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) may produce different diseases such as Parkinson, Alzheimer, atherosclerosis, cancer and neurological degenerative as well as cardiovascular diseases. Therefore, the use of antioxidants may prevent the development of these diseases by counteracting ROS toxicity levels. In addition, there is an increasing interest in natural antioxidants due to they are safer for consumers than synthetic antioxidants as they occur in nature. In this work we have studied the antioxidant activity of peptide fractions purified by copper affinity and size exclusion chromatography from a chickpea seed protein hydrolysate. Moreover, peptide sequences included in fractions with antioxidant activity were identified. The main identified sequences were ALEPDHR, TETWNPNHPEL, FVPH and SAEHGSLH, corresponded to fragments of legumin, the main seed storage protein. Most of them contained histidine, which has shown antioxidant effects due to its imidazole ring that acts as donor and acceptor of protons. Furthermore, two peptides, in addition to histidine, included the aromatic residues tryptophan and phenylalanine, in which the phenolic group could also serve as hydrogen donor. Hence, these results show that legumin is a source of antioxidant peptides that may be of high interest for food and pharmaceutical industries to develop new nutraceuticals and functional food with antioxidant properties.

KEY WORDS: Chickpea, Protein hydrolysate, Antioxidant peptides, Chelating peptides, Peptide sequencing.

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 (Ito et al 1983, 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, Slavin & Lloyd 2012). 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 et al 1998, Marcuse 1960). Hence, several proteins and peptides from different plant sources, such as soybean, potato, sunflower or rapeseed, have shown antioxidant properties (Garcia et al 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 et al 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 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 et al 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 et al 1991). Moreover, chickpea proteins have demonstrated to be a notable source of bioactive peptides with antioxidant, ACE inhibitory and hypocholesterolemic activities (Li et al 2008, Pedroche et al 2002, Yust et al 2012, Yust et al 2003, Zhang et al 2011).

In our previous study, we have analyzed the antioxidant activity of peptide fractions previously purified by copper affinity and size exclusion chromatography from a chickpea protein hydrolysate produced by sequential hydrolysis with pepsin and pancreatin (Torres-Fuentes et al 2011). These peptides fractions have also shown the capability to inhibit the copper-mediated lipid peroxidation (Torres-Fuentes et al 2014). Thus, in this study, we have carried out a further analysis of their antioxidant activity which no copper implication which allow to a better understanding of their precise mechanism. Moreover, we have achieved the characterization of new amino acids sequences within the most active fractions by reversed phase-high-performance liquid chromatography coupled to tandem mass spectrometry (RP-HPLC-MS/MS).

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 Na2HPO4, 1.4 mM NaH2PO4, 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 ultra-gradient) from Teknokroma (Barcelona, Spain).

2.2. Methods

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 by affinity chromatography using a FPLC AKTA-purifier system (GE Healthcare, Buckinghamshire, United Kingdom) 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.

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, TCA 2.5% (w/v) (final concentration) was added. Afterward, the solution was incubated with ferric chloride 0.01% (w/v) at 50 °C for 10 min. Finally, absorbance was read at 700 nm. Blank sample included neither sample nor ferric chloride and positive control includedethe 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).

2.3. Free Radical Scavenging Activity (FRSA)

FRSA was analyzed using the stable free radical DPPH as described by Shimada et al. (Shimada et al 1992). Chickpea protein hydrolysate and peptide fractions were mixed with 0.1 mM DPPH in 95% ethanol (1:1, v/v) 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 was included as positive control. The FRSA was calculated as follow:

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

where A_0 is the absorbance of control at 517 nm and As 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.).

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) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco, Invitrogen), 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.

2.5. 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 x 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. Fluorescence emission at 555 nm was determined with excitation at 485 nm every 15 min during 1 h using a Fluoroskan Ascent plate-reader (Thermo Fisher Scientific) at 37°C. Positive control (cells treated only with ABAP) and negative control with untreated cells (only HBSS) were included. The CAA was expressed as follows:

$$\textbf{CAA unit} = 100 - (\int SA / \int CA) * 100$$

where ∫SA is the integrated area under the sample fluorescence versus time curve and ∫CA is the integrated area from the positive control curve. Assay was carried out at least in triplicate and data were analyzed using GraphPad Prism software (PRISM 5.0; GraphPAD Software Inc.).

2.6. Peptide sequencing by RP-HPLC-MS/MS

RP-HPLC-MS/MS analysis of the most antioxidant chickpea peptide fractions was performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) equipped with a quaternary gradient pumping system, a variable wavelength detector and an autosampler.. The HPLC system was connected to an Esquire 3000 quadrupole ion trap (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionization source as previously described (Contreras et al 2010).The most active

chickpea peptide fractions were applied to a XBridge BEH300 C_{18} column (250 x 4.6 mm d.i., 5 µm of particle size, Waters Corporation, Milford, MA, USA). Solvent A was a mixture of Milli-Q water and TFA (1000:0.37, v/v) and solvent B contained acetonitrile and TFA (1000:0.27, v/v). Peptides were eluted with a linear gradient of solvent B in A from 0% to 70% over 75 min, at a flow rate of 0.8 mL/min. Sample peptide content was 150 µg. UV detection was carried out at 214 nm. The flow was split postdetector in a proportion 1:3 by placing a T-piece (Valco, Houston, TX) connected to a 75-μm i.d. peek outlet tube of an adjusted length to give approximately 265 µL/min of flow entering directly into the mass spectrometer via the electrospray interface. Nitrogen was used as nebulizing and drying gas (60 psi, 8 L/min, 350ºC). Helium was used as collision gas with an estimated pressure of 5×10^{-3} bar. The capillary was held at 4 kV. Spectra were recorded over the mass/charge (*m*/*z*) range 100-1500. About 15 spectra were averaged in the MS analyses and about 5 spectra in the MS(n) analyses. The signal threshold to perform auto MS(n) analyses was 10000 and the precursor ions were isolated within a range of 4.0 *m*/*z* and fragmented with a voltage ramp going from 0.3 to 2.0 V. The software Compass HyStar (Bruker Daltonik GmbH) was used for analysis and data collection. Using Data Analysis (version 3.0; Bruker Daltoniks), the *m*/*z* spectral data were processed and transformed to spectra representing mass values. BioTools (version 3.1; Bruker Daltoniks GmbH) was used to process the MS(n) spectra and to perform peptide sequencing. The tool SequenceEditor™ was used to build a database with the sequences of proteins from chickpea and other leguminous plants, which were obtained from UniProtKB (http://www.uniprot.org/help/uniprotkb).

