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Purification and identification of novel antioxidant peptides from enzymatic hydrolysate of chickpea (*Cicer arietinum L.*) protein concentrate

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ABSTRACT

Enzymatic hydrolysis of chickpea protein concentrate (CP) by Alcalase® and some physiochemical and antioxidant properties of the resulting hydrolysate (CPH) were characterised. CPH displayed higher antioxidant activity than CP. This hydrolysate was fractionated by size exclusion chromatography on a Sephadex G-25 into four major fractions (Fra.I, Fra.II, Fra.III, and Fra.IV). Fraction III, which exhibited the highest DPPH scavenging activity (54% at 1 mg/ml), was then fractionated by reversed-phase high performance liquid chromatography (RP-HPLC). Eleven antioxidant fractions were isolated and two peptide subfractions show antioxidant activity (P3 and P8). The P8 displayed the highest DPPH radical-scavenging activity (67%; at 200 μg/ml) among these peptides subfractions. The molecular masses and amino acids sequences of the purified peptides were determined using ESI-MS and ESIMS/MS, respectively. Their structures were identified as Asp-His-Gly and Val-Gly-Asp-Ile. These peptides did not show haemolytic activity towards bovine erythrocytes. The results suggest that CPH are good source of natural antioxidants.

1. Introduction

Oxidation of biomolecules has many undesirable impacts on food because it is a major cause of deleterious quality changes that can influence the colour, flavour and texture. Furthermore, oxidative stress can also modify proteins, DNA 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

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disorders and Alzheimer's disease (Halliwell, 2002; Stadtman, 2006). Recently, hydrolysis of proteins from plants and animals to generate bioactive peptides has attracted much attention. Antioxidative peptides have been purified from many protein hydrolysates such soybean (Moure, Domínguez, & Parajó, 2006), rice bran (Parrado et al., 2006), canola (Cumby, Zhong, Naczk, & Shahidi, 2008), Chinese leek (Hong, Chen, Hu, Yang, & Wang, 2014), sweet potato (Zhang, Mu, & Sun, 2014) and fish protein (Bougatef et al., 2010).

Peptides derived from natural sources have been the focus of great interest because of their potential health benefits associated with low molecular weight, low cost and high activity (Sarmadi & Ismail, 2010). These antioxidants can act as inhibitors of lipid peroxidation, as direct scavengers of free radicals and as agents to chelate the transition metal ions that catalyse the generation of radical species (Zhang, Sun et al., 2011).

Chickpea seeds are considered a good source of dietary proteins because of their well-balanced amino acid composition, high protein bioavailability, and relatively low levels of antinutritional factors (Friedman, 1996). Various biological activities, including antioxidant activity, antifungal activity, reduction of antigenic activity, metal-chelating ability and angiotensin I-converting enzyme (ACE) inhibition, have been reported for chickpea protein hydrolysate (Kou et al., 2013; Torres-Fuentes, Alaiz, & Vioque, 2011; Yust et al., 2003; Zhang, Li, Miao, & Jiang, 2011). Moreover, the use of natural protein hydrolysates have been the subject of several research works, because of their antioxidant potential. However, to the best of our knowledge, there is little information concerning the antioxidant properties of chickpea protein hydrolysate and the sequence of chickpea-derived peptides.

Therefore, the objective of the present study was to identify the antioxidative potentials and the structure (molecular weight and amino acid sequence) of peptides derived from chickpea. For this purpose, the extracted chickpea was enzymatically hydrolysed to obtain antioxidant peptides, and the antioxidant potential of chickpea hydrolysate (CPH) was investigated using several measurements, including scavenging activity on DPPH, the reducing power, metal-chelating activity and β-carotene bleaching inhibition activity. Furthermore, two new antioxidative peptides were isolated from the hydrolysate and their amino acid composition was also evaluated to elucidate their relationship with antioxidant activity.

2. Materials and methods

2.1. Reagents

Common chemicals and solvents of analytical grade were obtained from different commercial sources. Chemicals required for the assays including 1,1-diphenyl-2-picrylhydrazyl (DPPH) a butylated hydroxyanisole (BHA), β-carotene, ethylenediaminetetraacetic acid (EDTA) and linoleic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Sephadex G-25 was purchased from Pharmacia (Uppsala, Sweden). Water was obtained from a Culligan system; the resistivity was approximately 18 MΩ. All other chemicals and reagents used were of analytical grade.

2.2. Enzyme

The serine protease "Alcalase" from *B. licheniformis* (Novozymes®, Bagsvaerd, Denmark) was used for the production of hydrolysate. Protease activity was determined according to the method of Kembhavi, Kulkarni, and Pant (1993) using casein as a substrate. One unit of protease activity was defined as the amount of enzyme required to liberate 1 μg of tyrosine per min under the experimental conditions used.

