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Antioxidant and metal chelating activities of *Phaseolus vulgaris* L. var. Jamapa protein isolates, phaseolin and lectin hydrolysates

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ABSTRACT

A search in a database of potential bioactive short sequences in food proteins reveals that bioactive peptides with a variety of beneficial effects for cardiovascular health are present in the sequence of common bean proteins, including bioactive sequences with antioxidant properties. A protein isolate, the storage protein phaseolin and a lectin extract from *Phaseolus vulgaris* L. var. Jamapa, were hydrolyzed by treatment with pepsin and pancreatin in order to investigate the possible release of peptides with antioxidant and metal chelating properties. Antioxidant activity was determined in Caco-2 cells exposed to a free radical generator, and iron and copper chelating activities were determined using colorimetric methods. The highest antioxidant activity, 71% inhibition, was found in the hydrolyzed protein isolate. Copper and iron chelating activities were highest in the lectin and phaseolin hydrolysates, 53% and 81%, respectively. Thus, experimental data indicates, as suggested by the database search, that antioxidant peptides are abundant in pepsin–pancreatin hydrolysates, which may represent a valuable health-promoting property in common bean.

1. Introduction

The genus *Phaseolus* belonging to the leguminosae family, includes about 70 species. The most cultivated species of this genus in Mexico is the common bean (*Phaseolus vulgaris* L.), which constitutes a staple food of great economic, social, and cultural importance. Common bean is only second to soybean in legumes world production and it represents 85% of cultivated *Phaseolus* in the world (Celis-Velázquez, Peña-Valdivia, Luna-Cavazos, & Aguirre-Rivera, 2008).

Several studies have shown a correlation between consumption of beans and a reduced risk of suffering cardiovascular diseases, obesity, and diabetes. These health benefits have been at least partially attributed to the presence of antioxidant components in beans, especially polyphenols (Xu & Chang, 2008).

In addition to polyphenols, bioactive peptides within the sequence of native proteins may also have antioxidant activity, and could be used as functional ingredients in food formulations in order to prevent oxidative stress related diseases, and/or to improve the shelf life of foods (Di Bernardini et al., 2011).

Metal chelation appears to be at least partly responsible for the antioxidant activity that has been found in several amino acids, including Tyr, Met, His, Lys, Arg, and Trp (Huang, Majumder, & Wu, 2010; Zhu, Chen, Tang, & Xiong, 2008). Furthermore, antioxidant peptides containing Pro and His have a synergistic effect in the presence of other antioxidants such as polyphenols (Kitts & Weiler, 2003). In addition, chelation of iron by His, Glu, Asp, and Cys results in enhanced iron absorption, and can also result in reduction of ferric to ferrous ion (Storcksdieck, Bonsmann, & Hurrell, 2007). An earlier study showed that acidic and/or basic amino acids may play an important role in Fe^{2+} and Cu^{2+} chelation by peptides (Saiga, Tanabe, & Nishimura, 2003).

Common bean proteins, as compared to the FAO/WHO reference data (FAO/WHO, 1985), have a good content of aromatic amino acids, Lys, Leu, Ile, Asp and Glu, but is low in Met, Cys, Trp, Val and Thr. The contents in acid and aromatic amino acids suggest that common bean protein may represent a source of antioxidant peptides.

Common bean seed is about 20–25% protein, a large fraction of which is represented by the storage protein phaseolin (40–50% of the total) and lectins (10–27% of the total). Phaseolin is a 160 kDa trimeric protein belonging to the 7S vicilin class. It is glycosylated mostly by mannose residues and consist of three polypeptide subunits, α-, β- and γ, with molecular weights

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between 43 and 53 kDa (Montoya et al., 2006). Its nutritive value is limited by a low content in sulphur amino acids, and a high relative resistance to proteolysis. Thermal treatment greatly improves digestibility of phaseolin as well as functional and physicochemical properties, indicating a good potential for its application in food formulations (Yin, Tang, Wen, Yang, & Yuan, 2010). The lectin family of proteins in common beans includes among others the α-amylase inhibitor (α-AI), arcelin and the true lectin, phytohemagglutinin (PHA). Although members of this family reduce protein digestibility and present toxicity at the intestinal level, they can also exert antiviral, antibacterial, antimutagenic and anticarcinogenic effects at certain concentrations (González de Mejía, Valadez-Vega, Reynoso-Camacho, & Loarca-Pina, 2005).

Antioxidative and antihypertensive activities have previously been found in bean protein hydrolysates that were produced by treatment with the commercial, food-grade enzymatic preparations Alcalase® and Flavourzyme® (Akıllıoğlu & Karakaya, 2009; Torruco-Uco, Chel-Guerrero, Martínez-Ayala, Dávila-Ortíz, & Betancur-Ancona, 2009). These studies were focused on the release of bioactive peptide from total protein. The present work is focused on the release of antioxidant and chelating bioactive peptides from the most abundant proteins in common bean by the physiological enzymes pepsin and pancreatin.

