Journal of Functional Foods 27 (2016) 262–273

Available online at www.sciencedirect.com

ScienceDirect

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

Identification of three novel angiotensin-converting enzyme inhibitory peptides derived from cauliflower by-products by multidimensional liquid chromatography and bioinformatics

*Riccardo Zenezini Chiozzi, Anna Laura Capriotti *, Chiara Cavaliere, Giorgia La Barbera, Susy Piovesana, Aldo Laganà*

Dipartimento di Chimica, Sapienza Università di Roma, Piazzale Aldo Moro 5, 00185 Rome, Italy

ARTICLE INFO

Article history:
Received 18 July 2016
Received in revised form 8 September 2016
Accepted 12 September 2016
Available online

Keywords:
ACE-inhibitory peptides
Cauliflower by-products
Off-line multidimensional dimensional chromatography
High resolution mass spectrometry
Bioinformatic tools

ABSTRACT

The aim of this paper was the development of an analytical strategy for the production of purified bioactive peptides from cauliflower waste proteins, by testing two different extraction protocols and screening different enzymes for protein hydrolysis. The purification of peptides was carried out by multidimensional liquid chromatography employing reversed phase chromatography and hydrophilic interaction chromatography; the resulting fractions were tested for antihypertensive and antioxidative activities. The most active ones were characterized by nano-liquid chromatography-tandem mass spectrometry and identified by database search. The identified peptides were further mined by *in-silico* analysis using PeptideRanker to ascribe a bioactivity rank to each peptide. Thus, six potential ACE-inhibitory peptides were synthesized and validated checking their retention times and fragmentation patterns for consistency. Pure peptide standards were finally *in-vitro* tested for the specific bioactivity. In this way, three novel ACE-inhibitory peptides were successfully identified and validated from cauliflower waste hydrolysate, showing good IC_{50} values.

1. Introduction

Brassica crops, a huge vegetable family mainly constituted by cauliflower and broccoli, have a world production of about 22.3 million tons. Most of the production comes from Asia (with 82% of the total production), whereas Italy is the second world producer (with 0.4 million tons), followed by Spain and Mexico (http://faostat3.fao.org/). As a consequence of this intensive production, tons of cauliflower by-products are generated every year, and they are mainly made up of leaves (50% of the total) and stems. Currently, the use of cauliflower by-products is restricted only to flour and fibre (Hwang & Lim, 2015). These residues might cause huge economic and environmental problems, and that is the reason why it is important to discover new uses of these waste materials, outside the agricultural

* Corresponding author: Dipartimento di Chimica, Sapienza Università di Roma, Box no 34 - Roma 62, Piazzale Aldo Moro 5, 00185 Rome, Italy. Fax: +39 06 4906 31.
E-mail address: annalaura.capriotti@uniroma1.it (A.L. Capriotti).

Abbreviation: ACE, angiotensin-converting enzyme; AO, antioxidant; BAPs, bioactive peptides; BSA, bovine serum albumin; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HHL, N-hippuryl-L-histidyl-L-leucine; HILIC, hydrophilic interaction chromatography; IAA, iodacetamide; SDS, sodium dodecyl sulphate; SPE, solid phase extraction; RP, reversed phase; TCA, trichloroacetic acid; TFA, trifluoroacetic acid

http://dx.doi.org/10.1016/j.jff.2016.09.010
1756-4646/

sector as well, in order to create sustainable value chains in the farming and processing sectors (Herrero, del Pilar Sánchez-Camargo, Cifuentes, & Ibáñez, 2015).

A promising future application to valorize such by-products could be their exploitation as an economical source of active ingredients for production of animal feed and functional food ingredients (Llorach, Espín, Tomás-Barberán, & Ferreres, 2003).

Cruciferous (*Brassicaceae*) vegetables contain essential mineral elements, such as iron, calcium, selenium, copper, manganese and zinc (Singh, Kawatra, & Sehgal, 2001); furthermore, it is a fact that *Brassica* crops are sources of nutrients and health promoting phytochemicals, such as fibre, proteins, glucosinalates, phenolic acid, flavonoids and vitamins (Ahmed & Ali, 2013).

So far, the edible parts of *Brassica* vegetables have been the most investigated and the research has been mainly focused on the study of bioactive properties, due to their antioxidant (AO) and anti-carcinogenic compounds content (Hwang & Lim, 2015; Llorach, Gil-Izquierdo, Ferreres, & Tomás-Barberán, 2003; Mahn & Reyes, 2012). The research published by Talalay and his co-workers is a milestone in the area of cancer cruciferous chemoprevention, demonstrating a higher induction of cancer protective enzymes by bioactive molecules of broccoli sprouts. (Fahey, Zhang, & Talalay, 1997). Starting from this study, the interest of the scientific community on broccoli vegetables, as a natural source of anti-carcinogenic compounds and more in general as a health beneficial food, has considerably increased (Manchali, Chidambara Murthy, & Patil, 2012).

