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Isolation and Characterization of Bioactive Pro-Peptides with *in Vitro* Renin Inhibitory Activities from the Macroalga *Palmaria palmata*

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ABSTRACT: Renin is the initial rate limiting step in the renin angiotensinogen system (RAS). To combat hypertension, various stages of the RAS can be positively affected. The aim of this study was to isolate and characterize renin inhibitory peptides from the red seaweed *P. palmata* for use in functional foods. *Palmaria palmata* protein was extracted and hydrolyzed with the food grade enzyme Papain to generate renin inhibitory peptides. Following proteolytic hydrolysis of *P. palmata* protein, reverse phase-high performance liquid chromatography (RP-HPLC) was employed to enrich for peptides with renin inhibitory activities. Fraction 25 (Fr-25) inhibited renin activities by 58.97% (±1.26) at a concentration of 1 mg/mL. This fraction was further characterized using nano-electrospray ionization quadropole/time-of-flight mass spectrometry (ESI-Q/TOF MS). A number of novel peptide sequences were elucidated, and the parent protein from which they were derived was determined using MS in tandem with protein database searches. All sequences were confirmed using *de novo* sequencing. The renin inhibitory peptide Ile-Arg-Leu-Ile-Ile-Val-Leu-Met-Pro-Ile-Leu-Met-Ala (IRLIIVLMPILMA) was chemically synthesized and its bioactivity confirmed using the renin inhibitory assay. Other stages of the RAS have recently been inhibited by bioactive peptides sourced from macroalgae, but this is the first study to isolate and characterize renin inhibitory peptides from the macroalgae.

KEYWORDS: *macroalgae, P. palmata, renin inhibitory peptides, mass spectrometry, chemical synthesis*

INTRODUCTION

After World War II, cardiovascular disease (CVD) quickly replaced infectious diseases as the top cause of mortality in the developed world. CVD is the main cause of death in Europe, accounting for over 4.3 million deaths each year.[1] Hypertension is one of the major yet controllable risk factors in CVD.[1] To combat hypertension, various stages of the renin angiotensinogen system (RAS) can be positively affected. By inhibiting angiotensin converting enzyme-I (ACE-I) and renin, two rate limiting enzymes involved in the RAS, high blood pressure can be controlled. The renin inhibitory drug Aliskiren was placed on the market in 2007.[2] Although drugs that inhibit renin have only been commercialized in recent times, renin has long been recognized as the monospecific, initial, and rate-limiting enzyme involved in the RAS.[3] Some recent studies have identified renin inhibitory compounds from plant sources including peas,[4] soybean,[5] and the herb baicailin.[6] Furthermore, structure−activity relationship modeling of renin inhibitory dipeptides derived from these sources was carried out previously.[7,8] However, Li and Aluko (2011) are the only other group to characterize and synthesize renin inhibitory peptides from plant proteins (pea protein) to date.[4]

Macroalgae have for centuries been a staple food in the diet of East Asian populations.[9] In the western world, macroalgae are used predominantly as a source of functional and technological ingredients in the food, pharmaceutical, and cosmetic industries.[9] *P. palmata* belongs to the group of macroalgae known as Rhodophyta or red algae and is native to Europe. It is a known source of protein and contains between 9% and 25% protein of its dry weight.[10] This protein content in *P. palmata* varies based on the seasonality of collection and the geographical location of collection. However, figures obtained from the literature suggest that the highest percentage protein per dry weight is normally found in *P. palmata* collected during the winter season (October−January), while the lowest protein content per dry weight is found in *P. palmata* harvested during the summer months (July−September).[11] *P. palmata* is also rich in the essential amino acids Ile, Leu, and Val, which cannot be synthesized by humans but which are required for healthy growth and development.[10] The average levels of Leu, Val, and

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Met found in *P. palmata* are close to those found in the egg protein ovalbumin, while the levels of Ile and Thr are similar for those recorded in leguminous vegetables.[10]

Bioactive peptides are food derived peptides that exert a physiological, "hormone-like", beneficial health effect following consumption beyond those associated with basic nutritional values.[12] Bioactive peptides have been isolated from a variety of macroalgal species previously.[13,14] However, *P. palmata* derived peptides have not been reported in the literature to date.[15,16] Bioactive peptides which lower blood pressure through inhibition of ACE-I were discovered following digestion of macroalgal proteins with proteolytic enzymes.[17] Bioactive peptides are often initially encrypted within the parent protein molecule and may be released by *in vitro* proteolytic processes using exogenous enzymes, chemical hydrolysis, or bacterial cultures during processing or fermentation.[18]