2. Materials and methods

2.1. Materials

Black Jamapa bean (*P. vulgaris* L. var. Jamapa, anthracnose-resistance cultivar, grown in 2008 at "Santa Lucia" Experimental Station of INIFAP) seeds were kindly donated by CEVAMEX and stored at 4 °C. Pepsin A (E.C.3.4.23.1, PP-77163, 800–2500 units/mg protein, from hog stomach), pancreatin (P-1750, 4XUSP, from porcine pancreas), $FeCl_2$, ferrozine, pyrocatechol violet, EDTA (ethylenediaminetetraacetic acid), ABAP (2,2′-azobis (2-amidinopropane) dihydrochloride) and DCFH-DA (2′,7′-dichlorofluorescein diacetate) were purchased from Sigma–Aldrich (Sigma Chemical Co., St. Louis, MO, USA). Diethyl ethoxymethylenemalonate was obtained from Fluka (Buch, Switzerland). HBSS (Hank's Balanced Salt Solution), DMEM (Dulbecco's Modified Eagle's Medium), foetal bovine serum and antibiotics for cell culture were from GIBCO (Barcelona, Spain). All other chemicals were of analytical grade provided by JT Baker (Phillipsburg, NJ, USA), Merck (Darmstadt, Germany) or Bio-Rad (Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.2. Profile of potential biological activity in P. vulgaris proteins

Seed protein sequences reported for *P. vulgaris* were obtained from the UniProt database (http://www.uniprot.org/). Potential bioactive peptides and their function were determined by searching the Biopep database of bioactive amino acid sequences in proteins (http://www.uwm.edu.pl/biochemia/index_en.php).

The frequency of occurrence of the identified active peptides (*A* value) was defined in the following:

$$A = \frac{a}{N} \quad (1)$$

where *a* is the number of amino acid residues included in fragments with a given activity in the protein sequence, and *N* is the number of amino acid residues in the protein (Dziuba, Iwaniak, & Minkiewicz, 2003).

2.3. Production of P. vulgaris protein isolates

Whole seeds were ground to powder and passed through a 0.2 mm-mesh sieve. The resulting flour was defatted by extraction with hexane for 24 h at 4 °C and extracted five times with acetone 75% (v/v), 30 min at 4 °C in order to remove polyphenols. Proteins were extracted by suspending the flour in distilled water (1:10, w/v) adjusted to pH 9.5 with 1 N NaOH, and agitation for 30 min at 40 °C. The supernatant resulting from centrifugation at 5000 × *g* for 30 min was adjusted to pH 4.5 using 1 N HCl in order to precipitate protein. The protein precipitate was recovered by centrifugation at 10,000 × *g* for 30 min and lyophilized.

Purification of phaseolin was based on the method described by Montoya et al. (2006) with modifications. The flour, prepared as described above, was suspended in 0.5 M NaCl, 0.025 M HCl, pH 2 (1:20, w/v) for 1 h, and then centrifuged at 13,500 × *g* for 30 min. The supernatant was centrifuged again for 30 min at 4 °C and 13,500 × *g* after addition of five volumes of distilled water at 4 °C. The precipitate was washed with distilled water and centrifuged again. The final precipitate was dialyzed against distilled water at 4 °C for 24 h and lyophilized.

2.4. Extraction of lectins

Common bean lectins were extracted according to González de Mejía et al. (2005). Extraction was carried out by overnight incubation of flour 1:10 (w/v) in 10 mM phosphate buffered saline (PBS), pH 7.4 at 4 °C. The supernatant resulting from centrifugation at 12,000 × *g* was brought to 79% $(NH_4)_2SO_4$ saturation. The precipitate recovered by centrifugation at 12,000 × *g* for 20 min was redissolved in PBS, and dialyzed overnight against distilled water and lyophilized.

2.5. Determination of total phenolic content

Polyphenols were extracted according to Xu and Chang (2008) and determined using the Folin–Ciocalteu reagent as described using gallic acid as standard (Singleton, Orthofer, & Lamuela-Raventós, 1999). Polyphenols concentrations are given as mg gallic acid equivalents/g (mg GAE/g).

2.6. Electrophoresis (SDS–PAGE)

SDS–PAGE was carried out according to Laemmli (1970) using a Minin-Protean 3 Gel Electrophoresis Unit (BioRad). Gels consisted of a 13% polyacrylamide resolving gel (pH 8.8) and a 5% stacking gel (pH 6.8). Samples were dissolved in sample buffer (0.1 M Tris–HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol and 0.02% bromophenolblue) and loaded onto the gels (15 µl, 75 µg protein/well). Gels were stained using 0.125% (w/v) Coomassie brilliant blue R-250 in 7% acetic acid and 40% methanol (v/v) solution and destained in 7% acetic acid and a 30% ethanol (v/v) solution.

2.7. Enzymatic protein hydrolysis

Protein fractions were hydrolyzed by treatment with pepsin for 90 min followed by treatment with pancreatin for another 120 min according to Megías et al. (2008). Protein suspension in water (5%, w/v) were adjusted to pH 2.5 using HCl. Pepsin at a 1:20 (w/w) enzyme to substrate ratio was added at time 0, and pancreatin, at the same enzyme to substrate ratio, was added 120 min later after adjusting to pH 7.5. Temperature was kept at 37 °C throughout the whole process, and pH was kept at pH 2.5 or 7.5 for hydrolysis with pepsin or pancreatin, respectively. Aliquots of the reaction mixtures were withdrawn at regular intervals and heated at 90 °C for 10 min in order to inactivate enzymes. Aliquots at time

0 represent non- hydrolyzed protein. Hydrolysates were clarified by centrifugation at 10,000 × *g* for 15 min and kept at −20 °C.

2.8. Protein determination

Protein was determined by elemental analysis using a LECO CHNS-932 analyzer (St. Joseph, MI, USA) and calculated as % nitrogen content × 6.25.