2.7. Statistical analysis

Statistical analyses were performed using SPSS software (IBM SPSS statistics 20). One-way analysis of variance (ANOVA) followed by Bonferroni's Post Hoc Test was carried out to determine significant statistical differences in reducing power and free radical scavenging activity analysis. ANOVA followed by LSD Post Hoc Test was used to determine significant statistical differences in cellular antioxidant activity analysis. Statistical significances are subsequently depicted as follows: *indicating $p\leq0.05$, ** indicating $p\leq0.01$ or *** indicating $p\leq0.001$.

3. RESULTS

3.1. Reducing Power

The reducing power is the capability of certain biomolecules to act as donors of electrons. The antioxidant activity of a compound and its reducing power are related (Gulcin 2012). Hence, the determination of the reducing power is widely used to evaluate the antioxidant activity of a compound. In this study, the reducing power of chickpea protein hydrolysate and peptide fractions was analyzed through the determination of their capability to reduce Fe^{3+} to Fe^{2+} by monitoring the formation of ferric ferrocyanide at 700 nm (Ferreira et al 2007).

Figure 1A shows the reducing power, expressed as absorbance at 700 nm, of chickpea protein hydrolysate and peptide fractions, F1, F2 and F3, purified after copper affinity chromatography. Chickpea protein hydrolysate was assayed at 100-fold higher concentration (25 mg/mL) than the peptide fractions and it showed a reducing power of 30.3% as compared to standard BHT (250 µg/mL). Chickpea peptide fractions, F1, F2 and F3, showed a reducing power of 9.2, 22.6 and 29.4%, respectively, as compared to BHT at the maximum assayed concentration (250 µg/mL).

The reducing power of size exclusion peptide subfractions purified from F1, F2 and F3 was also studied. Peptide subfractions purified from F1 did not show absorbance at 700 nm and, therefore, no reducing power was observed. Only F1D showed a slight activity which was no significant as compared to BHT (**Figure 1B**). Peptide subfractions F2B to F2D showed an absorbance at 700 nm comparable to BHT and similar reducing power, which explain the activity found in fraction F2. Concretely, F2C and F2D were the most active fractions with a significant increase in reducing power compared to BHT at 12 µg/mL ($p<0.001$) and 25 µg /mL ($p<0.05$), respectively (**Figure 1C**). Finally, only F3D and F3E showed reducing power activity within peptide subfractions purified from F3 and, therefore, they were identified as the responsible of the activity found in F3 (**Figure 1D**). Hence, these subfractions showed a significant increase in reducing power at 12 µg/mL compared to BHT ($p<0.05$ and $p<0.001$, respectively).

3.2. Free Radical Scavenging Activity

Free radical scavenging assay has been widely used in natural antioxidant studies due to its simple and highly sensitivity and it is based on the theory that a hydrogen donor is an antioxidant. DPPH radical is characterized by a delocalization of the spare electron which produces a deep violet color and shows an absorption maximum at 517 nm in ethanol solution. This distinctive color of the radical form

turns to yellow upon absorption of hydrogen from an antioxidant (Shimada et al 1992). This reaction is stoichiometric with respect to the number of hydrogen atoms absorbed. Therefore, the antioxidant effect can be easily evaluated by following the decrease of UV absorption at 517 nm (Moon & Shibamoto 2009).

Figure 2 shows the free radical scavenging activity for the synthetic antioxidant BHT, chickpea protein hydrolysate and peptide fractions and subfractions purified by affinity and size exclusion chromatography, respectively. Chickpea protein hydrolysate exhibit antioxidant activity but it showed 2-fold lower efficacy (Emax) and 1000-fold higher half maximal effective concentration (EC_{50}) compared to BHT (**figure 2A**). On the other hand, chickpea peptide fractions purified by copper affinity chromatography, F1, F2 and F3, showed a free radical scavenging activity of approximately 35, 31 and 51% as compared to BHT, respectively, at assay concentration (0.1 mg/mL) (**figure 2B**).

Free radical scavenging activity of chickpea peptide subfractions purified by size exclusion chromatography from F1, F2 and F3 were also analysed. Within peptide fractions purified from F1, only F1D showed a considerable free radical scavenging activity of 73.2% (**figure 2C**). On the other hand, F2B, F2C and F2D were the most active within peptide fractions purified from F2 (**figure 2D**) and showed a free radical scavenging activity around 87%. The activity in all these cases was significantly lower ($p \leq 0.001$) compared to BHT. Finally, within peptide fractions purified from F3, it should be pointed out that F3D and F3E showed a similar activity than BHT with a free radical scavenging activity around 95% (**figure 2E**).

3.3. Cellular Antioxidant Activity

Cellular antioxidant activity was determined using the DCFH-DA assay, which is a fluorescence method to detect intracellular reactive oxygen species generation (Hipler et al 2001). The non-fluorescent DCFH-DA diffused into the cell where is deacetylated by cellular esterases to form the non-fluorescent DCFH, which is trapped inside of the cells due to its more polar nature. In presence of ROS, DCFH is oxidized to the fluorescent DCF. To increase ROS production, cells are treated with ABAP which is able to diffuse into cells and spontaneously form peroxyl radicals. Although ABAP is not a relevant physiological compound, the peroxyl-like radicals that are generated from its breakdown are very abundant *in vivo*. Therefore, ABAP has been widely used in cell-based antioxidant activity studies where it has shown an increased DCFH oxidation in a dose-response manner (Adom & Liu 2005, Garrett et al 2010, Stoddard et al 2013, Wolfe & Liu 2007). Peroxyl-like radicals attack the cell membrane producing

more radicals and oxidizing the intracellular DCFH to the fluorescent DCF (Wolfe & Liu 2007). Antioxidants prevent ABAP breakdown and ROS production reducing oxidation of DCFH and membrane lipids and, thus, decreasing the formation of the fluorescent DCF.

Figure 3 shows the inhibition of peroxyl radical-induced oxidation of DCFH to the fluorescent DCF in Caco-2 cells by chickpea protein hydrolysate (**figure 3A**) and its dose–response curve over 60 min (**figure 3B**). Chickpea protein hydrolysate inhibited DCFH oxidation in a dose-response manner up to a dose of 5 mg/mL. Thus, a decreased fluorescence from DCF and an increased CAA unit was observed at higher hydrolysate protein concentrations. Cellular antioxidant activity of chickpea peptide fractions is shown in **figure 4**. F2 and F3 peptide fractions purified by affinity chromatography showed a higher fluorescence decrease than F1 and, therefore, a higher antioxidant activity (**figure 4A**). Also, cellular antioxidant activity of the most antioxidant peptide fractions in previous assays from F1 (**figure 4B**), F2 (**figure 4C**) and F3 (**figure 4D**) was also studied. All these peptide fractions showed inhibition of peroxyl-induced oxidation of DCFH, and therefore a decreased fluorescence, compared to the positive control over 60 min incubation.