The objective of this study was to generate, enrich, isolate, and characterize renin inhibitory peptides from *P. palmata* protein by hydrolysis of the protein with food grade Papain. Previously, renin inhibitory hydrolysates have been isolated from other plant sources such as pea[4] and hemp seed,[19] but renin inhibitory macroalgal proteins and peptides are reported here for the first time. Following proteolytic hydrolysis of crude *P. palmata* protein with Papain, reverse phase-high performance liquid chromatography (RP-HPLC) was employed to enrich for peptides with renin inhibitory activities. Further characterization using electrospray ionization quadropole time-of-flight mass spectrometry (ESI-Q-TOF MS) and *de novo* sequencing was used to characterize active RP-HPLC fractions. Potential renin inhibitory peptides were synthesized using microwave-assisted solid phase peptide synthesis (MW-SPPS) and tested using the renin inhibitory assay to further confirm their bioactivity. The peptides identified in this study were not, to the best of the author's knowledge, reported previously in peptide databases such as BIOPEP (www.BIOPEP.com) or other peptide databases.[15,16] However, dipeptides that correspond to amino acids found within the identified bioactive peptide were reported previously in the literature as renin inhibitory, antioxidant, and ACE-I inhibitory peptides.[4,25]

MATERIALS AND METHODS

Materials and Reagents. *P. palmata* was collected at Spiddal, Co., Galway, Ireland, on the 4th of November 2010. Ammonium sulfate, Papain from *Carica papaya*, and the specific renin inhibitor Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe, which was used as a positive control, were supplied by Sigma Aldrich (Steinheim, Germany). The renin inhibitory screening assay kit was supplied by Cambridge BioSciences (Cambridge, England, U.K.). Acetonitrile and water were supplied by Romil Ltd., (Cambridge, England, U.K.). All other chemicals used were of analytical grade.

Protein Extraction. Crude protein was extracted using the method previously described by Galland-Irmouli et al. (1999).[11] Briefly, 10 g of dried *P. palmata* was suspended in 1 L of ultrapure water. After ultrasonication for 1 h, the seaweed solution was left to stir overnight on a magnetic stirrer plate (C-MAGHS 7KAMAG, IKA-Werke GmbH & Co. KG, Staufen, Germany) at 4 °C. The solution was then centrifuged at 10 000*g* for 1 h and the supernatant decanted. The pellet fraction was suspended in 200 mL of ultrapure water and subjected to a second extraction procedure as described above. Both supernatants were pooled together and subsequently brought to 80% ammonium sulfate saturation, stirred for an hour at 4 °C, and centrifuged at 20 000*g* for 1 h to precipitate the protein fraction. The precipitates were subsequently dialyzed using 3.5 KDa MWCO dialysis tubing (Fisher Scientific, New Hampshire State, USA) against ultrapure water at 4 °C overnight. The precipitates were subsequently freeze-dried and stored at −80 °C until further use. The concentration of the crude *P. palmata* protein was quantified using the QuantiPro BCA Assay Kit according to the manufacturers' instructions (Sigma, Saint Louis, MO, USA).

Enzymatic Hydrolysis. Papain hydrolysates (X 3) of the crude *P. palmata* protein were prepared using a New Brunswick (Cambridge, U.K.) 1.5 L bioreactor with temperature and pH control. The crude protein was dispersed in Romil HPLC grade water at a concentration of 0.015 g/mL at a total volume of 1 L. The temperature was adjusted to 60 °C and the pH to 6.0. The pH was regulated using 0.01 M NaOH. Once the appropriate conditions were achieved, the enzyme Papain was added at a concentration of 20.7 U/mg protein to initiate hydrolysis. Temperature and pH were kept constant for 24 h, and the hydrolysis of *P. palmata* protein was carried out with stirring in the New Brunswick bioreactor (Cambridge, U.K.) at 300 rpm. The hydrolysis was stopped by heating the mixture at 95 °C for 10 min in a water bath.

Renin Inhibitory Bioassay of Hydrolysates and RP-HPLC Fractions. This assay was carried out according to the manufacturers' instructions. Briefly, prior to carrying out the assay, the renin assay buffer was diluted by adding 3 mL of 50 mM Tris-HCl, pH 8.0 buffer to 27 mL of HPLC grade water to give a final assay buffer concentration of 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl. The renin protein solution was diluted 20-fold with assay buffer before use, and the assay buffer was prewarmed to 37 °C in accordance with the manufacturers' instructions. Background wells were prepared by adding 20 μL of substrate, 160 μL of assay buffer, and 10 μL of HPLC grade water. Negative control wells were prepared by adding 20 μL of substrate, 150 μL of assay buffer, and 10 μL of HPLC grade water. Inhibitor wells were prepared by adding 20 μL of substrate, 150 μL of assay buffer, and 10 μL of sample. The reaction was initiated by adding 10 μL of renin to the control and sample wells. Fluorescence intensity was recorded using an excitation wavelength of 340 nm and an emission wavelength of 490 nm. All macroalgal extracts were assayed at a concentration of 1 mg/mL in triplicate. The known specific renin inhibitor, Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe, was used as a positive control. Percentage inhibition was calculated using the following equation:

$$\%\text{renin inhibition} = (100\%\text{initial activity (AF)} - \text{inhibitor (AF)}) / 100\%\text{initial activity (AF)}) \times 100)$$

where AF is the average fluorescence. Initial activity is the assay performed without the presence of an inhibitor. Data were compared using the Students' *t*-test and considered significantly different if $P < 0.05$.

