Antioxidant and metal chelating activities of peptide fractions from phaseolin and bean protein hydrolysates

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ABSTRACT

Bean protein isolate and phaseolin were hydrolysed using pepsin and pancreatin, and the resulting hydrolysates were filtered through a 1 kDa cut-off membrane and fractionated by size exclusion chromatography. Three fractions corresponding to MW 0.7–1.0 kDa, 0.43–0.7 kDa and <0.43 kDa (A1, A2, and A3 for protein isolate fractions, and B1, B2, and B3 for phaseolin fractions) were assayed for antioxidant and metal chelating activity and they were also subjected to amino acid and SDS–PAGE analysis. Fractions A1 and B1 had the highest copper chelating activity (78% and 82%, respectively), while iron chelating activity was the highest in fractions A1 and B3 (36% and 16%, respectively). Fractions A2 and B3 had the highest antioxidant activity as determined by inhibition of reducing power and β-carotene bleaching, while the highest ABTS radical scavenging activity was found in A3 and B3. Thus, fractions coming from the isolate and phaseolin had similar activities except for iron chelation, suggesting that phaseolin is the major contributor to the antioxidant and copper chelating activities of the hydrolysed protein isolate.

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1. Introduction

Common bean (*Phaseolus vulgaris* L.) is grown and consumed in various regions of the world, providing an inexpensive source of protein in the diet (20–25%). It is becoming increasingly popular because of its health benefits (Campos-Vega, Loarca-Piña, & Oomah, 2010), and it is also an important model for research on seed storage proteins (Marsolais et al., 2010). Globulins (65% w/w) are the major protein components in beans, followed by albumins, glutelins, and prolamins (15, 10, and 2% w/w, respectively) (Montoya, Lallès, Beebe, & Leterme, 2010). Beans have a good amino acid composition, although its nutritional value is limited by a relatively low protein digestibility, and by deficiency in sulphur-containing amino acids (Met and Cys) and Trp. Enzyme inhibitors and lectins that are present in beans reduce protein digestibility and nutrient absorption respectively, and lectins may also exhibit certain toxic effects at the intestinal level. Nevertheless, these undesirable effects are mostly eliminated by cooking (Campos-Vega et al., 2010).

Phaseolin, a 7S globulin, constitutes up to 50% of total seed protein. It is a 160 kDa trimeric protein containing three polypeptide subunits, α-, β- and γ-, with molecular weights between 43 and 53 kDa. The subunits display molecular heterogeneity, which has been attributed to different degrees of glycosylation, mostly by mannose residues (Montoya et al., 2010). The sequence of the α and β subunits and the high-resolution X-ray structure of phaseolin have been reported (Lawrence, Izard, Beuchat, Blagrove, & Colman, 1994). In contrast to other 7S proteins, phaseolin is highly resistant to proteolytic attack, which is probably explained by several properties of the phaseolin molecule: a compact and rigid structure, a secondary structure rich in β-sheet domains, a high degree of glycosylation and high hydrophobicity (Montoya et al., 2010). Also, it seems that splitting of the EF loop, which protrudes on the surface of the subunit and limits access to the protein by proteinases, is essential for further hydrolysis of 7S proteins (Jivotovskaya, Senyuk, Rotari, Horstmann, & Vaintraub, 1996; Lawrence et al., 1994).

In recent years, much research has been focused on the generation of bioactive peptides from food sources. Antioxidant and metal chelating activities have been previously reported in bean protein hydrolysates. Protein isolates from hard-to-cook Jamapa bean were hydrolysed using pepsin-pancreatin and Alcalase™-Flavourzyme™ systems and antioxidant activity was measured using the ABTS·+ decolorisation assay (Ruiz-Ruiz, Dávila-Ortíz, Chel-Guerrero, & Betancur-Ancona, 2011). Also, some of the hydrolysates resulting

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from digestion of protein concentrates from three cultivars of Azufrado (sulphur yellow) beans using Thermolysin™, Alcalase™ or Pancreatin™ exhibited a high DPPH and ABTS scavenging activity (Valdez-Ortiz, Fuentes-Gutiérrez, Germán-Báez, Gutiérrez-Dorado, & Medina-Godoy, 2012). Antioxidant and metal chelating activities in the hydrolysates resulting from hydrolysis of a protein isolate, purified phaseolin, and a lectin fraction using pepsin and pancreatin have also been described (Carrasco-Castilla et al., 2012).

Different amino acid residues may be responsible for the antioxidant activity in peptides, which is usually due to chelation of transition metals and scavenging of free radicals. Nucleophilic sulphur-containing side chains in Cys and Met residues and aromatic side chains in Trp, Tyr, and Phe residues can easily donate hydrogen atoms. Thus, these residues are usually considered to have a potential antioxidant activity, although they may also have pro-oxidant effects under certain conditions (Chen, Muramoto, Yamauchi, & Nokihara, 1996). In addition to being susceptible to oxidative reactions, the imidazole group in His has metal chelating activity. Acidic and basic amino acids may also play an important role in Fe^{2+} and Cu^{2+} chelation (Saiga, Tanabe, & Nishimura, 2003). Because of their higher solubility in lipids, the hydrophobic amino acid residues Val and Leu seem to be responsible for high antioxidant effects (Chen et al., 1996).

