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Isolation and characterization of angiotensin I-converting enzyme (ACE) inhibitory peptides from *Ulva rigida* C. Agardh protein hydrolysate

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ABSTRACT

Ulva rigida protein was hydrolysed with pepsin plus bromelain after a screening of nine enzymes for optimal proteolysis. This hydrolysate, presenting ACE-inhibitory activity with an IC_{50} value of 0.483 mg/mL, was fractionated by ultrafiltration membranes into three molecular weight ranges (<1 kDa, 1–3 kDa and >3 kDa). The <1 kDa fraction that exhibited the highest activity (IC_{50}: 0.095 mg/mL) was purified using size-exclusion chromatography and reversed-phase high-performance liquid chromatography, yielding two active ACE-inhibitory purified peptides. Edman degradation revealed its amino acid sequences to be IP and AFL with IC_{50} values of 0.020 and 0.023 mg/mL, respectively. Both peptides were synthesized to confirm the structure and to validate their ACE-inhibitory activities. Lineweaver–Burk plots suggest that IP acts as a non-competitive and AFL as a competitive ACE-inhibitors. Stability assays showed that both peptides are heat-stable and AFL is hydrolysed by intestinal mucosa peptidases to FL with IC_{50} value of 0.004 mg/mL that acts as a non-competitive ACE-inhibitor. The results suggest that these peptides might have a potential use in the preparation of antihypertensive drugs or functional foods.
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1. Introduction

Macroalgae have been consumed in Asian countries since ancient times and their dietary ingestion has been shown to decrease blood pressure in humans (Hata, Nakajima, Uchida, Hidaka, & Nakano, 2001; Wada et al., 2011). Angiotensin-converting enzyme (ACE), a zinc-containing protease located in different mammalian tissues (Li, Le, Shi, & Shrestha, 2004), plays a key role in the regulation of blood pressure, since it catalyses the conversion of angiotensin I to a potent vasoconstrictor angiotensin II and it also promotes the degradation of the vasodilator bradykinin (Soffer, 1976). Therefore, the ACE inhibition has become a major target control for hypertension, a common progressive disorder leading to several chronic diseases such as cardiovascular disease, stroke, renal disease and diabetes (Zhang et al., 2006). In fact, according to Mittal and Singh (2010), one-quarter of the world's adult population has hypertension, and this is likely to increase to 29% by 2025. A wide variety of synthetic ACE-inhibitors have been currently used as clinical antihypertensive drugs. However, these synthetic drugs can cause certain adverse side effects such as cough, headaches,

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insomnia, allergic reactions, taste disturbances, skin rashes, angioedema, hyperkalaemia and drug–drug interactions (Antonios & MacGregor, 1995; Atkinson & Robertson, 1979). Therefore, searching for ACE-inhibitors from natural resources, such as marine organisms, has become a major area of research in the field of nutraceutical and pharmaceutical industries (e.g. Wijesekara & Kim, 2010), and some ACE-inhibitory peptides have already been isolated from the enzymatic hydrolysates of macroalgae species, especially from brown and red algal species (e.g. Qu et al., 2010; Sato et al., 2002). Many ACE-inhibitory peptides have been recently isolated from other protein hydrolysates, such as milk and dairy products, eggs, meat and plants (for a review, see Li et al., 2004). Indeed, there is substantial scientific evidence that enzymatic hydrolysis of food protein sources is an efficient method to recover potent bioactive peptides that may present lower side effects (Korhonen & Pihlanto, 2003; Lordan, Ross, & Stanton, 2011).

The Azores Islands (Portugal), due to their isolated location in the middle of the Atlantic Ocean associated with pristine seawaters (Neto et al., 2009), are a very promising location for marine natural resources that may produce new compounds with medicine-like effects in treating or preventing certain diseases. Macroalgae are abundant and structuring organisms on the coastal areas of the Azores Islands. Some species have a markedly seasonal pattern, whereas others are available throughout the year. This is the case of the green seaweed *Ulva rigida* C. Agardh, one of the consumed edible algae by the local population, which is abundant in the Azorean intertidal zone (Neto, Tittley, & Raposeiro, 2006). To the best of our knowledge, no study has been reported on the isolation of ACE-inhibitory peptides from species belonging to *Ulva* genus, which is ubiquitous, easily cultivated and an important food source in many countries.

The present study aimed to: (1) screen for effective enzymes and to find the enzymatic hydrolysis conditions for producing *U. rigida* protein hydrolysates with the higher ACE-inhibitory activity, (2) isolate and identify ACE-inhibitory peptides from *U. rigida* protein hydrolysed with pepsin plus bromelain enzymes, and (3) investigate the purified peptides in terms of ACE-inhibitory potency, inhibition pattern, and stability against temperature and gastrointestinal proteases, in order to evaluate its potential use in the preparation of antihypertensive drugs or functional foods.

2. Material and methods

2.1. Collection and preparation of U. rigida sample

Chlorophyta *U. rigida* sample was collected in April 2013 from São Miguel Island littoral zone (namely, Forno da Cal, São Roque) of Azores Archipelago (37° 40′ N and 25° 31′ W), Portugal. A voucher specimen was prepared (voucher number SMG-13-12) and deposited in the Herbarium AZB – Ruy Telles Palhinha of the Department of Biology at the University of Azores.

Within 24 h of collection, the *U. rigida* sample was first washed in seawater to remove encrusting material and epiphytes, carefully rinsed with distilled water to remove salts and then air-dried and stored in an air-tight container in a freezer (–80 °C) until further analysis. Prior to the analytical procedures, the algae sample was defrosted and dried at 40–45 °C for 48 h (avoiding overheating that could lead to oxidation) and then was cut into small pieces, grounded into a fine powder, re-dried at 40 °C and stored in the dark under N_2 in a desiccator at a refrigerated temperature.

2.2. Chemicals and reagents

Acetonitrile (ACN), methanol (MeOH), HPLC grade, trizma base, zinc chloride, hippuric acid (HA), hydrochloric acid (HCl), trifluoroacetic acid (TFA), heptafluorobutyric isobutyl ester (HFB-IBA), ethyl acetate, acetyl chloride, isobutanol, sulphuric acid, boric acid, Kjeldahl catalyst, *o*-phthaldialdehyde (OPA), *β*-mercaptoethanol, sodium dodecyl sulphate, dithiothreitol, Sephadex G-25, glutathione, captopril, hippuryl-L-histidyl-L-leucine (HHL) as substrate peptide of angiotensin I-converting enzyme (ACE), and the commercial enzymes ACE from porcine kidney, bromelain from pineapple stem (B-4882), chymotrypsin from bovine pancreas (C-9381), ficin (F-6008), pancreatin from porcine pancreas (P-1750), pepsin from porcine gastric mucosa (P-77161), peptidases from porcine intestinal mucosa (P-7500), protease from *Bacillus licheniformis* (P-5380), protease from *Streptomyces griseus* (P-5380) and trypsin from bovine pancreas (T-8003) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The synthetic tripeptide AFL (HPLC purity 98.21%) and dipeptide IP (HPLC purity 95.63%) were obtained from ProteoGenix SAS (Schiltigheim, France). Sodium chloride, sodium hydroxide, sodium tetraborate decahydrate, ethanol, phenol and 85% phosphoric acid were obtained from E. Merck (Darmstadt, Germany). Amino acid standard mixture and sequanal grade 6 N HCl were purchased from Pierce Chemicals (Rockford, IL, USA). All other reagents used in this study were reagent grade chemicals. Ultrafiltration (UF) membrane system and membranes were purchased from Millipore Co (Bedford, MA, USA). Deionized water was obtained from a Milli-Q water purification and filtration system with an 18 MΩ.cm resistivity (Millipore, Bedford, MA, USA). All samples were filtered through 0.45 µm nylon filter for the HPLC analysis.