RP-HPLC Analysis of Algal Extracts. The *P. palmaria* Papain hydrolysate was further purified using the Varian Pro-Star Reverse phase high performance liquid chromatography (RP-HPLC) system coupled to a photodiode array detector (SpectraLab Scientific Inc., Ontario, Canada). Prior to analysis, samples were filtered using 0.22 μm filters (Millipore, Billerica, Massachusetts, USA) and a concentration of 1 mg/mL was injected onto a reverse phase Phenomenex (Torrance, California, USA) C_{18} column with 5 μm particle size (100 μm × 21.2 mm). The column was equilibrated with TFA/H_2O (0.1% v/v) at a flow rate of 1.0 mL/min. Using trifluoroacetic acid (TFA)/acetonitrile (0.1% v/v), the concentration of the eluting solvent was raised from 0% to 100% over 60 min. Chromatogram peaks were integrated at an absorbance of 214 nm. Fractions eluted were collected every minute. This was repeated three times before pooling corresponding fractions. Acetonitrile and TFA were then removed under nitrogen and the fractions freeze-dried.

Peptide Identification by Tandem Mass Spectrometry. The most active fraction was further purified and analyzed using an electrospray ionization quadrupole time-of-flight (ESI-Q-TOF) mass spectrometer coupled to a nano-ultraperformance liquid chromatography system (Waters Corporation, Milford, MA, USA) using positive ionization mode.

The freeze-dried sample was dissolved in water/acetonitrile (90:10, v/v) with 0.1% formic acid (FA) and filtered through a 0.22 μm syringe filter (Millipore). After filtering, 1 μL of the redissolved fraction was loaded onto a nanoacquity UPLC column BEH130 C_{18} (100 μm × 100 mm, 1.7 μm particle size), preceded by a Symmetry C_{18} (180 μm × 20 mm, 5 μm particle size) trapping column. Mobile phases consisted of solvent A, which contained 0.1% FA in water, and solvent B, which contained 0.1% FA in acetonitrile. Trapping of the peptides was achieved using a loading time of 3 min at a flow rate of 5 μL/min with 97% of solvent A and 3% of solvent B and then elution onto the analytical column at 250 nL/min. Chromatographic conditions consisted of 95% of solvent A and 5% of solvent B isocratically for 3 min, followed by a linear gradient from 95 to 50% of solvent A over 48 min.

Mass spectral data were acquired in MS^e mode with collision energy for a full mass scan of 6 V and a collision energy ramp of 15−35 V. In the DDA mode, a 1 s TOF MS scan from m/z 100 to m/z 1500 was performed. The Q-TOF was calibrated externally using Glu-fibrinopeptide (Glu-Fib) for the mass range m/z 100 to 1500.

Database Search, Confirmation of Sequences, and de Novo Sequencing. Automated spectra processing and peak list generation was performed using the software Protein Lynx Global Server, v2.4 (Waters Corporation). Database searches were performed using Mascot interface 2.2 in combination with the Mascot Daemon interface 2.2.2 (Matrix Science, Inc., Massachusetts, USA) (http://www.matrixscience.com), against the UniProt and NCBI non-redundant databases. Mascot searches were done with none enzymatic specificity and with a tolerance on the mass measurement of 100 ppm in the MS mode and 0.6 Da for MS/MS ions. Oxidation of Met was used as variable modification. Comparison between the sequences of proteins to determine the protein origin of peptides was done using the UniProtKB/TrEMBL database.

Microwave-Assisted Solid Phase Peptide Synthesis. The bioactive peptides were synthesized using microwave-assisted solid phase peptide synthesis (MW-SPPS) performed on a Liberty CEM microwave peptide synthesizer (Mathews, North Carolina, USA). Peptides were synthesized on H-Ala-HMPB-ChemMatrix and H-Ile-HMPB-ChemMatrix resins (PCAS Biomatrix Inc., Quebec, Canada). Synthetic peptides were purified using RP-HPLC on a Semi Preparative Jupiter Proteo (4u, 90A) column (Phenomenex, Cheshire, U.K.) developed in a gradient of 30−58% acetonitrile/0.1% TFA over 35 min for IRLIIVLMPILMA. Fractions containing the desired molecular mass were identified using MALDI-TOF mass spectrometry and were pooled and lyophilized on a Genevac HT 4X (Genevac Ltd., Ipswitch, U.K.) lyophilizer. The process of bioassay guided fractionation of crude *P. palmata* protein hydrolysates coupled with ESI-Q-TOF MS is summarized in Figure 1 (Figure 1).