Finally, CAA unit of peptide fractions was calculated and compared to that of the chickpea protein hydrolysate (**figure 5**). Within peptide fractions purified by affinity chromatography, F2 and F3 showed a significant increased CAA unit (**P≤0.01) compared to chickpea hydrolysate protein while F1 did not show significant differences. Regarding the peptide subfractions purified by size exclusion chromatography, only the most active in reducing power and free radical scavenging assays were analysed. Thus, F1D, F1E, F2B and F2C showed the highest CAA unit compared to chickpea hydrolysate protein (***P≤0.001) followed by F2D (**P≤0.01), F3D (**P≤0.01) and F1F (**P≤0.05).

3.4. Peptide sequencing by RP-HPLC-MS/MS

The characterization of the most active chickpea peptide subfractions purified by size exclusion chromatography was carried out by RP-HPLC-MS/MS and these results are summarised in **Table 1**. A total of 11 different peptide sequences were successfully characterized as fragments of legumin, one of the major chickpea seed storage proteins (Chang et al 2012). Four peptides matched well with fragments of provicilin that has been evidenced at transcript level but not at protein level (see UniProtKB), and therefore, the assignment of the fragments was just tentative. Since chickpea seed proteome is not at all completely established, several peptides were partially sequenced taking into account Fabaceae seed proteins. It was found three and eight peptides that matched with fragments from 11S globulins legumin

A and legumin J, respectively, from *Pisum sativum, and* one peptide from legumin type B from *Vicia faba*. Moreover, a total of two and five peptides were sequenced as of 7S globulins vicilin and convicilin from *Pisum sativum*, respectively, and two from *Phaseolus vulgaris* phaseolin, □-type. It seems that these sequences could be highly conserved along legumes.

Among the identified sequences, those corresponding to peptides with major relative abundance in each fraction may be relevant for the observed activity. These sequences are shown in bold in **table 1** and were ALEPDHR in subfraction F1D, TETWNPNHPEL in F2B, FVPH in F2C, F2D, F3D and F3E, and SAEHGSLH in F3C. In the case of the subfractions F1E and F1F, the chromatographic profile over the retention time and the MS signal over the *m/z* were homogeneous and no major ion was found.

As example, **Figure 6** shows the MS/MS spectrum of the major molecular ions found along the chickpea subfractions. For clarity, only b and y fragment ions resulting from the cleavage of peptide bonds were labelled following the nomenclature of Roepstorff & Fohlman (Roepstorff & Fohlman 1984). Specifically, b ions are those where the charge remains with the N-terminal portion of the peptide ion and y ions are those product ions in which the charge is retained on the C-terminal portion of the ion (Roepstorff & Fohlman 1984). Overall, most abundant ions in the MS/MS spectrums of these peptide fragments correspond with b and y ions. Moreover, general rules previously described about peptide fragmentation helped us to assess the assignment of the peptides. In this regard, it is known that the presence of proline in a peptide favours cleavages of peptide bonds N-terminally to this residue (Breci et al 2003), e.g. product ions b_3 and y_4 in ALEPDHR (**figure 9A**), y_6 and b_8 in TETWNPNHPEL (**figure 9B**) and y_2 in FVPH (**figure 9C**). Furthermore, position of basic residues influences fragmentation (Contreras et al 2010, Tabb et al 2004). As example, several *b* and *y* product ions were formed surrounding of histidine residues in the peptide SAEHGSLH (**figura 9D**).

Interestingly, several sequences such as AHH and FVPH were identified along different fractions. It could be explained by the chromatography selectivity obtained by the previous purification processes by affinity and size exclusion chromatography (see (Torres-Fuentes et al 2011)). In the affinity chromatography step, peptides were separated according to their copper affinity by pH gradient. Hence, peptides eluted progressively along a pH gradient and reached a maximum elution and maximum abundance at a specific pH but part of these peptides could elute with lower abundance in other different fractions. Afterwards, in the size exclusion chromatography, peptides were eluted according to their molecular size. In this manner, peptides with similar size eluted at similar time reaching a maximum

elution at specific time. However, for low molecular weight peptides, it is possible that they elute in different subfractions with different abundance. Nevertheless, the contribution to the global antioxidant activity of the active subfractions of these peptides depends on their relative abundance.

4. DISCUSSION

In a previous work, we have purified different peptide fractions from a chickpea protein hydrolysate by copper affinity and size exclusion chromatography (Torres-Fuentes et al 2011). Some of these fractions showed copper (Torres-Fuentes et al 2011) and iron chelating (Torres-Fuentes et al 2012) activities and inhibition of copper-mediated lipid peroxidation process (Torres-Fuentes et al 2014). However, peptide sequences were not determined. In the present work we have further investigated the antioxidant activity of these peptide fractions and by a no metal chelation-mediated manner. In addition, 48 different peptide sequences were identified within the most active fractions using RP-HPLC-MS/MS (**Table 1**). Others studies have also shown antioxidant properties of chickpea proteins (Arcan & Yemenicioglu 2007, Kou et al 2013, Li et al 2008, Megias et al 2007, Torres-Fuentes et al 2014, Yili et al 2012, Yust et al 2012, Zhang et al 2012, Zhang et al 2011) but only few of them identified the potential bioactive peptides (Kou et al 2013, Yili et al 2012, Zhang et al 2011). Moreover, this information is also scarce in others plant sources (Garcia et al 2013) because plant proteins are not completely sequenced and sometimes no full genome sequence available. Furthermore, whereas most of the studies employed commercial non-digestive enzymes, such as alcalase and flavourzyme (Kou et al 2013, Li et al 2008, Yust et al 2012) to produce chickpea hydrolyzates, we used digestive enzymes. It is interesting since the potential antioxidant peptides characterized might be relevant in vivo as similar peptides could be released during the gastro intestinal digestion of chickpea proteins.