In a previous study, a bio-informatic analysis showed that common bean proteins contain a high number of antihypertensive and antioxidant peptides. Determination of antioxidant and metal chelating activity in Black Jamapa bean protein hydrolysates that were produced by treatment with pepsin and pancreatin revealed that the highest iron and copper chelating activities were found in phaseolin hydrolysates as compared to lectin extract hydrolysates (Carrasco-Castilla et al., 2012). The goal of this work was to determine antioxidant and metal chelating activities in low molecular weight peptidic fractions resulting from hydrolysis of purified phaseolin as compared to the low molecular weight fractions resulting from hydrolysis of protein isolates. Hydrolysis using pepsin and pancreatin was followed by filtration using a 1 kDa cut-off membrane and size exclusion chromatography.

2. Materials and methods

2.1. Materials

Black Jamapa bean (*P. vulgaris* L. var Jamapa, 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), FeCl₂, ferrozine, pyrocatechol violet, EDTA (ethylenediaminetetraacetic acid), ABTS (2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) and TNBS (2,4,6-trinitrobenzenesulphonic acid solution, 5% in H_2O) were purchased from Sigma–Aldrich (Sigma Chemical Co., St. Louis, MO, USA). Diethyl ethoxymethylenemalonate was obtained from Fluka (Buch, Switzerland). 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. Production of P. vulgaris protein isolates

Whole seeds were ground to a powder which was 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), over 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 agitating for 30 min at 40 °C. The supernatant resulting from centrifugation at 5,000g for 30 min was adjusted to pH 4.5 using 1 N HCl in order to precipitate the proteins, which were recovered by centrifugation at 10,000g for 30 min and lyophilised.

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 13,500g for 30 min. The supernatant was centrifuged again for 30 min at 4 °C and 13,500g 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 dialysed against distilled water at 4 °C for 24 h and lyophilised.

2.3. 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% bromophenol blue) and loaded onto the gels (10 μL, 65 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 de-stained in 7% acetic acid and a 30% ethanol (v/v) solution.

2.4. Enzymatic protein hydrolysis

Protein fractions were hydrolysed 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) was 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-hydrolysed protein. Hydrolysates were clarified by centrifugation at 10,000g for 15 min and kept at −20 °C.

2.5. Degree of hydrolysis

The degree of hydrolysis was calculated by determination of free amino groups by reaction with TNBS according to Adler-Nissen (1979). Total number of amino groups were determined in a sample 100% hydrolysed by treatment with 6 N HCl at 110 °C for 24 h.

2.6. Size exclusion chromatography

The hydrolysates resulting from treatment with pepsin for 90 min, followed by treatment with pancreatin for 120 min, were filtered through 1 kDa MWCO (molecular weight cut-off membrane, Millipore USA) using an Amicon system, and fractionated by gel filtration on a Sephadex G-10 column (2.5 × 30 cm) using phosphate buffer (10 mM, pH 7.4). Samples (1–1.5 mL, 45 mg protein/mL) were applied to the column and eluted at a flow rate of 0.5 mL/min with monitoring at 215 nm. Blue dextran (MW 2000,000), HHL (MW 429), and tryptophan (MW 204) were used as molecular weight standards (Peng, Xiong, & Kong, 2009).

2.7. Amino acid analysis

Protein samples (2 mg) were hydrolysed in 6 N HCl (4 mL) at 110 °C for 24 h in tubes sealed under nitrogen. Amino acids were

determined after derivatisation 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 300×3.9 mm i.d. reversed-phase column (Novapack C_{18}, 4 μm; Waters, Milford. MA, USA). Tryptophan was analysed by HPLC after basic hydrolysis according to Yust et al. (2004).

2.8. Antioxidant activity

2.8.1. β-carotene bleaching method

Antioxidant activity was determined by measuring inhibition of β-carotene bleaching as described by Marco (1968) with modifications. The reagent consisted of 1 mL β-carotene (2 mg/mL in chloroform), 20 mg of linoleic acid, and 100 mg of Tween 40, that were vigorously mixed by vortexing, and flushed with nitrogen in order to remove chloroform before addition of oxygen-sparged distilled water (100 mL) Samples (200 μL, 0.5 mg/mL protein) were added to 800 μL of the β-carotene reagent and incubated in the dark at 50 °C for 60 min before determination of absorbance at 450 nm. Antioxidant activity (AA) was calculated according to the following equation (Al-Saikhan, Howard, & Miller, 1995):

$$\% \text{ Antioxidant activity} = \frac{\text{Degradation Rate}_{control} - \text{Degradation Rate}_{sample}}{\text{Degradation Rate}_{control}}$$

And the degradation rate (DR) was calculated as:

$$\text{DR} = \frac{Ln \frac{\text{Abs at 0 min}}{\text{Abs at 62 min}}}{60}$$

2.8.2. Reducing power

Reducing power was determined according to Oyaizu (1968) with modifications. Hydrolysates (100 μg) or peptide fractions (50 μg) were added to 50 μL 0.2 M phosphate buffer (pH 6.6) and 50 μL 1% potassium ferricyanide and incubated at 50 °C for 20 min. Plates were incubated for another 10 min at 50 °C after addition of 50 μL 10% tricholoacetic acid and 10 μL 0.1% ferric chloride, and absorbance was read at 700 nm.