In Silico Analysis To Determine Potential Survival of Synthesized Peptides in Vivo. The most active peptide sequence was assessed for potential cleavage by gastrointestinal tract (GI) enzymes using the program Expasy Peptide Cutter (http://ca.expasy.org/cgi-bin/peptidecutter/peptidecutter.pl). The peptide was evaluated against a number of enzymes that are found in the GI tract using *in silico* analysis including pepsin (pH > 1.3 and pH 2.0), thermolysin, chymotrypsin, and trypsin.

RESULTS

Protein extracts were obtained using the method of Galland-Irmouli et al. (1999).[11] The extraction procedure outlined in this study yielded protein levels of 10.01% (±0.24) protein of dry mass. This is in close correlation to data found by Galland-Irmouli et al. (1999) in their seasonal variation study of the protein content of *P. palmata* harvested in late October, where *P. palmata* harvested off the west coast of France was found to have protein levels at approximately 10% of the dry mass of *P. palmata*.[11]

A Papain hydrolysate of the crude, *P. palmata* protein extract was generated in order to release renin inhibitory peptides from the parent proteins. When assayed for renin inhibition, the Papain hydrolyzed protein extract inhibited renin by 41.89% (±3.22) compared to the specific renin inhibitor, Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe, which was used as the positive control. This positive control was tested at two different concentrations: 1 μmol/L (half the maximal inhibitory concentration (IC_{50})) and 10 μmol/L, respectively. These concentrations were selected due to previous studies where the IC_{50} value for Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe was obtained and also where this compound demonstrated >90% inhibition of renin at a concentration of 10 μmol/L.[20] In this study, Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe, assayed at 10 μmol/L and 1 μmol/L, inhibited renin by 94.71% (±0.87) and 57.35% (±0.91), respectively, as shown in Figure 2.

To further isolate renin inhibitory peptides released during Papain hydrolysis, RP-HPLC was performed and resultant fractions collected every minute were tested for their renin inhibitory activities. Figure 3 shows the RP-HPLC chromatogram obtained for the Papain hydrolysate of the crude *P. palmata* protein. The highly concentrated plateau of peaks observed between 10 and 25 min were assayed for renin inhibition. The fraction collected at minute 25 (Fr-25) gave the highest renin inhibitory activity of all fractions tested and inhibited renin by 58.97% (±1.26) at a concentration of 1 mg/mL. This fraction was further characterized.

To identify the renin inhibitory peptides from Fr-25, ESI-Q-TOF MS was performed and 11 peptides were elucidated using *de novo* sequencing (Table 1). All 11 peptides identified had no homology to previously sequenced proteins from *P. palmata.* However, only 35 proteins from *P. palmata* have been characterized to date and all peptides identified from Fr-25 when aligned with BLAST show 100% homology with sections of the proteins; Photosystem II protein Y from *Cyanidium caldarium*; *Galdieria sulphuraria*, Photosystem I reaction center subunit VII from *Chlorella vulgaris*; and Cytochrome b6-f subunit 7 from *Cyanidioschyzon merolae.* Related proteins are found in seaweed species closely related to *P. palmata*, including *Cyanidioschyzon merolae*, *Cyanidium caldarium*, *Galdieria sulphuraria*, and the microalga *Chlorella vulgaris.* For example, the protein Cytochrome c oxidase (accession number D5I1R8) isolated from *P. palmata* has 80.50% homology with Cytochrome c oxidase (accession number Q9ZZQ9) isolated from *Cyanidioschyzon merolae.* Furthermore, the protein *Photosystem II D2* (accession number Q52W49), identified in *P. palmata* previously, is 93.20% homologous, with the same protein (accession number P28253) isolated from *Cyanidium caldarium* and *Galdieria sulphuraria* (accession number Q9TM47). Finally, the protein *Photosystem I P700 chlorophyll A apoprotein (accession number Q8MAG9)* isolated from *P. palmata* is *85.30%* homologous, with the same protein isolated from *Chlorella vulgaris* (accession number P56341).

The chemically synthesized peptides (Table 1) were tested for renin inhibitory activities using the Caymann renin assay kit. Of the 11 chemically synthesized peptides, the peptide corresponding to the amino acid sequence IRLIIVLMPILMA had a renin inhibitory IC_{50} value of 3.344 mM (±0.31). This corresponds well to previously published research which identified renin inhibitory peptides from pea proteins.[4]

This tridecopeptide is longer in length than most previously identified heart health peptides, such as Ile-Pro-Pro and Val-Pro-Pro, and may be considered a propeptide. Therefore, this peptide was subjected to *in silico* analysis using an Expasy peptide cutter (http://ca.expasy.org/cgi-bin/peptidecutter/peptidecutter.pl)

