Journal of Pharmaceutical and Biomedical Analysis xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Angiotensin I-converting enzyme (ACE) inhibitory activity of *Fucus spiralis* macroalgae and influence of the extracts storage temperature—A short report

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ARTICLE INFO

Article history:
Received 31 May 2016
Received in revised form 14 August 2016
Accepted 27 August 2016
Available online xxx

Keywords:
ACE-inhibitory activity
Total phenolic content
Phlorotannins
Sample stability studies
HPLC
Hypertension

ABSTRACT

Recently, increasing attention has been paid to the marine algae as a natural source of novel angiotensin-I converting enzyme (ACE) inhibitors, such as the phlorotannins that are the predominant polyphenols in brown algae. This study reports, for the first time, the ACE inhibition of methanol extract/fractions from Azorean brown algae *Fucus spiralis* (Fs) determined by HPLC-UV method, their total phenolic content (TPC) quantified as phloroglucinol equivalents (PE) and the effect of the Fs dry powder methanol extracts (Fs-DME) storage temperature on ACE inhibition. The results indicate that the ACE inhibition of Fs-DME decreased by 28.8% and 78.2% when stored during 15 days at −80 °C and −13 °C, respectively, as compared with the activity of Fs-DME at a refrigerated temperature of 6 °C and assayed immediately after extraction that showed a value of 80.1 ± 2.1%. This Fs-DME sample was fractionated by ultrafiltration membranes into three molecular weight ranges (<1 kDa, 1–3 kDa and >3 kDa), presenting the fraction >3 kDa remarkably high ACE inhibition (88.8 ± 2.4%), TPC value (156.6 ± 1.4 mg PE/g of dry weight fraction) and yield. Furthermore, chromatographic and spectrophotometric analyses corroborate that phenolic compounds were present in Fs methanol extract/fractions, and also revealed that phloroglucinol occurs in Fs. The results seem to suggest that Azorean Fs can be a source of powerful ACE-inhibitory phlorotannins with potential impact on public health, particularly on hypertensive patients.

1. Introduction

Macroalgae are living in highly competitive and aggressive surroundings, which are very different in many aspects from terrestrial environment. Such situations demand the production of quite specific and potent bioactive compounds which may lead to the development of novel drugs and/or functional foods or nutraceuticals [1]. Macroalgae have been consumed in Asian countries since ancient times and their dietary ingestion has been shown to decrease blood pressure in humans [2]. Angiotensin-I converting enzyme (ACE), a zinc metal protease, plays a key role in the control of blood pressure since catalyzes the conversion of angiotensin I to a potent vasoconstrictor angiotensin II and also promotes the degradation of the vasodilator bradykinin [3]. 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 [4]. In recent years, much attention has been paid to the potential of natural marine products as alternatives to synthetic drugs for the treatment of hypertension, due to their adverse side effects, [1,5] and some novel ACE-inhibitory compounds have already been isolated from algae species, such as the phlorotannins that are the predominant polyphenols found in brown algae [5,6]. These algae accumulate a variety of phlorotannins of low, intermediate and high molecular weights, ranging between 126 Da to 650 kDa, although they are more commonly found in the 10–100 kDa range. It is also known that the ACE-inhibitory activity may depend on the degree of polymerization of phlorotannin derivatives [5].

The Azores Islands, due to their location in the middle of the Atlantic Ocean associated with pristine seawaters, are a very promising location for marine natural resources that may pro-

Abbreviations: ACE, angiotensin I converting enzyme; DW, dry weight; Fs, *Fucus spiralis*; Fs-DME, Fs dry powder methanol extract; HA, hippuric acid; HHL, hippuryl-L-histidyl-L-leucine; PE, phloroglucinol equivalents; S/N, signal to noise; TPC, total phenolic content.

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http://dx.doi.org/10.1016/j.jpba.2016.08.029
0731-7085/

duce new compounds with medicine-like effects in treating or preventing certain diseases. Traditionally, the Azorean population has gathered macroalgae either to eat or for chemicals extraction. The brown algae *Fucus spiralis* (Fs) is a local delicacy particularly the frond tips (the receptacles) that are picked and eaten fresh [7]. The present study aimed to: (1) determine the ACE-inhibitory activity of crude and size-fractionated methanolic extract from Fs using HPLC-UV method, (2) estimate their total phenolic content (TPC) expressed as phloroglucinol equivalents, (3) analyze them by chromatography (TLC and HPLC-DAD) and spectrophotometry (UV and IR) methodologies and (4) show the effect of the Fs dry powder methanol extracts (Fs-DME) storage temperature on ACE inhibition, in order to maximize its potential use in the preparation of antihypertensive drugs or functional foods.

2. Material and methods

2.1. Chemicals and reagents

Methanol (MeOH) and acetonitrile (ACN), HPLC grade, were purchased from Fluka Chemika (Steinheim, Switzerland). Chloroform ($CHCl_3$), etanol (EtOH), ethyl acetate (EtOAc), acetic acid, hydrochloric acid (HCl), orthophosphoric acid, sulphuric acid (H_2SO_4), sodium chloride, sodium hydroxide (NaOH), potassium bromide (KBr), trizma base, zinc chloride, hippuric acid (HA), hippuryl-L-histidyl-L-leucine (HHL), phloroglucinol (1,3,5-trihydroxybenzene), Folin-Ciocalteu reagent, vanillin and angiotensin I-converting enzyme (ACE) from porcine kidney were obtained from Sigma-Aldrich (St. Louis, MO, USA). Ultrafiltration membrane system and membranes were purchased from Millipore Co (Bedford, MA, USA). Silica gel TLC plates were purchased from Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q water purification and filtration system with 18 MΩ cm resistivity (Millipore, Bedford, MA, USA).

