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Protein hydrolysates obtained from Azufrado (sulphur yellow) beans (*Phaseolus vulgaris*): Nutritional, ACE-inhibitory and antioxidative characterization

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ABSTRACT

The goals of the present work were to evaluate the nutraceutical and functional properties of protein hydrolysates obtained from three cultivars of Azufrado (sulphur yellow) beans. Bean protein concentrates were obtained and digested with Alcalase™, Thermolysin™ and Pancreatin™. The Azufrado Higuera cultivar showed the best protein yield/concentration ratio, having a higher amount of hydrolysates compared to the rest of the varieties. All treatments presented ACE-Inhibitory activity, these values ranged from 0.109 to 319.96 μg/ml. Regarding to antioxidant activity, the Az. Higuera/alcalase treatment showed the highest DPPH scavenging activity (40%), however with the ABTS method the Az. Regional '87-alcalase was the best treatment (99.89% of scavenging). Overall, the results of our work showed that Azufrado beans proteins hydrolysates are suitable for development of a nutraceutical product for prevention and control of degenerative diseases such as hypertension and those derived from cellular oxidation.

1. Introduction

Functional foods have compounds that posses certain biological activity, also known as nutraceutic compounds. There are many ways to make functional foods, most of them include adding nutraceutic compounds, removing undesired components, the modification of native compounds or processing foods to interfere with native compounds that have certain biological activity, making them bioavailables when they are consumed (Webb, 1990). Bioactive peptides are nutraceutic compounds, normally released during gastric hydrolysis of many proteins. Many of these peptides have the ability to reduce blood pressure through inhibition of the modular blood pressure control enzyme called Angiotensin Converting Enzyme (ACE). Thus, these peptides could be an alternative for hypertension (HT) treatment (Vioque & Millán, 2006). Several reports have demonstrated that bioactive peptides showed antioxidative activity, with possibility to delay cellular oxidation process *in vivo* lowering the risk of development of chronic diseases (Gerster, 1995; Halliwell, 2000, 2001). Nowadays there is an increasing interest in finding nutraceutic compounds from natural sources that until now have been unexploited; like common bean (*Phaseolus vulgaris*), a legume whose grain is an important source of protein for Mexican population. Common bean contains high digestibility protein (up to 22 g/100 g seed) making this, a good candidate to explore as a source of bioactive peptides. Mexican population is severely affected by HT, the goal of this work was to obtain protein hydrolysates from three types of Azufrado beans grown in Northwestern of Mexico, and to analyze ACE-Inhibitory (ACE-I) and antioxidative potential with *in vitro* assays.

2. Materials and methods

Common bean cultivars grains: Azufrado Higuera (Reg. No. FRI-045-170205), Azufrado Noroeste (Reg. No. FRI-044-170205) and Azufrado Regional '87 (Reg. No. FRI-008-251104) were donated by the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Valle del Fuerte, México. These genotypes account up to 70% of total Sinaloa state production, than in 2010 reach up to 300 million tons of grains. ACE (from rabbit lung), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), hippuryl–histidyl–leucine (HHL), alcalase, thermolysin and pancreatin were purchased from Sigma (Sigma Chemicals, St. Louis, Mo., U.S.A.).

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2.1. Protein isolations

The grains were grounded and flours kept at −20 °C until use. Protein concentrates were obtained as described by Morales-de León, Vázquez-Mata, Torres, Gil-Zenteno, and Bressani (2007) with modification. Flours were mixed with desionizated water (10 g/100 ml), pH was adjusted to 9 with 2 mol/L NaOH and then, soaked for 1 h, after this time, the solution was centrifuged at 10,000 × *g* at 4 °C for 30 min, afterwards the supernatant was recovered and its pH was adjusted to 4.3 with 2 mol/L HCl, to reach proteins isoelectric point and left at 4 °C for 1 h, then it was centrifuged at 10,000 × *g* at 4 °C for 10 min; afterwards it was washed with desionizated water and centrifuged again under the same conditions, the supernatants were discarded and the pellets collected and freeze-dried at −47 °C and 300 × 10^{-3} MPa.

2.2. Enzyme hydrolysis

Protein hydrolysis was carried out according to Humiski and Aluko (2007) and Adler-Nissen (1986) with modifications. Briefly, 3 g from each protein concentrate were mixed with 30 ml of deionized water. Reaction conditions are shown in Table 1. Hydrolysis reaction time was fixed at 2 h maintaining temperature (with a thermostated hotplate stirrer IKA™ C-MAG HS-7) and pH (Mettler Toledo T-50) conditions appropriated for enzymes optimum activity. After the hydrolysis, the slurries were adjusted to pH 4.0 with 2 mol/L HCl and were kept in a water bath at 95 °C for 10 min to inactivate the enzymes, after that, they were centrifuged at 10,000 × *g* at 25 °C for 30 min; the supernatant containing the protein hydrolysates was recovered and preserved at −20 °C. Degree of hydrolysis (DH) was determined, with the o-phthaldialdehyde (OPA) method (Nielsen, Petersen, & Dambmann, 2001). The reaction is specific for primary amines in amino acids, peptides. The OPA reagent in the presence of β-mercaptoethanol developing a coloured compound detectable at 340 nm in a spectrophotometer, using serine as standard.