2.3. Preparation of protein concentrates (CP)

One hundred grams of chickpea flours were placed in a dark flask and homogenised using Ultra-Turrax T25 homogeniser set at 17.500 rpm (Janke & Kunkel IKA Labortechnik, Staufen, Germany) with 300 ml of hexane. After mixing for 4 h in a shaker (Selecta, Barcelona, Spain) at a rate of 180 rpm/min, the mixture was centrifuged for 15 min at 3000 *g* at ambient temperature (20 °C). The extraction procedure was repeated twice and the pellet was used to produce protein extract. One hundred grams of defatted flour were mixed with water and adjusted to pH 9.0 with 1 M NaOH, to facilitate protein solubilisation. After centrifugation using a refrigerated centrifuge (Hettich Zentrifugen, ROTINA 380R, Tuttlingen, Germany), the supernatant was adjusted to pH 4.0 to precipitate the protein concentrate. To eliminate all soluble components, the pellet was washed with distilled water. The protein was resuspended in distilled water and adjusted to pH 7.0.

2.4. Preparation of chickpea protein hydrolysate (CPH)

Chickpea protein concentrate (500 g) was first 'resuspended' in 500 ml distilled water and then was cooked at 80 °C for 5 min to inactivate endogenous enzymes. The cooked protein sample was then homogenised at high speed for about 2 min using a Moulinex R62 homogeniser (Organotechnie, Courneuve, France). The sample was adjusted to pH 8.0 and 50 °C for optimal Alcalase activity.

The protein solutions were allowed to equilibrate for 30 min before hydrolysis was initiated. After the equilibrium was reached, the hydrolysis reaction was started by the addition of the enzyme at a 1:1 (U/mg) enzyme/protein ratio. The protein content of CP was determined by the Kjeldahl method. The reaction was conducted at 50 °C and pH 8.0 for 210 min. During the reaction, the pH of the mixture was maintained at the desired value by continuous addition of NaOH (4 M). After the required digestion time, the enzymatic hydrolysis was stopped by heating the solution for 20 min at 80 °C to inactivate enzyme. Protein hydrolysates were then centrifuged at 5000 *g* for 20 min to separate soluble and insoluble fractions. Finally, the soluble fractions, referred to as protein hydrolysates, were freeze-dried at –50 °C and 121 mbar (CHRIST, ALPHA 1–2 LD plus, Osterode am Harz, Germany).

2.5. Determination of the degree of hydrolysis

The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds cleaved (h) to the total number of peptide bonds in the substrate studied (h_{tot}), was calculated from the amount of base (NaOH) added to keep the pH

constant during the hydrolysis (Adler-Nissen, 1986) according to the following equation:

$$DH\,(\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times Nb}{MP} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100$$

where B is the amount of NaOH consumed (ml) to keep the pH constant during the proteolysis of the substrate. Nb is the normality of the base, MP is the mass (g) of the protein (N × 6.25: 'N' content of the CPH was determined by the Kjeldahl method), and α represents the average degree of dissociation of the α-NH2 groups in the protein substrate expressed as:

$$\alpha = \frac{10^{pH\text{-}pK}}{1 + 10^{pH\text{-}pK}}$$

where pH and pK are the values at which the proteolysis was conducted. The total number of peptide bonds (h_{tot}) in the protein substrate was assumed to be 7.22 mmol/g (Kou et al., 2013).

2.6. Antioxidant activity

2.6.1. DPPH radical-scavenging capacity

The DPPH radical-scavenging capacity of samples was determined as described by Bersuder, Hole, and Smith (1998). A volume of 500 µl of each sample at different concentrations was added to 375 µl of 99% ethanol and 125 µl of DPPH solution (0.02% in ethanol) as free radical source. The mixtures were shaken and then incubated for 60 min in a dark room at room temperature. Scavenging capacity was measured spectrophotometrically (UV mini 1240, UV/VIS spectrophotometer, SHIMDZU, Kyoto, Japan) by monitoring the decrease in absorbance at 517 nm. In its radical form, DPPH has an absorption band at 517 nm which disappears upon reduction by an antiradical compound. Lower absorbance of the reaction mixture indicated higher DPPH free radical-scavenging activity. BHA was used as positive control. DPPH radical-scavenging capacity was calculated as follows:

$$DPPH\ radical-scavenging\ activity\ (\%) = \frac{A\ blank - A\ sample}{A\ control} \times 100$$

where A blank is the absorbance of the control reaction (containing all reagents except the sample) and A sample is the absorbance of Bersuder (with the DPPH solution). The experiment was carried out in triplicate and the results are mean values.

2.6.2. Reducing power assay

The ability of samples to reduce iron (III) was determined according to the method of Yildirim, Mavi, and Kara (2001). An aliquot of 1 ml sample of each hydrolysate at different concentrations (1–5 mg/ml) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) potassium ferricyanide solution. The mixtures were incubated for 30 min at 50 °C. After incubation, 2.5 ml of 10% (w/v) TCA was added and the reaction mixtures were then centrifuged for 10 min at 10,285 *g*. Finally, 2.5 ml of the supernatant solution from each sample mixture were mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% (w/v) ferric chloride. After a 10 min reaction time, the absorbance of the resulting solutions was measured spectrophotometrically (UV mini 1240, UV/VIS spectrophotometer, SHIMDZU) at 700 nm. Higher absorbance of the reaction mixture indicated higher reducing power. The control was conducted in the same manner, except that distilled water was used instead of sample. Values presented are the mean of triplicate analyses. BHA was used as reference antioxidant.