2.8.3. Scavenging of ABTS·+ radical

The ABTS radical cation (ABTS·+) decoloration assay was performed according to Re et al. (1999) with some modifications. ABTS·+ was produced by reaction of ABTS (7 mM in water) with potassium persulfate (2.45 mM final concentration) in the dark at room temperature for more than 12 h. Prior to the assay, the solution was diluted in ethanol and equilibrated at room temperature to give an absorbance of 0.70 ± 0.02 at 734 nm. Samples (10 μL of a protein hydrolysate or peptide fraction, 1 mg protein/mL) were incubated for 6 min with 990 μL ABTS·+ radical cation solution before measuring absorbance at 734 nm. The activity was expressed as mM Trolox equivalent antioxidant capacity (TEAC)/mg protein. Percentage inhibition of the ABTS·+ radical was calculated using the equation:

$$\% \text{ Inhibition} = \frac{\text{Abs}_{control} - \text{Abs}_{sample}}{\text{Abs}_{control}} \times 100$$

2.9. Metal chelating activity

2.9.1. Iron chelating activity

Fe^{2+}-chelating activity was determined by measuring the formation of the Fe^{2+}-ferrozine complex (Carter, 1971). Hydrolysates (100 μg) or peptide fractions (50 μg) were mixed with 250 μL 100 mM Na acetate buffer at 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. Binding of Fe(II) ions to ferrozine

generates a coloured complex that was measured at 562 nm using a microplate reader (xMark™ Spectrophotometer, Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Iron chelating activity was calculated as:

$$\% \text{ Chelating activity} = \frac{\text{Abs}_{control} - \text{Abs}_{sample}}{\text{Abs}_{control}} \times 100$$

2.9.2. Copper chelating activity

$Cu^{2\pm}$ 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 $CuSo_4 \cdot 5H_2O$ (10 μg) were added to hydrolysates (100 μg) or peptide fractions (50 μg). Absorbance at 632 nm was measured using a microplate reader (xMark™ Spectrophotometer, Bio-Rad Laboratories, Inc., Hercules, CA, USA). Copper chelating activity was calculated as described above for iron.

2.10. Statistical Analysis

All results are presented as mean ± standard deviation of triplicate assays.

3. Results and discussion

3.1. Enzymatic hydrolysis

Hydrolysis of the protein isolate and purified phaseolin (protein content 88 and 89% w/w, respectively) were carried out by treatment with pepsin for 90 min followed by treatment with pancreatin for 120 min as previously reported (Carrasco-Castilla et al., 2012). Degree of hydrolysis (DH) increased up to 16% and 37% for the protein isolate and phaseolin preparations, respectively (Fig. 1). The DH value for the protein isolate hydrolysate was within the range, 12–34%, previously reported for Azufrado bean protein concentrates that were hydrolysed by treatment with pancreatin for 120 min (Valdez-Ortiz et al., 2012), but was lower than the 26% DH reported for the hydrolysis of protein isolates from hard to cook bean by incubation with pepsin for 90 followed by incubation with pancreatin for 45 min (Ruiz-Ruiz et al., 2011). These variations are probably due to differences in the methods that were used for protein extraction, or may be also due to differences in protein profile resulting in cultivars more or less susceptible to proteolysis. The variability of beans depends on genetic diversity. Cultivars are clas-
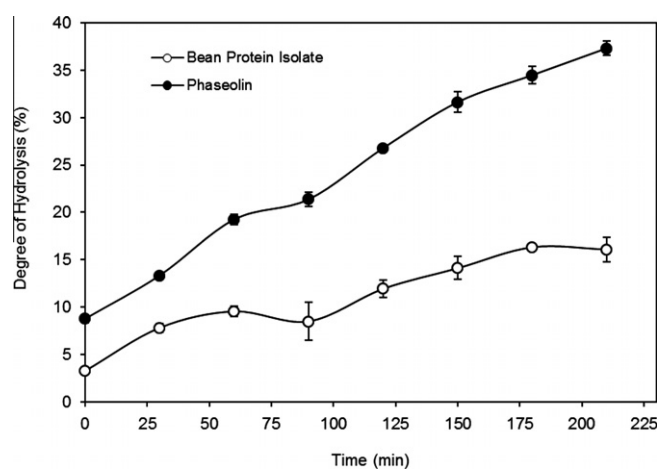