2.3. Enzyme screening for ACE-inhibitory peptides production from U. rigida protein

Nine commercial proteases (bromelain, chymotrypsin, ficin, pancreatin, pepsin, peptidases, protease from *Bacillus licheniformis*, protease from *Streptomyces griseus* and trypsin) were used for digestion of *U. rigida* that presented a protein content of 15.78 ± 0.10% (on a dry basis) as determined by Kjeldahl method (AOAC, 1990) and a moisture content of 84.50 ± 0.70% of the fresh algal material, calculated by subtracting the weight of dry sample from the wet weight. The digestion assay was performed according to the method of Sato et al. (2002a) with slightly modifications. One gram of dried *U. rigida* sample was suspended in 25 mL of ice-cold distilled water in order to prevent the sample heating and then homogenized with an Ultra-turrax T50 at 5635 × *g* for 10 min. Ten milligrams of each protease were individually added to the homogenate (substrate/enzyme ratio to 100:1, w/w) and the mixture was incubated for 20 h using the conditions (pH and temperature) suggested by the manufacturer for each enzyme reaction. After hydrolysis, the samples were heated for 10 min in boiling water

in order to inactivate the proteases. The precipitates were removed by centrifugation at 2504 × g for 10 min at 4 °C. The supernatants were deproteinized as described below, and then concentrated and lyophilized in a freeze-drier to obtain powder products that were assayed for ACE-inhibitory activity using the method described in section 2.6. The yield percentage of *U. rigida* hydrolysates was calculated according to Sato et al. (2002a). The unreacted proteins of the supernatants were removed by centrifugation (10000 × g, 5 min at 4 °C) after ethanol precipitation and then the supernatants were dried and the amount of solid contents measured. The yield was defined as the weight of the solid contents obtained from 1 g of dried *U. rigida* (*U. rigida* without enzymatic hydrolysis).

2.4. *Preparation of* U. rigida *enzymatic hydrolysate*

Twenty grams of dried *U. rigida* sample were suspended in 300 mL of distilled water to induce cell lysis by osmotic shock that facilitated subsequent protein extraction. Then, the suspension of ice-cold distilled water in order to prevent the sample heating was homogenized with an Ultra-turrax T50 at 5635 × g for 10 min and then hydrolysed by pepsin (substrate/enzyme ratio to 100:1, w/w) for 20 h to maximize the hydrolysis process at the conditions recommended by the enzyme manufacture (37 °C and pH 2.0, adjusted using 2 M HCl). After twenty hours, the digestion solution was boiled for 10 min in order to inactivate the enzyme, adjusted to pH 7.0 using 5 M NaOH and immediately concentrated until an approximate volume of 150 mL. The concentrated digestion solution (substrate) was subsequently hydrolysed with bromelain (substrate/enzyme ratio to 100:1, w/w) at 37 °C for 20 h. The digest was boiled for 10 min and immediately transferred to a 12 kDa molecular weight cut-off (MWCO) dialysing tube. The outer solutions (permeate) from five changes (approximately 3 litres) were combined, concentrated, lyophilized, weighted and kept at −80 °C for further fractionation and corresponding ACE-inhibitory assays using the method described in section 2.6. The protein concentration (unreacted protein plus the hydrolysis products content) of the lyophilized hydrolysate was determined by the Kjeldahl method (AOAC, 1990) and its peptide content was measured by OPA method (Church, Swaisgood, Porter, & Catignani, 1983) using glutathione as the calibration standard, according to Ghanbari et al. (2015). The extent of protein degradation by pepsin-bromelain enzymes was estimated by assessing the percent degree of hydrolysis (DH) using the OPA method described by Nielsen, Petersen, and Dambmann (2001).

2.5. *Purification of ACE-inhibitory peptides*

The *U. rigida* protein hydrolysate (URPH) digested with the pepsin and bromelain enzymes was fractionated through three different UF membranes in a cell dialyser system having a range of MWCO of 10, 3 and 1 kDa. All the pooled URPH fractions were lyophilized in a freeze-drier and assayed for ACE-inhibitory activity, as described in section 2.6. The percent yields of the UF fractions were calculated as the ratio of peptide weight of the lyophilized peptide permeate to peptide weight of the URPH, as described by Girgih, Udenigwe, Li, Adebiyi, and Aluko (2011). The protein and peptide content of the UF fractions were also determined by Kjeldahl and OPA methods, respectively, as described in section 2.4. The fraction (25 mg) with the highest activity was dissolved in 5 mL of distilled water, loaded onto a Sephadex G-25 size exclusion column (20 × 1.6 cm i.d.), eluted with water at a flow rate of 1.16 mL/min under UV detection at 220 nm and separated into several fractions, using medium pressure liquid chromatography (MPLC). These fractions were pooled, concentrated in a rotatory evaporator at 40 °C, lyophilized and the ACE-inhibitory activities were further determined in each fraction using the same concentration (2 mg/mL). The most active fraction was then separated on a HPLC reverse phase Ultrasphere ODS semi-preparative column (250 × 10 mm i.d., 5 µm particle size) (Beckman Coulter, Miami, USA), using a linear gradient of phase (A) 0.05% TFA in water and phase (B) 90 mL ACN plus 160 mL of phase A (t = 0 min – 0% B, t = 25 min – 25% B and t = 60 min – 100% B) at a flow rate of 2 mL/min and UV detection at 220 nm. In order to obtain higher degree of purification, the most active ACE-inhibitory fractions were again pooled, lyophilized immediately, and rechromatographed on a HPLC Vydac C4-protein reverse phase semi-preparative column (250 × 10 mm i.d., 5 µm particle size) from the Grace (The Nest Group, Southborough, MS, USA), using the same mobile phase in isocratic conditions A:B (85:15, v/v) at a flow rate of 1.0 mL/min. Finally, the active fractions were further re-purified on a HPLC Zorbax Eclipse XBD-C18 reverse phase analytical column (250 × 4.6 mm i.d., 5 µm particle size) (Agilent Technologies, Santa Clara, CA, USA) using a linear gradient of phase (A) 0.1% TFA in water and phase (B) 0.1% TFA in ACN (t = 0 min – 20% B, t = 25 min – 45% B and t = 25.01 min – 100% B) at a flow rate of 0.7 mL/min and UV detection at 220 nm. The purified active peptides were pooled for the determination of its amino acid composition and amino acid sequence, and properties (ACE-inhibitory potency, inhibition pattern, and stability against temperature and gastrointestinal proteases). The peptide content of the most active fractions obtained during the purification procedure by size-exclusion chromatography and RP-HPLC was also quantified by the OPA method, as described in section 2.4. The percent yields of the purification steps were calculated using a gravimetric method, according to Ko et al. (2012).