The most active chickpea peptide subfractions were F1D, F2B, F2C, F2D, F3D and F3E and they contained numerous peptides fragments (**Table 1**). However, major peptides from each subfraction may be the most relevant to the total activity due to their abundance. Hence, the most abundant sequences were ALEPDHR in F1D, TETWNPNHPEL in F2B, FVPH in F2C, F2D, F3D and F3E and SAEHGSLH in F3C, all of them belonging to the seed storage protein legumin (**Table 1**). These peptides are rich in amino acids with antioxidant properties: hydrophobic amino acids (Mendis et al 2005), acidic amino acid residues (Saiga et al 2003) and basic residues (Li et al 2011, Saiga et al 2003). In addition, the specific position of amino acids in the peptide sequence may also contribute to its bioactivity. Thus, hydrophobic amino acids at both N-terminus and C-terminus are considered significant (Chen et al 1995, Li & Li 2013,

Tsuge et al 1991). Moreover, polar/charged amino acids such as histidine (H) or arginine (R) at the C-terminus position also contribute to the antioxidant activity (Li & Li 2013). Hence, these sequences may be important for the antioxidant activity of these subfractions Moreover, these subfractions was previously reported to show copper and iron chelating activities (Torres-Fuentes et al 2011, Torres-Fuentes et al 2012) and capability to inhibit copper mediated oxidation in β-carotene and LDL lipid systems (Torres-Fuentes et al 2014). Hydrophobic amino acids are particularly important for antioxidant effects in lipid systems as they increase lipid solubility (Li et al 2011, Saiga et al 2003). In the case of the cellular antioxidant activity, high lipid solubility may be decisive to show antioxidant effects as peptides are able to bind to the cell membrane and/or pass through it. Therefore, they may act preventing the peroxyl radicals-mediated cell membrane oxidation and/or inhibiting DCFH intracellular oxidation (Wolfe & Liu 2007). On the other hand, aspartic acid and glutamic acid may be important for inhibition of metal-mediated oxidation process and metal chelating properties due to their capability to bind metals by their charged residues (Saiga et al 2003, Torres-Fuentes et al 2014). Finally, the basic amino acids histidine and arginine contribute to the antioxidant effects though the capability of these amino acids to act as donors or acceptors of protons through their imidazole (Kohen et al 1988, Murase et al 1993, Suetsuna et al 2000) and guanidine groups (Miliutina et al 1990, Nigris et al 2003, Wallner et al 2001) respectively. In particular, histidine has shown strong radical scavenging activity due to its imidazole ring decomposition (Yong & Karel 1978). Moreover, histidine may be the main amino acid implicated in the metal chelating activity and inhibition of metal-mediated oxidation process as it may bind metals through its imidazole ring (Burkitt 2001). In addition, F2C and F2D, F3D and F3E were the most metal chelating and lipid antioxidant fractions in overall (Torres-Fuentes et al 2011, Torres-Fuentes et al 2012) and, therefore, FVPH may be one of the most relevant peptides for antioxidant activity.

According with these results, the single charged ion m/z 110 is observed in the mass spectrum of each fraction. This ion matches with the immonium ion for histidine. These kind of ions correspond with the internal fragment with a single chain generated by a combination of *a* type and *y* type cleavage and it is specific for each amino acid (Medzihradszky 2005). Therefore, its presence in the mass spectrum indicates these peptide fractions contain histidine. Moreover, this ion was more intense along fractions purified from F3 which was the fraction that showed higher copper affinity. Hence, this verifies that histidine plays a crucial role in the peptide binding to the copper column during the affinity chromatography. Indeed, most of the sequences identified contained at least one histidine. Therefore,

histidine may be the main amino acid implicated in the antioxidant activity of these peptide fractions. Several histidine-containing peptides have shown antioxidant properties (Chen et al 1998). Some examples are VNPHDHQN, LVNPHDHQN, LLPHH, LLPHHADADY, and LNSGDALRVPSGTTYY isolated from soy bean hydrolysate (Chen et al 1995), carnosine (beta-alanyl-L-histidine) (Decker et al 1992) and AH, VHH, and VHHANEN isolated from egg white albumin hydrolysate (Tsuge et al 1991). Various peptide sequences identified along chickpea peptide subfractions are similar to some of these histidine-containing peptides: LLPH included in F2C and AHH included in F1E, F1F, F2D, F3D and F3E (**table 1**).

5. CONCLUSIONS

In this study, we have demonstrated that peptide fractions from chickpea purified by copper affinity and their derived subfractions by size exclusion chromatography have antioxidant properties, exhibiting different modes of action, such as donating electrons and hydrogen and scavenging peroxyl-like radicals. Moreover, some of these fractions showed a potent activity, as effective as the synthetic antioxidant BHT. Moreover, several peptides sequences were successfully characterized from the most antioxidant chickpea peptide subfractions. In overall, identified peptide fragments were rich in hydrophobic and polar amino acids, which have previously shown antioxidant effects. In particular, histidine was one of the most abundant amino acids appearing in the majority of the identified peptides and explaining in part the antioxidant activity. This is in accordance with the purification process by copper affinity since this amino acid is able to bind metals by its imidazole ring. These sequences also presented aromatic amino acids that also could contribute to the antioxidant activity. In addition, most antioxidant peptides will be very abundant in chickpea seeds as they belong to the major seed storage protein legumin.

This work provides new bioactive peptide sequences obtained from plant products. This is important to better understand the correlation between bioactivity (antioxidant) and peptides structure. The characterization and identification of bioactive peptides is also important as it may allow their incorporation into functional food with beneficial health effects. Therefore, this study may be of high interest for the food industry to develop new functional and medicinal food with antioxidant properties.

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Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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TABLES LEGENDS

Table 1. Identification of peptides contained in the most antioxidant chickpea peptide subfractions. Most abundant peptides in each subfraction are given in bold.

FIGURE LEGENDS

Figure 1. Reducing power activity of the synthetic antioxidant butylated hydroxytoluene (BHT), chickpea protein hydrolysate (CPH), chickpea peptides fractions purified by copper affinity chromatography (F1, F2 and F3) (**A**) and chickpea peptides subfractions purified by size exclusion chromatography from F1 (**B**), F2 (**C**) and F3 (**D**). Graph represents the mean ± SEM with each concentration point performed in duplicate. Reducing power was depicted as absorbance at 700 nm. Significant increased reducing power is depicted as ***P≤0.001, *P≤0.05 as compared to BHT (one-way ANOVA followed by Bonferroni post hoc test).

Figure 2. Free Radical Scavenging Activity of the synthetic antioxidant butylated hydroxytoluene (BHT) and chickpea protein hydrolysate (CPH) (**A**), chickpea peptides fractions purified by copper affinity chromatography (F1, F2 and F3) (**B**) and chickpea peptides subfractions purified by size exclusion chromatography from F1 (**C**), F2 (**D**) and F3 (**E**). Graph represents the mean ± SEM with each point performed in duplicate. Free Radical Scavenging Activity was depicted as absorbance at 700 nm. Significant decreased reducing power is depicted as ***P≤0.001 as compared to BHT (one-way ANOVA with Bonferroni correction post hoc test, $p<0.05$). All peptides fractions assayed at 0.1 mg/mL.

Figure 3. Cellular Antioxidant Activity of chickpea protein hydrolysate in caco-2 cells (**A**) and its dose–response curve (**B**) over 60 min. Graph represents the mean ± SEM (n=4).