2.9. FPLC-Gel filtration chromatography

Gel filtration chromatography was carried out in an AKTA-purifier FPLC system equipped with a Superdex Peptide, 10/300 GL column (Cat: 17-5176-01, GE Healthcare). Injection volume and protein concentration were 500 μl and 0.2 mg/ml, respectively. The eluent was 0.75 M ammonium bicarbonate and elution was monitored at 215 nm. Molecular masses were determined using blue dextran (2000 kDa), cytochrome C (12.5 kDa), aprotinin (6512 Da), bacitracin (1450 Da), cytidine (246 Da) and glycine (75 Da) as molecular weight standards (Amersham Pharmacia LKB Biotechnology, Uppsala, Sweden).

2.10. Amino acid analysis

Protein samples (2 mg) were hydrolyzed in 6 N HCl (4 ml) at 110 °C for 24 h in tubes sealed under nitrogen. Tryptophan was analyzed by HPLC after basic hydrolysis according to Yust et al. (2004). Amino acids were determined after derivatization with diethyl ethoxymethylenemalonate by HPLC according to the method of Alaiz, Navarro, Girón, and Vioque (1992), using D,L-α-aminobutyric acid as an internal standard and a 300 mm × 3.9 mm i.d. reversed-phase column (Novapack C_{18}, 4 μm; Waters, Milford, MA, USA).

2.11. Antioxidant activity in Caco-2 cells

Caco-2 cells were obtained from the European Cell Culture Collection and cultured under standard cell cultured conditions (5% CO_2 at 37 °C) in DMEM medium supplemented with 10% foetal bovine serum, 1% non-essential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin and cells were subcultured twice a week. Antioxidant activity was determined by measuring the 2,2′-Azo-bis-aminopropane (ABAP)-induced production of free radicals using the dichlorofluorescein (DCF) assay (Wang & Joseph, 1999). Cells were seeded in 96 well microplates (100 μl, 10^4–2×10^4 cells/well) 48 h before assays. Growth medium was removed and wells were washed with HBSS before addition of hydrolysates (50 μl in HBSS) and DCFH-DA (50 μl, 25 μM in HBSS). Plates were preincubated at 37 °C for 1 h and washed with HBSS (100 μl) followed by addition of 285 μM ABAP in HBSS (100 μl). Plates were then incubated in a Fluoroskan Ascent plate-reader (Thermo Scientific, MA, USA) at 37 °C and fluorescence (excitation at 485 nm, emission at 555 nm) was measured every 10 min for 30 min. Each plate included a positive control (cells treated with DCFH-DA and the free radical generator ABAP) and negative control (cells treated only with DCFH-DA). The negative control was included in order to prevent overestimation of free radicals due to photo oxidation of DCFH. Lower fluorescence emission in the presence of hydrolysates indicates a lower free radical concentration due to the antioxidant activity of the hydrolysates. Antioxidant activity was calculated as follows as a function of emitted fluorescence (% IF), shown in the following equation:

$$\%\,\mathrm{IF} = \frac{\text{Fluorescence at time 30 min} - \text{Fluorescence at time 0 min}}{\text{Fluorescence at time 0 min}} \times 100 \quad (2)$$

2.12. Metal chelating activity

2.12.1. Iron chelating activity

Fe^{2+}-chelating activity was determined by measuring the formation of the Fe^{2+}-ferrozine complex (Carter, 1971). Samples (100 μg) were mixed with 250 μl 100 mM Na acetate buffer pH 4.9, and 30 μl $FeCl_2$ (0.01%, w/v). Ferrozine (12.5 μl, 40 mM) was added after incubation for 30 min at room temperature. EDTA was used as a positive control. Binding of Fe(II) ions to ferrozine generates a coloured complex that was measured at 562 nm using a microplate reader (Multiskan Spectrum, ThermoLab Systems, MA, USA).

Iron chelating activity was calculated using the following equation:

$$\%\ \text{Chelating Activity} = \frac{\mathrm{Abs}_{\text{control}} - \mathrm{Abs}_{\text{sample}}}{\mathrm{Abs}_{\text{control}}} \times 100 \quad (3)$$

2.12.2. Copper chelating activity

Cu^{2+}-chelating activity was determined according to Saiga et al. (2003). Na acetate buffer, pH 6 (290 μl, 50 mM), 6 μl 4 mM pyrocatechol violet prepared in the same buffer, and $CuSo_4{\cdot}5H_2O$ (1 μg) were added to the hydrolysates (100 μg). Absorbance at 632 nm was measured using a microplate reader (Multiskan Spectrum, ThermoLab Systems, MA, USA).

Copper chelating activity was calculated as described above for iron.

2.13. Statistical analysis

Results were expressed as mean ± standard error. The means comparison was subjected to ANOVA and differences among means were determined using Fisher's least significant difference (LSD) at $p < 0.05$ by using G-Stat Student version 1.0.

3. Results and discussion

3.1. Profile of potential bioactive peptide in P. vulgaris proteins

A search of bioactive sequences within selected proteins was carried using the Biopep database of bioactive peptides, and common bean protein sequences from the UniProt database. Analysis of the sequence corresponding to 15 seed proteins identified bioactive sequences with 12 different biological activities (Fig. 1). The most frequent activities (highest *A* values) were antihypertensive, antioxidant and inhibitory (dipeptidyl-aminopeptidase IV, CaMP-DE and neuropeptide inhibitor) activities. The highest *A* value (0.36) was found in the phytohemagglutinins, followed by phaseolin with an *A* value of 0.32, suggesting that these proteins may be a better source of bioactive peptides especially antihypertensive and antioxidant peptides, than α-amylase and the Bowman–Birk type protease inhibitor with *A* values of 0.2 and 0.17, respectively. Arcelin proteins showed *A* values similar to those of phytohemagglutinins. Arcelins are glycoproteins related to the lectin family and have been found in wild accessions of beans (Sathe, 2002). They are important in pest resistance against Mexican bean weevil (*Zabrotes subfasciatus*) and their presence has been related to a decrease of phaseolin expression (Pueyo, Hunt, & Chrispeels, 1993).

