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Nutritional evaluation and research and characterization of peptides with inhibitory activity of angiotensin I-converting enzyme (ACE) in macroalgae of the Azores.

Dissertation for PhD degree in Biology
presented to the University of the Azores

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“Mestre é aquele que estende a mão, inicia o diálogo e encaminha para a aventura da vida. Não é só o que ensina fórmulas, regras, raciocínios, mas o que questiona e desperta para a realidade. Àqueles que nos ensinam muito mais que teorias e que nos preparam também para a vida.”

Autor desconhecido

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II. Paiva L., Lima E., Neto A. I., Massimo M., Baptista J. (2016). Health-promoting ingredients from four selected Azorean macroalgae. *Food Research International*, http://dx.doi.org/10.1016/j.foodres.2016.08.007. [IF2015: 3.182] (Annex II)

III. Paiva L., Lima E., Neto A. I., Massimo M. J. Baptista J. (2016). Nutritional and functional bioactivity value of selected Azorean macroalgae. Submitted to *European Journal of Phycology*. (Annex III)

IV. Paiva L., Lima E., Neto A. I., Baptista J. (2016). Isolation and characterization of angiotensin I-converting enzyme (ACE) inhibitory peptides from *Ulva rigida* C. Agardh protein hydrolysate. *Journal of Functional Foods, 26,* 65–76. [IF2015: 3.973] (Annex IV)

V. Paiva L., Lima E., Neto A. I., Baptista J. (2016). Angiotensin I-converting enzyme (ACE) inhibitory activity of *Fucus spiralis* macroalgae and influence of the extracts storage temperature – A short report. *Journal of Pharmaceutical and Biomedical Analysis, 131*, 503-507. [IF2015: 3.169] (Annex V)

VI. Paiva L., Lima E., Neto A. I., Baptista J. (2016). Angiotensin I-converting enzyme (ACE) inhibitory activity, antioxidant properties and total phenolic content of *Fucus spiralis* L. protein hydrolysate fractions. Submitted to *Marine Biotechnology*. (Annex VI)

RESUMO

As macroalgas *Fucus spiralis*, *Ulva rigida*, *U. compressa*, *Porphyra* sp. e *Osmundea pinnatifida* são consumidas como alimento em algumas das ilhas dos Açores, enquanto que *Gelidium microdon* e *Pterocladiella capillacea* são coletadas para a produção de agar. No entanto, pouca informação está disponível sobre o seu valor como fonte natural de compostos nutricionais e funcionais.

Esta tese investigou principalmente a composição nutricional e os aspetos promotores de saúde destas macroalgas, bem como o seu potencial como fonte de frações peptídicas e/ou de péptidos purificados inibidores da ECA.

Em relação aos aspetos nutricionais das macroalgas, esta tese investigou, pela primeira vez, as proteínas, o perfil de aminoácidos, a digestibilidade das proteínas *in vitro*, os lípidos, o perfil de ácidos gordos (saturados, monoinsaturados, polinsaturados e a razão *n*6/*n*3 e h/H), os hidratos de carbono solúveis, a fibra alimentar, as cinzas, os minerais e a razão Na/K e Ca/Mg, as vitaminas, a coenzima Q_{10}, a humidade, o teor de fenólicos totais, as atividades antioxidante e inibitória da ECA *in vitro* e o valor energético. Os resultados sugerem que um consumo regular destas algas, quer diretamente ou através de suplementos alimentares, pode melhorar a saúde humana ou pode ter um efeito protetor sobre algumas doenças degenerativas e, consequentemente, sobre o processo de envelhecimento.

As macroalgas podem também ser utilizadas para a produção de produtos farmacêuticos com potencial valor económico. Como é do conhecimento geral, a enzima conversora da angiotensina I (ECA) tornou-se um importante alvo para o controlo da pressão arterial, uma vez que catalisa a conversão de angiotensina I num potente vasoconstritor a angiotensina II. Recentemente, cada vez mais atenção tem sido dada às algas marinhas como fontes naturais de novos inibidores da ECA. Neste trabalho foram isolados e caracterizados alguns péptidos em *U. rigida* e os resultados revelaram, pela primeira vez, que péptidos inibidores da ECA podem ser eficientemente obtidos a partir das proteínas hidrolisadas pelas enzimas pepsina-bromelaína. Dois péptidos inibidores da ECA (IP e AFL) foram isolados e purificados com sucesso a partir deste hidrolisado. Os seus modos de inibição e estabilidade a diferentes temperaturas e os estudos *in vitro* do efeito das proteases gastrointestinais na atividade destes péptidos foram também caracterizados pela

primeira vez. Além disso, o tripéptido AFL foi hidrolisado por peptidases da mucosa intestinal dando origem a um dipéptido FL, que apresentou uma maior inibição da ECA relativamente ao seu percursor.