Figure 4. Cellular antioxidant activity of chickpea peptide fractions purified by copper affinity chromatography (F1, F2 and F3) (**A**) and chickpea peptides subfractions purified by size exclusion chromatography from F1 (**B**), F2 (**C**) and F3 (**D**) over 60 min. Graph represents the mean ± SEM (n=3). Peptides fractions: 0.3 mg/mL.

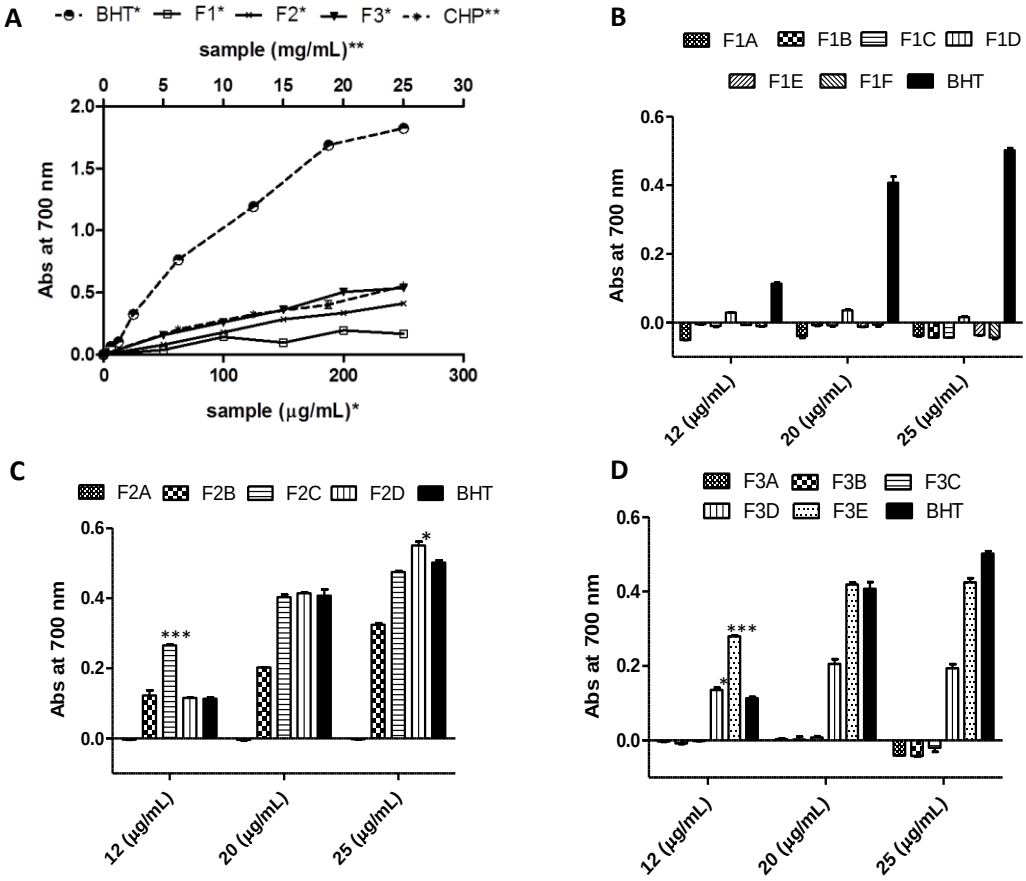
Figure 5. Cellular antioxidant activity of chickpea protein hydrolysate (CPH), chickpea peptide fractions purified by copper affinity chromatography (F1, F2 and F3) and size exclusion chromatography (F1D, F1E, F1F, F2B, F2C, F2D, F3C, F3D and F3E). Graph represents the mean ± SEM (n=3). All peptides fractions assayed at 0.3 mg/mL. CPH: 0.5 mg/mL. Significant increased CAA is depicted as ***P≤0.001, **P≤0.01, *P≤0.05 as compared to CPH (one-way ANOVA followed by LSD post hoc test). CPH: 0.5 mg/mL. Peptide fractions: 0.3 mg/mL.

Figure 6. Tandem mass spectrum of main characterized sequences. (**A**) Singly charged ion m/z 837.2 included in F1D. (**B**) Singly charged ion m/z 1337.3 included in F2B. (**C**) Single charged ion m/z 499.1 included in F1E, F1F, F2C, F2D, F3C, F3D and F3E. (**D**) Single charged ion m/z 837.1 included in F3C. Following sequence interpretation and data base searching, peptides were identified as Legumin J f(18-24), Legumin J f(32–42), Legumin f(350-353) and Legumin A f(359-366) respectively. The sequences of these peptides are displayed with the fragment ions observed in the spectra. Fragment ions are labelled according to the nomenclature proposed by Roepstorff and Fohlman (1984). For clarity, only b and y product ions are labelled.

