Antihypertensive effect of protein hydrolysate from Azufrado beans in spontaneously hypertensive rats

Article *in* Cereal Chemistry · October 2016

DOI: 10.1094/CCHEM-04-16-0105-FI

CITATIONS
0

READS
69

7 authors, including:

Norma A Bobadilla
Universidad Nacional Autónoma de México
141 PUBLICATIONS **3,502** CITATIONS
SEE PROFILE

Victoria Ramirez
Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán
35 PUBLICATIONS **1,466** CITATIONS
SEE PROFILE

Andrés León Villanueva
Instituto Politécnico Nacional
2 PUBLICATIONS **0** CITATIONS
SEE PROFILE

Ismael Lares-Asseff
Instituto Politécnico Nacional
103 PUBLICATIONS **374** CITATIONS
SEE PROFILE

Antihypertensive Effect of Protein Hydrolysate from Azufrado Beans in Spontaneously Hypertensive Rats

Paola Valenzuela-García,[1] Norma A. Bobadilla,[2,3] Victoria Ramírez-González,[2,3] Andrés León-Villanueva,[1] Ismael A. Lares-Asseff,[4] Angel Valdez-Ortiz,[5] and Sergio Medina-Godoy[1,†]

Cereal Chem. 94(1):117–123

ABSTRACT

The objective of this study was to evaluate the antihypertensive potential of common bean protein hydrolysate. Protein concentrates were obtained, followed by Alcalase enzymatic hydrolysis, and then ultrafiltrated (3,000 molecular weight cutoff); the lyophilized product was named BP3. The angiotensin converting enzyme (ACE) inhibitory activity was determined as IC_{50} (3.68 ± 0.07 μg/mL). The antihypertensive effect was evaluated in spontaneously hypertensive rats (SHR) by two assays; Captopril ACE inhibitor was used as a reference compound and water as a control. A short-term assay showed a maximum decrease in mean arterial pressure of –41 ± 5 mmHg in SHR, 3 h after oral administration of 500 mg of BP3/kg of body weight (bw). In a long-term assay, a significant decrease in systolic blood pressure of –24 ± 5 mmHg was observed in SHR, after 45 days of oral administration of 500 mg of BP3/kg of bw/12 h. In both assays, BP3 treatment showed antihypertensive effect over SHR, similar to Captopril treatment. The sequences of the most abundant peptides present in BP3, determined by mass spectrometry, were identified as KFPWVK, GADFRKK, and PQSPCKRVNRHS. These peptides are reported for the first time in Azufrado Higuera common beans, and they are most likely responsible for the antihypertensive effect of BP3.

Systemic hypertension is one of the major risk factors for the development of cardiovascular diseases, stroke, and chronic kidney diseases. High blood pressure affects 15–20% of the adult population in Western countries (Jung et al. 2006). Angiotensin converting enzyme (ACE), a zinc metallopeptidase, plays an important role in regulating blood pressure. ACE catalyzes the conversion of angiotensin from an inactive decapeptide called angiotensin I to a potent vasoconstrictor octapeptide called angiotensin II, and it inactivates antihypertensive vasodilator bradykinin (Ondetti 1977). Thus, many laboratories have attempted to synthesize new and better ACE inhibitors, as an alternative to Captopril, Enalapril, Alacepril, and Lisinopril ACE inhibitors, which are currently used in the treatment of essential hypertension and heart failure in humans (Patchett et al. 1980).

The search for natural ACE inhibitors as an alternative to synthetic drugs is of great interest among researchers, and many natural ACE inhibitors have been isolated from functional foods and bioresources. The isolated bioactive peptides from food sources are beginning to have an impact in the treatment of hypertension owing to multifunctional properties such as fewer side effects, good antihypertensive properties, and their easy absorption (Udenigwe and Aluko 2012).

Different studies have identified several ACE inhibitor peptides obtained from enzymatic hydrolysis of various natural sources such as dairy products (Yamamoto et al. 1994), marine products (Jung et al. 2006), vegetable products such as soybeans (Wu and Ding 2001), and rice (Li et al. 2007).

Pulses are inexpensive and affordable source of proteins. Currently, major research efforts have been focused on isolating ACE inhibiting peptides from legumes such as peas (Li et al. 2011; Jakubczyk and Baraniak 2014), lentils (Boye et al. 2010), chickpeas (Medina-Godoy et al. 2012), mung bean (Li et al. 2006), and common beans (Valdez-Ortiz et al. 2012; Rui et al. 2013; Mojica et al. 2015).

Common bean (*Phaseolus vulgaris* L.) is a pulse cultivated worldwide, and it is considered a nutraceutical food and a good source of protein. In the last decade, pulses have gained attention to produce bioactive hydrolysates and peptides (Luna-Vital et al. 2015). The authors recognize the efforts of several researchers for studies focused on the search for other potential biological uses of protein hydrolysates and peptides from common bean: antioxidant capacity and antidiabetic properties (Oseguera-Toledo et al. 2015), antimicrobial activity (Ariza-Ortega et al. 2014), and tumor cell inhibition activity (Luna Vital et al. 2014). It is important to mention that there are a greater number of reports focused on in vitro assays and few studies focused on in vivo assays, such as the antihypertensive effect evaluated in spontaneously hypertensive rats (SHR).

Azufrado Higuera bean (*P. vulgaris* L.) is a sulfur yellow common bean variety from the northwestern part of Mexico that is considered an important source of protein. Previously, Valdez-Ortiz et al. (2012) demonstrated that three varieties of Azufrado Higuera bean hydrolysates exhibited ACE-inhibitor activity. The aim of this study was to evaluate the antihypertensive effect of Azufrado Higuera bean protein hydrolysate administrated orally in SHRs.