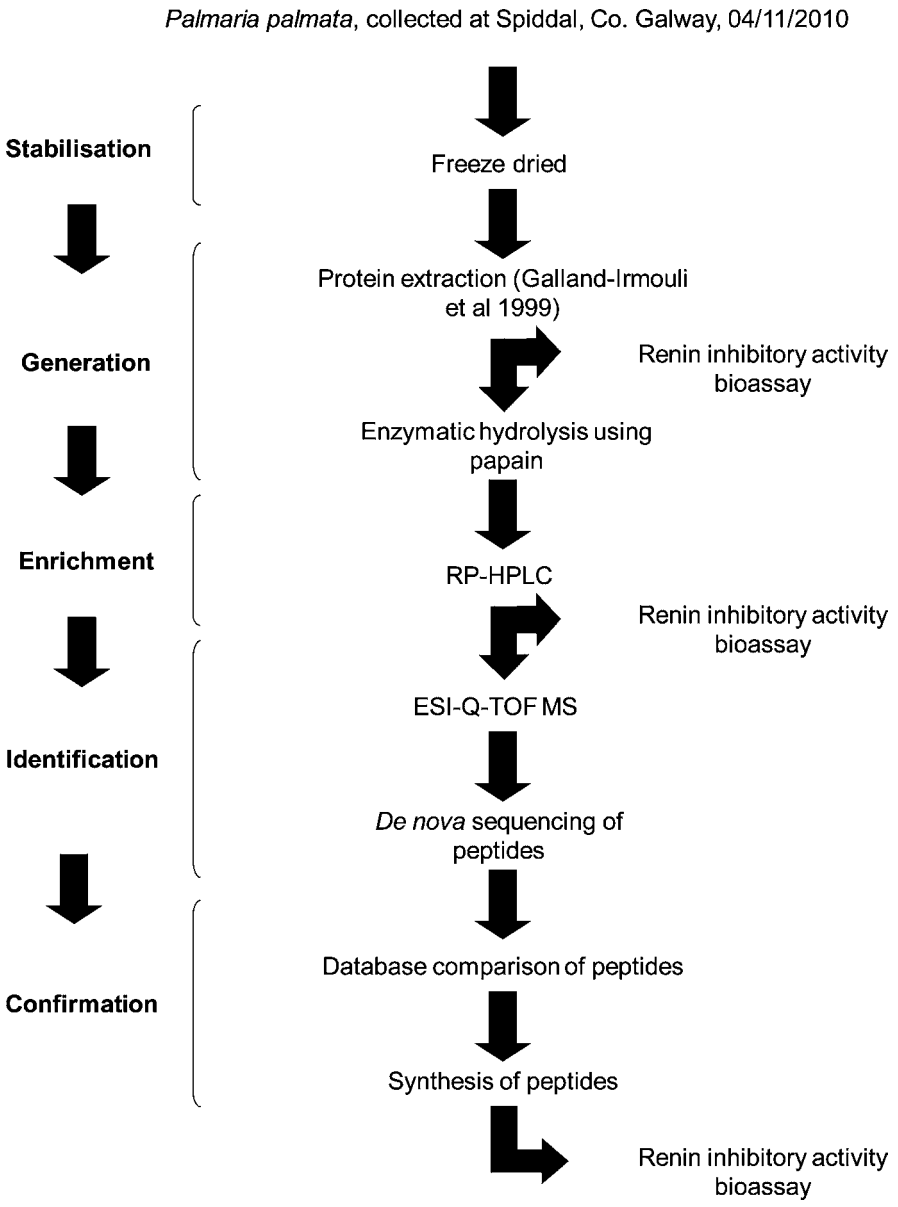

Figure 1. Schematic representation of the bioassay guided isolation and characterization approach used in this study to isolate renin inhibitory peptides from *P. palmata*.

to determine potential cleavage peptides and amino acids that may result from potential digestion of the peptide *in vivo* with enzymes and acids. The option of all enzymes and chemicals from the list displayed in the Expasy peptide cutter was chosen, and the results of cleavage analysis are shown in Table 2.

DISCUSSION

Inhibition of the RAS is a well-established approach in the treatment of hypertension. In this study, the enzyme Papain was used to release bioactive peptides from *P. palmata* protein. Papain was chosen to perform hydrolysis, as it has generally recognized as safe (GRAS) status. Furthermore, previous literature detailed the peptide generating capacity of Papain, and it has been used previously to generate peptides from algal protein waste capable of disrupting the RAS through ACE-I inhibition.[22] Another reason for choosing Papain as a hydrolytic enzyme is that while having broad substrate specificity it also exhibits specific substrate preferences for bulky hydrophobic or aromatic residues,[23] which may correlate with renin inhibitory peptides.[4]