Esta tese também apresenta, pela primeira vez, as atividades inibitória da ECA e antioxidante *in vitro* por frações proteicas hidrolisadas de *F. spiralis*, bem como o perfil de aminoácidos e o conteúdo em fenólicos totais destas frações. Os resultados revelaram que não só os péptidos ativos, mas também os compostos fenólicos contribuem para as elevadas atividades inibitória da ECA e antioxidante das fracções proteicas hidrolisadas de *F. spiralis*. Avaliou-se, ainda, pela primeira vez, o efeito inibitório da ECA por extratos/frações metanólicas de *F. spiralis*, o respetivo conteúdo em fenólicos totais e o efeito da temperatura de armazenamento do extrato metanólico seco de *F. spiralis* na inibição da ECA. Os resultados sugerem que esta macroalga é muito rica em florotaninos, os polifenóis mais abundantes em algas castanhas e que têm sido referidos como sendo uma fonte potencial de compostos inibidores da ECA.

Em conclusão, as macroalgas estudadas podem ser usadas como alimentos funcionais e são uma fonte natural e potencial de frações/péptidos inibidores da ECA que podem ser utilizados para a produção de nutracêuticos e farmacêuticos para prevenir e/ou tratar a hipertensão. A sua utilização seria uma terapia natural alternativa, económica e mais segura, aos medicamentos comerciais sintéticos e atuaria como um incentivo para a conservação da biodiversidade e para a manutenção de um ambiente limpo nos Açores.

Palavras-chave: Macroalgas; Inibidores naturais da ECA; Péptidos bioativos; Alimentos nutricionais e funcionais; Composição bioquímica; HPLC.

ABSTRACT

The macroalgae *Fucus spiralis*, *Ulva rigida, U. compressa, Porphyra* sp. and *Osmundea pinnatifida* are consumed as food in some of the Azorean Islands, whereas *Gelidium microdon* and *Pterocladiella capillacea* are collected for agar production. Little information is, however, available on their value as natural sources of nutritional and/or functional metabolites content.

This thesis investigated mainly the nutritional composition and health-promoting aspects of those selected macroalgae as well as their potential as source of protein-derived ACE-inhibitory peptide fractions and/or purified peptides.

Regarding the macroalgal nutritional aspects, this thesis evaluated, for the first time, the proteins, amino acid profiles, *in vitro* protein digestibility, lipids, fatty acid profiles (SFA, MUFA, PUFA and *n*6/*n*3 and h/H ratios), soluble carbohydrate, dietary fiber, ash, minerals and Na/K and Ca/Mg ratios, vitamins, coenzyme Q_{10}, moisture, total phenolic content, *in vitro* antioxidant and ACE inhibition activities and energy values. The results suggested that a regular consumption of these macroalgae, either directly or through food supplements, may improve human health or may have a protective effect on some of the degenerative diseases and consequently on the ageing process.

Macroalgae can also be used for the production of pharmaceuticals with potential economic value. It is well known that angiotensin-I converting enzyme (ACE) became a major target control for high blood pressure, since catalyzes the conversion of angiotensin I to a potent vasoconstrictor angiotensin II. Recently, increasing attention has been paid to the marine algae as natural sources of novel ACE-inhibitors. In this thesis the isolation and characterization of some peptides from *U. rigida* were achieved and the results revealed, for the first time, that ACE-inhibitory purified peptides could be efficiently generated from *U. rigida* protein hydrolyzed by the pepsin-bromelain enzymes. Two ACE-inhibitory peptides (IP and AFL) were successfully isolated and purified from this hydrolysate. Their inhibition patterns and stability at different temperatures and the *in vitro* study of the gastrointestinal proteases effect on the activity of these peptides were firstly characterized. Furthermore, AFL was hydrolyzed by intestinal mucosa peptidases to a dipeptide FL with a more potent ACE inhibition relatively to its percurssor.