MATERIALS AND METHODS

Materials. Azufrado Higuera beans (registration number FRI-045-170205) were donated by the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Valle del Fuerte, Mexico. ACE (from rabbit lung), hippuryl-L-histidyl-L-leucine as a substrate for ACE, and Alcalase enzyme (from *Bacillus licheniformis*) were purchased from Sigma-Aldrich (Saint Louis, MO, U.S.A.). An ultrafiltration system and ultrafiltration membranes (catalog number PLBC07610) were purchased from Millipore (Billerica, MA, U.S.A.). All chemicals used in this study were of analytical grade.

Preparation of Bean Protein Concentrates. Azufrado Higuera beans were milled (3100 laboratory mill, Perten, Hamburg, Germany), and the resultant flours were kept at –20°C until use. Protein concentrates were obtained as described by Valdez-Ortiz et al. (2012). The supernatants were discarded, and the pellets, which contained the protein concentrate, were lyophilized and stored at –20°C.

*The *e*-Xtra logo stands for "electronic extra" and indicates that one supplementary figure is published online.

† Corresponding author. Phone: +52 687 87 2 96 26. E-mail: smedinam@ipn.mx

[1] Instituto Politécnico Nacional, CIIDIR-Unidad Sinaloa, Departamento de Biotecnología Agrícola, Guasave, Sinaloa, Mexico.
[2] Unidad de Fisiología Molecular, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico.
[3] Departamento de Nefrología, Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán, México D.F., Mexico.
[4] Instituto Politécnico Nacional, CIIDIR-Durango, Departamento de Farmacogenómica y Biomedicina Molecular, Durango, Durango, Mexico.
[5] Maestría en Ciencia y Tecnología de Alimentos, Facultad de Ciencias Químico-Biológicas, Universidad Autónoma de Sinaloa, Culiacán, Sinaloa 80000, Mexico.

http://dx.doi.org/10.1094/CCHEM-04-16-0105-FI

Preparation of Protein Hydrolysate. Protein hydrolysis by Alcalase enzyme was carried out according to the method of Valdez-Ortiz et al. (2012). Hydrolysates were filtered in an ultrafiltration system, in which they were fractionated through ultrafiltration membranes with a 3,000 molecular weight cutoff (MWCO), and the ultrafiltered product was lyophilized and stored at –20°C. The hydrolysate obtained contained peptides of different molecular sizes smaller than 3,000, and this fraction was named as bean peptides <3,000 (BP3).

Proximate Analyses. Standard methods (AOAC 2005) were used to determine nitrogen (976.05), fat (920.39), ash (942.05), fiber (978.10), and moisture (930.15) contents of the bean flour, bean protein concentrate, and BP3. Carbohydrate content was estimated as nitrogen-free extract and calculated as the difference between 100 and the sum of percentages of moisture, fat, protein, fiber, and ash.

Amino Acid Analyses. Triplicate samples of protein concentrate and BP3 were analyzed for amino acids. Samples were hydrolyzed at 110°C for 24 h in 6N HCl and prepared for amino acid assay according to method 994.12 (AOAC 2005). Amino acid assays were performed by reversed-phase HPLC (Wu et al. 1997) with an Agilent 1100 HPLC (Agilent Technologies, Santa Clara, CA, U.S.A.) equipped with a fluorescence detector with a 350 nm excitation filter and a 455 nm emission filter. Amino acid standard (1 nmol/μL in 0.1M HCl) (Sigma-Aldrich, lot BCBJ2713V) was used. To avoid partial loss of methionine, samples were oxidized with performic acid to obtain methionine sulfone prior to acid hydrolysis (Baker 1997). Derivatization was performed with *o*-phthalaldehyde/3-mercaptopropionic acid and 9-fluorenylmethylchloformate. Amino acids were separated on a Hypersil ODS 5 μm (200 × 2.1 mm) column, preceded with a Hypersil ODS (20 × 2.1 mm) guard column.

Measurement of ACE-Inhibitory Activity. Antihypertensive potential in vitro of the protein hydrolysate was determined by the ACE-inhibitory activity according to the method of Valdez-Ortiz et al. (2012). ACE-inhibitory activity of BP3 was evaluated at different concentrations (0.0001, 0.001, 0.01, 0.1, 1, 10, 100, and 1,000 μg/mL). Data were adjusted to a linear regression model. The half maximal inhibitory concentration (IC_{50}) value was calculated as the concentration of BP3 required to inhibit 50% of the ACE activity.

Short-Term Antihypertensive Effect in SHR. All experiments involving animals were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* (National Research Council 1996) and were approved by the Animal Care and Use Committee at Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán (NMM-47-11-12-1). For this study, 20 male SHRs (10–16 weeks old) were included. Rats were purchased from Harlan Laboratories (Indianapolis, IN, U.S.A.) and reproduced in the animal facility. The rats were housed in polycarbonate cages regulated with a cycle of 12 h of light and 12 h of darkness. All rats were fed a standard chow diet and tap water ad libitum. The SHRs were randomly divided into three groups: the first group was treated with deionized water, SHR+water (n = 7), and was designated as the control group; the second group was treated with 50 mg of Captopril ACE inhibitor/kg of body weight (bw) (Sigma-Aldrich), SHR+Cap (n = 5); and the third group was treated with 500 mg of BP3/kg of bw (diluted in 800 μL), SHR+BP3 (n = 8). A single dose of each treatment was orally administered by gastric gavage. Rats were placed on a heating pad to maintain core body at 37°C and monitored with a rectal thermometer. Then, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg of bw), approximately every 2 h, to remain anesthetized throughout the experiment. The femoral arteries and jugular veins were catheterized with polyethylene tubing (PE-50). Isotonic SHR plasma obtained from a donor SHR was infused (10 mL/kg of bw) for 15 min to maintain euvolemic conditions. The mean arterial pressure (MAP) was monitored with a P23Gb pressure transducer (Gould, Cleveland, OH, U.S.A.) and recorded on a polygraph (Grass Instruments, Quincy, MA, U.S.A.) throughout the study. An ultrasound transit-time flow probe was placed around the left artery and filled with ultrasonic coupling gel (HR lubricating jelly, Carter-Wallace, New York, NY, U.S.A.) to detect renal blood flow, which was translated into MAP by the pressure transducer (Barrera-Chimal et al. 2011). With the supervision of SHR under anesthesia and after 30 min (time required for the finished plasma infusion and stabilization of MAP), MAP was recorded and set as the basal MAP, and control (water) or Captopril ACE inhibitor or BP3 were administrated by gavage. MAP was recorded every 10 min for 5 h, and three readings at each point were averaged to provide one recording for every 30 min. Blood samples were taken (approximately 12 mL) at the end of the study. The change in MAP (ΔMAP) was calculated by subtracting the data for the different time points from their respective basal values at time zero.