2.6.3. Antioxidant assay using the ß-carotene bleaching method

The ability of the chickpea concentrate and hydrolysate to prevent the bleaching of ß-carotene was determined as described by Koleva, van Beek, Linssen, de Groot, and Evstatieva (2002). Briefly, 0.5 mg ß-carotene in 1 ml chloroform was mixed with 200 µl of Tween-40 and 25 µl of linoleic acid. The chloroform was completely evaporated under vacuum (rotary evaporator, Heidolph, Schwabach, Germany) at 45 °C, then 100 ml of double distilled water was added and the resulting mixture was vigorously stirred. The emulsion obtained was freshly prepared before each experiment. An aliquot (2.5 ml) of the ß-carotene–linoleic acid emulsion was transferred to tubes containing 0.5 ml of each sample at different concentrations. The tubes were placed in water bath and incubated at 50 °C for 2 h. Thereafter, the absorbance of each sample was measured at 470 nm. A blank consisted of 0.5 ml of distilled water instead of the sample solution. BHA was used as positive standard.

2.6.4. Metal chelating activity

The chelating activities of the sample towards ferrous ion (Fe^{2+}) were determined according to the methods of Decker and Welch (1990) with slight modifications. A volume of 0.1 ml of each sample at different concentrations was mixed with 0.1 ml of 2 mM $FeCl_2$, $4H_2O$ and 0.2 ml of 5 mM 3-(2-pyridyl)-5,6-bis(4-phenyl-sulphonic acid)-1,2,4-triazine (ferrozine). The blank was conducted in the same manner except that distilled water was used instead of the sample. EDTA was used as reference. The metal ion chelating activity exhibited by the sample was calculated by the following equation

$$\text{Chelating activity}\ (\%) = \left[1 - \frac{\text{A562 of sample}}{\text{A562 of blank}}\right]$$

2.7. Purification of antioxidant peptides

2.7.1. Fractionation of protein hydrolysate with Sephadex G-25 gel filtration

The lyophilised hydrolysate (1 g), with a final DH of 14.67%, obtained by treatment with Alcalase® for 210 min, was suspended in 5 ml of distilled water, then separated onto a Sephadex G-25 gel filtration column (5.2 × 56 cm) pre-equilibrated and eluted with distilled water. Fractions (5 ml) were collected at a flow rate of 30 ml/h. Fractions (5 ml each) were collected and elution curves were obtained by measuring absorbance at 280 nm using an online spectrophotometer. Fractions that showed antioxidant activity were pooled and lyophilised (CHRIST, ALPHA 1–2 LD plus, Osterode am Harz, Germany).

2.7.2. *Reversed-phase high pressure liquid chromatography*

The most active fraction was dissolved in Milli Q water, filtered through 0.22 μm filters, and then separated by reverse-phase high-pressure liquid chromatography (RP-HPLC) on a Waters C18 column (4.6 mm × 250 mm) (XBridegTM, Dublin, Ireland). Peptides were eluted with eluent A (water containing 0.1% trifluoroacetic acid (TFA)) for 5 min, then with a linear gradient of acetonitrile (40–100% for 60 min) containing 0.1% TFA at a flow rate of 1 ml/min. On-line UV absorbance scans were performed between 200 and 300 nm at a rate of one spectrum per second with a resolution of 1.2 nm. Chromatographic analyses were completed with Millennium software. The antioxidant activities of the eluted peaks were determined. The liquid chromatographic system consisted of a Waters 600E automated gradient controller pump module, a Waters Wisp 717 automatic sampling device and a Waters 996 photodiode array detector. Spectral and chromatographic data were stored on a NEC Image 466 computer. Millennium software was used to plot, acquire and analyse chromatographic data.

2.8. *Identification of antioxidant peptides*

The molecular mass and peptide sequencing were conducted in a positive ion mode using electrospray ionisation-mass spectrometry (ESI-MS) and the tandem mass spectrometry (MS/MS), respectively. Concerning the ESI mass spectrometry, it was performed using a triple quadruple instrument Applied Biosystems API 3000 (PE Sciex, Toronto, ON, Canada), equipped with an electrospray ion source. The system is controlled by the Analyst Software 1.4, allowing the control of the spectrometer, the analysis and the processing data. The interpretations of MS-MS spectra were performed with the Bioanalyst software. The freeze-dried samples from RP-HPLC were dissolved in acetonitrile/water (20/80; v/v), containing 0.1% formic acid for the positive mode. The solution was injected (nebulised) uninterrupted by a pump (Model 22, Harvard Apparatus, South Natick, MA, USA) with a flow rate of 5 μl/min. The potential of ionisation was of 5000 V in a positive mode. At the time of the recording of the spectrum, 30 scans on average were added (MCA mode) for each spectrum. The used gases (nitrogen and air) were pure (up to 99%) and produced by a compressor Jun-Air 4000-40M and a nitrogen generator Whatman model 75–72 (Whatman, Inc., Haverhill, MA, USA). The polypropylene glycol (PPG) was used for the calibration and the optimisation of the machine. The peptide sequence was determined from the CID spectrum of the protonated analysis $[M + H]^+$ by MS/MS experiments. Peptide sequences were determined using the bioanalyst software (Applied Biosystems, South San Francisco, CA, USA).