Phaseolin and lectin extracts were selected for determination of antioxidant and metal chelating activities after hydrolysis using pepsin and pancreatin, in addition to a protein isolate, because of the potential bioactive activities that were found in this search of the Biopep database.

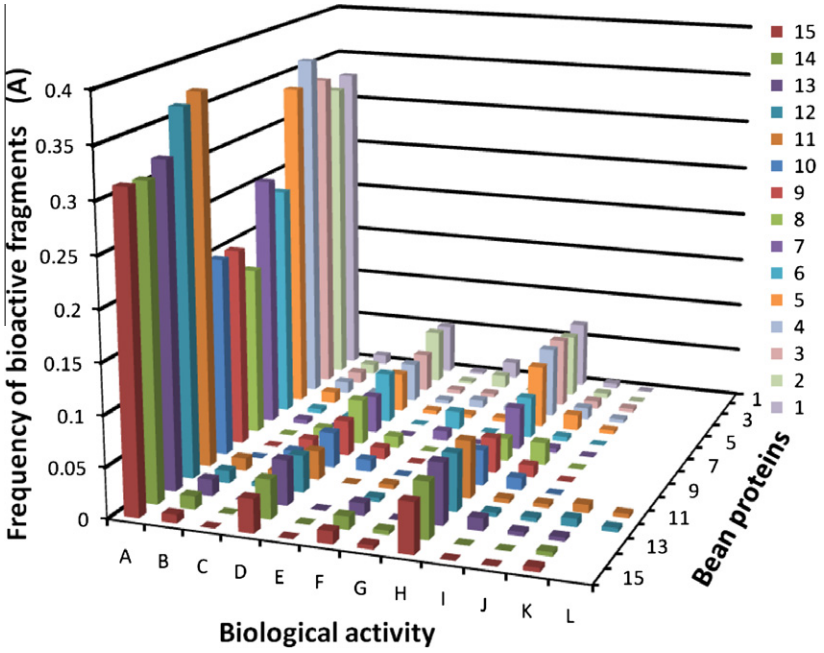

Fig. 1. Predicted profiles of bioactive peptides in common bean according to the Biopep database. The sequences of selected proteins were obtained from UniProt database. The frequency of the bioactive sequences (*y* axis) was plotted vs. the specific biological activities (A–L in *x* axis) and proteins (1–15 in *z* axis). Proteins: 1 = phaseolin, alpha-type; 2 = phaseolin, beta-type; 3 = erythroagglutinating phytohemagglutinin; 4-5 = leucoagglutinating phytohemagglutinin; 6 = alpha-amylase inhibitor 1; 7 = alpha-amylase inhibitor 2; 8 = Bowman–Birk type protease inhibitor 2; 9 = Bowman–Birk type protease inhibitor PVI-3(2); 10 = Bowman–Birk type protease inhibitor PVI-4; 11 = arcelin-1; 12 = arcelin-2; 13 = arcelin-4; 14 = arcelin-5A; 15 = arcelin-5B. Activities: A = ACE inhibitor; B = activating ubiquitin-mediated proteolysis; C = antiamnestic; D = antioxidative; E = antithrombotic; F = hypotensive; G = immunomodulating; H = inhibitor (dipeptidyl-aminopeptidase IV, CaMPDE and neuropeptide inhibitor are included); I = regulating; J = opioid; K = bacterial permease ligand; L = immunostimulating.

3.2. Preparation and characterization of protein isolate, lectins and phaseolin

Polyphenols were removed from the flour before preparation of protein isolates because these components have antioxidant and metal chelating activities (Xu & Chang, 2008). Extraction using 75% (v/v) acetone as described in materials and methods allowed for elimination of 95% of the original polyphenol content (7.49 ± 0.25 mg GAE/g flour). The protein isolate, phaseolin and lectin preparation had 88%, 89% and 14% (w/w) protein, respectively, as compared to 26% (w/w) protein in the original flour. These values compare very favourably with other previously reported for *P. vulgaris* (Morales-de-León, Vázquez-Mata, Torres, Gil-Zenteno, & Bressani, 2007; Torruco-Uco et al., 2009) and are similar to those reported for other grain legumes such as chickpea (Clemente et al., 1999).