Fig. 1. Enzymatic hydrolysis of Jamapa bean (*Phaseolus vulgaris* L.) protein isolate and phaseolin by treatment with pepsin (added at time 0 min) and pancreatin (added 90 min later). Data correspond to the average ± SD of three determinations.

sified according to geographic origin. Thus, Jamapa beans belong to the Mesoamerican breed and Azufrado beans belong to Nueva Granada race of Andean germplasm according to morphological evidences and phaseolin and alloenzymes composition (Celis-Velázquez, Peña-Valdivia, Luna-Cavazos, & Aguirre-Rivera, 2008). On the other hand, DH for phaseolin hydrolysates are slightly higher than those reported by Montoya et al. (2008) using a pepsin-pancreatin system (DH 11–27%). Variability in phaseolin susceptibility to *in vitro* proteolysis by pepsin and pancreatin was attributed to differences in amino acid composition and protein glycosylation across bean varieties (Montoya et al., 2008).

Thus, hydrolysis of phaseolin was much more effective than hydrolysis of the isolate, and leveled off at the end of the initial treatment with pepsin, and again at the end of the treatment with pancreatin (Fig. 1). On the other hand, hydrolysis of the protein isolate only leveled off a little at the end of the treatment with pepsin, and kept going at approximately the same rate throughout the whole treatment. Considering that phaseolin constitutes about 50% (w/w) of the isolate, these data show that hydrolysis of purified phaseolin is much more effective than hydrolysis of this protein when it is part of the isolate. This could be due to the presence of some residual Bowman–Birk type protease inhibitor (trypsin inhibitor) in the bean protein isolate. Thus, it has been reported that heat inactivation of protease inhibitors increased DH for hydrolysis of phaseolin from 57% to 96% (Montoya et al., 2008). It is also possible that limited access to phaseolin due to formation of protein aggregates may contribute to the reduced hydrolysis of phaseolin in the isolate.

3.2. SDS–PAGE of protein hydrolysates

Hydrolysis of the protein isolate and purified phaseolin was also monitored by SDS–PAGE. Results for both hydrolysis of the isolate (Fig. 2a) and purified phaseolin (Fig. 2b) are consistent with DH data as shown in Fig. 1. Thus, hydrolysis of the isolate is shown by a gradual decrease in the intensity of the higher molecular weight bands and an increase in lower molecular weight bands. Major bands were identified according to Rui, Boye, Ribereau, Simpson, and Prasher, (2011) (Fig. 2a, bands A to E). Bands A and C, corresponding to phaseolin, are still quite apparent even after treatment with pancreatin is completed. Bands B, D, and E would correspond to phytohemaglutinins PHA-L and PHA-E, α amylase inhibitor, and α amylase β subunit, respectively. On the other hand, hydrolysis of purified phaseolin (Fig. 2b) shows a sharp change in its polypeptide profile after treatment with pancreatin is started. This is consistent with the increase in DH values leveling-off at the end of treatment with pepsin, and sharply increasing again when treatment with pancreatin is started, as shown in Fig. 1. It has been reported that the first peptidic bond in phaseolin β type that is hydrolysed by trypsin is R212-K213 in the middle of the subunits causing the formation of two fragments that show as bands at 22 and 33 kDa in SDS–PAGE followed by the bond between residues K217 and Q218 (Jivotovskaya et al., 1996). Assuming that most of the peptide bonds were cleaved by pancreatin, the 210 min hydrolysates were chosen for separation by gel filtration (see Fig. 3).

3.3. Size exclusion chromatography

Previous studies have demonstrated that the lower molecular weight fractions (<1 kDa) resulting from hydrolysis of hard to cook *P. vulgaris* using pepsin and pancreatin had the highest antioxidant activity (1985.5 mM TEAC/mg protein) (Ruiz-Ruiz et al., 2011). Thus, the hydrolysates resulting from treatment with pepsin and pancreatin for a total of 210 min were filtered through a 1-K MWCO membrane before fractionation by gel filtration.
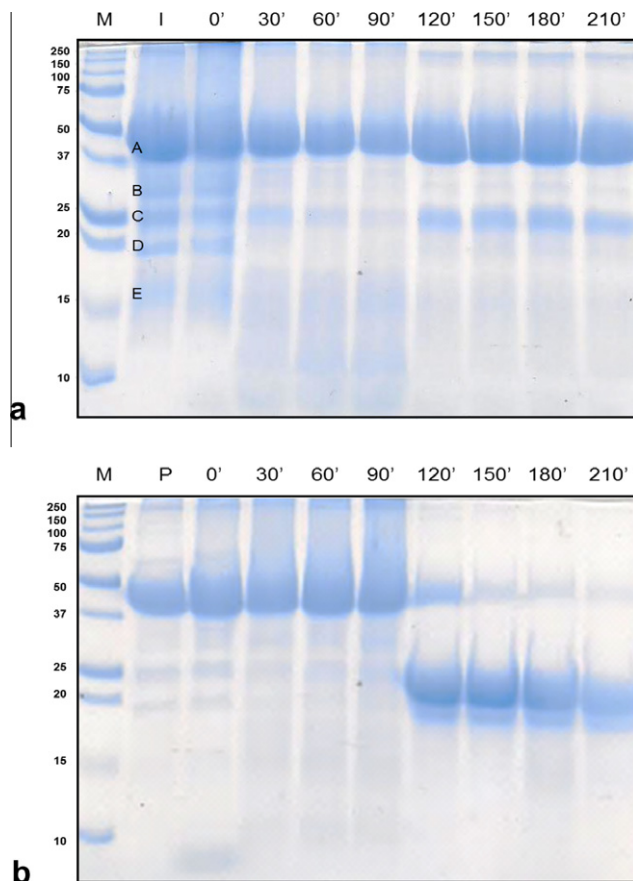