2.6. *Determination of ACE-inhibitory activity*

The determination of ACE-inhibitory activity was performed *in vitro* by RP-HPLC adapted from the spectrophotometric method described by Cushman and Cheung (1971) with slight modifications. This method is based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (Hip-His-Leu) catalysed by ACE. For the assay, 42.5 µL of the sample solution (2 mg/mL) was pre-incubated at 37 °C for 5 min with 10 µL ACE (0.6 mU/mL) enzyme. The mixture was subsequently incubated at the same temperature for 60 min with 20 µL of the substrate (5 mM HHL in 10 µM zinc chloride containing 100 mM sodium trizma base and 300 mM NaCl at pH 8.3). The reaction was terminated by adding 12.5 µL of 5 M HCl. The percentage of ACE inhibition was determined by an HPLC system from Waters equipped with a 626 pump and 600S controller coupled to a 486 tunable UV detector. An aliquot of 20 µL from the reaction mixture was analysed on a reverse-phase Ultrasphere ODS analytical column (250 × 4.6 mm i.d., 5 µm

particle size) (Beckman Coulter, Miami, USA), using an isocratic elution of MeOH:ACN:0.1% HCl (25:25:50, v/v/v) at a constant flow-rate of 0.6 mL/min, and HA and HHL were detected by UV at 228 nm. The average value from three determinations at each concentration was used to calculate the ACE inhibition (%) rate as follows:

$$\text{ACE inhibition}(\%) = [B - A/B - C] \times 100,$$

where A is the absorbance of HA generated in the presence of ACE inhibitor, B the absorbance of HA generated without ACE inhibitor and C the absorbance of HA generated without ACE (corresponding to HHL autolysis in the course of enzymatic assay). The IC_{50} value (mg/mL) was defined as the concentration of inhibitor required to reduce the HA peak by 50% (corresponding to 50% inhibition of ACE activity). The captopril was used as a positive control for ACE inhibition.

2.7. Determination of amino acid composition of the purified ACE-inhibitory peptides

The amino acid composition of the purified peptides was determined by the Pons et al. (2003) methodology with some modifications. The dried purified peptides samples (*ca* 0.2 mg) were placed in a small reaction vials, exposed to a stream of dry nitrogen, then capped and submitted to acid hydrolysis at 100 °C for 24 h with 100 μL 6 N HCl containing 0.1% phenol for tyrosine protection. After cooling until room temperature, the samples were evaporated under a stream of dry nitrogen and then transesterified slowly by adding the mixture of acetyl chloride:isobutanol (1.25:5 mL, v/v, the reagent was obtained by adding the acetyl chloride in isobutanol precooled to −20 °C) and heating at 100 °C for 60 min. Next, the sample vials were opened and the mixtures were exposed to a stream of dry nitrogen to remove excess reagent. After cooling in an ice bath, the samples were supplemented with 200 μL of acetonitrile and 50 μL of the derivatization reagent HFB-IBA, and heated again at 100 °C for 15 min. After evaporation of the excess reagent at 115 °C under a stream of dry nitrogen and cooling to room temperature, samples were dissolved in 300 μL of ethyl acetate and an aliquot (1 μL) were used for GC analysis. The GC analysis was performed using a Bruker GC model 450-GC gas chromatograph equipped with a split/splitless injector and a flame ionization detector (FID) using a wall-coated open tubular (WCOT) fused silica AT-Amino acid capillary column (250 × 0.53 mm i.d., 1.2 μm film thickness) from Heliflex/Alltech (San Jose, CA, USA). The temperature started at 60 °C for 3 min, programmed at a rate of 4 °C/min to 210 °C and then held at this temperature for further 20 min. The injector and detector temperatures were held constants at 260 °C and 280 °C, respectively. Helium was the carrier gas at a flow rate of 28 cm/sec.

2.8. Identification of molecular mass and amino acid sequence of the purified ACE-inhibitory peptides

To identify the molecular mass, the purified peptides were subjected to a quadrupole time-of-flight mass spectrometer (Q-TOF-2 MS; Micromass Co., Manchester, UK) equipped with an electrospray ionization (ESI) source in positive and/or negative ion mode. To determine the amino acid sequence, the purified peptides were subjected to the automated Edman degradation on a protein sequencer from Applied Biosystem model Procise (Foster City, CA, USA) according to the manufacturer's instructions. Synthetic peptides (purity >95% by HPLC) using a solid-phase method were obtained from a peptide synthesizer from ProteoGenix SAS, and used as an external validation data set for the ACE-inhibitory activity of the purified peptides isolated from *U. rigida*.

2.9. Stability of the purified ACE-inhibitory peptides

For the study of the temperature effect on the ACE-inhibitory activity, the purified peptide solutions (2 mg/mL, 0.06 mL) were incubated at different temperatures (20, 40, 60, 80 and 100 °C) for 2 h, and after cooling down until room temperature (20 °C) the residual ACE-inhibitory activity was determined using the method described in section 2.6. For the study of the gastrointestinal proteases effect on the ACE-inhibitory activity, the purified peptides were assayed *in vitro* following the method of Vercruysse, Smagghe, Beckers, and Van Camp (2009) with little modification. The purified peptides (2 mg/mL, 0.1 mL) were subsequently digested with pepsin, trypsin and chymotrypsin as follows: pepsin solution (0.05%, 0.2 mL) was prepared in 0.1 M glycine-HCl buffer adjusted to pH 2.0 using 4 M HCl and incubated for 2h at 37 °C, while trypsin and chymotrypsin solutions (0.025%, 0.2 mL) were prepared in 0.1 M potassium phosphate buffer adjusted to pH 7.0 with 1 M NaOH and incubated for 2.5 h at 37 °C. Furthermore, the porcine mucosal peptidases, containing a general proteolytic and aminopeptidase activity similar to the enterocytes in the human body, were subsequently used (0.05%, 0.2 mL) at pH 7.0 during 2 h at 37 °C. The reactions were stopped by boiling for 10 min. After drying with dry nitrogen, the residues were re-suspended in 100 μL of buffer and assayed for ACE-inhibitory activity using the method described in section 2.6. In addition, the hydrolysed digests that presented an increased ACE-inhibitory activity were purified on a RP-Zorbax C18 column (using the analytical conditions described in section 2.5), and the purified active peptides were pooled for the determination of its amino acid composition by GC analysis (as described in section 2.7) and its ACE-inhibitory activity.

