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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.

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 $ABTS^{\cdot+}$ 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 prooxidant 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), $FeCl_2$, 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 5,000*g* 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 10,000*g* 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 13,500*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 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 destained 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 10,000*g* 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