This thesis also reports, for the first time, the *in vitro* ACE-inhibitory and antioxidant activities by *F. spiralis* protein hydrolysate fractions and their amino acid profiles and total phenolic content. Results suggest that not only the active peptides, but also the phenolic compounds contribute to the strong ACE-inhibitory and antioxidant activities of *F. spiralis* protein hydrolysate fractions. Furthermore, this is the first study reporting the ACE inhibition by methanol extract/fractions from *F. spiralis*, their total phenolic content and the effect of the storage temperature of *F. spiralis* dry powder methanol extract on ACE inhibition. Results suggest that this macroalgae is very rich in phlorotannins, the most abundant polyphenols in brown algae that have been reported to be a potential source of powerful ACE-inhibitory compounds.

In conclusion, the studied Azorean macroalgae can be used for functional foods supplementation and are a natural potential source of ACE-inhibitory fractions/peptides that may be used for the production of nutraceuticals and pharmaceuticals to prevent and/or to treat hypertension. Their use would be economical, safer and a natural alternative therapy to commercial synthetic drugs, and would make an incentive for the biodiversity conservation and for the maintenance of a clean environment in the Azores.

Keywords: Macroalgae; Natural ACE-inhibitors; Bioactive peptides; Nutritional and Functional foods; Biochemical composition; HPLC.

FRAMEWORK AND THESIS OBJECTIVES

The angiotensin I-converting enzyme (ACE) inhibition has become a major target to control hypertension, which is a main risk factor for the development of cardiovascular diseases that are a significant public health problem worldwide. Natural products have been investigated in order to prevent or treat high blood pressure as an alternative potentially safer than the use of synthetic drugs that can have some adverse effects. Indeed, searching for ACE-inhibitors from natural resources, such as marine organisms including macroalgae, has become one of the major areas of research in the field of nutraceutical and pharmaceutical industries, and some antihypertensive products such as peptides and phenolic compounds have already successfully been isolated. Recently, some researchers have reported that the ACE-inhibitory compounds also present other significant bioactivities, including antioxidant properties.

Marine macroalgae are undoubtedly a valuable sustainable resource of "unique" bioactive phytochemical structures as well as sources of nutritious food components that have a high potential for producing health-care products for the pharmaceutical, medical, cosmetic and food industries. However, little information is available on the potential of macroalgae from Azores Islands (Portugal) as natural sources of functional metabolites.

The main aim of this thesis was to evaluate the nutritional and health-promoting aspects of selected Azorean marine macroalgae species, and to investigate their potential as sources of protein-derived ACE-inhibitory peptide fractions and purified peptides. The goal was mainly to gather knowledge on the important nutrients contents, and on the structure and activity of the ACE-inhibitory peptides, having in mind the possibility of producing, in the future, new health-promoting products for nutraceutical, pharmaceutical and cosmeceutical marine biotechnology markets.

More specifically, the objectives of this thesis were:

- ✓ To investigate the biochemical composition and the health-promoting aspects of the selected macroalgae in terms of moisture, proteins, lipids, amino acids and fatty acids profiles, soluble carbohydrates, dietary fiber, vitamins, coenzyme Q_{10}, ash, minerals and total phenolic contents, and also the energy value, the *in vitro* protein digestibility, and

the *in vitro* antioxidant and ACE inhibition properties. In this study seven common species of macroalgae from the Azorean littoral zone were investigated: *Fucus spiralis* (Ochrophyta, Phaeophyceae), *Ulva rigida*, *Ulva compressa* (Chlorophyta) and *Porphyra* sp., *Osmundea pinnatifida*, *Pterocladiella capillacea* and *Gelidium microdon* (Rhodophyta).

- ✓ To investigate and to evaluate the potential of the selected edible macroalgae *U. rigida* as a source of ACE-inhibitory peptide fractions and purified peptides, namely:
  - to optimize the protein extraction process, to screen for the most effective enzymes and to find the enzymatic hydrolysis reaction conditions in order to maximize the number of ACE-inhibitory peptides in the macroalgae protein hydrolysates;
  - to isolate the ACE-inhibitory peptide fractions from the selected enzymatic protein hydrolysate for further purification in order to obtain the purified bioactive peptides;
  - to determine the yield, protein and peptide content of the selected enzymatic protein hydrolysate and also from the ultrafiltration fractions;
  - to evaluate *in vitro* the ACE-inhibitory activity of the macroalgae hydrolysate fractions and the purified peptides;
  - to determine the amino acids composition and sequences of the purified ACE-inhibitory peptides;
  - to synthetize the most active ACE-inhibitory peptides to compare their activities with the isolated natural ones and also to the synthetic antihypertensive drugs usually prescribed by physicians;
  - to investigate the purified ACE-inhibitory peptides in terms of yield and *in vitro* properties, e.g. inhibition patterns and stability against temperature and gastrointestinal enzymes (pepsin, trypsin, chymotrypsin and intestinal mucosa peptidases).