2.10. Determination of the inhibition pattern on ACE of the purified peptides

To investigate the inhibitory mechanisms of the purified peptides on ACE (means to know that peptides bind or not competitively with substrate on the active sites of ACE), different concentrations of ACE-inhibitory purified peptides were co-incubated with different substrate (HHL) concentrations, and each reaction mixture was assayed as described in section 2.6. The resulting data were reciprocally plotted where the reciprocal of HHL concentration is used as an independent variable (X-axis) and the reciprocal of production rate of HA as a dependent variable (Y-axis). The ACE-inhibitory types of the purified peptides were estimated by comparing the curves of these data in the presence of the peptides with those in the absence of the peptides. The K_m and V_{max} values for the reactions

at different concentrations of purified peptides were determined according to Lineweaver–Burk plots.

2.11. Statistical analysis

The IC_{50} values were calculated from ACE inhibition curves obtained with increasing amounts of inhibitor (*U. rigida* hydrolysates or peptides and captopril). All determinations were performed at least in triplicate, and the results were expressed as means ± standard deviations (SD). The significance of the differences between the control and *U. rigida* hydrolysate groups was analysed using the Student's *t*-test. One-way analysis of variance test (ANOVA) was carried out to assess for any significant difference between the means. Differences between the means at the 5% ($p < 0.05$) level were considered significant.

3. Results and discussion

3.1. Enzyme screening for ACE-inhibitory peptides production from U. rigida protein

Peptides are inactive within the parent protein and could be released by *in vitro* enzyme-catalysed proteolysis. However, the peptides bioactivity is mainly dependent on its primary structure, as a consequence of the protein substrate, the specificity of the protease used, the degree of hydrolysis, the conditions used during hydrolysis (such as temperature, hydrolysis time, substrate to enzyme ratio, pH), and the pretreatment of protein before hydrolysis (Kristinsson & Rasco, 2000; Udenigwe & Aluko, 2012). Results of the present study for the ACE-inhibitory activities and yield of the most active *U. rigida* protein hydrolysates, after a preliminary screening of nine proteases, are shown in Fig. 1A and Fig. 1B, respectively. Bromelain hydrolysate exhibited the highest specificity in the generation of ACE-inhibitory peptides followed by pepsin, chymotrypsin and trypsin with percentage values of 86.7 ± 1.1%, 78.4 ± 0.9%, 70.6 ± 1.0% and 68.2 ± 0.9%, and IC_{50} values of 0.719 ± 0.032, 1.167 ± 0.041, 1.256 ± 0.076 and 1.387 ± 0.054 mg/mL, respectively. The yield percentage of hydrolysates presented values of 31.5 ± 0.6%, 27.8 ± 0.8%, 28.5 ± 0.7% and 28.1 ± 0.6% for bromelain, pepsin, chymotrypsin and trypsin, respectively. The values showed significant difference from control at $p < 0.05$. Other reports indicate that pepsin was capable of producing ACE-inhibitory peptides from other macroalgae (e.g. Sheih, Fang, & Wu, 2009; Suetsuna & Nakano, 2000). The first authors, using commercial enzymes to digest algae protein waste from the microalgae *Chlorella vulgaris*, had similar results to the present study, with ACE-inhibitory activities values ranging from 20 to 70%, the higher value being recorded in pepsin hydrolysate. To the best of our knowledge, there is no report by others authors on the preparation of ACE-inhibitory peptides from algae protein via hydrolysis by bromelain. However, previous studies on other marine resources, such as sea cucumber, revealed that bromelain is one of the most efficient protease for the production of hydrolysates with high ACE-inhibitory activity (e.g. Ghanbari et al., 2015).

3.2. Preparation of U. rigida protein hydrolysate (URPH) and purification of their ACE-inhibitory peptides

In this study, *U. rigida* protein after a two-step hydrolysis with a combination of the two most active proteases pepsin and bromelain (see Fig. 1A) resulted in URPH with a high DH (59.85 ± 1.73%) and an IC_{50} value of 0.483 mg/mL (see Table 1), which was used to isolate the bioactive peptides. A two-step hydrolysis was also been previously used in other food protein sources, to promote the release of low-molecular-weight peptides (Girgih et al., 2011; Lin et al., 2011; Zhao et al., 2009).

It is well known that molecular weights of peptide fragments are crucial for their biological activities. In this study, after the application of an ultrafiltration to separate the URPH into three fractions, as shown in Table 1, the fraction corresponding to MW < 1 kDa (URPH-III) presented the highest ACE-inhibitory activity (IC_{50} value of 0.095 mg/mL) and recovery yield (75.50%). These results indicated the efficiency of the enzyme hydrolysis process used, and also the economic viability of this peptide fraction commercialization as an ingredient for anti-hypertensive functional foods and nutraceuticals formulation, since higher yields will be more beneficial for processing and marketing of new products (Girgih et al., 2011). However, being the URPH-III the first permeate it may also contained non proteinous low molecular weight compounds, such as soluble sugars and salts formed during acid-base neutralization in the digestion process. Protein and peptide content of URPH and their UF fractions are also shown in Table 1, being the higher values also presented in URPH-III fraction.

To enhance the ACE inhibition, the URPH-III fraction was separated into six fractions after being loaded onto a Sephadex G-25 column (Fig. 2A). The fraction Fr 4 exhibited the strongest ACE-inhibitory activity (Fig. 2B) with an IC_{50} value of 0.068 mg/mL and a peptide content of 2.35 mg/mL. This fraction, further separated by RP-HPLC on an Ultrasphere ODS semi-preparative column, was divided into four fractions (Fig. 2C). The fraction Fr 4.3, the most potent ACE-inhibitory activity fraction (Fig. 2D) with an IC_{50} of 0.058 mg/mL and a peptide content of 0.98 mg/mL, was further purified on a Vydac protein C4 reverse phase semi-preparative column (data not shown), resulting in a fraction (Fr 4.3') with an IC_{50} value of 0.047 mg/mL and a peptide content of 0.53 mg/mL. This fraction was finally separated on a Zorbax C18 reverse phase analytical column into two purified peptides (Fig. 2E) with IC_{50} values of 0.020 and 0.023 mg/mL. The results obtained during the purification steps are summarized in Table 2. The ACE-inhibitory peptides from the pepsin-bromelain URPH were purified 24.15 fold for IP and 21.00 fold for AFL using five-step purification procedure, with 0.08% and 0.06% yield, respectively. A previous report in another algae species (Ko et al., 2012) had similar yield to the present study.