Fig. 2. Electrophoretic (SDS–PAGE) pattern of *P. vulgaris* protein isolate (a) and phaseolin (b) hydrolysed with pepsin (added at time 0 min) and pancreatin (added after 90 min) at different reaction times. Lane M = MW standards (kDa); Lane I = bean protein isolate; Lane P = phaseolin. Protein bands: A: Phaseolin (43–47 kDa); B: PHA-E, PHA-L (31 kDa); C: Phaseolin β type (fragment, 25 kDa); D: Phaseolin α type (fragment, 21 kDa), α amylase inhibitor (18 kDa); E: α amylase inhibitor β subunit (15 kDa).
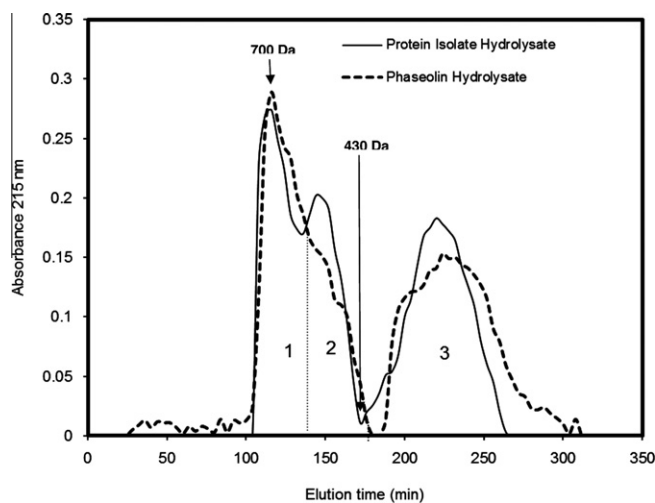

Fig. 3. Elution profile of <1 kDa-hydrolysates of protein isolate and phaseolin from *P. vulgaris* L. var. Jamapa, produced by treatment with pepsin and pancreatin by 210 min, on Sephadex G10 gel filtration chromatography. Bed size, 2.5 × 30 cm; flow rate, 0.5 mL/min; mobile phase, phosphate buffer solution (10 mM, pH 7.4), NaN₃ 0.02%; fraction size, 2 mL.

Gel filtration chromatography using a Sephadex G-10 column separated the low molecular weight fraction of the isolate hydroly-

Table 1
Antioxidant and metal chelating activities of fractions purified by Sephadex G10 gel filtration chromatography from the <1 kDa fraction of 210 min protein isolate and phaseolin hydrolysate.

Sample	β-carotene bleaching method (% AA)	Reducing power $Abs_{700\ nm}$	Scavenging of ABTS^{+} radical (mM TEAC/mg protein)	Cu^{2+} chelating activity (%)	Fe^{2+} chelating activity (%)
210 min-protein isolate hydrolysate	72.98 ± 1.08	0.249 ± 0.020	1.82 ± 0.11	50.50 ± 0.35	22.87 ± 2.71
Fraction > 1 kDa	59.82 ± 1.27	0.118 ± 0.007	8.77 ± 1.74	49.65 ± 3.80	16.04 ± 3.56
Fraction < 1 kDa					
A1	12.41 ± 0.67	0.093 ± 0.001	101.55 ± 5.47	78.21 ± 2.23	35.83 ± 1.35
A2	18.03 ± 1.41	0.213 ± 0.018	105.55 ± 4.88	39.00 ± 5.02	8.85 ± 1.16
A3	6.04 ± 1.39	0.099 ± 0.008	933.61 ± 49.78	nd	8.56 ± 2.15
210 min-phaseolin hydrolysate	69.51 ± 1.33	0.062 ± 0.005	5.51 ± 0.28	37.54 ± 0.73	81.22 ± 2.55
Fraction > 1 kDa	53.12 ± 2.29	0.048 ± 0.002	3.35 ± 0.39	38.89 ± 5.06	nd
Fraction < 1 kDa					
B1	11.87 ± 0.50	0.080 ± 0.003	82.59 ± 3.57	81.63 ± 1.66	6.48 ± 0.84
B2	18.92 ± 0.01	0.130 ± 0.007	33.64 ± 3.66	65.45 ± 2.17	2.95 ± 1.78
B3	80.26 ± 2.54	0.073 ± 0.007	999.33 ± 14.89	21.09 ± 6.28	15.68 ± 1.02

Data are the mean ± standard deviation of three replicates.
Nd: Not determined.

