Screening for angiotensin I-converting enzyme (ACE) inhibitory activity of enzymatic hydrolysates obtained from Azorean macroalgae

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As part of ongoing studies into the isolation of antihypertensive peptides, 12 common macroalgae species from Azores were investigated for their potential angiotensin-converting enzyme (ACE) inhibitory properties. After optimization of enzymatic hydrolysis conditions, the anti-ACE activity was determined by high performance liquid chromatography (HPLC). The species under study include four Rhodophyta (*Gelidium microdon*, *Osmundea pinnatifida*, *Porphyra* sp. and *Pterocladiella capillacea*), four Chlorophyta (*Chaetomorpha linum*, *Codium adhaerens*, *Ulva compressa* and *Ulva rigida*) and four Heterokontophyta (*Cystoseira humilis*, *Fucus spiralis*, *Padina pavonica* and *Sargassum cymosum*). The bromelain hydrolysates derived from *P. capillacea*, *Porphyra* sp., *F. spiralis, U. compressa*, *O. pinnatifida* and *U. rigida* were the most active presenting an ACE inhibitory activity of 48.73 ± 1.58%, 50.96 ± 1.82%, 56.77 ± 1.80%, 59.80 ± 0.95%, 62.59 ± 1.60% and 65.68 ± 0.72%, respectively. These results suggest that some macroalgae from the littoral Azorean sea are potential sources of powerful ACE inhibitor peptides, with potential impact on public health, particularly on the hypertensive patients. These features make these algae valuable to the regional economy and may also provide an incentive for marine biodiversity conservation and valuation of marine algal resources.

Key words: Azores, marine macroalgae, antihypertensive peptides, ACE inhibition, enzymatic hydrolysis, pepsin, bromelain, HPLC

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INTRODUCTION

Hypertension, one of the most common serious chronic health problems, affects about 25% of the world's adult population (Mittal & Singh 2010). This problem of epidemic proportions carries a high risk factor for arteriosclerosis, stroke, myocardial infarction and end-stage renal disease (Zhang et al. 2006). It is well known that the angiotensin I-converting enzyme (ACE), a zinc-containing metalloenzyme, plays a key role in the control of blood pressure by the rennin-angiotensin system (Fujita et al. 2000). It acts as an exo-peptidase that cleaves a dipeptide of

C-terminal of angiotensin I to produce the octapeptide angiotensin II, which is a potent vasoconstrictor (Curtiss et al. 1978). Generally, ACE synthetic inhibitors cause a number of undesirable side effects, like cough, hyperkalemia, renal failure, fetal anomalies, angioedema, dysgeusia and skin rashes (Brown & Vaughan 1998). For this reason, there is a trend towards searching for natural ACE inhibitors as alternatives to synthetic drugs. Recently, much attention has been paid to the potential of marine organism constituents as novel therapeutic drug candidates for the treatment of hypertension (e.g. Wijesekara & Kim 2010), and some ACE inhibitory peptides have already been isolated from the hydrolyzates of algae species (e.g. Sato et al. 2002). Enzymatic hydrolysis has become a valuable tool for modifying the functionality of proteins resulting in a good source of bioactive peptides that may be beneficial for the organism's functions (Korhonen et al. 1998).

The Azores Islands, due to their isolated location in the middle of the Atlantic Ocean associated with a low pollution levels of their seawater (Neto et al. 2009), are a very promising location for marine natural resources that may produce new ingredients with medicine-like effects in treating or preventing certain diseases. Ongoing research investigates the isolation of natural antihypertensive agents to search for novel ACE inhibitory activity peptides from marine algae (Paiva et al. 2014a,b). The present study analyses, for the first time, the *in vitro* ACE inhibitory properties of enzymatic hydrolysates obtained from 12 macroalgae species which are abundant in the Azorean intertidal zone (Neto et al. 2006).

MATERIAL AND METHODS

CHEMICALS AND REAGENTS

Acetonitrile (ACN) and Methanol (MeOH), HPLC grade, were purchased from Fluka Chemika (Steinheim, Switzerland). Sodium chloride was obtained from E. Merck (Darmstadt, Germany). Deionized water was obtained from a Millipore water purification system (Millipore, Bedford, MA, USA). Trizma base, zinc chloride, hippuric acid (HA), hippuryl-L-histidyl-L-leucine (HHL), hydrochloric acid (HCl) and various commercial enzymes, such as angiotensin I-converting enzyme (ACE) from porcine kidney, bromelain (B4882), chymotrypsin (C-9381), ficin (F6008), pancreatin (P1750), pepsin (77161), peptidase (P7500), protease from *Bacillus licheniformis* (P5380), protease from *Streptomyces griseus* (P6911) and trypsin (T8003) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used in this study were reagent grade chemicals.