Long-Term Antihypertensive Effect in SHR. For this study, 17 male SHRs (10–16 weeks old) were included. Rats were regulated with a cycle of 12 h of light and 12 h of darkness, and all animals were fed a standard chow diet and tap water ad libitum. The SHRs were randomly divided into three groups: a control group with water, SHR+water (n = 6); a group treated with 25 mg/kg of bw/12 h of Captopril ACE inhibitor, SHR+Cap (n = 6); and a group treated with 500 mg/kg of bw/12 h of bean peptides, SHR+BP3 (n = 5), diluted in deionized water. A dose of each treatment was orally administered every 12 h by gastric gavage for 45 days. The systolic blood pressure (SBP) was measured by the tail-cuff method (noninvasive) with a blood pressure analyzer (model 179, IITC, Woodland Hills, CA, U.S.A.) (Chirino et al. 2008). SBP measurements were made during the light period at time zero, 15 days, 30 days, and 45 days after treatment. The change in SBP (ΔSBP, mmHg) was calculated by subtracting the data for the different time points from their respective basal values at time zero.

Mass Spectrometry and Data Analysis. To get the peptide mass fingerprint of the bean protein hydrolysate (BP3), matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF-TOF) mass spectrometry was used; molecular mass and molecular mass distributions were determined. Mass spectra were obtained on a Bruker Autoflex MALDI-TOF-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Soluble sample of BP3 (1 mg/mL) was dissolved in acetonitrile/water (1:1, v/v). Soluble BP3 (1 μL) was mixed with 1 μL of matrix solution (α-cyano-4-hydroxycinnamic acid in acetonitrile/trichloroacetic acid 0.1%). The sample mixture was placed on the sample desorption plate (T_0209520_0022513_49, Bruker, Billerica, MA, U.S.A.). MALDI conditions were varied widely to obtain optimal spectra by adjusting laser power (30–90%), reflection voltage gain (12×–60×), and pulsed ion extraction (140 ns). Acquired raw data were analyzed with Novo Sequencing by BioTools 3.2 software (Bruker Daltonics). The potential biological activity of the peptides was predicted by using the BIOPEP database (www.uwm.edu.pl/biochemia).

Statistical Analysis. Generalized linear models were applied to compare changes in blood pressure from the baseline with three predictive factors (treatment groups, time of treatment, and subjects). The multiple sample comparison test was used to find significant differences between means at different times. To discriminate among the means, the least significant difference (LSD) method of Fisher was performed by the multiple range test. Generalized linear models, multiple sample comparison tests, and LSD analyses were conducted with Statgraphics Centurion XVII software (version 17.1.06) with significance set at $P < 0.05$.

RESULTS AND DISCUSSION

Protein Hydrolysate. The composition of moisture, protein, fat, crude fiber, ash, and carbohydrates in different Azufrado bean products was characterized. Table I shows that the greatest protein content was determined in the protein concentrate (83.8%) and BP3 (71.4%), as might be expected. In the process of obtaining the bean protein concentrate it was observed that protein yield was 13%, similar as that reported by Valdez-Ortiz et al. (2012). Also, in order

to obtain 30 g of BP3, 100 g of protein concentrate was hydrolyzed, which represented a 30% yield.

Amino Acid Composition. The amino acid profiles determined in the protein concentrate and BP3 and their distribution classified according to the properties of amino acid side chains are shown in Table II, for which almost all concentrations of amino acids in the protein concentrate were shown to be higher compared with those determined for BP3; the only exception was observed for the essential amino acid Lys (K), which presented values of 41.8 and 48.6 mg/g for protein concentrate and BP3, respectively. BP3 was obtained after several larger peptide fractions with molecular weight >3,000 were discarded through ultrafiltration of hydrolyzed protein concentrate. Therefore, the observed lower concentration of amino acids in BP3 was attributed to the loss of amino acids, through the indicated analytical processes of hydrolysis and ultrafiltration. Both the protein concentrate and BP3 were found to be rich in the amino acids Asp (D) and Glu (E), which impart acidic characteristic to the proteins. The distribution of amino acids with different characteristics (hydrophilic, hydrophobic, basic charged, and acidic charged) was also observed, and amino acids with hydrophobic properties were present at the highest concentrations of 329.5 and 267.1 mg/g in protein concentrate and BP3, respectively; it has been previously indicated that the hydrophobic property of amino acids is a very important characteristic of ACE-inhibitory peptides (Pihlanto-Leppälä 2000).

ACE-Inhibitory Activity of BP3. The ACE-inhibitory activity of Azufrado Higuera protein hydrolysate (BP3) was determined. Data were fitted to a lineal equation: ACE-inhibitory activity (%) = 7.92(log BP3) + 45.51. The IC_{50} value of BP3 was 3.68 ± 0.07 μg/mL, which was much higher in comparison with the IC_{50} value of Captopril ACE inhibitor of 0.0015 μg/mL reported by Tsai et al. (2008). However, Ariza-Ortega et al. (2014) reported an ACE-inhibitory activity of IC_{50} = 4.82 ± 1.59 μg/mL in an Alcalase-Flavorzyme hydrolysate of Azufrado Higuera bean protein, which is higher than the value obtained in the present study.