2.2. Collection, preparation, extraction and fractionation of F. spiralis (Fs) sample

The *F. spiralis* Linnaeus (Phaeophyceae, Ochrophyta) sample was collected in January 2013 from the littoral of São Miguel Island of Azores Archipelago (37° 40′ N and 25° 31′ W), Portugal, and a voucher specimen was prepared (voucher number SMG-13-04) 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 Fs sample was first washed in seawater followed by distilled water to remove encrusting material, epiphytes and salts, and then air-dried and stored in an air-tight container in a freezer (−80 °C). Prior to the analytical procedures, the Fs sample was defrosted and dried at 40–45 °C for 48 h (avoiding overheating that could lead to oxidation), and then was grounded into a fine powder of 0.5 mm particle size, re-dried at 40 °C and stored in the dark under N_2 in a desiccator at a refrigerated temperature. The Fs-DME was prepared by suspending 1 g of dried algae powder in 100 mL solution of methanol:water (80:20 v/v) under continuous stirring at room temperature during 24 h. After centrifugation, the supernatant was dried under vacuum in a rotary evaporator at 40 °C and then dissolved in deionized water to adjust to 2 mg/mL concentration. The solution was then divided into three samples (ref. A, B and C), where the first one was used for the direct determination of ACE-inhibitory activity, immediately after extraction, and the other two were frozen at −80 °C and −13 °C, respectively, during 15 days for further determinations. The most active Fs-DME sample (ref. A) was further separated by ultrafiltration membranes with molecular weight cut-off of 1 and 3 kDa to obtain ultrafiltrates (Fr_{A1} <1 kDa, 1< Fr_{A2} <3 kDa and Fr_{A3} >3 kDa) that were then lyophilized.

2.3. ACE-inhibitory activity determination of Fs methanol extract/fractions

The determination of ACE-inhibitory activity was performed in vitro by RP-HPLC adapted from the spectrophotometric method described by Cushman and Cheung [8] with slight modifications. This method is based on the liberation of HA from HHL catalyzed by ACE. For the assay, 80 μL of the sample solution was pre-incubated at 37 °C for 5 min with 20 μL of ACE (2 mU/mL) enzyme. The mixture was subsequently incubated at the same temperature for 60 min with 200 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 250 μL of 1 M HCl and the percentage of ACE inhibition was determined by an HPLC system. An aliquot of 10 μL from the reaction mixture was analyzed on a reverse-phase Ultrasphere C_{18} column (250 × 4.6 mm i.d., 5 μm particle size) (Beckman Coulter, Miami, FL, 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 was calculated as follows:

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

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). Captopril was used as a positive control for ACE inhibition. 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. The linearity of HA was evaluated by the calibration curve constructed in triplicate with five different concentrations.

2.4. Total phenolic content (TPC) determination of Fs methanol extract/fractions

The TPC was determined according to the Folin–Ciocalteu method as previously described [9], using phloroglucinol (a basic structural unit of phlorotannins) as a standard and expressing the results as mg of phloroglucinol equivalents (PE) per g of dry weight (DW) sample.

2.5. Chromatographic and spectrophotometric analysis of Fs methanol extract/fractions

The thin-layer chromatography (TLC) was performed on a silica gel plate (Kieselgel 60F, 0.25 mm). An aliquot of each Fs sample and phloroglucinol standard was spotted on the silica gel plate with a solvent system of $CHCl_3$:MeOH:water:acetic acid (65:25:4:3, v/v). The spots were visualized by spraying the plates with vanillin-H_2SO_4 solution. Red color spots produced indicate the presence of phenolic compounds [10]. The HPLC-DAD analysis of Fs-DME was performed using the following analytical conditions: column Prevail C_{18} (250 × 4.6 mm i.d, 5 μm particle size) from the Grace (The Nest Group, Southborough, MS, USA), mobile phase (A) 89.5% orthophosphoric acid 0.1% + 2% EtOAc + 8.5% ACN and phase (B) ACN:H_2O (1:1 v/v), gradient elution (t = 0–10 min, 0% B and t = 20 min, 60% B) and flow-rate of 0.6 mL/min. Spectra data were sweeped from 220 to 400 nm, and the chromatogram was recorded at 280 nm. Phloroglucinol was identified by retention time based on comparison with the authentic standard and by spiking the sample with standard. The UV spectra (in EtOH) of Fs fractions were recorded on a Shimadzu model 1800 UV/VIS spectrophotometer.

Please cite this article in press as: L. Paiva, et al., Angiotensin I-converting enzyme (ACE) inhibitory activity of *Fucus spiralis* macroalgae and influence of the extracts storage temperature—A short report, J. Pharm. Biomed. Anal. (2016), http://dx.doi.org/10.1016/j.jpba.2016.08.029