein hydrolysates generated with Newlase F, Umamizyme, Promod 278 and Alcalase. DPP

IV inhibitory activities have been reported for a range of protein hydrolysates generated from sodium caseinate and whey protein isolate substrates (Lacroix and Li-Chan 2012a). DPP IV inhibition ranged from 15 % to 50 % when tested at 0.475 mg mL<sup>-1</sup>. DPP IV inhibitory values of approximately 16 %, 27 % and 39 % were also reported for Atlantic salmon skin gelatin hydrolysates (5 mg mL<sup>-1</sup>) generated with Alcalase, bromelain and Flavourzyme, respectively (Li-Chan et al. 2012). It would appear that *P. palmata* hydrolysates are more potent than Atlantic salmon skin gelatin hydrolysates. The inhibitory potency of the positive control (Diprotin A: 5  $\mu$ M) was in agreement with DPP IV  $IC_{50}$  values reported in the literature.

Numerous DPP IV substrate specificity studies using synthetic di- tri- and oligopeptides have yielded information in relation to the structural characteristics of DPP IV inhibitors (Lacroix and Li-Chan 2012b). Several of these inhibitory peptides contain proline and/or hydrophobic residues in their sequence. Furthermore, the position of these residues appears to significantly affect the potency of the peptides (Lacroix and Li-Chan 2012b; Li-Chan et al. 2012; Boots 2006). Further purification and characterisation studies are required in order to ascertain the sequence of the peptides in *P. palmata* hydrolysates herein exerting potential anti-diabetic activity

**Table 3** Ferric reducing antioxidant power (FRAP) and oxygen radical antioxidant capacity (ORAC) of *Palmaria palmata* protein hydrolysates

Sample	FRAP value ( $\mu$ mol TE g <sup>-1</sup> FDP)	ORAC value ( $\mu$ mol TE g <sup>-1</sup> FDP)
Aq-Ctl	$1.65 \pm 0.02^g$	$45.17 \pm 1.95^f$
Aq-Alc	$12.98 \pm 0.23^b$	$422.75 \pm 14.80^a$
Aq-Flav	$2.91 \pm 0.20^c$	$110.41 \pm 5.97^{eg}$
Aq-Coro	$21.59 \pm 0.55^a$	$467.54 \pm 26.73^a$
Alk-Ctl	$1.06 \pm 0.05^i$	$124.79 \pm 16.60^{ef}$
Alk-Alc	$6.89 \pm 0.14^d$	$270.42 \pm 4.10^d$
Alk-Flav	$2.66 \pm 0.01^f$	$188.93 \pm 17.20^e$
Alk-Coro	$9.64 \pm 0.11^c$	$340.91 \pm 14.07^{bc}$
Aq+Alk-Ctl	$1.41 \pm 0.02^h$	$81.35 \pm 14.44^{fg}$
Aq+Alk-Alc	$8.91 \pm 0.40^{cd}$	$323.22 \pm 11.17^c$
Aq+Alk-Flav	$2.76 \pm 0.09^{ef}$	$152.51 \pm 7.98^e$
Aq+Alk-Coro	$13.54 \pm 0.04^b$	$401.20 \pm 17.41^{ab}$

Mean $\pm$ SD ( $n=3$ ). For each assay, samples with different letters are significantly different at  $p<0.05$

Aq aqueous protein fraction, Alk alkaline protein fraction, Aq+Alk combined aqueous and alkaline protein fractions, Ctl Control (unhydrolysed), Alc Alcalase hydrolysates, Flav Flavourzyme hydrolysates, Coro Corolase PP hydrolysates

