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Identification and characterization of antioxidant peptides from chickpea protein hydrolysates

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ABSTRACT

Oxidative stress due to the excess of radical oxygen species (ROS) contribute to the development of different diseases. The use of antioxidants may prevent the development of these diseases by counteracting ROS levels. There is an increasing interest in natural antioxidants as they are safer for consumers than synthetic antioxidants. In this work, reducing power, free radical scavenging and cellular antioxidant activities of chickpea peptides fractions have been investigated. Peptide sequences included in fractions with antioxidant activity were identified. Main sequences, ALEPDHR, TETWNPNHPEL, FVPH and SAEHGSLH, corresponded to legumin, the main seed protein. Most peptides contained histidine, which has shown antioxidant activity. Two peptides also included tryptophan and phenylalanine, in which the phenolic group could also serve as hydrogen donor. These results show that legumin is a source of antioxidant peptides of high interest for food and pharmaceutical industries to develop new nutraceuticals and functional foods.

1. Introduction

Reactive oxygen species (ROS), such as free radicals and peroxides, may be generated naturally as by-product of different metabolic process or as consequence of environmental exposures such as tobacco smoke, radiation or pollution (Wu & Cederbaum, 2003). These ROS may reach toxicity levels due to an imbalance between their production and the detoxification biological system, leading to cellular damage that contributes to aging and increases the risk to develop different diseases such as Parkinson, Alzheimer, atherosclerosis, cancer and neurological degenerative as well as cardiovascular diseases (Moon & Shibamoto, 2009). Hence, there is an increased interest in the identification, characterization and application of antioxidants to prevent this oxidative stress in the organism. In addition, natural antioxidants are receiving special consideration as they seem safer for the consumers than synthetic antioxidants, such as butylated hydroxytoluene (BHT), which have shown carcinogenic effects (Pokorny, 2007).

In the last years, several epidemiologic studies have shown that people who consume diets rich in fruits and vegetables have lower risk of develop diseases related with the oxidative stress (Chen & Chen, 2013). Indeed, plants are one of the main natural sources of antioxidants as they are rich in ROS detoxification systems (Halliwell, 2009). Plants provide different antioxidant compounds such as ascorbate, α-tocopherol, tocotrienols, flavonoids and carotenoids (Halliwell, 2009). In addition to these compounds, plants are rich in proteins that may exert antioxidant activity through the capability of certain amino acids to act as metal chelating and hydrogen donors agents (Chen, Muramoto, Yamauchi, Fujimoto, & Nokihara, 1998). Hence, several proteins and peptides from different plant sources, such as soybean, potato, sunflower or rapeseed, have shown antioxidant properties (Garcia, Puchalska, Esteve, & Marina, 2013). The identification of their amino acid sequences is a challenging task and only few works have focused on it. In this context, mass spectrometry (MS), specially combined with high-performance liquid chromatography (HPLC), is one of the most widely used analytical methods for peptide characterization and quantification since it offers high selectivity and sensitivity (Contreras, Lopez-Exposito, Hernandez-Ledesma, Ramos, & Recio, 2008). Thus, an exhaustive peptide characterization would allow the identification of the peptides responsible for activity and their production can be further optimized through targeted hydrolysis processes. These hydrolysates or enriched fractions in

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these peptides could be used by the food industry as ingredients of functional foods.

Chickpea is the second most widely grown legume in the world. Chickpea seeds nutritional quality has been considered better than in other legumes (Jukanti, Gaur, Gowda, & Chibbar, 2012). Several studies have shown chickpea beneficial effects in the prevention of diseases such as type 2 diabetes, digestive diseases or cancer (Jukanti et al., 2012). Hence, chickpea is getting importance as functional food and the identification of the bioactive compounds implicated in these beneficial effects may be of high interest for the food industry. Most of the chickpea beneficial effects have been attributed to no proteins components such as fiber, starch, amylose, phytosterols or carotenoids (Jukanti et al., 2012). However, proteins are one of the main components of chickpea seeds with a crude content ranging from 15% to 30% (Paredes Lopez, Ordorica Falomir, & Olivares Vazquez, 1991). Moreover, chickpea proteins have demonstrated to be a notable source of bioactive peptides with antioxidant, ACE inhibitory and hypocholesterolemic activities (Pedroche et al., 2002; Yust, Millan-Linares, Alcaide-Hidalgo, Millan, & Pedroche, 2012; Yust et al., 2003).

In our previous study, we purified different peptide fractions by copper affinity and size exclusion chromatography from a chickpea protein hydrolysate (27.03% degree of hydrolysis) produced by sequential hydrolysis with pepsin and pancreatin (Torres-Fuentes, Alaiz, & Vioque, 2011). These peptides fractions have shown the capability to inhibit the copper-mediated lipid peroxidation (Torres-Fuentes, Alaiz, & Vioque, 2014). However, this antioxidant effect could be due to copper chelating activity rather than to antioxidant properties. In this study, we have carried out a further analysis of their non-copper-mediated antioxidant activity which allow to a better understanding of their precise mechanism and, moreover, new amino acids sequences within the most active fractions have been determined by reversed phase-high-performance liquid chromatography coupled to tandem mass spectrometry (RP-HPLC–MS/MS). This provides important relevant data as new bioactive peptides amino acids sequences have been detected.