2.9. *Haemolytic activity*

The haemolytic activity of the active peptide fractions was determined by method of Dathe et al. (1996). Five millilitres of bovine blood was centrifuged at 1260 *g* for 10 min to isolate erythrocytes, which were then washed three times with sodium phosphate (10 mM), containing NaCl 9 g/l, pH 7.5. The cell concentration stock suspension was adjusted to 10^9 cells/ml. The cell suspension (12 μl) along with varying amounts of fractions stock solution and the buffer were placed into tubes to give a final volume of 50 μl. The tubes with 2.5×10^8 cells/ml were then incubated at 37 °C during 40 min. After centrifugation (2570 *g*, 5 min), 30 μl of supernatant was diluted in 500 μl water. The absorbance of the diluted solution was measured at 420 nm. The absorbance obtained after treating erythrocytes with only NaCl/Pi and SDS (0.2%) was taken as 0% and 100%, respectively.

2.10. *Statistical analysis*

Analytical values were carried out using three independent determinations. Results were expressed as mean values ± standard error of three independent determinations. Statistical analyses were determined using a statistical software program (SPSS for Windows version 11.0). The data were subjected to analysis of variance using the general linear model to determine significant differences between samples ($p < 0.05$).

3. Results and discussion

3.1. *Production of CPH*

Alcalase®, an inexpensive endopeptidase, has been used for the production of protein hydrolysates with better functional and nutritional characteristics than the original proteins and for the generation of bioactive peptides (Yust et al., 2003).

The hydrolysis curve of deffated chickpea proteins after 220 min of incubation is shown in Fig. 1. Hydrolysis of chickpea proteins extract with Alcalase® was characterised by a high rate of hydrolysis during the initial 30 min. The rate of enzymatic hydrolysis was subsequently decreased, and then the enzymatic reaction reached a plateau when no apparent hydrolysis took place. After 220 min of hydrolysis, the DH value was 14.67%. The shape of hydrolysis curves is similar to those previously published for hydrolysates from buckwheat (He Tang, Peng, Zhen, & Chen, 2009), wheat germ (Zhu, Zhou, & Qian, 2006) and pumpkin oil cake (Vaštag, Popović, Popović, Krimer, & Peričin, 2011).

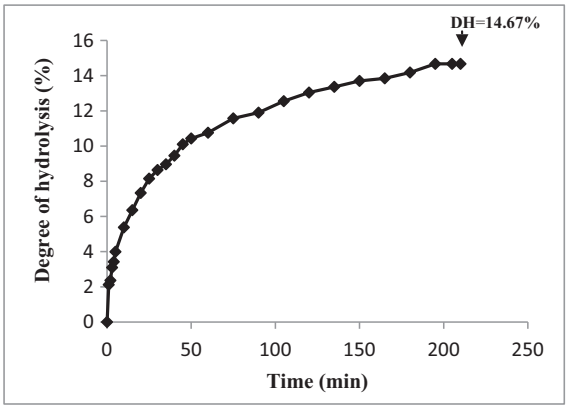

Fig. 1 – Hydrolysis curve of chickpea protein extract treated with Alcalase® from *B. licheniformis*. Hydrolysis was conducted at the following conditions: enzyme (U)/protein (mg) ratio of 1:1, pH 8.0 and at 50 °C.

Table 1 – Chemical composition of chickpea flours, protein extract and protein hydrolysates obtained by treatment with Alcalase®.

	Flour of chickpea	Protein extract	Hydrolysate
Dry matter (%)	92.96 ± 0.15[a]	93.75 ± 0.1[b]	95.13 ± 0.14[c]
Protein (%)	24.51 ± 0.27[a]	78.53 ± 0.7[b]	83.75 ± 0.86[c]
Crude fat (%)	8.20 ± 0.87[a]	4.5 ± 0.5[b]	1.14 ± 0.05[c]
Carbohydrates (%)	57.11 ± 0.92[a]	6.19 ± 0.1[b]	4.1 ± 0.13[b]
Ash (%)	3.14 ± 0.07[a]	4.53 ± 0.23[b]	6.14 ± 0.16[b]

All the data are expressed as mean ± SD and are the mean of three replicates.
Means with the different superscript letters within the same line are significantly different ($p < 0.05$).

3.2. *Chemical composition*

Table 1 shows the chemical composition of protein isolate compared with the composition of the original chickpea flour and protein hydrolysates obtained by treatment with Alcalase®. Flour of chickpea contains 24% of protein. From this flour, about 70% of total proteins were extracted at pH 9, at a solid-to-water ratio of 1:10 (w/v). The protein content data of CP are lower than that reported by Torres-Fuentes et al. (2011) but higher than that by Arcan and Yemenicioğlu (2007). Hydrolysates contained higher protein content than chickpea protein extract but lower moisture and lipid contents that might significantly contribute to their stability during storage. Ash content was higher in the hydrolysates due to the addition of NaOH to keep the pH constant during hydrolysis. These results are similar to those of other published studies on fish protein hydrolysates (Bougatef et al., 2010; Hmidet et al., 2011).