Inhibition of the enzyme ACE-I, with chemically synthesized ACE-I inhibitors including captopril (Capoten), enalopril, alcacepril, and lisinopril is well-known.[21] However, inhibition of renin has several advantages over ACE-I inhibition. Renin is the only known enzyme that converts angiotensinogen to angiotensin I. In the phenomenon, known as "ACE escape", angiotensin II levels increase in blood plasma due to other enzymes converting angiotensin I to angiotensin II during ACE-I inhibition.[20] Renin inhibition in turn eliminates angiotensin I from the bloodstream, preventing the formation of angiotensin II. Another advantage of renin inhibition over ACE-I inhibition is that renin inhibitors do not affect kinin metabolism and, hence, are not expected to cause dry cough or angioneurotic edema, which are characteristic side effects of ACE-I inhibitors.[2]

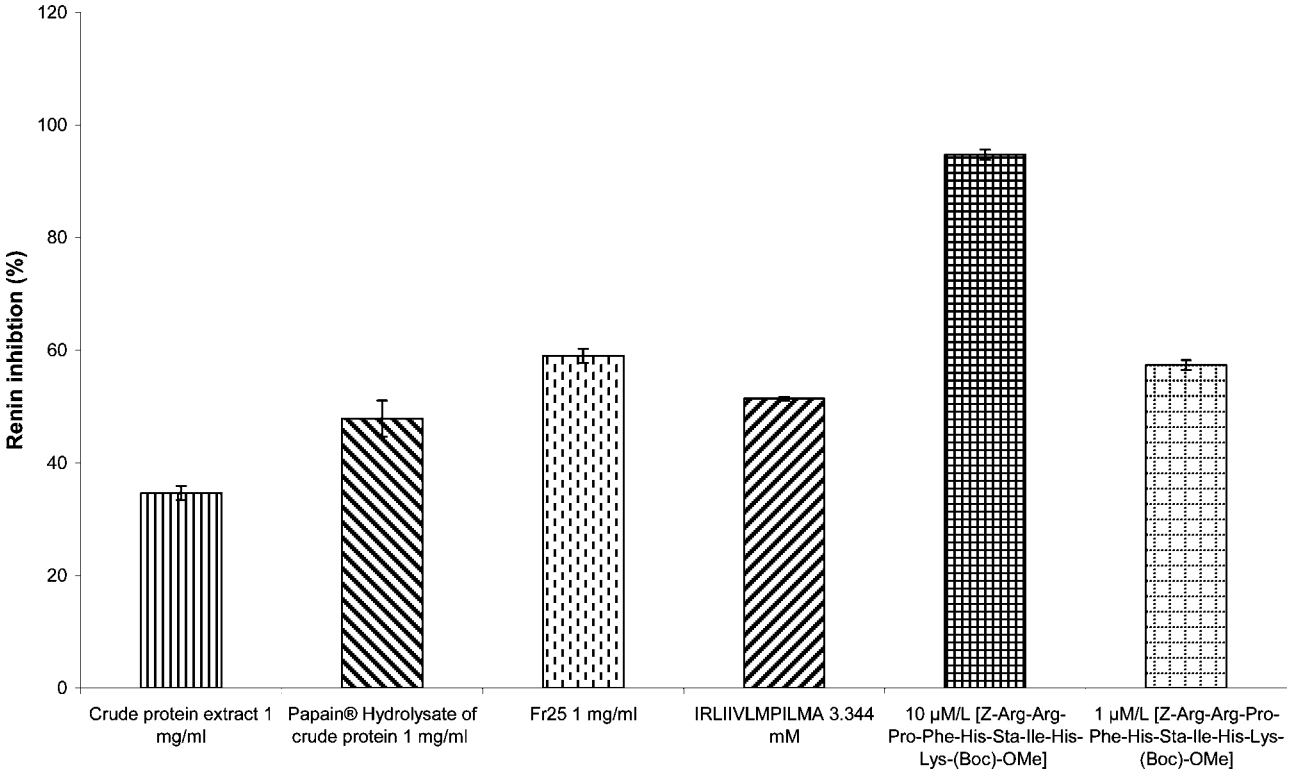

Figure 2. Renin inhibitory assay of the crude protein, Papain hydrolyzed crude protein, and Fr-25, all at 1 mg/mL. Also shown is the peptide IRLIIVLMPILMA seen at its IC_{50} of 3.344 mM (±0.31) and the positive control Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe seen at 1 μmol/L and 10 μmol/L. Values are mean ± SEM (n = 3). *P < 0.05 and **P < 0.01 and compared with initial activity.