Table 2
Amino acid composition (g/100 g protein) of *P. vulgaris* L protein isolate, phaseolin and their peptidic fractions obtained by gel filtration chromatography.

Amino acid	Isolate	A1	A2	A3	Phaseolin	B1	B2	B3
Asp + Asn	11.3 ± 0.2	16.2 ± 0.1	6.7 ± 0.0	3.5 ± 0.3	7.8 ± 0.3	14.2 ± 0.1	3.9 ± 0.1	3.6 ± 0.1
Glu + Gln	17.1 ± 0.1	19.5 ± 0.1	6.8 ± 0.1	1.0 ± 0.0	18.2 ± 0.4	23.2 ± 0.0	9.9 ± 0.0	3.0 ± 0.2
Ser	7.1 ± 0.0	9.1 ± 0.1	5.3 ± 0.2	4.1 ± 0.4	8.4 ± 0.1	12.3 ± 0.1	5.0 ± 0.1	3.6 ± 0.0
His	3.5 ± 0.0	2.4 ± 0.1	2.7 ± 0.1	1.1 ± 0.1	3.8 ± 0.0	3.2 ± 0.0	4.3 ± 0.0	2.0 ± 0.1
Gly	4.3 ± 0.0	5.7 ± 0.0	3.2 ± 0.0	2.9 ± 0.2	4.9 ± 0.0	6.7 ± 0.1	4.3 ± 0.0	1.7 ± 0.0
Thr	4.2 ± 0.0	6.3 ± 0.0	5.0 ± 0.0	1.0 ± 0.2	3.8 ± 0.0	4.9 ± 0.0	2.4 ± 0.1	1.3 ± 0.2
Arg	6.0 ± 0.0	4.2 ± 0.1	11.9 ± 0.6	2.1 ± 0.1	6.8 ± 0.1	2.5 ± 0.1	19.5 ± 0.3	6.7 ± 0.3
Ala	4.1 ± 0.0	6.0 ± 0.0	6.3 ± 0.1	2.0 ± 0.4	4.0 ± 0.0	4.6 ± 0.0	3.6 ± 0.0	1.8 ± 0.1
Pro	2.0 ± 0.0	1.8 ± 0.0	0.5 ± 0.1	nd	1.1 ± 0.2	1.9 ± 0.3	1.7 ± 0.3	nd
Tyr	4.1 ± 0.0	2.0 ± 0.0	1.8 ± 0.0	nd	3.5 ± 0.0	0.8 ± 0.1	0.8 ± 0.0	nd
Val	5.7 ± 0.0	6.2 ± 0.0	7.3 ± 0.4	15.8 ± 2.6	4.9 ± 0.0	5.3 ± 0.4	4.5 ± 0.0	1.5 ± 0.0
Met	0.8 ± 0.0	0.2 ± 0.0	2.0 ± 0.0	2.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.4 ± 0.0
Cys	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	nd	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	nd
Ile	5.5 ± 0.0	4.7 ± 0.1	6.9 ± 0.0	1.5 ± 0.0	4.8 ± 0.0	3.7 ± 0.0	4.7 ± 0.0	1.1 ± 0.4
Trp	0.2 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	4.9 ± 0.5	0.3 ± 0.0	0.3 ± 0.0	0.9 ± 0.0	5.0 ± 0.3
Leu	9.9 ± 0.1	5.1 ± 0.1	23.6 ± 0.5	6.5 ± 0.1	10.8 ± 0.1	5.4 ± 0.0	15.4 ± 0.1	4.5 ± 0.0
Phe	6.8 ± 0.6	2.8 ± 0.0	1.9 ± 0.0	50.9 ± 3.1	8.6 ± 0.0	2.0 ± 0.0	1.2 ± 0.0	61.7 ± 0.5
Lys	7.4 ± 0.1	7.0 ± 0.2	7.5 ± 0.0	0.6 ± 0.0	7.9 ± 0.1	8.6 ± 0.0	17.5 ± 0.0	2.0 ± 0.1
% Amino acid distribution								
Hydrophobic	35.0	27.3	48.9	83.7	34.7	23.3	32.1	76.0
(+) Charged	16.9	13.7	22.0	3.8	18.5	14.3	41.2	10.8
(−) Charged	28.4	35.8	13.5	4.5	26.0	37.4	13.8	6.6
Aromatic	11.1	5.3	4.2	55.8	12.4	3.1	3.0	66.8

Data are the mean ± standard deviation of two replicates.
Nd: Not determined.

sate into three fractions that were collected by pooling together the eluate corresponding to 0.7–1.0 kDa, 0.43–0.7 kDa, and lower than 0.43 kDa (fractions 1–3, respectively, in Fig. 3). Chromatography of phaseolin hydrolysate yielded a similar chromatogram, except that the absorbance corresponding to fraction 2 was much lower, resulting in two shoulders after fraction 1. Therefore, it seems that hydrolysis of phaseolin contributes mainly to peptides in fractions 1 and 3 of the isolate hydrolysate.