SDS–PAGE analysis of the three protein preparations is shown in Fig. 2. Ten protein bands with molecular weights (MW) ranging from 15 to 200 kDa, are present in the protein isolate (Fig. 2, lane 1). The 41 and 46 kDa bands, corresponding to phaseolin subunits (between 40 and 55 kDa according to Montoya et al. (2006)) are the most abundant proteins. The 15, 18, 25 and 32 kDa, bands correspond to proteins belonging to the lectin-family. Phytohemagglutinins, PHA-L and PHA-E, have 22-32 kDa subunits. Bands at 10 kDa corresponding to protease inhibitor, and 12.4, 15.2, 33.6 and 45 kDa corresponding to α-amylase inhibitor and its β subunits, are also visible (Montoya et al., 2006; Morales-de-León et al., 2007; Pueyo et al., 1993). Phaseolin (Fig. 2, lane 2) revealed two major bands at 42 and 46.5 kDa, as expected, in addition to a few minor bands at 20, 25 and 62 kDa. This electrophoretic pattern is similar to those previously reported by Montoya et al. (2006), and indicate that our phaseolin preparation was enriched in the main reserve protein of *P. vulgaris* and contains almost no other contaminating proteins. SDS–PAGE of the lectin extract

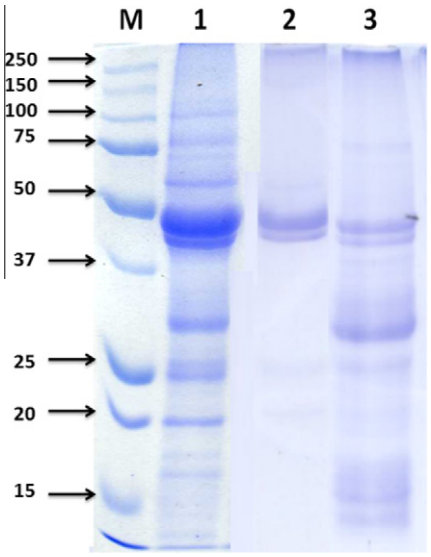

Fig. 2. SDS–PAGE analysis of *P. vulgaris* L. protein isolate (lane 1), phaseolin (lane 2), and lectin extract (lane 3). Lane M = MW standards (kDa).

(Fig. 2, lane 3) showed several bands from 14 to 45 kDa. The 15 and 25 kDa bands, which could correspond to α-amylase inhibitor and the subunits of PHA, respectively, were more noticeable than in the protein isolate and the phaseolin preparation (Fig. 3, lanes 1 and 2).

3.3. Amino acid composition

In addition to SDS–PAGE analysis, the amino acid compositions of the flour, the protein isolate, the lectin extract and the phaseolin

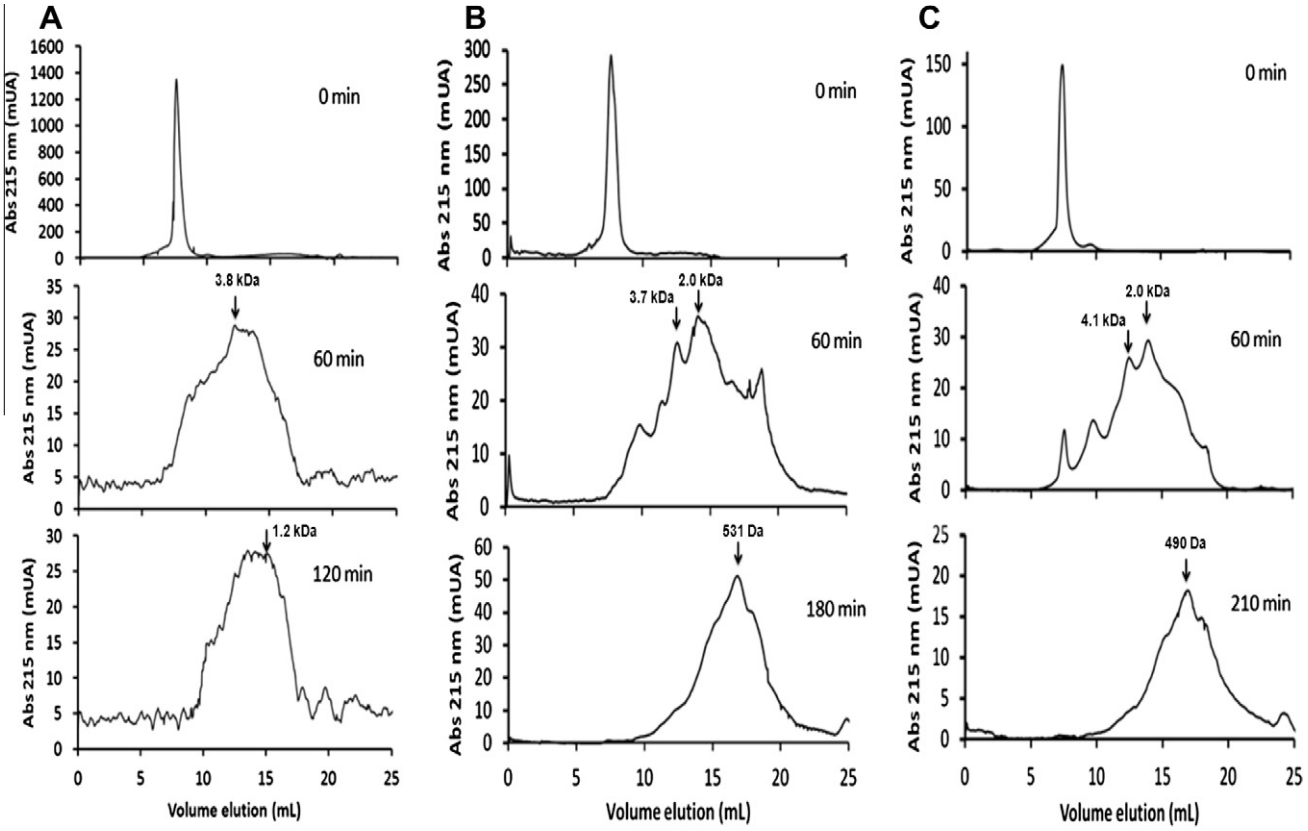

Fig. 3. FPLC gel filtration analysis of the hydrolysates produced by treatment with pepsin (added at time 0) and pancreatin (added 90 min later). Aliquots were taken at different time points as indicated. (A) Protein isolate, (B) phaseolin and (C) lectin extract.