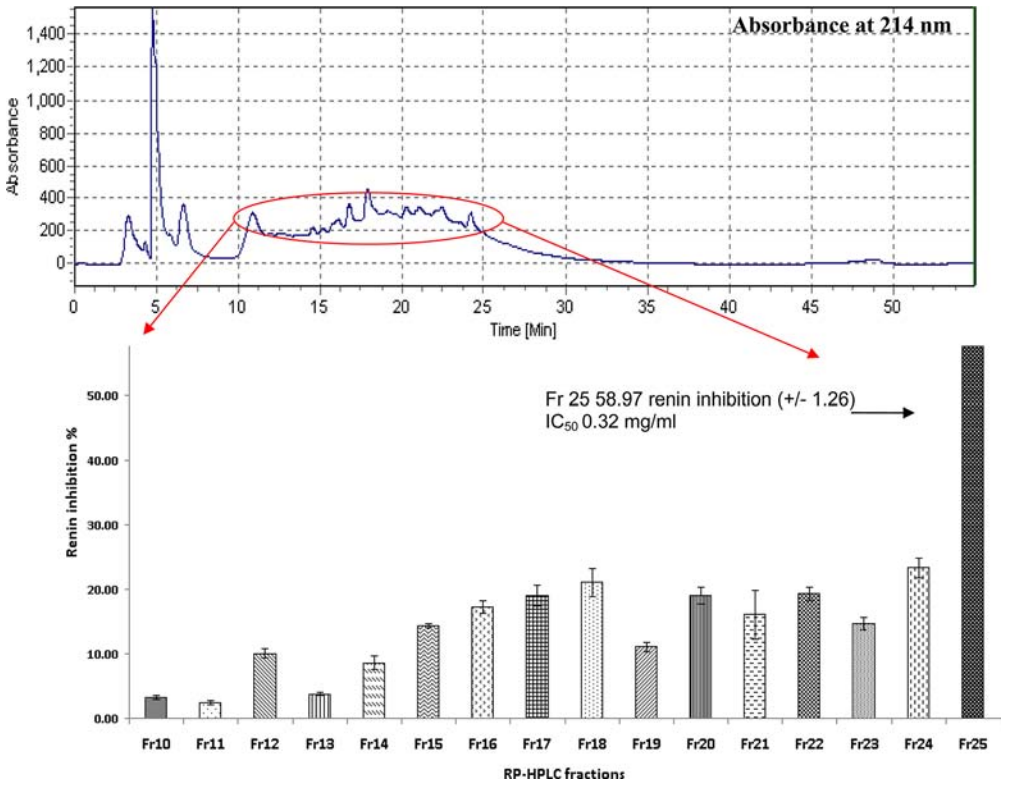

Figure 3. RP-HPLC chromatograph of a Papain hydrolysate of *P. palmata* protein. Absorbance plotted at 214 nm. The renin inhibitory assay results obtained from RP-HPLC fractions Fr10−Fr25 of the *P. palmata* Papain protein hydrolysate. Samples were tested at a concentration of 1 mg/mL. Data is displayed as % inhibition. Values are mean ± SEM (n = 3).

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Table 1. Peptides Identified in RP-HPLC Fr25 Enriched from the *P. palmata* Papain Hydrolysate of *P. palmata*

sequence[a]	phylum	protein (assession number)	species sharing 100% homology	obsd mass	calcd mass	charged state
D.IRLIIVLMPILMA.A	Rhodophyta	Photosystem II protein Y (O19893)	*Cyanidium caldarium,Galdieria sulphuraria*	1494.93	499.29	(+3)
MNEIVALMI.I	Rhodophyta	Cytochrome b6-f complex (Q85FX8)	*Cyanidioschyzon merolae*	1032.53	517.26	(+2)
P.ILMA.A	Rhodophyta	Photosystem II protein Y (O19893)	*Cyanidium caldarium,Galdieria sulphuraria*	446.25	447.1235	(+1)
I.LMAASWAIY.N	Rhodophyta	Photosystem II protein Y (O19893)	*Cyanidium caldarium,Galdieria sulphuraria*	1024.5	1025.48	(+1)
Q.ILPSILVPLV.G	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	1062.7	532.32	(+2)
L.PSIL.V	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	428.26	429.09	(+1)
I.LVPLVGLV.F	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	808.54	809.4	(+1)
V.PLVGLVFPAI.A	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	1024.63	1025.48	(+1)
L.VFPAIAM.A	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	747.39	748.4	(+1)
V.FPAI.A	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	446.25	447.12	(+1)
F.PAIA.M	Rhodophyta	Photosystem I reaction center subunit VII (P58214)	*Chlorella vulgaris*	370.22	371.11	(+1)

[a]. = cleavage point of Papain enzyme.