Table 1

	Ion (m/z)[a]	Observed mass	Calculated mass[b]	Protein	Sequence[c]
	742.1 (1)	741.3	741.1	Legumin (302-308)	HQNIGSS
F1D	**837.2 (1)**	**836.4**	**836.2**	**Legumin J (18-24)**	**ALEPDHR**
	842.1 (1)	841.4	841.1	Provicilin (271-277)	KNPQLQD
	364.3 (1)	363.1	363.3	Phaseolin (380-382)	AHH
F1E	499.2 (1)	498.2	498.2	Legumin (450-453)	FVPH
	551.3 (1)	550.3	550.3	Legumin (363-367)	YALKG
	428.2 (1)	427.2	427.3	Provicilin (149-151)	RPR
F1F	364.3 (1)	363.1	363.3	Phaseolin (380-382)	AHH
	499.2 (1)	498.2	498.2	Legumin (450-453)	FVPH
	897.3 (1)	896.4	896.3	Legumin (162-169)	LAGNHEQE
	837.2 (1)	836.4	836.2	Legumin A (359-366)	SAEHGSLH
	896.3 (1)	895.4	895.3	Vicilin (59-66)	LPQHTDAD
	837.3 (1)	836.4	836.3	Legumin J (18-24)	ALEPDHR
	1060.3 (1)	1059.4	1059.3	Legumin (161-169)	YLAGNHEQE
F2B	908.3 (1)	907.4	907.3	Convicilin (178-185)	LPQHIDAD
	1224.3 (1)	1223.5	1223.3	Legumin J (32-41)	TETWNPNHPE
	1096.3 (1)	1095.5	1095.3	Legumin J (60-69)	HLPSFSPSPQ
	1337.3 (1)	**1336.6**	**1336.3**	**Legumin J (32-42)**	**TETWNPNHPEL**
	1450.4 (1)	1449.6	1449.4	Legumin J (31-42)	LTETWNPNHPEL
	1266.4 (1)	1265.6	1265.4	Legumin J (58-69)	GLHLPSFSPSPQ
	904.2 (2)	1805.9	1806.4	Legumin J (53-69)	TIDPNGLHLPSFSPSPQ
	642.1 (1)	641.3	641.1	Legumin (105-109)	RDSHQ
	721.1 (1)	720.2	720.1	Convicilin (110-115)	EGEEEE
	697.1 (1)	696.3	696.1	Legumin type B (181-185)	QERHQ
	537.1 (1)	536.3	536.1	Legumin (274-277)	RQPH
	600.1 (1)	599.3	599.1	Legumin (345-349)	HKNAM
F2C	789.1 (1)	788.3	788.1	Provicilin (320-325)	RNENEQ
	629.1 (1)	628.3	628.1	Legumin (351-355)	VPHYN
	499.0 (1)	**498.2**	**498.0**	**Legumin (350-353)**	**FVPH**
	688.1 (1)	687.1	687.4	Convicilin (97-101)	QREKK
	479.1 (1)	478.3	478.1	Vicilin (271-274)	LLPH
	560.1 (1)	559.3	559.1	Legumin (341-347)	FGSLH
	714.2 (1)	713.3	713.2	Provicilin (294-299)	LPHFNS
	364.2 (1)	363.1	363.3	Phaseolin (380-382)	AHH
F2D	629.2 (1)	628.3	628.2	Legumin (351-355)	VPHYN
	499.1 (1)	**498.2**	**498.1**	**Legumin (350-353)**	**FVPH**
	560.2 (1)	559.3	559.2	Legumin (341-345)	FGSLH
	750.1 (1)	749.3	749.1	Legumin A (360-366)	AEHGSLH
	742.1 (1)	741.3	741.1	Legumin (302-308)	HQNIGSS
	837.1 (1)	**836.4**	**836.1**	**Legumin A (359-366)**	**SAEHGSLH**
F3C	706.1 (1)	705.4	705.1	Legumin J (37-42)	PNHPEL
	700.1 (1)	699.3	699.1	Legumin A (359-365)	SAEHGSL
	499.1 (1)	498.2	498.1	Legumin (350-353)	FVPH
	692.1 (1)	691.3	691.1	Legumin (307-313)	SSSSPDI
	803.2 (1)	802.5	802.2	Convicilin (445-452)	IIPAGHPV
	364.2 (1)	363.1	363.2	Phaseolin (380-382)	AHH
	537.3 (1)	536.3	536.3	Legumin (258-261	RQPH
	742.3 (1)	741.3	741.3	Legumin (302-308)	HQNIGSS
	837.3 (1)	836.4	836.3	Legumin A (359-366)	SAEHGSLH
F3D	366.3 (1)	365.2	365.3	Convicilin (389-391)	LPH
	629.3 (1)	628.3	628.3	Legumin (351-355)	VPHYN
	499.1 (1)	**498.2**	**498.1**	**Legumin (350-353)**	**FVPH**
	688.4 (1)	687.4	687.4	Convicilin (50-54)	QREKK
	560.3 (1)	559.3	559.3	Legumin (341-345)	FGSLH
	714.4 (1)	713.3	713.4	Provicilin (294-299)	LPHFNS
	627.4 (1)	626.3	626.4	Provicilin (294-298)	LPHFN
	364.2 (1)	363.1	363.2	Phaseolin (380-382)	AHH
	633.2 (1)	632.3	632.2	Phaseolin (310-315)	IKATSN
F3E	629.2 (1)	628.3	628.2	Legumin (351-355)	VPHYN
	499.1 (1)	**498.2**	**498.1**	**Legumin (350-353)**	**FVPH**
	457.2 (1)	456.2	456.2	Legumin J (136-139)	HGDE
	714.2 (1)	713.3	713.2	Provicilin (294-299)	LPHFNS

Figure 1


A
BHT*
F1*
F2*
F3*
CHP**
sample (mg/mL)**
Abs at 700 nm
sample (µg/mL)*
B
F1A
F1B
F1C
F1D
F1E
F1F
BHT
Abs at 700 nm
12 (µg/mL)
20 (µg/mL)
25 (µg/mL)
C
F2A
F2B
F2C
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BHT
Abs at 700 nm
D
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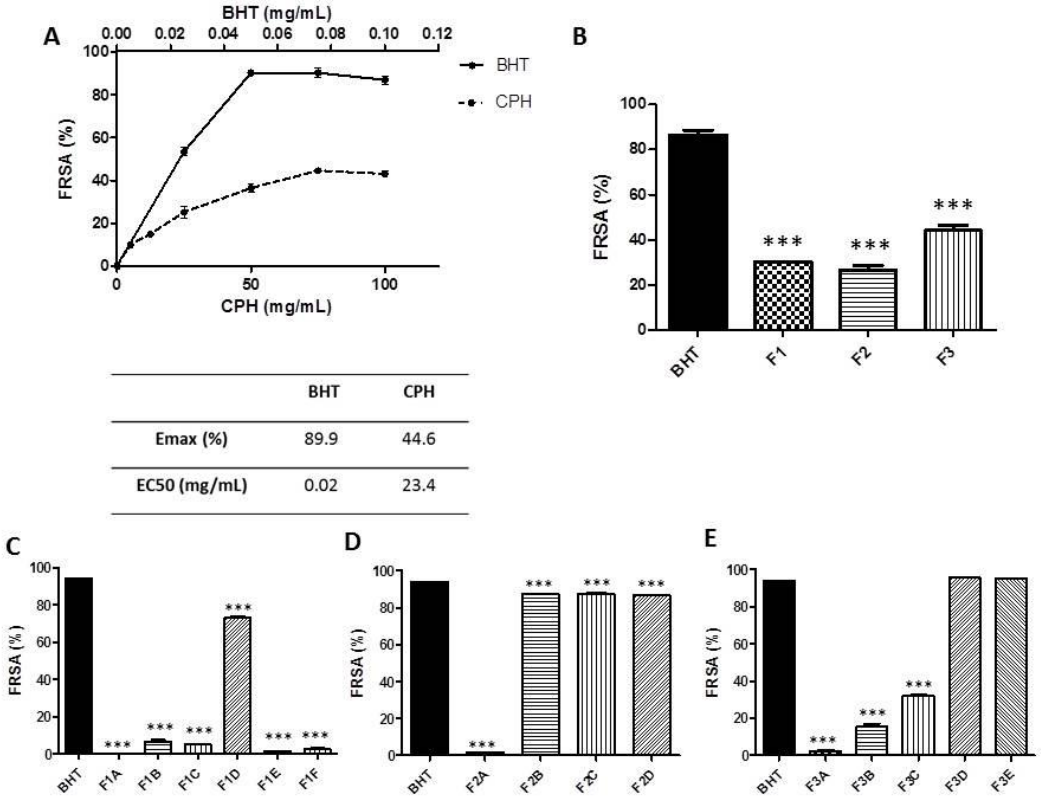
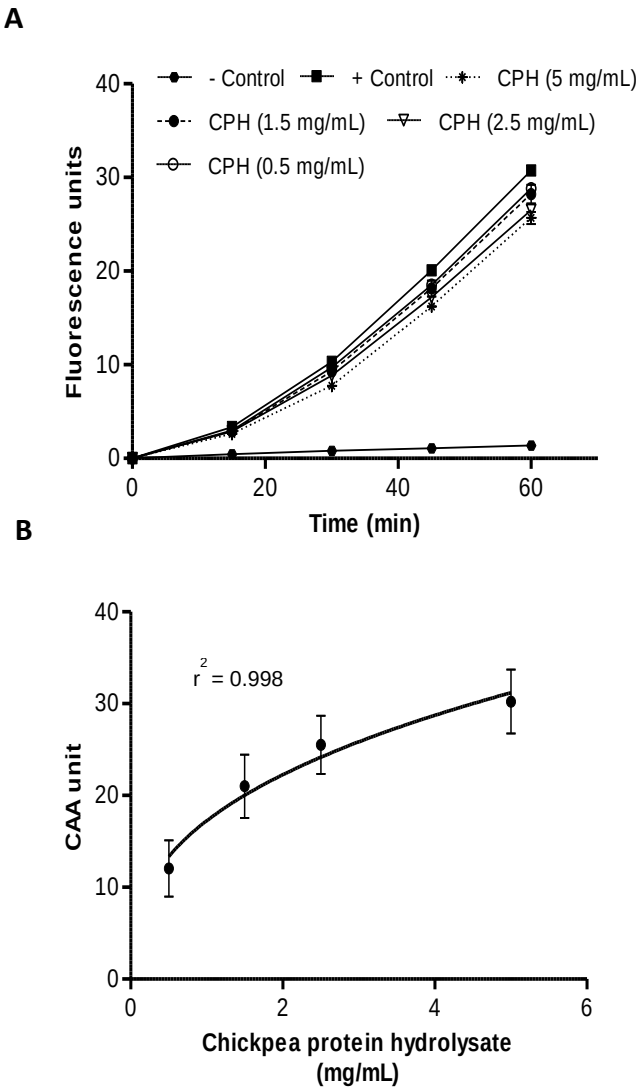
F3C
F3D
F3E
BHT
Abs at 700 nm