At the same time the use of food industry-related by-products is also gaining interest, since it is now well known that some specific residues generated during food manufacture possess significant amounts of bioactive compounds. In fact, recent works have shown that cauliflower by-products are a good source of phenolic compounds, mainly flavonol derivatives (Llorach et al., 2003).

While many studies reported the presence of AO and anticancer compounds in broccoli (Ávila et al., 2013; Hwang & Lim, 2015; Porter, 2012), the bioactive peptides (BAPs) remain poorly investigated, with just two studies described in the literature. One study was focused on identification of the endogenous ACE-inhibitory YPK peptide, which was detected in broccoli edible part (Lee, Bae, Lee, & Yang, 2006). The other study was performed on cauliflower by-products and identified the angiotensin-converting enzyme (ACE)-inhibitory VW dipeptide after digestion by Alcalase 2.4 L (Xu et al., 2016).

To the best of our knowledge, it is the only study related to BAPs performed on cauliflower waste products. As a consequence, nowadays this research field is highly challenging and that is the reason why the aim of this work is to propose a way to valorize cauliflower by-products as a source of BAPs, in perspective of their possible application as nutraceutical compounds. The main objective of this study was the development of a new systematic analytical approach for the recovery, separation, identification of BAPs. In particular, in this study, we employed a platform based on two different extraction methods, comparing a sodium dodecyl sulphate (SDS) and ethylenediaminetetraacetic acid (EDTA) based protocols, followed by an *in vitro* digestion by two different enzymes, trypsin and a mixture of pancreatin and pepsin. The four protocols were tested for the ACE-inhibitory and AO activity. The protocols providing the highest bioactivity were purified by a combination of consecutive chromatographic separations, first by RP liquid chromatography and then by hydrophilic interaction chromatography (HILIC). The most active fractions, obtained from the second chromatographic dimension, were further analysed by nanoRP-HPLC coupled with an Orbitrap mass spectrometry for peptide sequencing. After database search, the identified peptides were further mined by *in-silico* analysis using PeptideRanker, which provided a bioactivity score later used to select candidates for chemical synthesis. Finally, on the basis on the probability as calculated by PeptideRanker algorithm and on the area, 6 peptides were selected and synthesized. The synthesized peptides were validated compared to the natural occurring ones checking their retention times and fragmentation patterns in cauliflower by-product hydrolysates. Finally, their activities were determined by *in-vitro* bioassays on the individual peptide standards.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals, reagents, and organic solvents of the highest grade available were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), ACE from rabbit lung, N-hippuryl-L-histidyl-L-leucine (HHL) and hippuric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Deionized water was prepared by an Arium 611 VF system from Sartorius (Göttingen, Germany). SPE C18 cartridges were Bond Elut (Varian, Palo Alto, CA, USA).

2.2. Plant material and protein extraction

The typical cauliflower by-products (*Brassica oleracea L. var. botrytis*) mainly consist of leaves, which were supplied by a local farm. Fresh cauliflower by-products were washed with deionized water, chopped with a sharp stainless steel knife in small pieces and then ground to a fine powder with liquid nitrogen. One gram of ground material was extracted employing two different extraction buffers. The extraction buffer A was prepared as previously described (Capriotti et al., 2014) and was made up of 50 mmol L^{-1} Tris-HCl (pH 8.8), 1.5 mmol L^{-1} KCl, 0.07% (v/v), β-mercaptoethanol, 0.1% (v/v) protease inhibitor cocktail, and 1% (w/v) SDS. The extraction buffer B was prepared with 50 mmol L^{-1} Tris–HCl (pH 8.0), 1 mmol L^{-1} EDTA, 20 mmol L^{-1} dithiothreitol (DTT) and 0.1% (v/v) protease inhibitor (Liao, Yen, & Wang, 2009).

For both procedures, 1.0 g of ground leaves was extracted with 10 mL of extraction buffer A or B. Then, samples were incubated on ice for 1 h with intermittent vortexing (1 min) every 15 min, and finally the insoluble material was removed by centrifugation at 4 °C for 15 min at 11,000 × g. The two supernatants coming from experiments A and B were transferred into new centrifuge tubes and subjected to protein precipitation procedure.