3.4. Antioxidant and metal chelating activities

Antioxidant and metal chelating activities were determined in the whole isolate and phaseolin hydrolysates, in the fractions containing peptides with molecular weight higher than 1 kDa, and in the fractions resulting from gel filtration chromatography of the peptides with molecular weight lower than 1 kDa. Antioxidant activity was analysed by determination of the inhibition of β-carotene bleaching, reducing power, and scavenging of the ABTS

radical. Copper and iron chelating activities were determined using colorimetric methods (Table 1). Amino acid analysis of these fractions was also carried out in order to explore possible correlations between amino acid composition and antioxidant and metal chelating activities (Table 2).

3.5. β-carotene bleaching method

The β-carotene decoloration or bleaching method is based on the oxidative decomposition of β-carotene in the presence of the unsaturated fatty acid linoleic acid, resulting in a decrease of absorbance at 450 nm. An emulsion of β-carotene and linoleic acid was formed, and oxidation of linoleic acid was promoted by addition of oxygen-sparged distilled water. Inhibition of β-carotene bleaching was higher in the fractions including peptides with molecular weight higher than 1 kDa, and in fractions A2 and B3 from gel filtration chromatography of the low molecular weight peptides, but lower than in the whole hydrolysates, except for frac-

tion B3 with an activity of 80% (Table 1). Fractions A2 and B2, which have a similar activity, are rich in positively charged and hydrophobic amino acids, mainly Arg and Leu, and have a low content of aromatic residues, suggesting that the former may be responsible for the antioxidant activity. On the other hand, fraction B3 was enriched in Phe and Arg (Table 2). Megías et al. (2007) reported an increase in His and Arg in copper chelating peptides purified from sunflower hydrolysates that were also very effective at inhibiting the oxidation of β-carotene. The content of Arg and His in fractions A2 and B2 was also high (Table 2). In addition, it seems that the antioxidant mechanism in fraction B3, could be attributed to aromatic and hydrophobic amino acids, which facilitates accessibility to hydrophobic radical species (Sarmadi & Ismail, 2010). Although, fraction A3 and B3 are rich in Phe, these fractions show large differences in Val and Arg content, respectively. Also, phaseolin represents 50% of total protein; however, results in Table 2 suggest that Val content in A3, might not be released from phaseolin hydrolysis (Val could come from other bean proteins such as phytohemagglutinins, α-amylase inhibitors or trypsin inhibitors). Val content could increase hydrophobic interactions between peptides in the conditions assayed, thus promoting aggregation between peptides in A3 and limiting their ability to stabilize or terminate radicals and, as consequence, increasing the degradation of β-carotene.

3.5.1. Reducing power

Reducing power was determined by measuring reduction of the Fe^{3+}/ferricyanide complex to the ferrous form, which was monitored by measuring the formation of Perl's Prussian blue at 700 nm. Reducing power in all the fractions resulting from filtration and gel filtration chromatography of the isolate hydrolysate was lower than in the whole hydrolysate (Table 1), although the value for fraction A2 was close to that for the whole hydrolysate. On the other hand, all fractions resulting from gel filtration of the phaseolin hydrolysate had higher reducing power than the high molecular weight and the whole hydrolysates. Reducing power is a result of the presence of peptides that are electron donors and that can react with free radicals, thus terminating the free radical chain reaction. This activity has been attributed to Tyr, Met, Cys, His, Lys, and Trp residues. The content in these amino acid residues in A2 and B2 fractions is, with the exception of Lys, relatively low, which might explain its low reducing power as compared to the reducing power that was described for an alfalfa protein hydrolysate (absorbance 0.4) (Xie, Huang, Xu, & Jin, 2008).

3.5.2. Scavenging of ABTS·+ radical

The ABTS decolorization assay can be used to determine antioxidant activity of both lipophilic and hydrophilic molecules, and is based on the reaction of hydrogen donating antioxidants with the ABTS·+ radical, which is intensely coloured and is determined by measuring absorbance at 734 nm. Antioxidant activity as determined by decolorization of the ABTS radical was low in the isolate and whole phaseolin hydrolysates (1.82 and 5.51 mM TEAC/mg protein), and much higher in the fractions resulting from gel filtration chromatography. The highest activity was found in fractions A3 and B3 (933.61 and 999.33 mM TEAC/mg protein, respectively) (Table 1). These values are lower than those previously reported for the whole low molecular weight (<1 kDa) fraction from hard-to-cook bean hydrolysates (1985.5 mM TEAC/mg protein) (Ruiz-Ruiz et al., 2011). The active fractions had a high content of hydrophobic amino acid, particularly Phe, which increased seven fold in A3 and B3 as compared to the original protein isolate and phaseolin preparations (Table 2). Similar results were observed by Beermann, Euler, Herzberg, and Stahl, (2009) in fractions obtained by RP-HPLC of soybean protein hydrolysates. These data are consistent with reports that the radical scavenging mechanism depends on the presence of hydrophobic amino acids (Jiménez-Escrig, Alaiz, Vioque, & Rupérez, 2010).