## Antioxidant activity

It has been shown that modified oxygen employment and/or a rise in the formation of ROS can be a factor in the progression of CVD and type II diabetes (Papaharalambus and Griendling 2007; Fatehi-Hassanabad et al. 2010). All protein substrates hydrolysed with Alcalase and Corolase PP showed significantly higher antioxidant activity (FRAP and ORAC) compared to hydrolysates generated with Flavourzyme and the unhydrolysed control sample (Table 3). All hydrolysates generated with Corolase PP showed higher antioxidant activity in the FRAP assay than the equivalent protein hydrolysates generated with Alcalase. FRAP values of  $21.59 \pm 0.55$ ,  $9.64 \pm 11$  and  $13.54 \pm 0.04 \mu\text{mol TE g}^{-1}$  freeze-dried powder (FDP) were recorded for the aqueous, alkaline and combined aqueous and alkaline protein-derived Corolase PP hydrolysates, respectively. This compared to  $12.98 \pm 0.23$ ,  $6.89 \pm 0.04$  and  $8.91 \pm 0.40 \mu\text{mol TE g}^{-1}$  FDP for the equivalent protein hydrolysates generated with Alcalase, respectively. No significant difference in antioxidant activity was observed between aqueous protein hydrolysates generated with Alcalase and Corolase PP in the ORAC assay. In contrast, higher ORAC values were observed for alkaline and combined aqueous and alkaline hydrolysates generated with Corolase PP compared to equivalent hydrolysates generated with Alcalase. The ORAC value obtained for the aqueous protein control ( $45.17 \pm 1.95 \mu\text{mol TE g}^{-1}$  FDP) was comparable to that reported for aqueous extracts from *P. palmata* harvested off the coast of Iceland (Wang et al. 2010). Furthermore, the ORAC values reported in this study for combined aqueous and alkaline protein hydrolysates generated with Alcalase and Flavourzyme were significantly higher than those reported for hydrolysates generated from the Icelandic *P. palmata* by direct hydrolysis with the same enzymes. The difference in antioxidant activity may be due to the presence of different peptides in the Irish and Icelandic *P. palmata* hydrolysates as macroalgal protein level and type vary with season and geographical location. Furthermore, the higher ORAC values reported in this study for the combined aqueous and alkaline protein hydrolysates may be due to a greater extent of hydrolysis. This may arise due to the removal of proteinase inhibitory agents during protein extraction. However, it was not possible to compare the protein profiles of the starting substrates, the extent of hydrolysis and physiochemical characteristics of peptides present in the Icelandic and Irish *P. palmata* hydrolysates in order to verify this possibility.

This study has shown that extraction of different protein fractions rather than the total protein can act as a pre-enrichment step for isolation of peptides with specific biological activities. Furthermore, the specificity of the enzymes used to generate hydrolysates clearly had an important role in

dictating *in vitro* biological properties of resultant hydrolysates. Given the linkage between antioxidant status and etiology of CVD and type II diabetes the results herein indicate the potential of *P. palmata* protein hydrolysates to beneficially modulate markers associated with the control of blood pressure and type II diabetes. Further beneficial effects may be exerted if different *P. palmata* protein hydrolysates were combined. Challenges with the application of bioactive macroalgal protein hydrolysates/peptide as functional food ingredients include the development of food-grade enrichment strategies, compatibility with different food matrixes, gastrointestinal stability and bioavailability. Furthermore, carefully controlled animal and human trials are needed to demonstrate the efficacy of these bioactivities prior to specific health claim submissions.

## Conclusion

The results of this *in vitro* study clearly indicate that *P. palmata* protein hydrolysates have potential as functional food ingredients in the prevention/control of hypertension and type II diabetes. The observed ACE, renin and DPP IV inhibitory and antioxidant activities were dependent on the macroalgal protein substrate (aqueous and alkaline) and the proteolytic enzymes used for peptide release. In general, higher inhibitory activity was observed with the aqueous protein hydrolysates generated with Alcalase and Corolase PP. No synergistic effects were observed in hydrolysates generated from combined aqueous and alkaline protein substrates. Sequence analysis is required to identify the peptides exerting these activities, as are *in vivo* studies to verify their physiological effects.

**Acknowledgments** This project (Grant Aid Agreement No. MFFRI/07/01) was carried out under the *Sea Change* Strategy with the support of the Marine Institute and the Department of Agriculture, Food and the Marine, funded under the National Development Plan 2007–2013. The authors would like to acknowledge Dr. Anna Soler-Vila, NUI Galway for the freeze-dried *P. palmata* sample.

## References

- Boots JWP (2006) Protein hydrolysates enriched in peptides inhibiting DPP-IV and their use. PCT Patent Application WO/2006/068480
- Cha SH, Ahn GN, Heo SJ, Kim KN, Lee KW, Song CB, Cho SK, Jeon YJ (2006) Screening of extracts from marine green and brown algae in Jeju for potential marine angiotensin-I converting enzyme (ACE) inhibitory activity. *J Korean Soc Food Sci Nutr* 35:307–314
- Cian RE, Alaiz M, Vioque J, Drago SR (2012) Enzyme proteolysis enhanced extraction of ACE inhibitory and antioxidant compounds (peptides and polyphenols) from *Porphyra columbina* residual cake. *J Appl Phycol*. doi:10.1007/s10811-012-9913-2
- Fatehi-Hassanabad Z, Chan CB, Furman BL (2010) Reactive oxygen species and endothelial function in diabetes. *Eur J Pharmacol* 636:8–17