3.3. Amino acid composition and sequence of the purified ACE-inhibitory peptides

Most of the reported peptides exhibiting potent ACE-inhibitory activity contained 2–13 amino acids (Li et al., 2004). Results of the amino acid composition and amino acid ratio of the two purified peptides determined by GC shows that the first purified peptide (eluted with a shorter retention time – Fig. 2E)

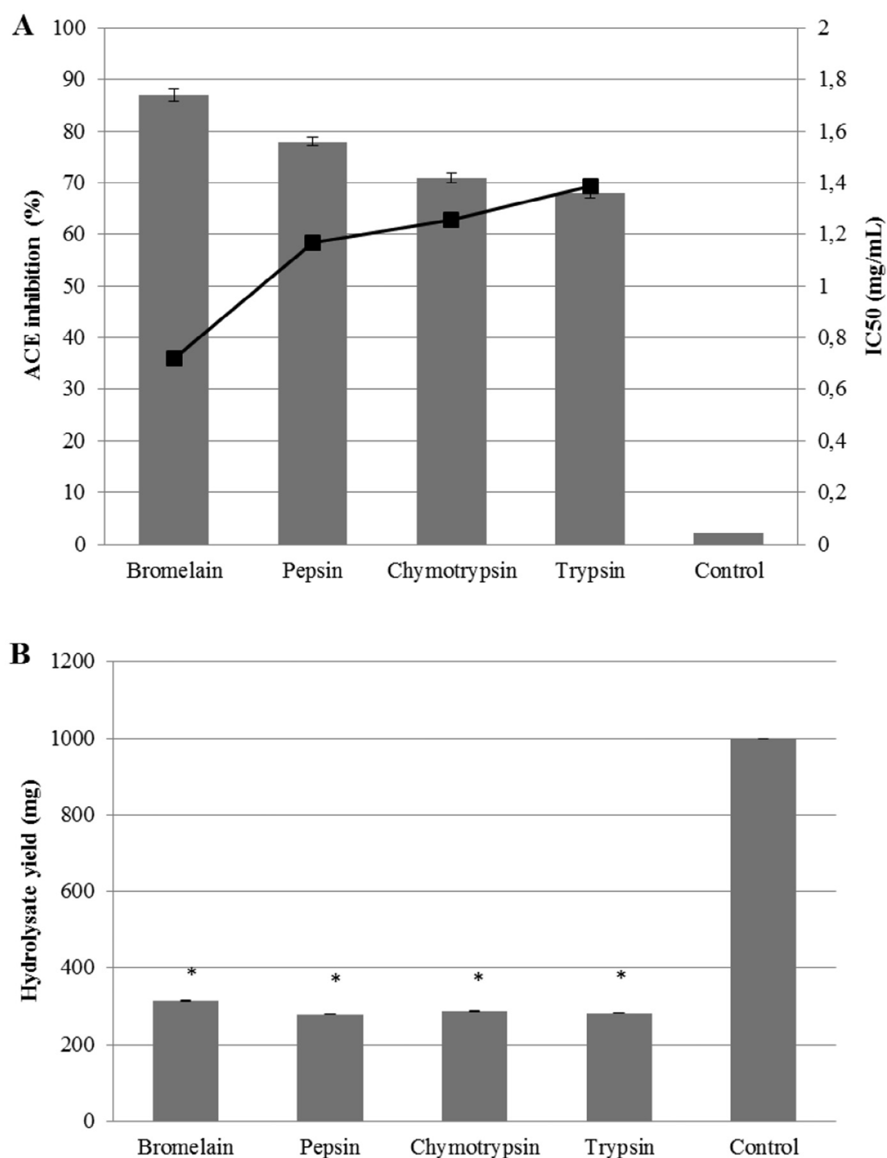

Fig. 1 – (A) Angiotensin-I converting enzyme (ACE) inhibitory activity in percentage (bar) and IC_{50} value (filled square) and (B) yield of *U. rigida* protein hydrolysed by various proteases. The hydrolysate yield was calculated as referred in Methods. Values are mean ± SD (n = 3). *Significant difference from control at $p < 0.05$. Tested concentration for anti-ACE assay = 2 mg/mL.

Table 1 – Angiotensin-I converting enzyme (ACE) inhibitory activity, yield, protein and peptide content of pepsin-bromelain URPH and their fractions obtained by ultrafiltration cell dialyser system.

Sample	Ref.	ACE inhibitory activity		Yield (mg)	Protein (mg/g)	Peptide content (mg/g)
		Percentage	IC_{50} value (mg/mL)			
Unfractionated	URPH*	65.68 ± 0.72^b	0.483 ± 0.041^b	737.01	275.50 ± 1.32	140.10 ± 1.10
$Fr_1 > 3$ kDa	URPH-I	38.90 ± 1.83^a	3.220 ± 0.060^a	22.11	285.15 ± 1.25	167.65 ± 1.50
$1 < Fr_2 < 3$ kDa	URPH-II	43.19 ± 0.54^a	2.750 ± 0.055^a	75.17	374.20 ± 1.96	207.78 ± 1.23
$Fr_3 < 1$ kDa	URPH-III	87.60 ± 1.38^c	0.095 ± 0.003^c	556.44	565.45 ± 2.78	289.37 ± 1.85

Values are mean ± SD (n = 3). Superscript letters are significantly different ($p < 0.05$). IC_{50} defined as the concentration which inhibits 50% of the ACE activity (tested concentration = 2 mg/mL).
* Corresponding to the permeate obtained after the 12 kDa dialysis of pepsin-bromelain URPH, as described in Methods.
URPH, *U. rigida* protein hydrolysate.