3.5.3. Metal chelating activity

Chelation of metal ions has an antioxidant effect because the transition metals iron and copper catalyse the generation of reactive oxygen species, including hydroxyl radical (·OH) and superoxide radical (O_2·−), leading to oxidation of unsaturated lipids and promoting oxidative damage at different levels (Saiga et al., 2003). In contrast to ABTS·+ scavenging activity, for both protein isolate and phaseolin fractions obtained, highest molecular weight corresponds with a high copper chelating activity, as observed in fractions A1 and B1 (Table 1). It has been reported that a "cage structure" in metallothionein, which excludes surrounding water, would facilitate binding of copper by thiol groups. In addition, carboxylic groups are considered as high- affinity metal binding sites through electrostatic and ionic interactions with copper (Zhu, Chen, Tang, & Xiong, 2008). Recently, it has been reported that high His contents (20–30%) and small peptides (105–1205 Da) provided the highest copper chelating activity (Torres-Fuentes, Alaiz, & Vioque, 2011). In contrast with this report, fractions A1 and B1 have low contents of Cys, Met and His. Thus, copper chelating activity in these fractions might be related to their content of negatively charged amino acids (Asp and Glu) (Table 2). Also, decreases in chelating activity of peptide fractions is proportional to their content of these amino acids.

Iron chelation was in general less efficient than copper chelation (Table 1). The whole isolate and phaseolin hydrolysates had higher iron chelating activity than any of the fractions that were prepared by filtration and gel filtration chromatography, except for A1 which had an activity of 35.8% as compared with 22.8% for the whole isolate hydrolysate. Iron and copper chelating activity followed a somewhat similar trend in the fractions coming from the isolate hydrolysate, suggesting that the mechanisms for iron and copper chelation might be related. A similar pattern can also be observed in the amino acid composition of these samples. Thus, chelation of iron was also associated to Asp + Asn, Glu + Gln, His, and Cys content. In consequence, Asp and Glu might be responsible for iron chelation in A1 as well. Iron binding was far weaker than copper binding, which is consistent with data previously described for whey protein hydrolysates prepared with Alcalase (Peng, Kong, Xia, & Liu, 2010). The cause for the poor iron binding was not clear, but it may be related to the greater number of coordination sites that are required for iron chelation as compared to those for copper chelation (Kong & Xiong, 2006).

On the other hand, iron chelating activity in the different fractions corresponding to the phaseolin hydrolysate showed a different trend from that for copper chelating. The highest activity was in fraction B3 (16%), although even in this fraction the activity was much lower than in the original whole hydrolysates (81%), suggesting a synergistic effect between peptides from phaseolin before chromatographic fractionation. Although the Asp + Asn and Glu + Gln content was higher in the B1 and B2 fractions, this did not result in higher iron chelating activity. Furthermore, the amino acid pattern of fraction B3 is quite similar to the pattern of A3 except for the content in Arg and Val, suggesting that other factors, including amino acid sequence, steric effects, and molecular weight, should be responsible for weak iron chelation in fraction B3. These results suggest that peptides released from phaseolin do not represent a major contribution to the iron chelating activity in isolate fractions.

4. Conclusions

Our results show that several of the small molecular weight peptide fractions that were purified from the *P. vulgaris* L. var

Jamapa protein isolate and phaseolin hydrolysates have antioxidant activity higher than the original whole hydrolysates, as determined using three different *in vitro* methods. According to the mechanism of reaction, the assays for measuring antioxidant capacity are classified into two groups: methods based on hydrogen atom transfer (HAT) and methods based on electron transfer (ET) (Sarmadi & Ismail, 2010). The β-carotene bleaching assay is a HAT- based assay, whereas determination of reducing power and scavenging of the ABTS·+ radical are ET- based assays. Our results suggest that the antioxidant activity in the peptide fractions arising from hydrolysis of the bean protein isolate and purified phaseolin may be due to multiple mechanisms, including donation of hydrogen atoms and electrons, stabilization of free radicals, and chelation of transition metals. Analysis of the amino acid composition of these fractions showed that some amino acids were present in large amounts as compared with the amino acid composition of the original unhydrolysed protein. These are the residues that are at the target sites for pepsin, trypsin and chymotrypsin, including Arg and Leu in A2 and B2; Phe and Trp in A3 and B3; and Lys in B2, indicating the release of peptides with these amino acids in their carboxy-terminal end. Considering our results, it appears that peptides released from phaseolin by treatment with pepsin and pancreatin are the primary contributors to the antioxidant and metal chelating activities of the whole isolate hydrolysate except for iron chelating activity which appears to derive from hydrolysis of other proteins.

Further purification should be done in order to identify the sequence of the peptides in isolate and phaseolin fractions that are responsible for high activity. Also, the relationship between structure of the peptide and specific antioxidant mechanisms needs to be studied in depth.

Acknowledgements

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