Meisel et al. (2006) indicated that the IC_{50} value is not always directly related to blood pressure reduction. For example, the tetrapeptide Tyr-Gly-Gly-Tyr having an IC_{50} of 16.2 μmol/L did not yield any change in SBP following oral administration at 100 mg/kg (Saito et al. 1994). Conversely, Lys-Val-Leu-Pro-Val-Pro-Gln (β-casein [*f*169–175]) having an IC_{50} of 1,000 μmol/L on oral administration at 1 mg/kg gave a maximal decrease in SBP of –31.5 mmHg (Maeno et al. 1996). Therefore, it was important to evaluate the antihypertensive effect of BP3 in an in vivo assay with SHR rats.

Short-Term Antihypertensive Effect on SHR. At the beginning of the study, the values of basal MAP were 161 ± 7 mmHg for the SHR in the control group, 174 ± 5.2 mmHg for the SHR in the Captopril group, and 164 ± 4.7 mmHg for the SHR in the BP3-treated group. After oral administration of the different treatments, changes in MAP were observed (Fig. 1). According to the generalized linear models, there was a statistically significant effect of the predictive factors (treatments groups, time of treatment, and subjects) on changes in blood pressure, F(49,170) = 13.37, $P = 0.0000$. After 2 h of study, a statistical difference between treatments was observed, F(2,17) = 7.43, $P = 0.0048$. To discriminate among the means, Fisher's LSD method was performed by the multiple range test. LSD results showed that Captopril and BP3 treatments were statistically similar but were significantly ($\alpha = 0.05$) different from the control group. Similar results were obtained at 2.5, 3, 4, and 5 h of study. However, at 3.5 and 4.5 h of study, all treatments were statistically different ($\alpha = 0.05$) from each other. The maximum antihypertensive effect of BP3 was observed at 3 h of study with a MAP of –41 ± 5 mmHg. Conversely, at this point the Captopril treatment presented its lowest antihypertensive effect with a MAP of –46 ± 8 mmHg. This may be owing to an adaptation period of SHR under the Captopril and BP3 treatments, because after 3 h of study both treatments were statistically different ($\alpha = 0.05$).

There are only limited reports on the antihypertensive effect of legume sources in SHR. One of the few studies was carried out on mung bean hydrolysate (<3,000) by Li et al. (2006); they reported a significant decrease in SBP after oral administration of 600 mg/kg of bw, with the highest reduction observed after 6 h (–30.8 mmHg). Based on this study, the effect of BP3 from Azufrado Higuera bean protein represents a more effective way to reduce SBP, at a lower dose and in a shorter period of time, than hydrolysate from mung beans. Li et al. (2011) reported a maximum reduction on SBP of –19 mmHg in SHR at 4 h of study, after oral administration of 200 mg/kg of bw of pea protein hydrolysate (<3,000); this corresponded to 46.34% of the hypotensive effect of BP3 (–41 mmHg) in SHR, which could indicate the beneficial effect of BP3 over SHR. However, because the pea protein hydrolysate was fed at a lower dose, it is difficult to compare the actual effectiveness of BP3 over that induced by pea protein hydrolysate.

Ariza-Ortega et al. (2014) reported the antihypertensive effect of Azufrado bean protein hydrolysate (3,000–10,000) at a dose of 4 mg/kg of bw in SHR ($n = 3$) administered intraperitoneally. The

TABLE I
Proximate Composition of Different Bean Products (Bean Flour, Protein Concentrate, and Common Bean Protein Hydrolysate [BP3])[a]

Composition	Bean Flour (%)	Protein Concentrate (%)	BP3 (%)
Moisture[b]	8.4	2.3	3.7
Protein[c]	27.5	83.8	71.4
Fat	1.5	0.6	0.05
Crude fiber	3.1	0.07	0.2
Ash	5.1	3.5	18.6
Carbohydrate	62	11.8	9.8

[a] All values are expressed as percentage (%) and are means of triplicate measurements. Carbohydrate is calculated by difference.
[b] Dry basis moisture.
[c] Nitrogen–protein conversion factor = 6.25.

TABLE II
Amino Acid Composition of Protein Concentrates and Common Bean Protein Hydrolysate (BP3) (mg of Amino Acid per Gram of Protein) and Their Distribution Classified According to the Properties of Amino Acid Side Chains[a]

Amino Acid Distribution	Amino Acid[b]	Protein Concentrate (mg/g)	BP3 (mg/g)
Hydrophilic	Ser (S)	45.4	44.2
	Thr (T)	33.2	27.1
	Tyr (Y)	33.3	29.3
	Total	111.9	100.6
Hydrophobic	Gly (G)	44.0	34.3
	Ala (A)	68.2	52.7
	Val (V)	NC	NC
	Phe (F)	39.1	34.9
	Ile (I)	48.6	42.3
	Leu (L)	71.0	64.2
	Pro (P)	54.8	37.2
	Met (M)	3.8	1.5
	Total	329.5	267.1
Basic charged	His (H)	ND	ND
	Arg (R)	41.4	19.7
	Lys (K)	41.8	48.6
	Total	83.2	68.3
Acidic charged	Asp (D)	166.3	142.3
	Glu (E)	84.7	77.2
	Total	251.0	219.5

[a] All values are in milligrams of amino acid per gram of protein and are means of triplicate measurements. The amino acid distribution was classified according to the properties of the side chain, and the total amount in milligrams of amino acids distributed according to classification was quantified. NC = not quantified, and ND = not detected. Nitrogen–protein conversion factor = 6.25.
[b] Amino acid standard (Sigma-Aldrich, lot BCBJ2713V).

maximum decline of SBP of –27.13 ± 11.17 mmHg was observed at 2 h of study. However, the authors emphasized that this study was only a preliminary test, in which a small number of rats ($n = 3$) were analyzed. In addition, intraperitoneal administration of protein hydrolysate was not common in similar in vivo studies. In our preliminary assays, a single oral administration of different doses (0, 50, 200, and 500 mg/kg of bw) of BP3 was evaluated for 5 h in SHR; we did not observe any antihypertensive effect, except in SHR fed 500 mg/kg of bw (Supplementary Fig. 1). Because of this reason, we fed SHR with BP3 (<3,000) at 500 mg/kg of bw in the present study.