3.3. *Antioxidant activity of chickpea protein hydrolysate*

Due to the diversity of antioxidant action of protein hydrolysates and oxidation processes, many methods were used to evaluate antioxidant activity and provide a clear idea about their real antioxidant potential. Therefore, four chemical in vitro assays based on different antioxidant mechanisms were used in this study to evaluate the antioxidant activity of the chickpea protein extract and chickpea protein hydrolysates obtained by treatment with Alcalase®.

3.3.1. *DPPH free radical-scavenging activity*

The DPPH radicals, which are stable in ethanol, show maximum absorbance at 517 nm and have widely been used in the evaluation of antioxidant capacity. The resulting hydrolysate was freeze-dried and assayed for antioxidant activity using DPPH radical-scavenging activity. The radical scavenging activities of the CP and CPH, tested at different concentrations, are shown in Fig. 2A.

The results (Fig. 2A) clearly indicated that hydrolysate obtained by treatment with Alcalase® exhibited a strong DPPH free radical-scavenging activity. In the range of concentration tested, DPPH radical scavenging activity of chickpea protein was lower than protein hydrolysates. This result shows that proteins have the ability to act as an antioxidative compound but because of their large size, they cannot cross cellular membranes. Therefore, hydrolysis is required to generate smaller peptides that possess biological activity. The antioxidant activity of chickpea protein hydrolysate increased with increasing CPH concentration. This activity was lower than that of BHA at all concentrations tested but higher than that of protein isolate of chickpea. Our findings are in line with previous works reported by He Tang et al. (2009) who reported that the DPPH-scavenging activity increased with increasing buckwheat protein hydrolysates concentrations.

3.3.2. *Reducing power*

Reducing power is used to evaluate the capacity of antioxidant to donate an electron to free radical and to convert them into more stable compounds. In this assay, the ability of CP and CPH to reduce the Fe^{3+}/ferric cyanide complex to the ferrous form was determined. Therefore, the Fe^{2+} complex can be monitored by measuring the formation of Perl's Prussian blue at 700 nm.

Fig. 2B shows the reducing power activities (as indicated by the absorbance at 700 nm) of the CP and CPH compared with BHA as standard. The highest reducing power was found in BHA, followed by CPH and CP, respectively. Chickpea protein hydrolysate possessed reducing power with concentration-dependent effects. Compared with wheat germ protein hydrolysate, the reducing power of the chickpea protein hydrolysate was much higher (Zhu et al., 2006). Chickpea hydrolysate may exhibit donating capacity by neutralising and converting free radicals to more stable products and, thereby, may terminate the chain reactions initiated by free radicals (Ardestani & Yazdanparast, 2007).

The reducing power increased with increasing the concentrations. A similar observation has been reported on wheat germ protein hydrolysate (Zhu et al., 2006) and on chickpea protein (Li, Jiang, Zhang, Mu, & Liu, 2008).

3.3.3. *β-carotene bleaching inhibition activity*

Lipid oxidation products react with proteins causing their oxidation. Carbohydrates are also susceptible to oxidation, but they are less sensitive than lipids and proteins (Zhuang, Tang, & Yuan, 2013). In this study, lipid peroxidation inhibition activity of CP and CPH was determined by assessing their ability to inhibit oxidation of linoleic acid in an emulsified model system.

The antioxidative activities of CP and CPH measured by the β-carotene bleaching assay are represented in Fig. 2C. All hydrolysates inhibited the oxidation of β-carotene at different degrees. CPH showed significantly ($p < 0.05$) higher antioxidant activities (66.92%) than CP, and BHA displayed a better antioxidant activity than all hydrolysates at the same concentration. The antioxidant activity index of peptides or proteins in the free radical-mediated lipid peroxidation system is influenced by molecular size, chemical properties and electron transferring ability of amino acid residues in the sequence (Qian, Jung, & Kim, 2008).

3.3.4. *Chelating activity*

EDTA, a known metal ion chelator, was therefore used as reference to compare with it the chelating effect of CP and CPH. The ferrous ion-chelating effects of CP, CPH and EDTA are shown in Fig. 2D. The CPH displayed between 21.15% and 50.45%

