LWT - Food Science and Technology 50 (2013) 591–598

Contents lists available at SciVerse ScienceDirect

LWT - Food Science and Technology

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

Purification and identification of antioxidant peptides from chickpea (*Cicer arietinum* L.) albumin hydrolysates

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ARTICLE INFO

Article history:
Received 28 September 2011
Received in revised form
28 July 2012
Accepted 2 August 2012

Keywords:
Chickpea albumin hydrolysate
Antioxidant peptide
Amino acid sequence
Free radical scavenging
LC–ESI–MS/MS

ABSTRACT

Albumin derived from chickpea was hydrolyzed sequentially using Alcalase and Flavorzyme proteases for production of antioxidant peptides. To identify antioxidant peptides, chickpea albumin hydrolysate (CAH) was fractionated using size exclusion chromatography (G-25) methods. Antioxidant and free radical-scavenging activities of peptides purified from the CAH were evaluated using reducing power assays with 1,1-diphenyl-2-pycrylhydrazyl (DPPH) and TEAC as well as with hydroxyl radical-scavenging assays. The results demonstrated that chickpea peptide (CPe)-III exhibited the highest antioxidant activity compared to the other hydrolysates. CPe-III was identified to be RQSHFANAQP (1155 Da) by LC–ESI–MS/MS.

1. Introduction

Oxidation is a major cause of deleterious quality changes in food products that can influence the flavor, color and texture, and it also leads to loss of nutritive value or food spoilage. In addition, oxidative stress can also modify DNA, proteins and small cellular molecules, and it is thought to have a significant role in the occurrence of diseases, such as cancer, arteriosclerosis, cardiovascular diseases, diabetes mellitus, neurological disorders and Alzheimer's disease (Halliwell, 2002; Stadtman, 2006).

In recent years, the antioxidant activity of bioactive peptides generated from the digestion of various proteins has attracted much attention. Previously, studies have shown that antioxidative peptides can be purified from many protein hydrolysates, such as fish protein (Bougatef et al., 2010), soybean (Moure, Domínguez, & Parajó, 2006), rice bran (Zhang et al., 2010), and milk (Tsopmo et al., 2011). Many antioxidant properties of these protein hydrolysates have been described, including their abilities to inactivate reactive oxygen species, scavenge free radicals, chelate pro-oxidative transition metals, in addition to the possibility of preventing the penetration of lipid oxidation initiators by forming a membrane around oil droplets (Moure et al., 2006; Zhu, Chen, Tang, & Xiong, 2008).

Several studies have reported that the antioxidative activity of protein hydrolysates and isolated peptides prepared from natural resources, in some cases, is similar or higher than that of commonly used synthetic antioxidants, such as butylated hydroxytoluene (BHA), butylated hydroxylanisole (BHT) and propyl gallate (Samaranayaka & Li-Chan, 2008). In addition, there are safety concerns over the use of synthetic antioxidants as food additives. Hence, recent attention has focused on antioxidants from natural resources.

Many studies have reported *in vitro* formation of antioxidative peptides from various legume bioresources and their potential use as alternative antioxidants. Chickpea (*Cicer arietinum* L.), an annual herbage plant, is the third most important grain legume in the world on the basis of total grain production (Newman, Roth, & Lockerman, 1987). Chickpea proteins have been considered to be a suitable source of dietary protein due to their well-balanced amino acid composition, high protein bioavailability, and relatively low level of antinutritional factors (Newman et al., 1987). Various biological activities, including antifungal activity, angiotensin I-converting enzyme (ACE) inhibition, metal-chelating ability, antioxidant activity, and reduction of antigenic activity, have been reported for chickpea protein hydrolysate (Torres-Fuentes, Alaiz, & Vioque, 2011; Yust et al., 2003; Zhang, Li, &

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http://dx.doi.org/10.1016/j.lwt.2012.08.002

Miao, 2011). Recently, chickpeas have also been studied for their antioxidant properties due to the increasing interest in the health benefits associated with antioxidants. Moreover, studies on the amino acid constituents in antioxidant compounds and on their antioxidant capacity have also gained interest. However, to the best of our knowledge, little research has been done on the sequence of chickpea-derived peptides.

Antioxidant peptides usually contain 3–20 amino acid residues, and their activity depends on their amino acid constituents, sequences and structure (Pihlanto-Leppälä, 2000). However, the protein source, type of protease and hydrolysis process determine the size, amino acid composition, hydrolysate peptide sequence and, subsequently, antioxidant activity. Most researchers believe that antioxidant peptides possess some metal chelation or hydrogen/electron donor activity, which may allow them to interact with free radicals, thereby, terminating the radical chain reaction or preventing their formation. Hydrophobic amino acids and one or more residues of His, Pro, Met, Cys, Tyr, Trp, Phe and Met are believed to enhance the activities of antioxidant peptides, and the most antioxidant peptides include the following amino acids: nucleophilic sulfur-containing amino acids (Cys and Met), aromatic amino acids (Trp, Tyr, and Phe), and imidazole-containing amino acids (His). However, some antioxidant peptides do not contain any of the abovementioned proton-donating amino acid residues in their sequence. For example, Gln-Gly-Ala-Arg (Li, Chen, Wang, Ji, & Wu, 2007) and Glu-Leu (Jun, Park, Jung, & Kim, 2004) have been reported to have an important role in radical scavenging. However, additional research is needed to clarify the structure/function relationship of antioxidative peptides.