Girgih et al. (2016) conducted a comprehensive study of the antihypertensive effect of a 5,000 membrane pea protein hydrolysate permeate (PPH-5) during short- and long-term assays in SHR. In the short-term assay, they reported that after 2 h of a single oral administration (100 mg/kg of bw) of PPH-5, the maximum effect of lowering SBP was –36 mmHg. In the long-term evaluation, authors tested the pea hydrolysate PPH-5 incorporated in casein substitute as 0.5 or 1% (w/w) in the SHR diet; they reported maximum SBP reductions of –22 and –26 mmHg, respectively, after 3 weeks. These data are not comparable with our results, because in our study we only evaluated the bean protein hydrolysate (3,000) orally without incorporating any diet in SHR.

Studies on absorption and bioactivity of the hydrolysates reported that low-molecular-weight peptides (<3,000 and <1,000) were better absorbed in the digestive tract and thus have better antihypertensive effect than high-molecular-weight peptides. In particular, the bioactive potential of small peptides was highest because they can be absorbed in the intestine without being broken down by digestive enzymes and thus reach the target sites in the body (Meisel et al. 2006).

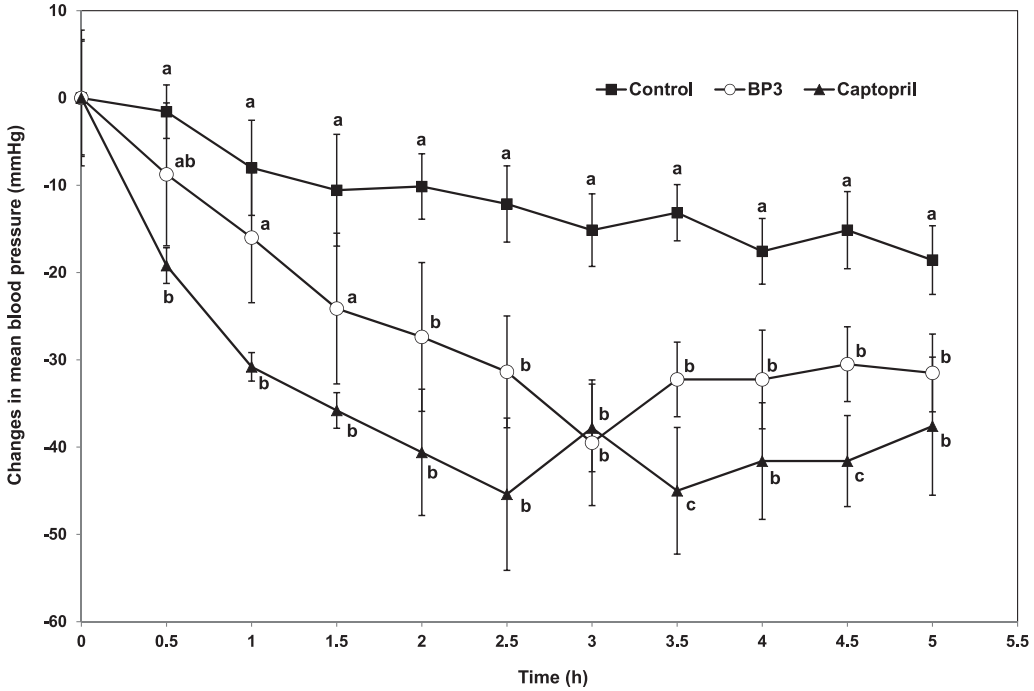

Fig. 1. Short-term antihypertensive effect of common bean protein hydrolysate (BP3) in spontaneously hypertensive rats, by a pressure transducer method for 5 h of evaluation. Different letters represent statistical difference according to the LSD method by multiple range test ($\alpha = 0.05$) with a confidence level of 95%.

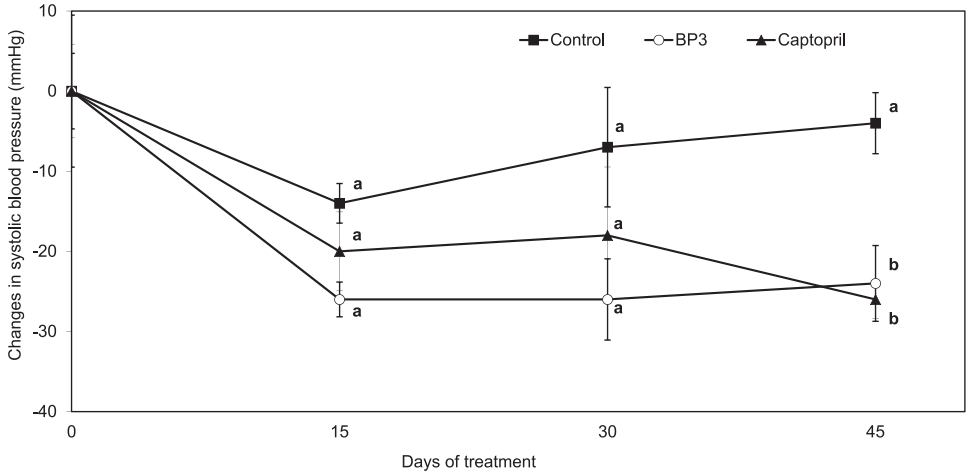

Fig. 2. Long-term antihypertensive effect of common bean protein hydrolysate (BP3) in spontaneously hypertensive rats, by a plethysmograph in the tail of the rat method for 45 days of evaluation. Different letters represent statistical difference according to the LSD method by multiple range test ($\alpha = 0.05$) with a confidence level of 95%.