- ✓ To investigate the ACE inhibition by methanol extract/fractions from *F. spiralis*, their total phenolic content and the effect of the storage temperature of *F. spiralis* dry powder methanol extract on ACE inhibition.

- ✓ To investigate and to evaluate the ACE-inhibitory and antioxidant activities of protein hydrolysate fractions from the selected edible macroalgae *F. spiralis*, namely:
  - to optimize the protein extraction process and the enzymatic hydrolysis reaction conditions to obtain higher yield of the macroalgae protein hydrolysates for further fractionation by ultrafiltration;
  - to determine the yield, protein, peptide, total phenolic content and amino acids profile of the macroalgae protein hydrolysate fractions;
  - to evaluate the *in vitro* ACE-inhibitory activity of the macroalgae protein hydrolysate fractions and also their *in vitro* antioxidant activities;
  - to determine the correlation between the various parameters (ACE inhibition, antioxidant activity and total phenolic content).

THESIS OUTLINE

This thesis is composed of eight chapters.

Chapter one is a general introduction that includes information on nutritional and health-promoting aspects of macroalgae species and on their potential as sources of antihypertensive compounds considering the relevant published literature. Chapter starts with the principal information of therapeutic and industrial applications of macroalgae, followed by the evaluation of their nutritional composition (proteins and amino acids, lipids and fatty acids, fibers, minerals, and vitamins and related compounds) and antioxidant potential. Then, it follows with the approach of hypertension disease and the explanation of the hypotensive action mechanism of bioactive peptides and their mechanism of transport and absorption. Chapter continues with more exhaustive explanation of the methods used in the bioactive peptides purification and identification in order to support the methods mentioned in chapter's five. It is also referred the studies with *in vitro* and *in vivo* bioactive peptides and a description of physical and chemical structure of the angiotensin I-converting enzyme (ACE) as well as its activity determination. Chapter ends with a description of the selected Azorean macroalgae.

Chapter's two to four (corresponding to manuscripts I to III) detail the information regarding the analytical methods used to determine the nutritional content of macroalgae. Chapter two provides, for the first time, full information on the biochemical and nutritional composition of the three most consumed edible Azorean macroalgae (*Osmundea pinnatifida*, *Fucus spiralis* and *Porphyra* sp.) in order to determine their nutritional value for human consumption and their potential impact on human health. Chapters three and four report, for the first time, the nutritional aspects, the health promoting ingredients content and the *in vitro* antioxidant and ACE-inhibitory activities of the Azorean macroalgae *Ulva compressa*, *U. rigida*, *Gelidium microdon* and *Pterocladiella capillacea*, in order to evaluate their use as functional foods and/or for producing health-care products for the pharmaceutical, medical, cosmetic and food industries.

Chapter five describes, for the first time, the investigation of the edible *U. rigida* protein hydrolysate as a source of ACE-inhibitory peptide fractions and their purified

peptides in order to evaluate its potential use in the preparation of antihypertensive drugs or functional foods. Chapter details the following steps: (a) screening for effective proteolytic enzymes and investigating the enzymatic hydrolysis conditions for producing *U. rigida* protein hydrolysates with the higher ACE-inhibitory activity, (b) isolating and identifying ACE-inhibitory peptides from *U. rigida* protein hydrolysed with pepsin plus bromelain enzymes, and (c) investigating *in vitro* the purified peptides in terms of ACE-inhibitory activity, inhibition pattern, and stability against temperature and gastrointestinal proteases.

Chapter six reports, for the first time, the studies of *in vitro* ACE-inhibitory activity of crude and size-fractionated methanol extracts from *F. spiralis*, detailing the following steps: (a) determining their total phenolic content (TPC), (b) analyzing them by chromatography (TLC and HPLC-DAD) and spectrophotometry (UV and IR) methodologies, and (c) investigating the effect of *F. spiralis* dry powder methanol extracts storage temperature on the ACE inhibition, in order to maximize its potential use in the preparation of antihypertensive drugs or functional foods.

Chapter seven describes, for the first time, the investigation of *F. spiralis* protein hydrolysate as a source of ACE-inhibitory and antioxidant peptide fractions, and also their protein, peptide, phenolics content and amino acids composition in order to evaluate its potential to be incorporated as multifunctional ingredients into foods as alternatives to conventional antihypertensive drugs or synthetic antioxidants.

Finally, chapter eight presents the final considerations and remarks, the limitations of the work and discusses future perspectives.