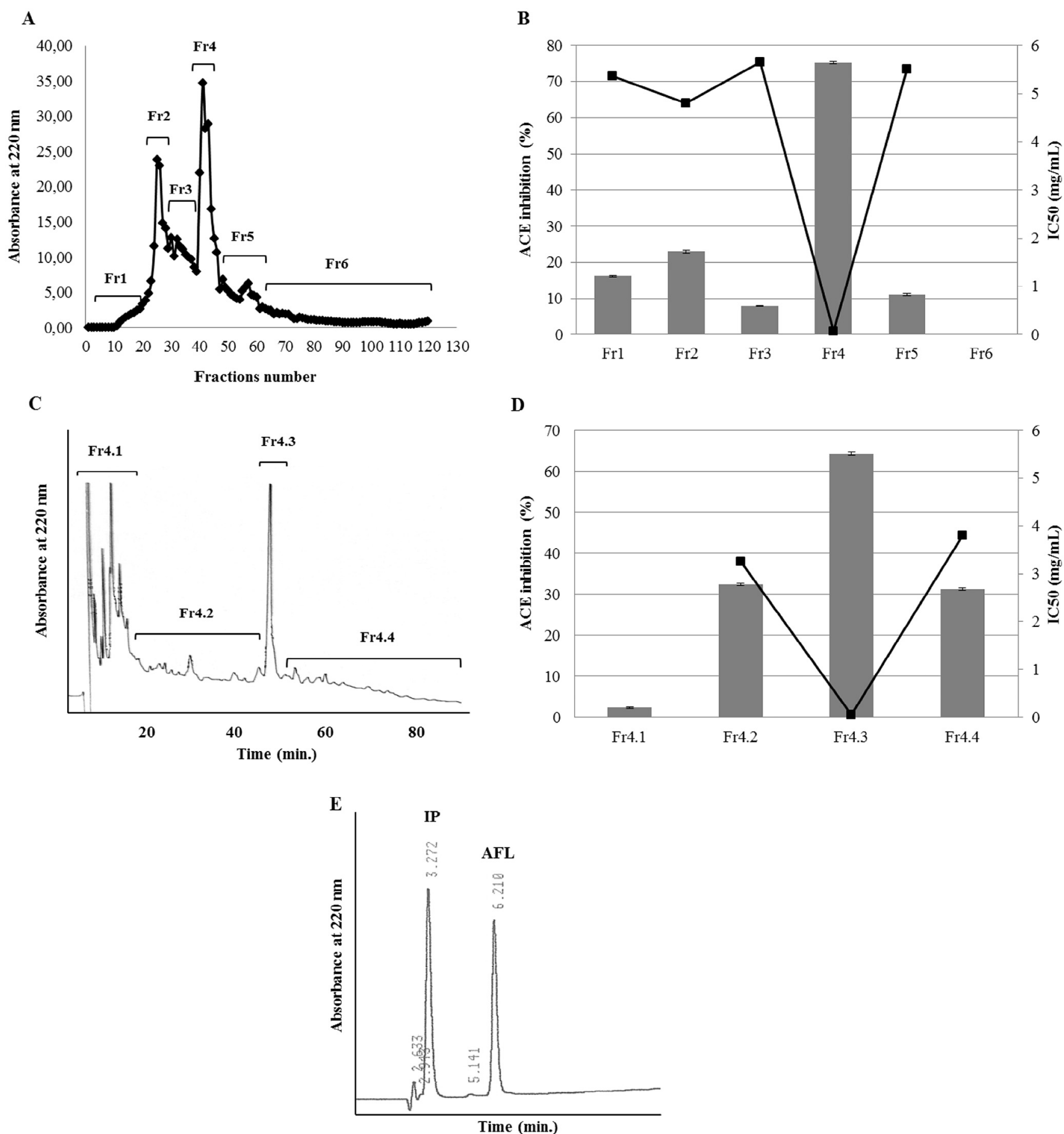

Fig. 2 – Purification of angiotensin-I converting enzyme (ACE) inhibitory peptides from *U. rigida* protein hydrolysate (URPH) digested with pepsin plus bromelain. (A) Elution of fraction URPH-III (MW < 1 kDa) on Sephadex G-25 column and (B) ACE-inhibitory activity of fractions Fr 1 to Fr 6. (C) Separation of fraction Fr 4 on RP-Ultrasphere ODS semi-preparative column and (D) ACE-inhibitory activity of fractions Fr 4.1 to Fr 4.4. (E) Separation of the purified peptides from fraction Fr 4.3 using a RP-Zorbax C18 analytical column, after pre-purification on a RP-Vydac protein C4 semi-preparative column. Values of ACE-inhibitory activity (in percentage and IC_{50} value, bar and filled square, respectively) are mean ± SD (n = 3); tested concentration = 2 mg/mL. Analytical chromatographic conditions as referred in Methods.

have only 2 amino acids, Ile and Pro in equimolar concentration (1.04–0.97 ratio) and that the second peptide (eluted with a longer retention time – Fig. 2E) have 3 amino acids, Ala, Leu and Phe also in equimolar concentration (0.97–1.10–1.08 ratio). The purified peptides sequences determined by N-terminal Edman degradation were Ile-Pro (IP) and Ala-Phe-Leu (AFL) with molecular mass of 228.23 Da and 349.18 Da, respectively, determined by a Q-TOF-2 MS, as shown in Fig. 3. It should be

Table 2 – Summary of the purification of ACE inhibitory peptides from URPH-III fraction by sequential chromatographic techniques.

Purification step	IC_{50} value (mg/mL)[a]	Purification fold[b]	Yield (mg/g URPH)	Peptide content (mg/mL)
Pepsin-bromelain URPH[c]	0.483 ± 0.041	1.00	1000.00	–
URPH-III (MW < 1 kDa)[d]	0.095 ± 0.003	5.08	851.19	–
Gel permeation (Fr 4)	0.068 ± 0.004	7.10	271.30	2.35 ± 0.06
1st RP-HPLC prep (Fr 4.3)	0.058 ± 0.003	8.33	59.85	0.98 ± 0.03
2nd RP-HPLC prep (Fr 4.3’)	0.047 ± 0.004	10.28	21.30	0.53 ± 0.03
RP-HPLC analytical	0.020 ± 0.002[e]	24.15	0.80	NA
	0.023 ± 0.001[f]	21.00	0.60	NA

Values are mean ± SD (n = 3).
[a] IC_{50} defined as the concentration which inhibits 50% of the ACE activity (tested concentration = 2 mg/mL).
[b] Relative value of reciprocal of ACE IC_{50}.
[c] URPH, *U. rigida* protein hydrolysate.
[d] Obtained by ultrafiltration of the URPH.
[e] Purified peptide IP (synthetic peptide, IC_{50} = 0.019 ± 0.001 mg/mL).
[f] Purified peptide AFL (synthetic peptide, IC_{50} = 0.022 ± 0.002 mg/mL).
NA, not applicable.

pointed out that these peptides cannot be derived from the proteolytic enzymes autolysis since they are not encrypted in the porcine pepsin and bromelain sequences. To the best of our knowledge, the tripeptide AFL was firstly found in the pepsin digest of *Chlorella vulgaris* (Suetsuna & Chen, 2001). The dipeptide IP was reported to be released by enzymatic β-casein hydrolysis using *Lactobacillus* peptidases and was detected by LC–MS, presenting a yield of 0.13% (Stressler, Eisele, & Fischer, 2013). In order to validate the ACE-inhibitory activity of the purified peptides, synthetic peptides with the same sequences were synthesized and tested. The synthetic peptides exhibited similar ACE-inhibitory activities as the purified peptides from *U. rigida* protein hydrolysate (see Table 2). Results of the present study on the IC_{50} values of the purified peptides showed that the IP (0.020 mg/mL equivalent to 87.6 μM) presented a lower value (expressed in mg/mL) in comparison with the AFL (0.023 mg/mL equivalent to 65.9 μM) and the two peptides showed a higher value when compared to the captopril (0.163 ng/mL equivalent to 0.77 μM). Natural ACE-inhibitory peptides were first discovered in snake venom (Ferreira, Bartelt, & Greene, 1970). Since then, several reports have been published on the ACE-inhibitory activity of purified peptides from diverse food protein sources (Li et al., 2004), and various low-molecular-weight ACE-inhibitory peptides have been isolated from a range of marine proteins (e.g. Wijesekara & Kim, 2010), including some algae. In fact, several ACE-inhibitory peptides contained 2–5 amino acids, with IC_{50} values ranging from 1.5 to 213 μM, were isolated from the macroalgae *Hizikia fusiformis*, *Porphyra yezoensis* and *Undaria pinnatifida*, and the microalgae *Chlorella ellipsoidea*, *Chlorella vulgaris* and *Spirulina platensis* (Norris, Harnedy, & FitzGerald, 2014; Pangestuti & Kim, 2013). Among these algae, *U. pinnatifida*, a known source of antihypertensive ingredients (Norris et al., 2014), is very rich in dipeptides isolated from pronase S hydrolysate (Sato et al., 2002), and tetrapeptides isolated from pepsin hydrolysate (Suetsuna & Nakano, 2000), with IC_{50} values ranging from 1.5 to 42.3 μM and 21 to 213 μM, respectively. Another recent study, reported the isolation of the tripeptide LEQ (IC_{50} value of 173 μM) from pepsin *Nannochloropsis oculata* hydrolysate (Samarakoon et al., 2013). The isolated peptides from *U. rigida*, with IC_{50} values of 65.9 and 87.6 μM, show comparable or higher ACE-inhibitory activity than some of the reported algae peptides.