Long-Term Antihypertensive Effect on SHR. Similar to the short-term antihypertensive effect on SHR study, the values of basal SBP were measured at the beginning of the study. The value of basal SBP was 192 ± 5 mmHg for the SHR in the control group, 197 ± 6 mmHg for the SHR in the Captopril group, and 200 ± 9 mmHg for the SHR in the BP3-treated group. Differences in baseline values between short-term and long-term assays were owing to the measures of blood pressure used for each assay (MAP and SBP). According to generalized linear models, there was a statistically significant effect of predictive factors (treatment groups, time of treatment, and subjects) on changes in blood pressure, $F(25, 42) = 4.25$, $P = 0.0000$. Also, Figure 2 showed that after 15 and 30 days of oral administration of 1 mL/12 h of distilled water (in the control treatment), or 25 mg/kg of bw/12 h of Captopril ACE inhibitor, or 500 mg/kg of bw/12 h of BP3, the SBP reduction on SHR under all indicated treatments was not significantly different ($\alpha = 0.05$). However, at 45 days of study, the BP3 and Captopril treatments were similar but different than the control. The analysis of variance by multiple sample comparison test at 15, 30, and 45 days confirmed the statistical differences of the treatments: $F(2, 14) = 0.98$, $P = 0.3989$; $F(2, 14) = 1.04$, $P = 0.3790$; and $F(2, 14) = 3.90$, $P = 0.0450$, respectively. Additionally, the LSD method showed that the SBP reduction on SHR under the Captopril (–26 ± 2 mmHg) and BP3 (–24 ± 5 mmHg) treatments at 45 days was similar ($\alpha = 0.05$), but both these treatments were significantly different from the control treatment (–4 ± 4 mmHg).

Only one study about long-term antihypertensive evaluation of legume hydrolysate sources was reported. Li et al. (2011) reported the antihypertensive effect of casein supplemented with pea protein hydrolysate on Han:SPRD-cy rats (a model of chronic kidney disease). They indicated that the maximum decrease in SBP of –35 mmHg was observed at 45 days of study. In the present study, a similar maximum decrease in SBP of –24 ± 5 mmHg was observed also at 45 days of study. However, the results obtained by Li et al. (2011) cannot be compared with those of the present research, because the rat model used was different in both studies. Additionally, they used an impure source of protein hydrolysate in the form of a mixture of the pea protein hydrolysate with casein, whereas in the present study a pure protein hydrolysate (BP3) from Azufrado bean was used. That difference in SBP values observed between the studies is attributed to the indicated differences in rat models and the source of protein hydrolysate used.

It is important to mention that there were two methods for measuring blood pressure in the SHR in vivo assays. The first method used in this study was the pressure transducer method (an invasive short-term assay), and the second one was by a plethysmograph in the tail of the rat method (noninvasive long-term assay). A large degree of error in the graphs of both assays was clearly observed; however, high uncertainty in a reported measurement of in vivo assays is also common, owing to multiple factors that affect the true value. Usually experiments with animal models give high variability, and it is normal to see greater variability with an invasive method owing to exposure of rats to stress, anesthesia, and surgery, which is why it is important to increase the number of individuals to analyze. However, data were analyzed with the generalized linear model followed by multiple sample comparison test, and both statistical tests were very effective for data analysis. The viability of these two methods was advocated in many literature reports. The