- Fitzgerald C, Mora-Soler L, Gallagher E, O'Connor P, Prieto J, Soler-Vila A, Hayes M (2012) Isolation and characterization of bioactive pro-peptides with *in vitro* renin inhibitory activities from the macroalga *Palmaria palmata*. *J Agric Food Chem* 60:7421–7427
- Fleurence J (2004) Seaweed proteins. In: Yada RY (ed) *Proteins in food processing*. Woodhead Publishing Limited, Cambridge, pp 197–213
- Girgih AT, Udenigwe CC, Li H, Adebisi AP, Aluko RE (2011) Kinetics of enzyme inhibition and antihypertensive effects of hemp seed (*Cannabis sativa* L.) protein hydrolysates. *JAACS* 88:1767–1774
- Green BD, Gault VA, O'Harte FP, Flatt PF (2004) Structurally modified analogues of glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) as future antidiabetic agents. *Curr Pharm Des* 10:3651–3662
- Harnedy PA, FitzGerald RJ (2011) Bioactive proteins, peptides and amino acids from macroalgae. *J Phycol* 47:218–232
- Harnedy PA, FitzGerald RJ (2013) Extraction of protein from the macroalga *Palmaria palmata*. *LET Food Sci Technol* 51:375–382
- 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
- Kelman D, Posner EK, McDermid KJ, Tabandera NK, Wright PR, Wright AD (2012) Antioxidant activity of Hawaiian marine algae. *Mar Drugs* 10:403–416
- Kurtz TW, Pravenec M (2004) Antidiabetic mechanisms of angiotensin-converting enzyme inhibitors and angiotensin II receptor antagonists: beyond the renin-angiotensin system. *J Hypertens* 22:2253–2261
- Lacroix IME, Li-Chan ECY (2012a) Dipeptidyl peptidase-IV inhibitory activity of dairy protein hydrolysates. *Int Dairy J* 25:97–102
- Lacroix IME, Li-Chan ECY (2012b) Evaluation of the potential of dietary proteins as precursors of dipeptidyl peptidase (DPP)-IV inhibitors by an *in silico* approach. *J Funct Foods* 4:403–422
- Lago RM, Singh PP, Nesto RW (2007) Diabetes and hypertension. *Nat Clin Pract Endocrinol Metab* 3:667–667
- Lee JM, You SG, Kim SM (2005) Functional activities of low molecular weight peptides purified from enzymatic hydrolysates of seaweeds. *J Korean Soc Food Sci Nutr* 34:1124–1129
- Li H, Aluko RE (2010) Identification and inhibitory properties of multifunctional peptides from pea protein hydrolysate. *J Agric Food Chem* 58:11471–11476
- Li H, Prairie N, Udenigwe CC, Adebisi AP, Tappia PS, Aukema HM, Jones PJ, Aluko RE (2011) Blood pressure lowering effect of a pea protein hydrolysate in hypertensive rats and humans. *J Agric Food Chem* 59:9854–9860
- Li-Chan EC, Hunag SL, Jao CL, Ho KP, Hsu KC (2012) Peptides derived from atlantic salmon skin gelatin as dipeptidyl-peptidase IV inhibitors. *J Agric Food Chem* 60:973–978
- Meisel H, Walsh DJ, Murray BA, FitzGerald RJ (2006) ACE inhibitory peptides. In: Mine Y, Shahidi F (eds) *Nutraceutical proteins and peptides in health and disease*, vol 4. CRC Press, Boca Raton, pp 269–315
- Mullally MM, O'Callaghan DM, FitzGerald RJ, Donnelly WJ, Dalton JP (1994) Proteolytic and peptidolytic activities in commercial pancreatic protease preparations and their relationship to some whey protein hydrolyzate characteristics. *J Agric Food Chem* 42:2973–2981
- Murray BA, Walsh DJ, FitzGerald RJ (2004) Modification of the furanacryloyl-L-phenylalanyl-glycylglycine assay for determination of angiotensin-I-converting enzyme inhibitory activity. *J Biochem Biophys Methods* 59:127–137
- Nongonierma AB, FitzGerald RJ (2013) Dipeptidyl peptidase IV inhibitory and antioxidative properties of milk protein-derived dipeptides and hydrolysates. *Peptides* 39:157–163