preparation were determined and compared to FAO recommendations (FAO/WHO, 1985) (Table 1). The amino acid profiles of the flour and the protein isolate were similar and met FAO requirements (FAO/WHO, 1985), except for a low content in sulphur amino acids (Met and Cys), Trp and Phe. Comparison of the compositions of phaseolin and the protein isolate reveals that Asp + Asn, Pro and Tyr are lower in phaseolin but Ser, Phe, Lys and Leu are higher. The amino acid composition of the lectin extract is characterized by a high content of Cys as compared to the flour and the protein isolate, which is probably due to the presence of high amounts of sulphur amino acid residues in protease and α-amylase inhibitors (Sathe, 2002). Other significant differences in amino acid composition can be observed in Table 1. Thus the contents of Ser, Thr and Ala were higher in the lectin extract than in

Table 1
Amino acid composition (g/100 g protein) of *P. vulgaris* L. flour, protein isolate, phaseolin and lectin extract.

Amino acid	FAO/WHO (1985)		*P. vulgaris* L. FAO	Flour	Isolate	Phaseolin	Lectin extract
	Children	Adults					
Asp + Asn				11.9 ± 0.6^{a}	11.3 ± 0.1^{a}	7.8 ± 0.2^{b}	10.1 ± 0.0^{c}
Glu + Gln				16.0 ± 1.0^{a}	17.1 ± 0.1^{a}	18.2 ± 0.3^{a}	14.0 ± 0.0^{b}
Ser				6.9 ± 0.5^{a}	7.1 ± 0.0^{a}	8.4 ± 0.1^{b}	9.2 ± 0.0^{b}
His			3.0	3.1 ± 0.2^{a}	3.5 ± 0.0^{b}	3.8 ± 0.0^{b}	2.9 ± 0.0^{a}
Gly			3.8	4.8 ± 0.3^{ac}	4.3 ± 0.0^{a}	4.9 ± 0.0^{bc}	4.9 ± 0.0^{bc}
Thr	3.4	0.9	4.4	4.6 ± 0.3^{a}	4.2 ± 0.0^{ab}	3.8 ± 0.0^{b}	7.2 ± 0.0^{c}
Arg			5.6	7.2 ± 0.4^{a}	6.0 ± 0.0^{b}	6.8 ± 0.1^{a}	5.4 ± 0.0^{b}
Ala			5.8	4.5 ± 0.1^{a}	4.1 ± 0.0^{b}	4.0 ± 0.0^{b}	6.5 ± 0.0^{c}
Pro				2.5 ± 0.1^{a}	2.0 ± 0.0^{b}	1.1 ± 0.1^{c}	2.3 ± 0.1^{b}
Tyr			4.2	2.5 ± 0.2^{a}	4.1 ± 0.0^{b}	3.5 ± 0.0^{c}	2.8 ± 0.0^{a}
Val	3.5	1.3		4.5 ± 0.3^{a}	5.7 ± 0.0^{b}	4.9 ± 0.0^{a}	4.8 ± 0.0^{a}
Met			0.3	0.2 ± 0.1^{a}	0.8 ± 0.0^{b}	0.2 ± 0.0^{a}	1.0 ± 0.0^{c}
Cys	2.5[A]	1.7[A]	1.1	0.2 ± 0.0^{a}	0.3 ± 0.1^{a}	0.2 ± 0.0^{a}	2.5 ± 0.1^{b}
Ile	2.8	1.3	5.9	5.9 ± 1.8^{a}	5.5 ± 0.0^{a}	4.8 ± 0.0^{a}	4.3 ± 0.0^{a}
Trp	0.8	0.5	1.7	0.9 ± 0.0^{a}	0.2 ± 0.0^{b}	0.3 ± 0.0^{c}	0.8 ± 0.0^{a}
Leu	6.6	1.9	9.4	8.1 ± 0.4^{a}	9.9 ± 0.1^{b}	10.8 ± 0.1^{c}	7.4 ± 0.0^{a}
Phe	6.3[B]	1.9[B]	5.8	5.9 ± 0.4^{ac}	6.8 ± 0.4^{a}	8.6 ± 0.0^{b}	6.0 ± 0.0^{c}
Lys	5.8	1.6	6.9	6.8 ± 0.4^{ab}	7.4 ± 0.1^{ab}	7.9 ± 0.1^{b}	8.0 ± 0.0^{b}

Data are the mean ± standard error of two replicates. Values with different letters are significantly different ($p < 0.05$).
[A] Met + Cys.
[B] Phe + Tyr.

the protein isolate and phaseolin, while contents in Glu + Gln, His, Ile and Phe were lower.