MACROALGAL COLLECTION

All macroalgae samples used in this study were collected from the littoral zone of São Miguel Island, Azores Archipelago (37°40′ N - 25°31′ W), Portugal, and voucher specimens were prepared and deposited in the Herbarium AZB - Ruy Telles Palhinha of the Department of Biology at the University of Azores, namely: *Chaetomorpha linum* (O.F. Müller) Kützing (SMG-12-29); *Codium adhaerens* C. Agardh (SMG-12-28); *Cystoseira humilis* Schousboe ex Kützing (SMG-12-26); *Fucus spiralis* Linnaeus (SMG-13-04); *Gelidium microdon* Kützing (SMG-13-03); *Osmundea pinnatifida* (Hudson) Stackhouse (SMG-13-02); *Padina pavonica* (Linnaeus) Thivy (SMG-13-13); *Porphyra* sp. C. Agardh (SMG-14-03); *Pterocladiella capillacea* (S.G. Gmelin) Santelices & Hommersand (SMG-13-05); *Ulva compressa* Linnaeus (SMG-13-15); *Ulva rigida* C. Agardh (SMG-13-12) and *Sargassum cymosum* C. Agardh (SMG-12-27).

MACROALGAE SAMPLE PREPARATION

Within 24 h of collection the macroalgae samples were 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 samples were defrosted and dried at 45 °C during 48 h (avoiding overheating that could lead to oxidation). Dried triplicate samples were grounded into a fine powder, redried at 40 °C and stored in desiccators at a refrigerated temperature.

ENZYME SCREENING FOR THE ESTIMATION OF THE HIGHER NUMBER OF PEPTIDES USING *FUCUS SPIRALIS*

Five grams of *Fucus spiralis* was suspended in 100 mL of distilled water and then homogenized with an Ultra-turrax T50 at 6.000 rpm for 10 min. Five milligrams of each protease were individually added to 10 mL of the homogenate, adjusting substrate/enzyme ratio to 100:1 (w/w) and the mixtures were incubated for 20 h at an appropriated pH and temperature for each enzyme reaction, using the conditions suggested by the manufacturer. After hydrolysis, the samples were heated for 15 min in boiling water in order to inactivate the proteases. The precipitates were removed by centrifugation (1100 x g) at 4 °C for 10 min. The supernatants were concentrated and lyophilized to obtain powder products that were used for the number of peptides determination using HPLC analysis under the following conditions: reverse-phase Zorbax Eclipse XDB-C_{18} column (4.6 x 150 mm i.d., 5 µm) (Agilent Technologies, USA) using a linear gradient of phase A - 0.1% TFA in water and phase B - 0.1% TFA in acetonitrile (t = 0 min - 0% B and t = 60 min - 40% B) at a flow rate of 1 mL/min and detection by UV at 220 nm.

PREPARATION OF MACROALGAE ENZYMATIC HYDROLYSATES FOR PRODUCTION OF ACE INHIBITORY PEPTIDES

Twenty grams of the macroalgae samples were suspended in 300 mL of distilled water, homogenized with an Ultra-turrax T50 at 6.000 rpm for 10 min and then hydrolysed by proteolytic active enzymes (pepsin and bromelain) using the same reaction conditions described above. The homogenates were concentrated until an approximate volume of 150 mL and immediately transferred to a 10,000 Da MWCO dialyzing tube. Each permeate was concentrated, lyophilized and kept at -80 °C for the ACE inhibitory activity determination.

DETERMINATION OF ACE INHIBITORY ACTIVITY

For the assay, 80 µL of the enzymatic hydrolysate solution was pre-incubated at 37 °C for 5 min with 20 µL ACE (2 mU/mL) enzyme. The mixture was subsequently incubated at the same temperature with 200 µL of the substrate (5 mM HHL in 100 mM sodium trizma base containing 300 mM NaCl at pH 8.3) during 60 min. The reaction was terminated by adding 250 µL of 1 M HCl. The percentage of inhibition was determined by HPLC analysis. An aliquot of 10 µL from the reaction mixture was analyzed on a reverse-phase Ultrasphere C_{18} column (250 x 4.6 mm i.d., 5 µm) (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 0.5 mL/min and HA and HHL were detected by UV at 228 nm. The percentage of ACE inhibition by macroalgae hydrolysate sample was calculated by the amount of the hippuric acid (HA) released from the substrate hippuryl-L-histidyl-L-leucine (HHL). The extent of inhibition was calculated as follows:

$$\text{ACE inhibition (\%)} = [1 - (A_s - A_b)/C] \times 100$$

where A_s = HA peak area of the sample, A_b = Peak area of blank (no ACE or with ACE inhibitor) and C = HA peak area (HHL plus ACE) without sample.