The antioxidant activity of low molecular weight chickpea peptides has been well-established. Moreover, the antioxidant activity of chickpea protein can be enhanced after enzymatic hydrolysis, and these peptidic antioxidants may have an impact on reducing oxidative stress and the risk of various degenerative diseases, such as cancer, cardiovascular disease, and inflammatory diseases, associated with oxidative stress (Chen, Muramoto, Yamauchi, & Nokihara, 1996; Li, Jiang, Zhang, Mu, & Liu, 2008; Peñta-Ramos & Xiong, 2002). However, antioxidant activities in chickpea peptides have not been adequately studied. Only a limited number of studies on the impacts of the peptide structures on biological antioxidative potential of peptide hydrolysates or isolated peptides have been reported.

Chickpea proteins are usually classified into two major fractions as follows: globulins and albumins. Globulins are storage proteins, and these proteins mainly consist of legumin and vicilin proteins. The albumin fraction, which is less abundant than globulins, represents approximately 15–25% of total cotyledonary proteins. However, albumins have an essential role in seeds because they include most of the enzymatic and metabolic proteins. For example, Tavano and Neves (2008) reported that trypsin inhibitor activity is more concentrated in the albumin fraction than in the globulin fraction. Moreover, albumins may be partially responsible for the poor bioavailability of legume proteins (Clemente et al., 2000).

Therefore, the objective of the present investigation was to identify the antioxidative potentials and the structure (molecular weight and amino acid sequence) of peptides derived from chickpea albumin. For this purpose, the extracted chickpea albumin was enzymatically hydrolyzed to obtain antioxidant peptides, and the antioxidant potential of different chickpea albumin hydrolysate (CAH) fractions was investigated using several measurements, including the reducing power and scavenging activity on DPPH, ABTS and hydroxyl radicals. Furthermore, amino acid composition and molecular weight distribution were also evaluated to elucidate their relationship with antioxidant activity.

2. Materials and methods

2.1. Materials

Chickpea was obtained from Xinjiang Market in China. Alcalase 2.4 L and Flavorzyme were purchased from Novo Enzyme (Bagsvaerd, Denmark). Sephadex G-25, 2,2-Diphenyl-picrylhydrazyl (DPPH), 2-thiobarbituric acid (TBA), 2-deoxyribose, ethylenediaminetetraacetic acid (EDTA), 6-hydroxy- 2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and L-ascorbic acid were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). All the other solvents and chemicals used were of analytical grade.

2.2. Extraction of chickpea albumin isolate (CAI)

Chickpea albumin isolate (CAI) was produced according to the method described by Xue et al. (2009). Briefly, chickpea seeds were ground and defatted with ether, then air-dried overnight at room temperature in a well vented place. Defatted chickpea flour (100 g) was stirred for 1 h at room temperature in distilled water (1000 mL). The resulting slurry was centrifuged at 2200 × *g* for 10 min and the supernatant was collected. The residue was extracted with a further 500 mL of distilled water for 1 h and separated as mentioned previously. Supernatants were pooled, and the pH was adjusted by a 0.5 mol/L HCl solution to pH = 4.3 where most proteins precipitated. The precipitate was removed and the pH of the supernatant was lowered to 3.7 to precipitate the albumin fraction. The precipitate obtained was washed three times with distilled water, and a 0.5 mol/L NaOH solution was used to adjust the pH to 7.0 to dissolve the precipitate. The dissolved albumin fraction was lyophilized and stored at −30 °C.

2.3. Preparation of chickpea albumin hydrolysate (CAH) by Alcalase and Flavorzyme

CAH was produced according to the method described by Xue, Liu, Wu, Zhuang, and Yu (2010). Briefly, CAI was resuspended in distilled water (5 g/100 mL), and the Alcalase/substrate ratio was adjusted to 0.3 AU enzyme/g (AU, Anson Units) under optimal conditions (50 °C, 1 h, and pH 8.0). During the course of the reaction, the pH was kept constant with a 1 mol/L NaOH solution in an appropriate concentration. Proteins were withdrawn at 1 h of hydrolysis, and the pH was adjusted to 7.0 followed by the addition of Flavorzyme at 50 LAPU/g ([*E*]/[*S*]) (LAPU, Leucine Amino Peptidase Unit). The sample was hydrolyzed for 2 h at pH of 7.0, which was adjusted with the 1 mol/L NaOH solution. The hydrolysate was subsequently transferred to a water bath (80 °C) for 10 min to inactivate the enzyme. After cooling, the resultant chickpea albumin hydrolysate was adjusted to the isoelectric point (pH = 4.5) to precipitate the protein which haven't been hydrolyzed. The supernatant was obtained by centrifugation at 2200 × *g* for 10 min. The supernatant was then lyophilized and used as the chickpea peptide (CPe).

The degree of hydrolysis (DH) of hydrolyzed CAI was determined using a pH-stat method (Adler-Nissen, 1986, pp. 110–125) based on the following equations: $DH = (h/h_{tot}) \times 100\%$ and $h = B \times N_b \times 1/\alpha \times 1/MP$; where B is the base consumption (mL); N_b is the concentration of base (1 mol/L NaOH); $1/\alpha$ is the calibration factor for pH-stat; MP is the mass of protein (g); and h is the hydrolysis equivalent. For whey, h_{tot} is equal to 7.22 mmol/g protein.

The bitter taste of chickpea albumin hydrolysates identified in the study was quantified using quinoline as a standard. For the