3.4. FPLC profile of P. vulgaris protein hydrolysates

In addition to SDS–PAGE, gel filtration chromatography using a FPLC system was carried out in order to analyze the molecular weight distribution of the hydrolysates in non-denaturing conditions. This analysis evidenced the gradual hydrolysis of proteins by treatment with pepsin followed by treatment with pancreatin (Fig. 3). It has been reported, that phaseolin and lectins are highly resistant to hydrolysis by proteases (Kelsall et al., 2002; Sathe, 2002). The initial substrates, protein isolate, lectins and phaseolin appear as a single peak before hydrolysis (time 0, upper panels in Fig. 3). The consecutive hydrolysis with pancreatin, increased the rate of hydrolysis in protein isolate, followed by phaseolin and lectins extract. No further changes in FPLC profiles were observed after 120 min for protein isolate hydrolysate, 180 min for phaseolin hydrolysates and 210 min for lectins extract hydrolysates (lower panels in Fig. 3). Treatment with pepsin for 60 min produces molecular weight profiles that span the whole range of separation of the column, although some of the starting material is still present. A large peptidic fraction with MW around 3.8 kDa can be seen at this time (Fig. 3, panels in the middle). Two major peaks can be observed in the phaseolin hydrolysate at 2.0 and 3.7 kDa (Fig. 3B, middle panel). The lectin extract was hydrolyzed at lower extent than the protein isolate and phaseolin at this time, although two major peptidic fractions at around 2.0 and 4.1 kDa can be observed (Fig. 3C, middle panel). Treatment with pancreatin further shifted the profiles to lower molecular weights (Fig. 3, lower panels).

3.5. Antioxidant activity of the hydrolysates in Caco-2 cells

The antioxidant activity of the protein hydrolysates was evaluated using Caco-2 cell cultures in which oxidative stress was induced by addition of the free radical generator ABAP (Wang & Joseph, 1999). Caco-2 cells are derived from a human colorectal carcinoma. Determination of antioxidant activity in these cells represents a more biologically relevant method than chemical assays of antioxidant activity, because it accounts for some aspects of the uptake, metabolism, and location of antioxidants within cells. The nonionic, nonpolar DCFH-DA substrate crosses the cell membrane and is hydrolyzed enzymatically by intracellular esterases to the nonfluorescent DCFH. It is oxidized to the fluorescent dichlorofluorescein (DFC) product in the presence of reactive oxygen species, so that DFC fluorescence represents a measure of reactive oxygen species within cells (Wang & Joseph, 1999).

Results showed that some of the hydrolysates had antioxidant activity, although others had a pro-oxidant effect in cells compared with the lack of antioxidant activity of non-hydrolyzed protein (0 min) except for a pro-oxidant effect in lectins (Fig. 4). Thus, all the hydrolysates resulting from treatment of the protein isolate for different times showed antioxidant activity, while the effect of the hydrolysates corresponding to treatment of phaseolin and lectin was dependent on the time of hydrolysis. Fluorescence in the cells treated with ABAP in the presence of the isolate hydrolysates varied between 29% and 73% of control. Addition of the 60, 90, and 210 min phaseolin hydrolysates reduced fluorescence to about 80–85% of the positive control. Addition of the 120 and 180 min lectin hydrolysates reduced fluorescence even more, to 27% and 46% of the positive control, respectively. A pro-oxidant effect, which is shown by fluorescence values higher than the positive control, was observed in the cells treated with ABAP in the presence of the 30, 120, and 180 min phaseolin hydrolysates, and the 0, 60, 90 and 210 min lectin hydrolysates. This pro-oxidant effect could be due to the presence of antioxidant peptides at high concentrations, and is consistent with the observation that some antioxidants can actually have pro-oxidant effects at concentrations higher than those at which they have an antioxidant effect (Antolovich, Prenzler, Patsalides, McDonald, & Robards, 2002). Although, the bioinformatic analysis (Fig. 1) indicated that peptides released from phaseolin and lectins have a high potential antioxidant activity, this potential can be affected in reality by factors such as synergistic antioxidant effects, the limited number of peptides liberated by the pepsin–pancreatin system, cell permeability and interactions of the peptides with the cell membrane limiting or promoting their absorption. Differences between the antioxidant activity that was expected in the hydrolysates and experimental results highlight the importance of using physiologically relevant systems such as the ABAP-Caco-2 model for *in vitro* evaluation of antioxidant activity.

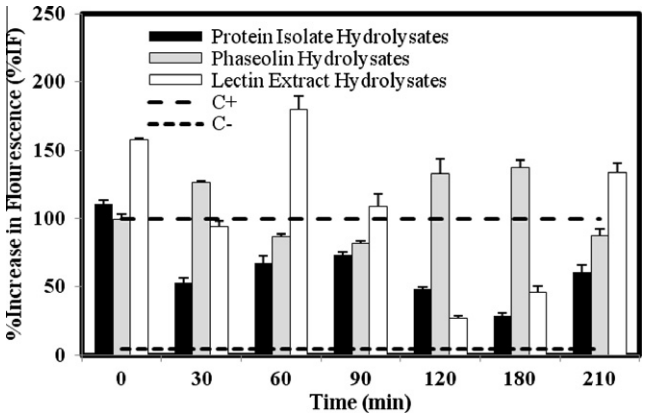

Fig. 4. Antioxidant activity of *P. vulgaris* L. hydrolysates in Caco-cells treated with the free radical generator ABAP. Protein isolate and phaseolin hydrolysates: 250 μg/well; lectin extract hydrolysates: 200 μg/well. Negative and positive controls consisted of cells treated only with DCFH and DCFH plus ABAP, respectively. Values represent the mean ± standard error of four determinations.

3.6. Metal chelating activity of the P. vulgaris hydrolysates

Antioxidant activity in the protein hydrolysates may not be attributed to a single mechanism. Chelation of metal ions has an antioxidant effect because the transition metals iron and copper promote oxidative damage at different levels (Saiga et al., 2003). These oxidative reactions *in vivo* appear to be involved in the pathogenesis of at least some neurodegenerative diseases (Mandel, Amit, Reznichenko, Weinreb, & Youdim, 2006).