The repeatability of the HA HPLC analysis was evaluated in intraday and interday measurements of the retention time by repeated injections (n = 5). The recovery was calculated based on the difference between the total concentration determined in the spiked samples and the concentration observed in the non-spiked samples.

STATISTICAL ANALYSIS

All determinations were performed in triplicate and the results expressed as means ± standard deviations (SD). Statistical comparison was performed by using one-way analysis of variance (ANOVA). A *P*-values of less than 0.05 ($P < 0.05$) were considered to be statistically significant.

RESULTS AND DISCUSSION

The HPLC methodology used to quantify the HA produced during the ACE-catalyzed reaction completely eliminates the interference from HHL during the quantification of the HA as compared with spectrophotometric method of Cushman &

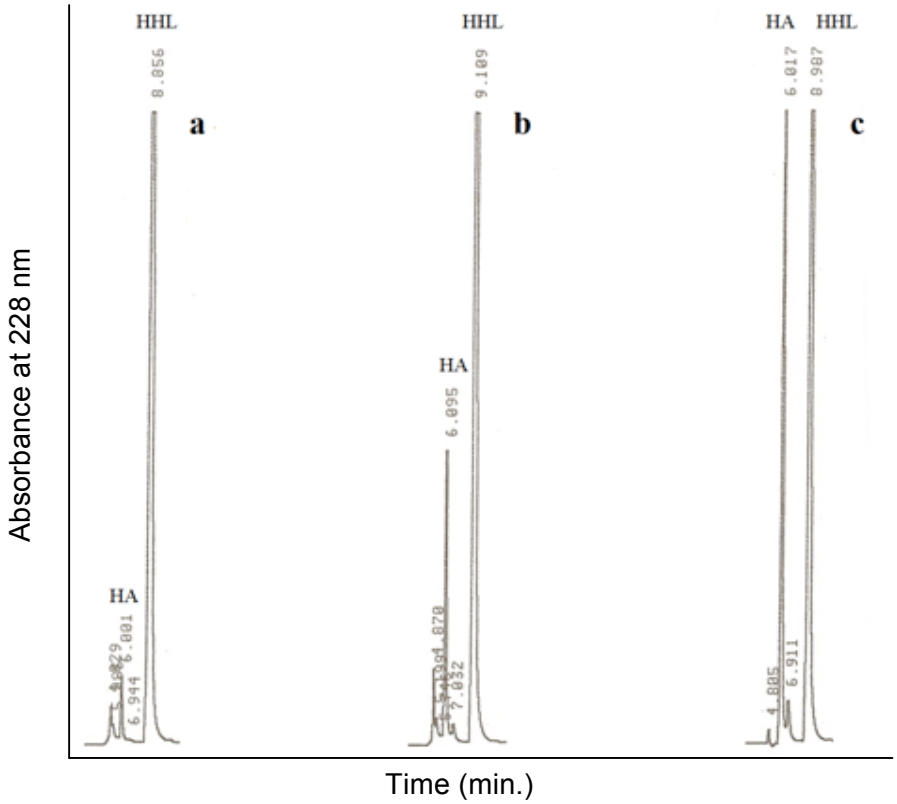

Fig. 1. Representative HPLC chromatograms of the angiotensin-converting enzyme inhibition assay. a) More active enzymatic hydrolysate; b) Less active enzymatic hydrolysate; c) Control sample (without inhibitors). Chromatographic conditions as referred in methods.

Cheung (1971). Complete baseline separation of HA and HHL was achieved by HPLC in isocratic elution conditions in less than 10 min under the analytical conditions described in methods (see Figure 1) showing a relative standard deviation (RSD) of 1.51% in multiple injections that represents a good repeatability. Another advantage of the HPLC method is the reduction of the time-consuming process of HA extraction into ethyl acetate that is required by the standard Cushman & Cheung (1971) method and also the standard spectrophotometric assay requires at least 250 to 500 µL of reaction mixture, compared to the 10 µL used in HPLC assay. Therefore, the HPLC provides a simple, rapid and accurate method for the assay of ACE-catalyzed reactions.

The estimation of the proteolytic activity (number of peptides) derived from nine commercial enzymes using *F. spiralis* revealed that pepsin and bromelain were the enzymes with higher proteolytic activity (Figure 2).

From the selected macroalgae species the bromelain hydrolysates derived from *P. capillacea*, *Porphyra* sp., *F. spiralis, U. compressa*, *O. pinnatifida* and *U. rigida* presented an ACE inhibitory activity similar and greater than 50% (ranging from 48.73% to 65.68%) (Figure 3).