Table 2. Cleavage of the Peptide IRLIIVLMPILMA by Proteolytic Enzymes and Acids Using an Expasy Peptide Cutter

enzyme	cleavages	position of cleavages
Arg-C proteinase	1	2
CNBr	2	8 12
chymotrypsin-low specificity (C-term to [FYWML], not before P)	4	3 7 11 12
clostripain	1	2
pepsin (pH 1.3)	3	3 6 11
pepsin (pH > 2)	3	3 6 11
proteinase K	9	1 3 4 5 6 7 10 11 13
thermolysin	9	2 3 4 5 6 9 10 11 12
trypsin	1	2

While ACE-I inhibitory peptides were discovered in macroalgae (*Undaria pinnatifida*) before,[17] this study is, to the best of the authors knowledge, the first to display renin inhibitory activities for peptides isolated from *P. palmata*, or indeed any macroalgae. Papain hydrolyzed *P. palmata* protein inhibited renin by 41.89% (±3.22). It is well documented that hydrolysis with proteolytic enzymes such as Papain is used frequently as a method to liberate bioactive peptides from plant and other proteins.[4,27] In this study, hydrolysis of *P. palmata* protein with Papain and further fractionation using RP-HPLC was found to enhance the renin inhibitory activity of the protein, as Fr-25 inhibited renin activity by 58.97% (±1.26) at a concentration of 1 mg/mL compared to the chemically synthesized positive control, Z-Arg-Arg-Pro-Phe-His-Sta-Ile-His-Lys-(Boc)-OMe, which inhibited renin by 50% at a concentration of 1 μmol/mL (Figure 2).

In order to confirm the renin inhibitory activity of Fr-25, the peptides within this fraction were identified and characterized using ESI-Q-TOF mass spectrometry, and as the peptides to be sequenced were unknown, they were elucidated using *de novo* sequencing. Eleven peptides (Table 1) were chosen for chemical synthesis based on their amino acid sequences. Sequences with amino acids containing bulky side chains at the C-terminus and hydrophobic amino acids at the N-terminus were chosen for chemical synthesis, as peptides containing these amino acid sequences were documented previously as potential enzyme (ACE-I and renin) inhibitors.[4,26] Chemical synthesis was necessary in order to confirm the observed renin inhibitory activities of Fr-25.

Table 1 displays the eleven peptides identified in Fr-25. It also shows the parent macroalgal proteins that contain the amino acid sequences elucidated for the identified peptides. These proteins were not characterized for *P. palmata.* The peptides elucidated in this study do not share homology with any peptides discovered in *P. palmata*, as to date there are only 35 proteins completely sequenced from *P. palmata*.[24] However, the eleven peptides identified in Fr-25 were found to correspond to the amino acid sequences of proteins found in seaweed species closely related to *P. palmata. P. palmata* proteins are closely related to proteins sequenced from the seaweed species *Cyanidioschyzon merolae*, *Cyanidium caldarium*, *Galdieria sulphuraria*, and *Chlorella vulgaris*. Inherent protein sequence homologies exist between *P. palmata* and these macroalgae species. All chemically synthesized peptides were assayed for their abilities to inhibit renin, and the tridecopeptide IRLIIVLMPILMA (Figure 2) inhibited renin by 50% at a concentration of 3.344 mM (±0.31). This compared favorably with the positive control, which was tested at 10 μM/L and 1 μM/L, respectively, and also to previously isolated peptidic renin inhibitors from plant pea sources that displayed IC_{50} values between 9.2 mM (±0.18) and 22.66 mM (±1.71).[4,7]

Several published works explore the potential of *in silico* methods for screening bioactive peptides.[4,26] In this study the propeptide IRLIIVLMPILMA identified in Fr-25 and confirmed to inhibit renin *in vitro* using the Caymann renin inhibitory bioassay method was subjected to *in silico* cleavage analysis using the computer program Expasy peptide cutter. Enzymes found in the GI tract were chosen for cleavage analysis. Enzymes that cleaved the propeptide IRLIIVLMPILMA were found to release di- and tripeptides. For example, Pepsin (pH 1.3 and pH > 2) cleaved this propeptide at

positions 3, 6, and 11, releasing the tripeptides Ile-Arg-Leu (IRL) and Ile-Ile-Val (IIV) and the dipeptide Met-Ala (MA). It also releases the peptide Leu-Met-Pro-Ile-Leu (LMPIL), which is five amino acids in length. In addition, the enzymes trypsin, thermolysin, and Arg-C-proteinase released the dipeptide Ile-Arg (IR) from IRLIIVLMPILMA (Table 2). The peptide IR has a documented IC_{50} value of 9.2 mM for renin.[4] Overall, the N-terminal amino acids of the propeptide remained largely uncleaved, with only Proteinase K capable of cleaving the peptide in the first position. These results suggest that this propeptide and the di- and tripeptides released from it may cross the lumen into the bloodstream and potentiate an antihypertensive effect. Indeed, certain peptides including IR and IRL were previously documented as having renin, antioxidant, and ACE-I inhibitory activities.[4,25] The use of this *in silico* Expasy peptide cutter program has some limitations, as some active peptides are not released, as such, after *in silico* digestion, and further work is required to determine the *in vivo* effects of this peptide, especially if it is to be used as an antihypertensive agent in the future.

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Notes

The authors declare no competing financial interest.

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