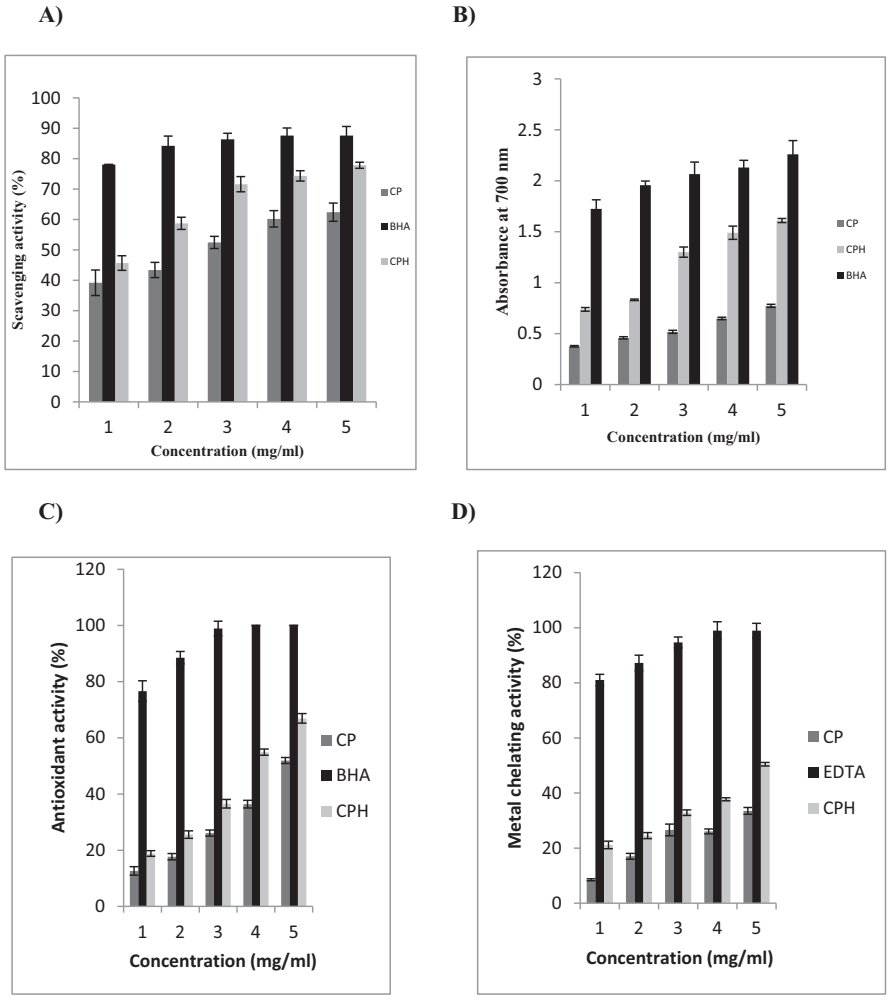

Fig. 2 – Antioxidant activities of CP and CPH at different concentrations. (A) DPPH scavenging activities. (B) Reducing power. (C) β-carotene bleaching inhibition. (D) Metal chelating activities. BHA and EDTA were used as positive controls (2 mM). All values are means ± standard deviation (±SD) of three determinations.

chelating effects on ferrous ion. Further, results reported in Fig. 2D show that metal chelating activity increased with increasing concentrations of hydrolysates and chickpea protein. However, the chelating ability was slightly lower than that of EDTA. For example at 5 mg/ml, the metal chelating activities of protein hydrolysate and EDTA were 50.45% and 98.96%, respectively. Although the chemical EDTA exhibited the highest ($p < 0.05$) metal chelating ability, natural antioxidants are of growing interest. Indeed, the incorporation of protein hydrolysate to foods could confer desirable nutritional and functional properties (Kim, Je, & Kim, 2007).

3.4. *Fractionation with Sephadex G-25 gel filtration*

During hydrolysis, a wide variety of smaller peptides and free amino acids are generated. Changes in size, level and composition of free amino acids and small peptides affect antioxidant activity (Kou et al., 2013).

The chickpea protein hydrolysate, obtained with a DH of 14.67%, was fractionated on a Sephadex G-25 gel filtration chromatography. As reported in Fig. 3, there are four major absorbance peaks (Fra.$_{I}$, Fra.$_{II}$, Fra.$_{III}$ and Fra.$_{IV}$) at 280 nm. Fractions associated with each peak were collected, concentrated by lyophilisation and then evaluated for antioxidant activity. All fractions (1 mg/ml) displayed antioxidant activities. Fraction Fra.$_{III}$ possessed the highest radical-scavenging activity (54%) among all fractions. At the same concentration of 1 mg/ml, Fra.$_{I}$, Fra.$_{II}$ and Fra.$_{IV}$ exhibited 1.21, 33.38 and 5.78% hydroxyl radical-scavenging activity, respectively. Because the direct correlation between antioxidant activity and reducing power of certain bioactive compounds, the reducing power test was investigated to evaluate the antioxidant activity of Fra.$_{II}$ and Fra.$_{III}$. At a concentration of 1 mg/ml, the absorbance at 700 nm was 0.857 for Fra.$_{III}$ followed by Fra.$_{II}$ ($A_{700} = 0.621$).

3.5. *Purification of the antioxidant peptides by HPLC*

Fraction Fra.$_{III}$, which exhibited the highest antioxidant activity, was further separated by RP-HPLC on a Waters C18 column and fractionated into 11 major sub-fractions (P_1–P_{11}). The elution profile of the peptides is shown in Fig. 4A. Fractions were collected separately through repeated chromatography using reversed-phase HPLC column and concentrated in vacuum prior