As shown in Fig. 5A, hydrolytic treatment increased the copper chelating activity of the protein isolate and the phaseolin preparation from 0% up to 30% and 35%, respectively. The lectin extract already had a 15% chelating activity before hydrolysis, and this value only experienced an increase up to 50% in the last aliquot taken from the hydrolysis reaction, corresponding to treatment with pepsin for 90 min followed by treatment with pancreatin for another 120 min. An overall trend of increasing copper chelating activity with time of hydrolysis was observed, although the activity decreased in the 90 min lectin hydrolysate and the 120 min isolate hydrolysate. These decreases were probably due to hydrolysis by pancreatin of some of the chelating peptides that were released by pepsin. Similar results were reported for whey protein isolate hydrolysates produced by treatment with Alcalase® for 1, 3, and 8 h, resulting in 22.4%, 56.4%, and 55.2% copper chelating activity, respectively (Peng, Kong, Xia, & Liu, 2010).

The Iron chelating activity of the hydrolysates, as shown in Fig. 5B, was somewhat different than the copper chelating activity.

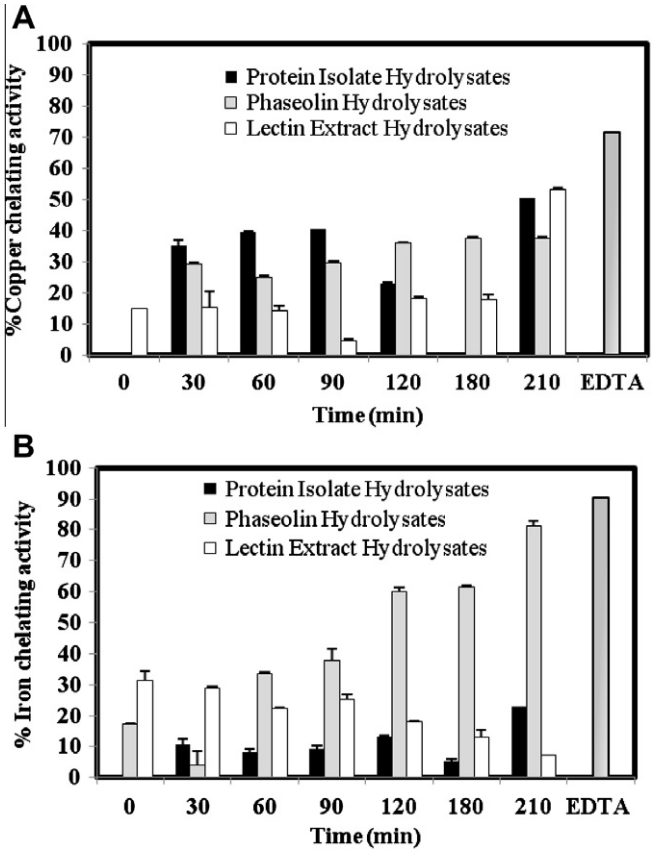

Fig. 5. Copper (A) and iron (B) chelating activity of *P. vulgaris* L. hydrolysates. Hydrolysates: 100 μg de protein/well; positive control EDTA: 5 and 6 μg/well, respectively. Values represent the mean ± standard error of three determinations.

Phaseolin and lectins already had an 18% and 32% chelating activity before hydrolysis. While the activity of phaseolin gradually increased with hydrolytic treatment up to an 81%, the activity of the lectin extract went down to 7%. The protein isolate did not have any activity before hydrolysis, and the hydrolysates had activities between 5% and 23%.

Thus, the phaseolin hydrolysates were the most effective iron chelators (81%), which is consistent with a 97% iron chelating activity that was previously described for pea protein hydrolysates after treatment with Thermolysin (Pownall, Udenigwe, & Aluko, 2010).

The low iron chelating activity in phaseolin and the lectin extract might be related to differences in amino acid composition. As shown in Table 1, the content of Asp + Asn, Glu + Gln, His and Cys in phaseolin and the lectin extract were significantly different. These amino acids have been reported to chelate iron ions (Storcksdieck et al., 2007). Cys content was higher in the lectin extract, although this did not result in higher iron chelating activity of the lectin hydrolysates as compared to the phaseolin hydrolysates. In addition to the amino acid composition, chelating activity of peptides also depends on other factors such as peptide structure, steric effects and molecular weight.

4. Conclusions

Bioinformatic analysis using a bioactive peptides database showed that common bean proteins contain many peptides with antihypertensive, antioxidant, and inhibitory (dipeptidyl-aminopeptidase IV, CaMPDE and neuropeptide inhibitor) type activities that can be released by enzymatic hydrolysis. Experimental results indicate that some of the common bean hydrolysates produced by *in vitro* digestion using pepsin and pancreatin have antioxidant activity in Caco-2 cell cultures treated with the free radical generator ABAP. The highest antioxidant activity was found in the hydrolysates resulting from hydrolytic treatment of whole protein isolates, as compared to a lectin extract and a phaseolin preparation. Nevertheless, the lectin and phaseolin hydrolysates, especially the latter, had higher copper and iron chelating activity, which constitutes a powerful antioxidant mechanism. Results of this study may be useful for explaining how peptides released by hydrolysis of different protein fractions contribute to antioxidant and chelating activities of whole protein hydrolysates. Further studies are needed to analyze interactions between peptides and the structure-activity relationships in purified peptides.

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