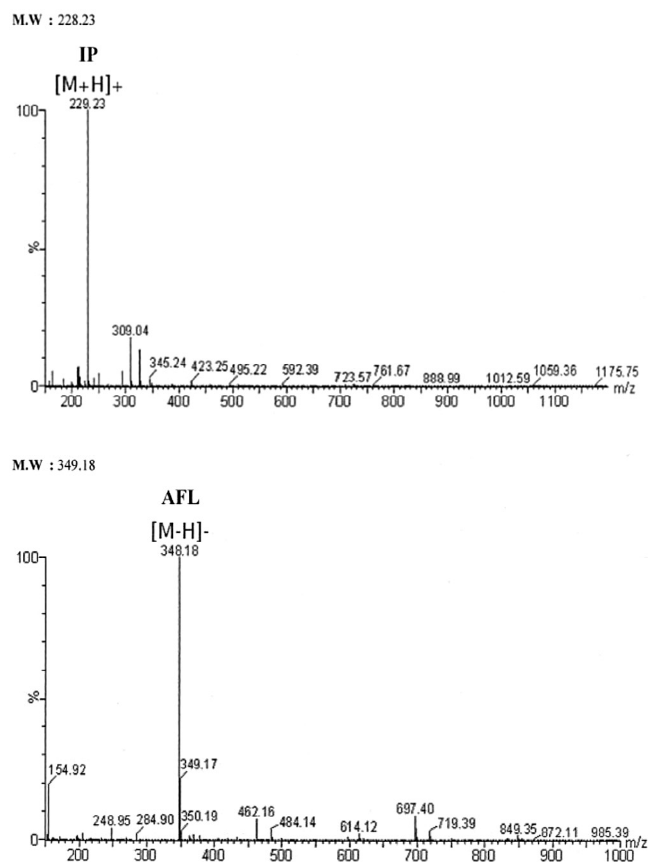

Fig. 3 – Determination of molecular mass of the purified peptides (IP and AFL) from URPH-III fraction (MW < 1 kDa), performed on a quadrupole time-of-flight mass spectrometer (Q-TOF-2 MS) equipped with an electrospray ionization (ESI) source.

Previous reports on the structure–activity correlations indicate that binding to ACE is strongly influenced by the

C-terminal tripeptide sequence of the substrate, and it is suggested that peptides which contain hydrophobic amino acids residues at these position are potent ACE-inhibitors (Wijesekara & Kim, 2010). Cheung, Wang, Ondetti, Sabo, and Cushman (1980) also suggested that ACE is highly specific with regard to the C-terminal dipeptide of substrates and inhibitors. According to these authors, the presence of hydrophobic Pro residues at one or more positions in the C-terminal tripeptide region seems to positively influence the ACE-inhibitory activity of a peptide. Other reports suggested that the presence of Leu as the C-terminal residue may also contribute to ACE inhibition (Ruiz, Ramos, & Recio, 2004). This is consistent with our results in which the isolated bioactive peptides contained Pro or Leu residues on their C-terminal. In addition, according to previous reports in other ACE-inhibitory peptides (e.g. Cheung et. al., 1980), the two isolated peptides from *U. rigida* have also favourable N-terminal amino acids (the hydrophobic Ile or Ala residues).

3.4. Stability of the purified ACE-inhibitory peptides

The stability of the *U. rigida* purified peptides was investigated with various temperature treatments in order to evaluate its potential use as a food additive. The results showed that the IP IC_{50} values ranged from 0.020 to 0.022 mg/mL and the AFL IC_{50} values ranged from 0.022 to 0.024 mg/mL for 20, 40, 60, 80 and 100 °C. These results indicated that the purified peptides retained its ACE-inhibitory activity ($p > 0.05$), revealing that they were heat-stable.

It is well known that some ACE-inhibitory substances failed to show the hypotensive activity after oral administration *in vivo*, due to the possible hydrolysis of these peptides by gastrointestinal proteases or eventually ACE (Li et al., 2004). The resistance of the *U. rigida* purified peptides to digestive proteases was evaluated by incubating the peptides with different gastrointestinal enzymes (pepsin, trypsin, chymotrypsin and intestinal mucosa peptidases) *in vitro* that provide an easy process to imitate the fate of these peptides under oral administration. The ACE-inhibitory activities of the digests were assayed using the HPLC profile comparison and are summarized in Table 3. The results indicated that no hydrolysis of IP and AFL occurred after the subsequent digestion with pepsin, trypsin and chymotrypsin, since the ACE-inhibitory activity of the unhydrolysed peptides and its digest samples were identical. This resistance of the purified peptides to digestion was consistent with previous reported studies that short-chained peptides have low susceptibility to hydrolysis by digestive enzymes due to their fast absorption in the small intestine, as compared with long-chained peptides. According to Ruiz et al. (2004), the presence of proline makes also the amino acid sequences less susceptible to hydrolysis during intestinal digestion and assimilation. In addition, Pro-rich short peptides are believed to be also resistant to protein-degrading enzymes within the circulatory system itself (Ohara, Matsumoto, Ito, Iwai, & Sato, 2007). As also showed in Table 3, the results indicated that, after intestinal mucosa peptidases digestion, the purified IP peptide maintained its resistant to hydrolysis, as expected, while, in contrast, the AFL was hydrolysed leading to an increase in the ACE-inhibitory activity. The GC analysis of the pooled peptide from the RP-HPLC of AFL digest shows the presence of 2 amino acids Phe and Leu in equimolar concentrations (1.05-0.98 ratio). The percent of residual AFL was estimated at 65.1 ± 0.5% by the peak area on the HPLC chromatogram. The purified FL peptide exhibited an IC_{50} value of 0.004 mg/mL (equivalent to 16.0 μM), which is similar to the previously reported by Wu, Aluko, and Nakai (2006) in a data set of dipeptides showing the log IC_{50} value of 1.20 (15.9 μM). The FL peptide has also been reported by Iwai et al. (2005) that showed that minor peptides (Ile-Hyp, Leu-Hyp, Phe-Hyp and Phe-Leu) in human blood have a resistance to digestion by the plasma and gastrointestinal peptidases.