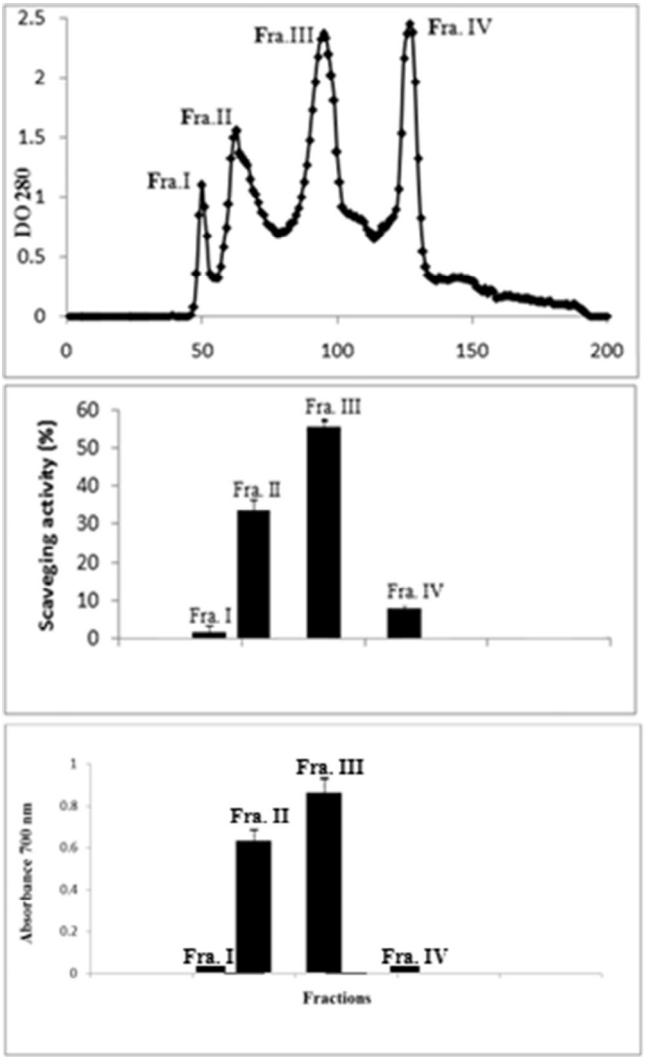

Fig. 3 – Elution profile of chickpea protein hydrolysates separated by size exclusion chromatography on Sephadex G-25 (upper panel) and the DPPH radical-scavenging activities and reducing power of the separated fractions (lower panel). The column (5.2 × 56 cm) was equilibrated and eluted with distilled water at a flow rate of 30 ml/h. Values presented are the mean of triplicate analyses.

to testing the antioxidant activities. As reported in Table 2, only, two peptide sub-fractions showed antioxidant activity and were designated P3 and P8. At 200 μg/ml, The P_8 had the highest DPPH radical-scavenging (67.32%). The other sub-fraction also showed good DPPH radical-scavenging activities (51.66%). These sub-fractions were further analysed for peptide identification.

Table 2 – Amino acid composition and antioxidant activity of HPLC fractions P_3 and P_8, at 200 μg/ml. The antioxidant activity was measured by DPPH radical-scavenging effect.

HPLC fractions	Amino acid composition	Molecular weight (Da)	DPPH scavenging (%)
P_3	Asp-His-Gly	327,33	51.66
P_8	Val-Gly-Asp-Ile	402,49	67.32

3.6. *Mass spectrometry analysis*

Antioxidative properties are related to composition, structure, hydrophobicity and amino acid sequence of the peptide.

Peptides in sub-fractions P_3 and P_8 were analysed by ESI-MS for molecular mass determination and ESI-MS/MS for the characterisation of peptides. Mass spectrum and ESI/MS/MS analysis of the purified antioxidant peptides from sub-fractions P3 and P8 were presented in Fig. 4B.

Two purified peptides were identified in sub-fractions P_3 (Asp-His-Gly) and P_8 (Val-Gly-Asp-Ile) (Table 2). Bioactive peptides usually contain 2–20 amino acid residues that could cross the intestinal barrier and exert biological effects (Shahidi & Zhong, 2008). Val-Gly-Asp-Ile displayed the highest DPPH radical-scavenging activity (67.32% at 200 μg/ml). In addition, sub-fraction P8 contains two hydrophobic peptides with Val at the N-terminal in the sequence. Hydrophobic peptides have the ability to enhance the solubility of peptide in lipid which facilitates the accessibility to hydrophobic radical species. Moreover, presence of valine at the N-terminal of peptide sequence has been reported to be antioxidative in an oil system (Chen, Muramoto, & Yamauchi, 1995). P_3 contained histidine residue within the sequence. Antioxidative activity of histidine-containing peptides has been reported (Murase, Nagao, & Terao, 1993; Park, Jung, Nam, Shahidi, & Kim, 2001; Uchida & Kawakishi, 1992). This activity may be attributed to the ability of this amino acid to convert radicals to stable molecules by donating electron via resonance structure (Rajapakse, Mendis, Jung, Je, & Kim, 2005). Both P_3 and P_8 contained aspartic acid .This acidic amino acid play an important role in the antioxidant activity by carboxyl groups in their side chains (Suetsuna, Ukeda, & Ochi, 2000). Previous research have also purified and identified the short-chain antioxidant peptides, e.g. peptides from Zizyphus jujuba protein hydrolysates (678.36 and 482.27 Da) (Memarpoor-Yazdi, Mahaki, & Zare-Zardini, 2013), corn gluten hydrolysates (375.46, 488.64 and 522.64 Da) (Zhuang et al., 2013) and sweet potato protein hydrolysates (640–930 Da) (Zhang et al., 2014). A peptide from chickpea protein hydrolysates with molecular weight of 717.37 Da and its amino acid sequence was identified as Asn-Arg-Tyr-His-Glu by Zhang, Li et al. (2011). These differences could be related to the origin of the used enzyme and hydrolysis conditions.