The *U. rigida* exhibited the strongest anti-ACE activity (65.68 ± 0.72%) and the other Chlorophyta, *U. compressa,* presented the third highest anti-ACE activity (59.80 ± 0.95%). The Rhodophyta *O. pinnatifida* presented the second highest anti-ACE activity (62.59 ± 1.60%) and the other red algal species showed lower activity (48.73 ± 1.58% and 50.96 ± 1.82% for *P. capillacea* and *Porphyra* sp., respectively). The Heterokontophyta *F. spiralis,* the only active of the selected brown algae, presented the fourth highest anti-

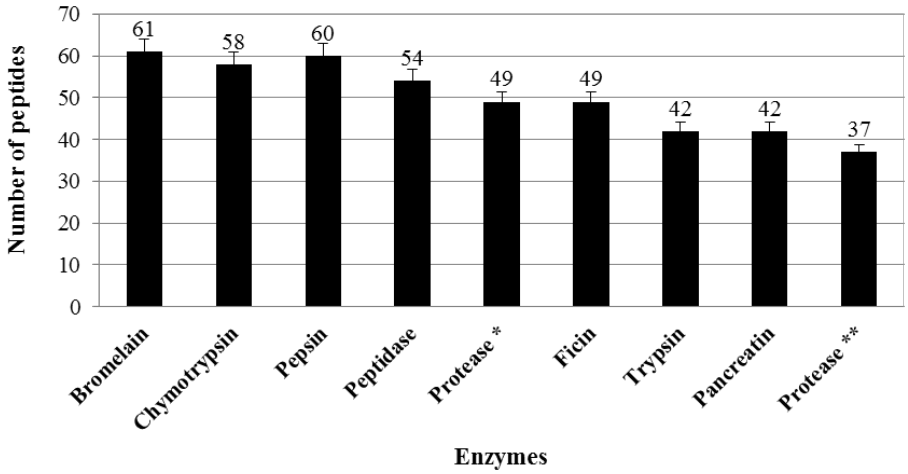

Fig. 2. HPLC estimation for the proteolytic activity (number of peptides) derived from different commercial enzymes using the Azorean macroalgae *Fucus spiralis*. Chromatographic conditions as referred in methods; * *Streptomyces griseus, ** Bacillus licheniformis.*

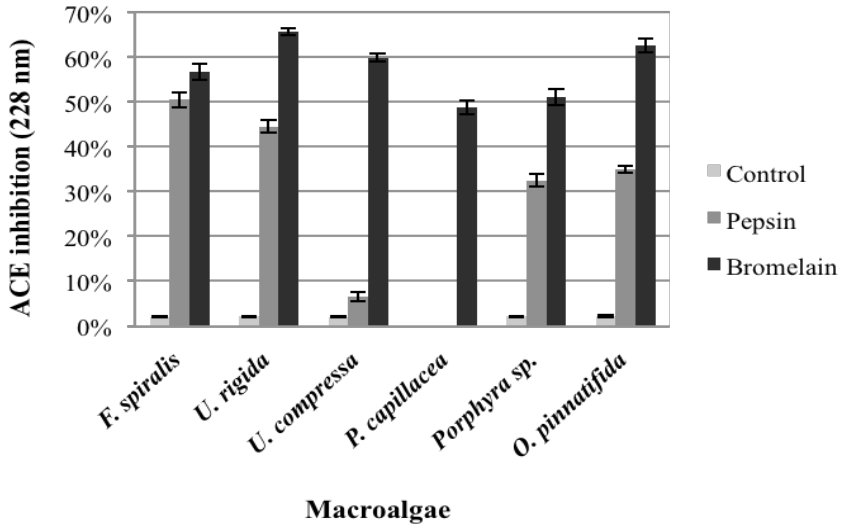

Fig. 3. Angiotensin-converting enzyme (ACE) inhibitory activity (%) of six selected Azorean macroalgae enzymatic hydrolysates. Algal protein not hydrolyzed was used for control. Data are mean ± SD of triplicate experiments.

ACE activity (56.77 ± 1.80%). Remarkable ACE inhibitory activities of enzymatic hydrolysates from some macroalgae have also been reported by other authors, especially from brown and red algal species (e.g. Sato et al. 2002; Athukorala & Jeon 2005; Qu et al. 2010). From these studies, Qu et al. (2010) reported for *Porphyra yezoensis* 55% of ACE inhibition that is similar to the value obtained for the studied *Porphyra* sp. (*ca* 51%). Research by Cha et al. (2006a,b), but using crude extracts from Korean *Porphyra tenera*, *Pterocladiella capillacea*, *Ulva conglobata* and *U.*

pertusa, also reported ACE inhibition but much lower values than the ones obtained for the same genus in the present study. These results emphasize the importance of the enzymatic hydrolysis in the process of ACE inhibitory activity.

In conclusion, this study shows a high activity in six Azorean macroalgae suggesting they can be excellent sources of ACE inhibitors, with potential pharmaceutical and nutraceutical applications. Therefore, purification and characterization of their ACE inhibitory peptides from the more potent hydrolysates is already in progress.

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