3.5. Determination of the inhibition pattern on ACE of the purified peptides

The ACE inhibition patterns of the purified peptides were estimated using the Lineweaver–Burk plots of ACE with and without inhibitors, and the kinetic parameters were calculated with the Michaelis–Menten equation. As shown in Fig. 4, IP and FL act as non-competitive and AFL as competitive ACE-inhibitors. Although competitive ACE-inhibitors have been most frequently reported, some non-competitive inhibitors have also been found in other algae, such as the IY and LW dipeptides from *Undaria pinnatifida* (Sato et al., 2002), and in another marine food, like sardine (Matsufuji et al., 1994), oyster (Wang et al., 2008) and tuna (Qian, Je, & Kim, 2007). Also captopril has been referred to show competitive inhibition with substrates for binding to active ACE sites (Tsai, Lin, Chen, & Pan, 2006).

4. Conclusion

Algae remain a relatively untapped reserve of antihypertensive peptides. This study revealed, for the first time, that ACE-inhibitory peptide fractions and purified peptides could

Table 3 – Simulated gastrointestinal digestion of the purified peptides.

Peptides	IC_{50} value in mg/mL (μM)		
	Before digestion	After digestion	
		P + T + C	P + T + C + PEP
IP	0.0200 ± 0.002 (87.6)	0.0199 ± 0.0002 (87.2)	0.0197 ± 0.0003 (86.3)
AFL	0.0230 ± 0.001 (65.9)	0.0220 ± 0.0003 (63.0)	0.0143 ± 0.0003 (40.9)

Values are mean ± SD (n = 3). IC_{50} defined as the concentration which inhibits 50% of the ACE activity.
P, pepsin. T, trypsin. C, chymotrypsin. PEP, peptidases from intestinal mucosa.

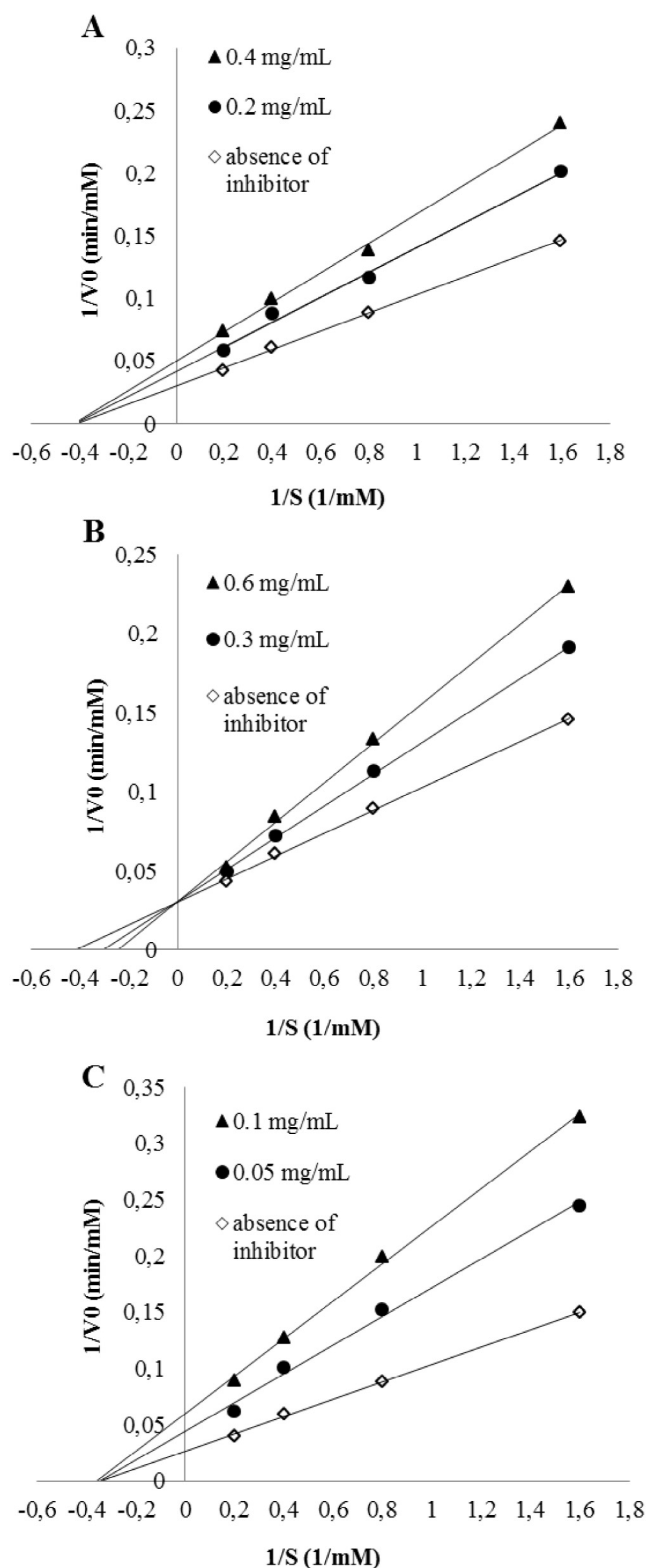

Fig. 4 – Lineweaver–Burk plots of angiotensin-I converting enzyme (ACE) inhibition by the two purified peptide from URPH-III fraction: the dipeptide IP (A) and the tripeptide AFL (B), and by the FL (C) released after *in vitro* digestion of AFL by intestinal mucosa peptidases. The ACE activities were measured in the absence or presence of the purified peptides. Values are mean ± SD (n = 3).

be efficiently generated from *U. rigida* protein hydrolysed by the pepsin-bromelain enzymes. Two ACE-inhibitory peptides (IP and AFL) were successfully purified from this hydrolysate using ultrafiltration membranes and sequential chromatographic methods, which suggests that *U. rigida* proteins can serve as an alternative raw material for producing these active peptides. Their ACE-inhibitory activities were accurately determined and validated using synthetic peptides with the same sequences, and showed higher potency than some of the purified peptides from other sources. In addition, their inhibition patterns and stability against temperature and gastrointestinal proteases were firstly characterized. Both peptides were heat-stable and the AFL was hydrolysed by intestinal mucosa peptidases to FL, a more potent ACE inhibitor. Lineweaver–Burk plots suggest that IP and FL act as non-competitive and AFL as competitive ACE-inhibitors. The results revealed that these peptides have the potential to be used as therapeutic candidates for prevent and/or treat hypertension and its related diseases, and their involvements in the future pharmaceuticals or in clinical nutrition are promising. Furthermore, the development of nutraceuticals and functional foods to prevent and/or to reduce hypertension would also be economical, innovative and a natural alternative therapy to commercial synthetic drugs and also may be safer for the consumers.

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