3.7. *Haemolytic activity of antioxidant peptides*

The haemolytic activity of all fractions was tested on bovine erythrocytes. Several concentrations for each peptide were tested. For all peptides, no haemolysis was observed. These results show that these peptides would be non-toxic even if used at high concentrations.

4. Conclusion

In this study, we purified and characterised, for the first time, two antioxidant peptides from chickpea protein isolates

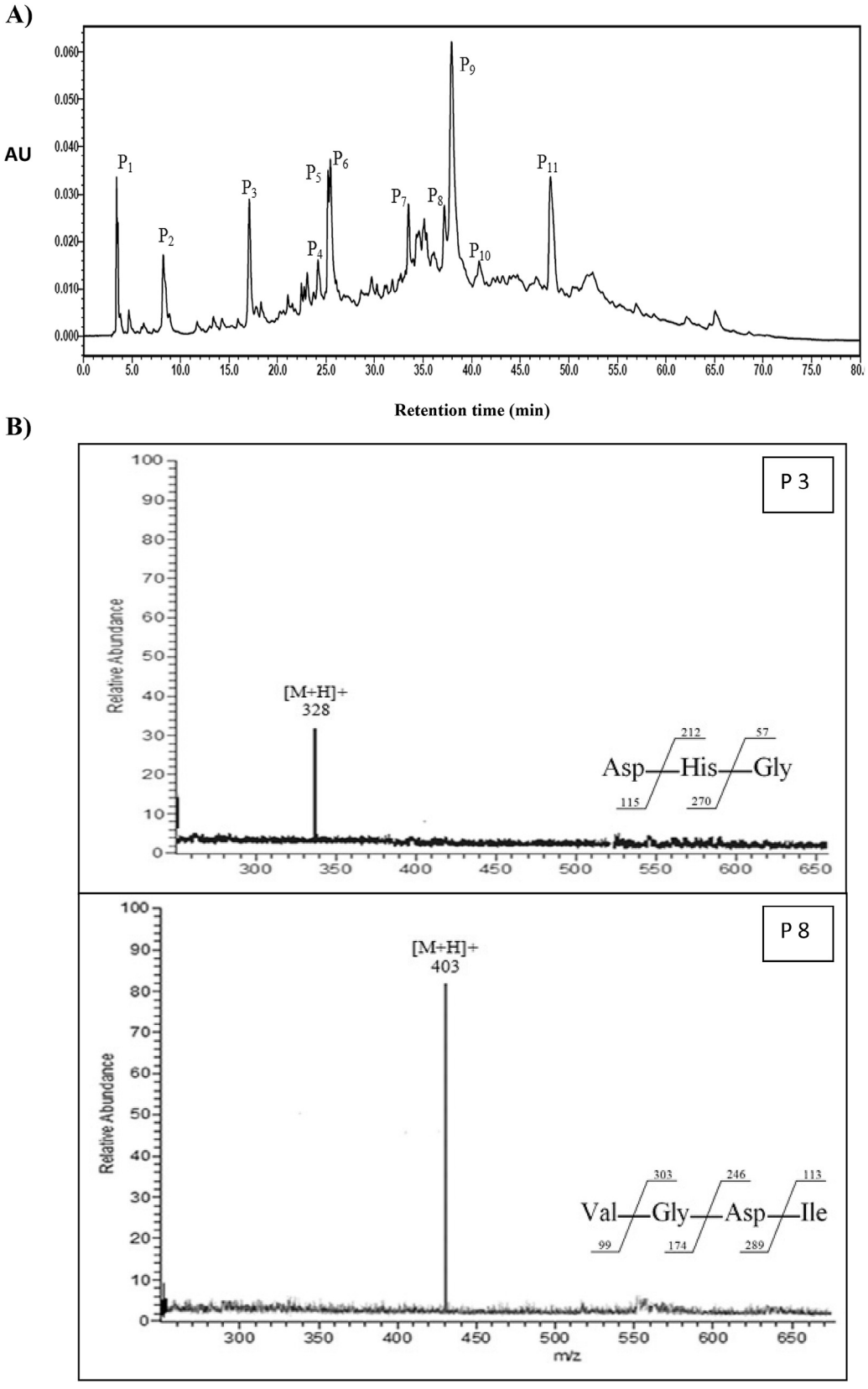

Fig. 4 – HPLC and MS spectrums: (A) Reversed-phase HPLC pattern on a Vydac C18 column of the Fraction.III obtained from Sephadex G-25 gel filtration. Flow rate, 1 ml/min; monitoring absorbance, 215 nm. (B) Mass spectrum and ESI/MS/MS analysis of the purified antioxidant peptides from sub-fractions P3 and P8.

hydrolysed by Alcalase®. The results of the present work indicate that Fra.III possessed high free radical scavenging activity and was fractionated into 11 major sub-fractions by HPLC. The molecular masses and amino acids sequences of the purified peptides were identified using ESI-MS and ESI-MS/MS, as Asp-His-Gly (327 Da) and Val-Gly-Asp-Ile (402 Da). The two purified peptides are new potent free radical scavengers that can be used as natural antioxidants in preventing the oxidation of related food. These results suggest that the peptide may be a promising antioxidant as functional food ingredients. However, further studies are required to investigate the in *vivo* antioxidant activities.

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