- Norris R, Casey F, FitzGerald RJ, Shields D, Mooney C (2012) Predictive modelling of angiotensin converting enzyme inhibitory dipeptides. *Food Chem* 133:1349–1354
- Papaharalambus CA, Griendling KK (2007) Basic mechanisms of oxidative stress and reactive oxygen species in cardiovascular injury. *Trends Cardiovasc Med* 17:48–54
- Saito M, Hagino H (2005) Antihypertensive effect of oligopeptides derived from nori (*Porphyra yezoensis*) and Ala-Lys-Tyr-Ser-Tyr in rats. *J Jap Soc Nutr Food Sci* 58:177–184
- Sato M, Hosokawa T, Yamaguchi T, Nakano T, Muramoto K, Kahara T, Funayama K, Kobayashi A, Nakano T (2002a) Angiotensin I-converting enzyme inhibitory peptides derived from brown alga (*Undaria pinnatifida*) and their antihypertensive effect in spontaneously hypertensive rats. *J Agric Food Chem* 50:6245–6252
- Sato M, Oba T, Yamaguchi T, Nakano T, Kahara T, Funayama K, Kobayashi A, Nakano T (2002b) Antihypertensive effects of hydrolysates of Wakame (*Undaria pinnatifida*) and their angiotensin I-converting enzyme inhibitory activity. *Ann Nutr Metab* 46:259–267
- Sentandreu MÁ, Toldrá F (2006) A rapid, simple and sensitive fluorescence method for the assay of angiotensin-I converting enzyme. *Food Chem* 97:546–554
- 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
- Sowers JR (2004) Treatment of hypertension in patients with diabetes. *Arch Intern Med* 164:1850–1857
- Spellman D, McEvoy E, O'Cuinn G, FitzGerald RJ (2003) Proteinase and exopeptidase hydrolysis of whey protein: comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *Int Dairy J* 13:447–453
- 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 (1998a) Purification and identification of angiotensin I-converting enzyme inhibitors from the red alga *Porphyra yezoensis*. *J Mar Biotechnol* 6:163–167
- Suetsuna K (1998b) Separation and identification of angiotensin I-converting enzyme inhibitory peptides from peptic digest of *Hizikia fusiformis* protein. *Nippon Suisan Gakk* 64:862–866
- Suetsuna K, Nakano T (2000) Identification of an antihypertensive peptide from peptic digest of wakame (*Undaria pinnatifida*). *J Nutr Biochem* 11:450–454
- Tulipano G, Sibilia V, Caroli AM, Cocchi D (2011) Whey proteins as source of dipeptidyl dipeptidase IV (dipeptidyl peptidase-4) inhibitors. *Peptides* 32:835–838
- Uchida M, Ohshiba Y, Mogam O (2011) Novel dipeptidyl peptidase-4-inhibiting peptide derived from  $\beta$ -lactoglobulin. *J Pharmacol Sci* 117:63–66
- Udenigwe CC, Lin Y-S, Hou W-C, Aluko RE (2009) Kinetics of the inhibition of renin and angiotensin I-converting enzyme by flaxseed protein hydrolysate fractions. *J Funct Foods* 1:199–207
- Uenishi H, Kabuki T, Seto Y, Serizawa A, Nakajima H (2012) Isolation and identification of casein-derived dipeptidyl-peptidase 4 (DPP-4)-inhibitory peptide LPQNIPPL from gouda-type cheese and its effect on plasma glucose in rats. *Int Dairy J* 22:24–30
- Van Amerongen A, Beelen-Thomissen MJC, Van Zeeland-Wolbers LAM, Van Gilst WH, Buikema JH, Nelissen JWPM (2009) Egg protein hydrolysates. PCT Patent Application WO/2009/128713
- Wang T, Jónsdóttir R, Kristinsson HG, Hreggvidsson GO, Jónsson JO, Thorkelsson G, Ólafsdóttir G (2010) Enzyme-enhanced extraction of antioxidant ingredients from red algae *Palmaria palmata*. *LWT - Food Sci Technol* 43:1387–1393
- Wijesekera I, Kim SK (2010) Angiotensin-I-converting enzyme (ACE) Inhibitors from marine resources: Prospects in the pharmaceutical industry. *Mar Drugs* 8:1080–1093