2.3. ACE-inhibitory activity

Antihypertensive potential of each protein hydrolysate was determined by their ACE-inhibitory (ACE-I) activity according to Miguel, Aleixandre, Ramos, and López-Fandiro (2006) with modifications. ACE hydrolyses HHL to generate hippuric acid and the peptide His–Leu. The reaction mixture, consisting of the substrate (HHL) and protein hydrolysate samples were prepared in ACE buffer (50 mol/L sodium borate, containing 0.5 mol/L sodium chloride, pH 8.3) were pre-incubated at 37 °C for 5 min, after that, ACE was added to a final concentration of 2.5 mU/ml. Different dilutions of each protein hydrolysate were added and incubated in the before mentioned reaction mixture for 30 min at 37 °C; then, the reactions were stopped by addition of 150 μl of 1 mol/L HCl solution, followed by addition of 1 ml of ethyl acetate, to extract hippuric acid, and mixed by vortex for 1 min. The mixture was centrifugated at 14,000 × *g* at 25 °C for 10 min, 750 μl of the organic phase was collected and transferred to a test tube and evaporated. The residue was dissolved in 600 μl water and concentration of hippuric acid was determined at 228 nm using water as a blank. In order to determinate the IC_{50} of each protein hydrolysate, data were adjusted to a non-linear regression model using Hill's equation (Motulsky & Christopoulos, 2003).

Significant differences between treatments were found using Fisher's test (LSD, $\alpha = 0.05$).

Table 1
ACE-inhibitory activity of enzymatic bean protein hydrolysates.

	Reaction conditions			Treatment	IC_{50} (μg/ml)
	T (°C)	pH	E/S*		
Alcalase	50	8.0	4.0%	Azufrado Higuera	0.19**[g]
				Azufrado Noroeste	0.45[f]
				Azufrado Regional '87	1.30[e]
Thermolysin	55	9.0	1.0%	Azufrado Higuera	0.11[g]
				Azufrado Noroeste	0.35[f]
				Azufrado Regional '87	2.78[d]
Pancreatin	39	8.0	3.3%	Azufrado Higuera	60.36[c]
				Azufrado Noroeste	203.65[b]
				Azufrado Regional '87	319.97[a]

*Ratio: enzyme:substrate (protein base) (g/100 g).
**Different letters indicate significant difference (LSD = 0.137 μg/ml, $\alpha = 0.05$).

2.4. Radical-scavenging activity

2.4.1. DPPH radical-scavenging activities

Scavenging activity of the protein hydrolysates against DPPH radical was performed according to Hou et al. (2001). Briefly, the samples were diluted in a 0.1 mol/L sodium phosphate buffer supplemented with Triton X-100 (1 g/100 ml), pH 7. DPPH was prepared in 100 μmol/L methanol, 1.5 ml of DPPH solution were mixed with 1.5 ml samples or 1.5 ml of buffer (control) and left for 30 min in the dark at room temperature. The absorbance was determined at 515 nm. Scavenging activity of the samples was expressed as the percentage of free radical-scavenging effect following the equation:

$$\text{Scavenging effect} = [(\text{Abs Blank} - \text{Abs sample})/\text{Abs Blank}] \times 100$$

Additional, the antioxidant activity obtained calculated was plotted as a function of antioxidant Trolox concentrations in the standard reference data. The Trolox equivalent antioxidant coefficient (TEAC) was calculated by dividing the absorbance percentage inhibition absorbance versus antioxidant concentration slope by the Trolox plot slope.

2.4.2. Antioxidant activity by the ABTS radical cation assay

Free radical-scavenging activity was determined by the ABTS method according to Re et al. (1999). This reaction is based on decolourization of the green colour of the free ABTS radical cation (ABTS*). The radical solution was prepared with ABTS and potassium persulfate, diluted in ethanol, at final concentration of 2.45 mmol and left at dark for 16 h to allow radical development. The solution was diluted to reach absorbance measures around 0.70 ± 0.02 at 734 nm. Protein hydrolysates were freeze-dried and diluted with deionized water to obtain various concentrations; 10.0 μl of each sample were mixed with 1 ml of the ABTS* radical solution and absorbance was measured at 734 nm exactly 1 min after initial mixing and up to 6 min. Deionized water was used as blank. Percentage inhibition of the ABTS* radical was then calculated using the equation:

$$\text{ABTS}^* \text{ radical inhibition percentage} = [(\text{Abs blank} - \text{Abs sample})/\text{Abs blank}] \times 100$$

The Trolox equivalent antioxidant coefficient (TEAC) was calculated as described above.

2.5. Amino acid composition

Amino acid composition of protein hydrolysates samples was determined by HPLC, after acid hydrolysis.