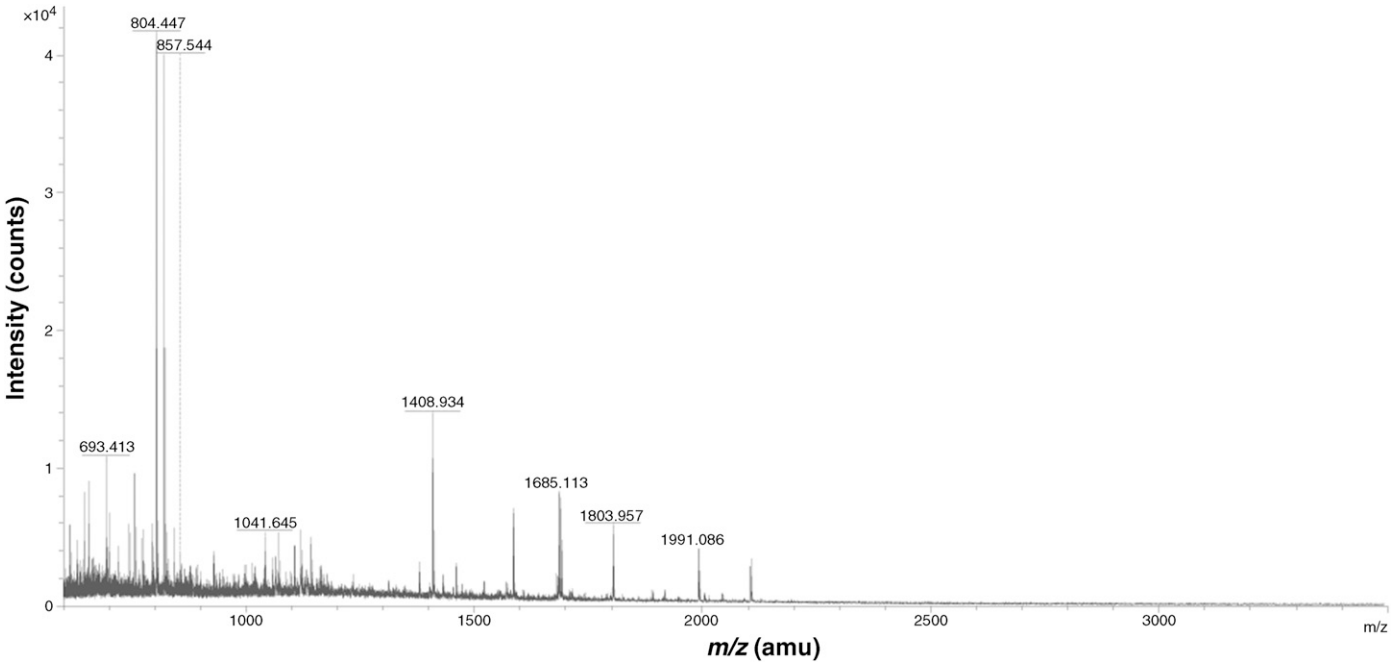

Fig. 3. Peptide fingerprint of common bean protein hydrolysate by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry.

TABLE III
Molecular Mass by MALDI-TOF-TOF Mass Spectrometry and Amino Acid Sequences by Novo Sequencing of Common Bean Peptides (BP3)[a]

Measured *m/z*	Peptide Sequence	Bioactive Sequence	Potential Bioactivity
804.40	KFPWVK	FP, VK, KF	ACE-I, hypotensive
821.40	GADFRKK	FR, GA	ACE-I
1,408.90	PQSPCKRVNRHS	KR, PQ	ACE-I

[a] Potential bioactivities were obtained from the BIOPEP database (www.uwm.edu.pl/biochemia). MALDI-TOF-TOF = matrix-assisted laser desorption/ionization time-of-flight/time-of-flight; and ACE-I = angiotensin converting enzyme inhibition.

aim of the present study was not to evaluate the effectiveness between these two methods. However, these two methods were employed successfully on each of our in vivo short- and long-term assays.

Mass Spectrometry and Data Analysis. The peptide mass fingerprint of BP3 was determined by MALDI-TOF-TOF mass spectrometry. Ions were monitored in the field 500–2,200 *m/z*, and 21 mass spectra were obtained (Fig. 3). Three mass spectra were detected as the most abundant peptides in the hydrolysate, and acquired raw data were analyzed with Novo Sequencing by BioTools 3.2 software (Bruker Daltonics). The sequences of the three major peptides were KFPWVK, GADFRKK, and PQSPCKRVNRHS. The potential biological activity of the peptides as bioactive peptides was predicted by using the BIOPEP database; the potential ACE-inhibitory effect was predicted in the three peptides (Table III). These sequences with ACE-inhibitory activity may be responsible for the antihypertensive activity in the in vivo assays. However further studies are needed to isolate and purify these peptides to test their ACE-inhibitory activity in vitro, and the effect in vivo assays could be correlated with the ACE-inhibitory activity.

In the case of ACE-inhibitory peptides, the most potent and specific peptide inhibitors with similar structures and ACE activity were strongly influenced by the C-terminal tripeptide sequence of those peptides. The tripeptides with W, Y, F, P, and a hydrophobic amino acid at the C-terminus were effective for ACE-inhibitory activity because of the interaction between three subsites at the active site of ACE (Pihlanto-Leppälä 2000).

Peptides from BP3 contained hydrophobic amino acids but not P at the C-terminal tripeptide sequence. Guan Hong et al. (2006) isolated ACE-inhibitory peptides from mung bean (LNYRL, VTPALR, and KLPAGTLF). Also, an octapeptide (PVNNPQIH) isolated from small red bean hydrolysate showed to be an ACE inhibitor (Rui et al. 2013). In research on pea protein hydrolysate, Jakubczyk and Baraniak (2014) reported five peptides isolated with strong ACE-inhibitory capacity (GGSGNY, DLKLP, GSSDNR, MRDLK, and HNTDSR), of which only one peptide contained P at the C-terminal tripeptide sequence; however, the same authors indicated that the occurrence of certain amino acids at the C-terminus or in a peptide does not necessarily mean that the relationship had a strong hypotensive effect.

Structure-activity data also suggest that the positive charge on the guanidine or ε-amino group of C-terminal arginine (R) and lysine (K) side chains contribute substantially to the ACE-inhibitory potency of several peptides (Meisel 1993). Mojica et al. (2015) isolated peptides of different varieties of common beans (black, pinto, red, navy, and great northern) with ACE-inhibitory activity as potential bioactivity in silico (RKRAAQ, RNEQMGAGRLGRLRK, RRQRRRRMRKDK, QQRRLLRRK, and YAGGS). In the present study, all peptide sequences of BP3 hydrolysate showed R or K at the C-terminus, indicating that these peptides could be potential inhibitors of ACE.

CONCLUSIONS

The Azufrado Higuera common bean protein hydrolysate (BP3) obtained after Alcalase hydrolysis and an ultrafiltration process (3,000 molecular weight cutoff) presented antihypertensive property. In vivo assays with SHR showed the effect of BP3 on blood pressure reduction. In a short-term assay, a decline in MAP was observed after a single oral administration of 500 mg/kg of bw of BP3, and the maximum decrease was at 3 h of study. In a long-term assay, a significant decrease on SBP was observed at 45 days of study, on SHR under the BP3 treatment. This is the first study showing the antihypertensive capacity of the oral administration of Azufrado Higuera common bean (*P. vulgaris* L.) protein hydrolysate in a short- and long-term assay with SHR. The sequences of the most abundant peptides of BP3 were detected and identified. It is important to highlight that those sequences are reported for the first time in Azufrado Higuera common beans and that they are most likely responsible for the antihypertensive effect of BP3. Based on the results of this study, it is concluded that these peptides represent valuable information to food scientists and may have a potentially significant impact on human health to prevent or control high blood pressure. Azufrado Higuera common beans could be used in the nutraceutical industry as a value-added ingredient in the form of protein hydrolysate.