Figure 2



	BHT	CPH
Emax (%)	89.9	44.6
EC50 (mg/mL)	0.02	23.4

Figure 3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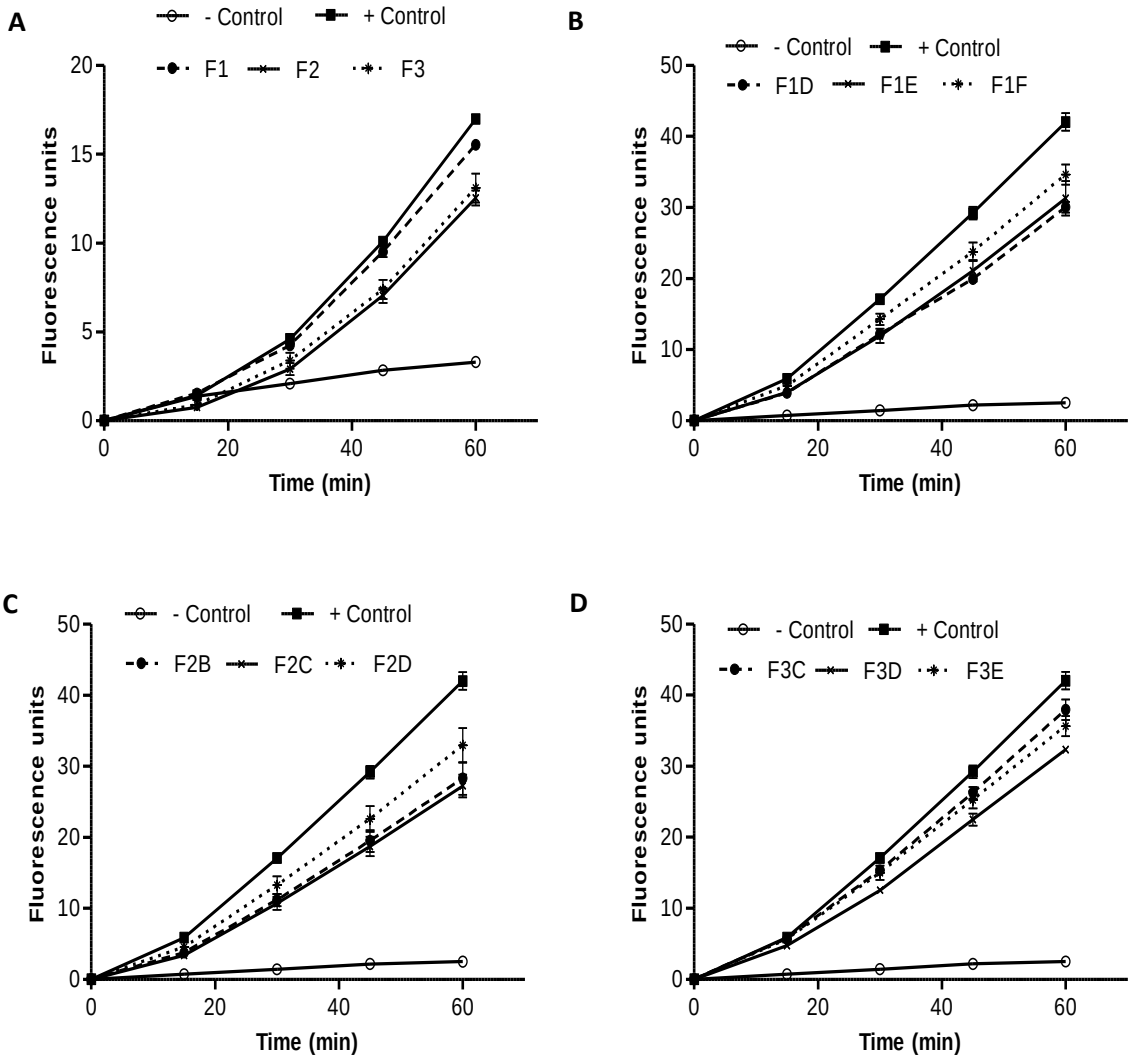
+ Control
CPH (5 mg/mL)
CPH (1.5 mg/mL)
CPH (2.5 mg/mL)
CPH (0.5 mg/mL)
Fluorescence units
Time (min)
B
r² = 0.998
CAA unit
Chickpea protein hydrolysate (mg/mL)