2. Material and methods

2.1. Materials

Chickpea seeds were purchased in a local market. Potassium ferricyanide, ferric chloride, trichloroacetic acid (TCA), trifluoroacetic acid (TFA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP) and phosphate buffered saline (PBS) (0.144 M NaCl, 5 mM KCl, 8.5 mM Na_2HPO_4, 1.4 mM NaH_2PO_4, pH 7.4) were provided by Sigma–Aldrich (St. Louis, MO, USA). Hanks' Balanced Salt Solution (HBSS), fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco (Invitrogen, Barcelona, Spain). Ultrapure water was obtained using a Mili-Q system (Millipore, Bedford, MA, USA) and acetonitrile (UpS ultragradient) from Teknokroma (Barcelona, Spain).

2.2. Methods

2.2.1. Purification of chickpea peptides fractions

Chickpea chelating peptides were purified as previously described (Torres-Fuentes et al., 2011). Briefly, chickpea protein isolates were sequentially hydrolyzed with the digestive enzymes pepsin and pancreatin. Peptide fractions were purified from the chickpea protein hydrolysate, dissolved in 50 mM pH 7.4 sodium acetate buffer, 0.5 M sodium chloride, by affinity chromatography using a FPLC AKTA-purifier system (GE Healthcare, Buckinghamshire, UK) with immobilized copper as ligand. Purified peptide fractions (F1, F2 and F3) were further fractioned (F1A–F1F; F2A–F2D; F3A–F3E) by size exclusion chromatography using a Superdex-peptide 10/300 GL column (GE Healthcare) coupled to the FPLC AKTA-purifier system. The eluent used was 0.75 M ammonium bicarbonate buffer at a flow rate of 0.5 mL/min. Then, all eluted peptide fractions were collected and concentrated in distillated water using a nanofiltration system (Amicon, Millipore Corporation, Bedford, MA, USA) with a nanofiltration membrane TFC-SR model 3 (Koch membranes).

2.2.2. Reducing power

Reducing power was analyzed according to (Oyaizu, 1986). Chickpea protein hydrolysate and peptide samples were incubated with potassium ferricyanide 1% (w/v) in 0.2 M phosphate buffer pH 6.6 at 50 °C for 20 min. Then, 2.5% (w/v) TCA was added. Afterward, the solution was incubated with 0.01% (w/v) ferric chloride at 50 °C for 10 min. Finally, absorbance was read at 700 nm. Blank sample included neither sample nor ferric chloride and positive control included the synthetic antioxidant BHT. The assay was carried out in duplicate and data were analyzed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., San Diego, CA, USA).

2.2.3. Free radical scavenging activity (FRSA)

FRSA was analyzed using the stable free radical DPPH as described by Shimada, Fujikawa, Yahara, and Nakamura (1992). Chickpea protein hydrolysate and peptide fractions were mixed with 0.1 mM DPPH in 95% (1:1, v/v) ethanol and incubated with shaking for 30 min at room temperature and then the absorbance was read at 517 nm. Control (without sample) and blank (95% ethanol) were included. BHT (butylated hydroxytoluene) was included as positive control. The FRSA was calculated as follow:

$$\text{FRSA}\ (\%) = ((A_0 - A_S)/A_0) \times 100$$

where A_0 is the absorbance of control at 517 nm and A_s the absorbance in the presence of sample. The assay was carried out in duplicate and data were analyzed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc., La Jolla, CA, USA).

2.2.4. Cell culture

Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC; number 86010202, Salisbury, UK) and cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (1000 mg/mL glucose, 110 mg/mL pyruvate, 850 mg/mL glutamine; Gibco, Invitrogen, Madrid, Spain) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco, Invitrogen, Madrid, Spain), 1% non-essential amino acids (NEAA), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were grown at culture conditions (37 °C and 5% CO_2 in a humidified atmosphere) to a confluence of 70% and afterwards split to a lower density.

2.2.5. Cellular antioxidant activity (CAA): DCFH-DA assay

CAA of chickpea protein hydrolysate and peptides fractions was investigated by monitoring the decrease in fluorescence from dichlorofluorescein (DCF) as previously described (Wolfe & Liu, 2007) with modifications. Cells were seeded at a density of 2×10^4 cells/well in 96-well microplates in DMEM and incubated for 48 h at culture conditions. The outside wells of the plate were not used. Then, culture medium was removed and cells were washed twice with 1% PBS. Next, samples plus 25 μM DCFH-DA in HBSS were added and cells were incubated for 1 h at cultured conditions. Afterwards, cells were washed twice with 1% PBS and incubated with 600 μM ABAP in HBSS for 1 h at cultured conditions. Some peptides may be lost in these washes as they are not able to cross the cell membrane due to their big size and polarity,