Figure 4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Time (min)
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- Control
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F1D
F1E
F1F
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Time (min)
C
- Control
+ Control
F2B
F2C
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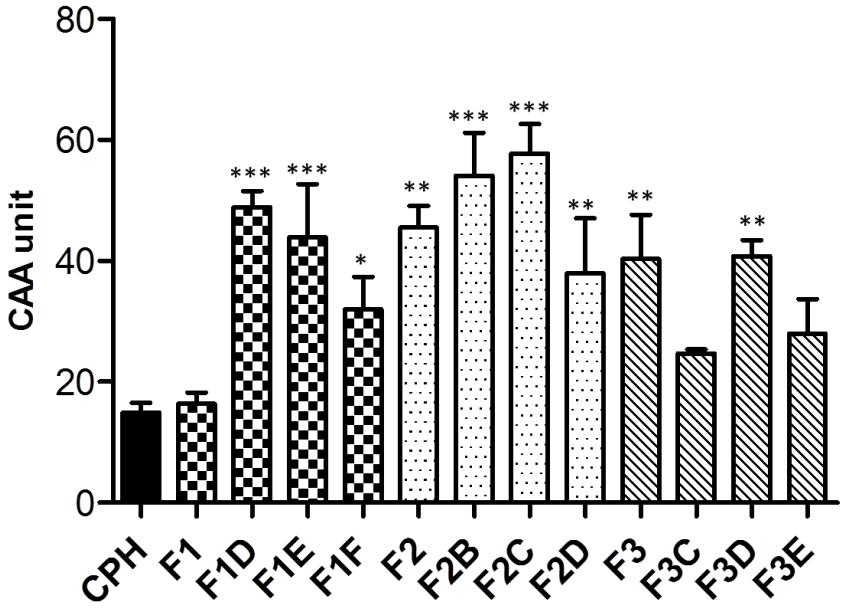
F3E
Fluorescence units
Time (min)

Figure 5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CPH
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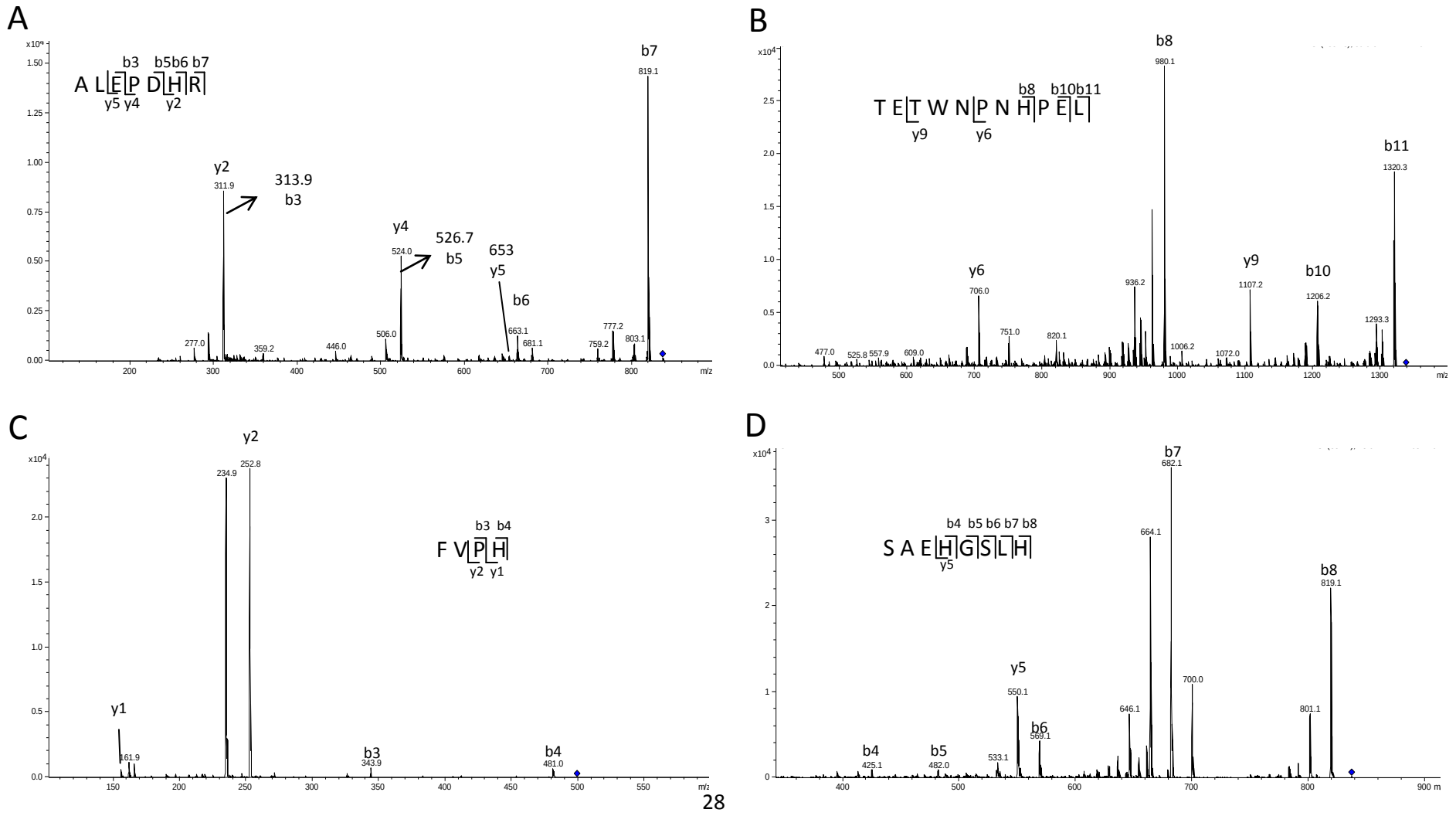
F3D
F3E

Figure 6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y5 y4 y2
b7
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y2
311.9
313.9
b3
y4
524.0
526.7
b5
653
y5
b6
277.0
359.2
446.0
506.0
663.1
681.1
759.2
777.2
803.1
B
T E T W N P N H P E L
b8 b10b11
y9 y6
b8
980.1
b11
1320.3
y6
706.0
y9
1107.2
b10
1206.2
936.2
1293.3
751.0
820.1
1006.2
1072.0
C
F V P H
b3 b4
y2 y1
y2
234.9
252.8
y1
161.9
b3
343.9
b4
481.0
D
S A E H G S L H
b4 b5 b6 b7 b8
y5
b7
682.1
664.1
b8
819.1
y5
550.1
700.0
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b6
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b4
425.1
b5
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533.1