ACKNOWLEDGMENTS

The authors thank Fundación Produce Sinaloa for funding support. Also, thanks to Octavio Sanchez Villanueva, Rosalva Pérez Villalba, and Cristino Cruz Rivera for support in the in vivo assays with SHR. Thanks to Jose De J. Berrios and Emma P. Báez Valdez for support in the production of the manuscript draft. P. Valenzuela-García thanks to CONACYT-México, PIFI-IPN, and Colegio de Sinaloa for the scholarship support. The authors also thank CNMN-IPN for mass spectrometry analysis.

LITERATURE CITED

AOAC. 2005. Official Methods of Analysis of the Association of the Official Analytical Chemists, 18th Ed. Methods 930.15 Moisture, 942.05 Ash, 976.05 Nitrogen, 920.39 Fat, 978.10 Crude fiber, and 994.12 Amino acids. W. Horwitz, ed. AOAC: Gaithersburg, MD.

Ariza-Ortega, T. J., Zenón-Briones, E. Y., Castrejón-Flores, J. L., Yáñez-Fernández, J., Gómez-Gómez, Y. M., and Oliver-Salvador, M. C. 2014. Angiotensin-I-converting enzyme inhibitory, antimicrobial and antioxidant effect of bioactive peptides obtained from different varieties of common beans (*Phaseolus vulgaris* L.) with in vivo antihypertensive activity in spontaneously hypertensive rats. Eur. Food Res. Technol. 239:785-794.

Baker, D. H. 1997. Ideal amino acid profiles for swine and poultry and their applications in feed formulation. FERMEX report no. 9. Nutri-Quest: Chesterfield, MO.

Barrera-Chimal, J., Pérez-Villalva, R., Cortés-González, C., Ojeda-Cervantes, M., Gamba, G., Morales-Buenrostro, L. E., and Bobadilla, N. A. 2011. Hsp72 is an early and sensitive biomarker to detect acute kidney injury. EMBO Mol. Med. 3:5-20.

Boye, J. I., Roufik, S., Pesta, N., and Barbana, C. 2010. Angiotensin I-converting enzyme inhibitory properties and SDS-PAGE of red lentil protein hydrolysates. Int. J. Food Sci. Technol. 43:987-991.

Chirino, Y. I., Trujillo, J., Sánchez-González, D. J., Martínez-Martínez, C. M., Cruz, C., Bobadilla, N. A., and Pedraza-Chaverri, J. 2008. Selective iNOS inhibition reduces renal damage induced by cisplatin. Toxicol. Lett. 176:48-57.

Girgih, A. T., Nwachukwu, I. D., Onuh, J. O., Malomo, S., and Aluko, R. E. 2016. Antihypertensive properties of a pea protein hydrolysate during short- and long-term oral administration to spontaneously hypertensive rats. J. Food Sci. 81:H1281-H1287.

Jakubczyk, A., and Baraniak, B. 2014. Angiotensin I converting enzyme inhibitory peptides obtained after in vitro hydrolysis of pea (*Pisum sativum* var. Bajka) globulins. J. Biomed. Biotechnol. Res. Int. 2014: 438459.

Jung, W. K., Mendis, E., Je, J. Y., Park, P. J., Son, B. W., and Kim, H. C. 2006. Angiotensin I converting enzyme inhibitory peptide from yellowfin sole (*Limanda aspera*) frame protein and its antihypertensive effect in spontaneously hypertensive rats. Food Chem. 94:26-32.

Li, G.-H., Qu, M.-R., Wan, J.-Z., and You, J.-M. 2007. Antihypertensive effect of rice protein hydrolysate with in vitro angiotensin I-converting enzyme inhibitory activity in spontaneously hypertensive rats. Asia Pac. J. Clin. Nutr. 16:275-280.

Li, G.-H., Shi, Y.-H., Liu, H., and Le, G.-W. 2006. Antihypertensive effect of Alcalase generated mung bean protein hydrolysates in spontaneously hypertensive rats. Eur. Food Res. Technol. 222:733-736.

Li, H., Prairie, N., Udenigwe, C. C., Adebiyi, A. P., Tappia, P. S., Aukema, H. M., Jones, P. J. H., and Aluko, R. E. 2011. Blood pressure lowering effect of a pea protein hydrolysate in hypertensive rats and humans. J. Agric. Food Chem. 59:9854-9860.

Luna Vital, D. A., González de Mejía, E., Dia, V. P., and Loarca-Piña, G. 2014. Peptides in common bean fractions inhibit human colorectal cancer cells. Food Chem. 157:347-355.

Luna-Vital, D. A., Mojica, L., González de Mejía, E., Mendoza, S., and Loarca-Piña, G. 2015. Biological potential of protein hydrolysates and peptides from common bean (*Phaseolus vulgaris* L.): A review. Food Res. Int. 76:39-50.

Maeno, M., Yamamoto, Y., and Takano, T. 1996. Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from *Lactobacillus helveticus* CP790. J. Dairy Sci. 79:1316-1321.

Medina-Godoy, S., Ambriz-Pérez, L., Fuentes-Gutiérrez, C. I., Germán-Báez, L. J., Gutiérrez-Dorado, R., Reyes-Moreno, C., and Valdez-Ortiz, A. 2012. Angiotensin-converting enzyme inhibitory and antioxidative activities and functional characterization of protein hydrolysates of hard-to-cook chickpeas. J. Sci. Food Agric. 92:1974-1981.

Meisel, H. 1993. Casokinins as bioactive peptides in the primary structure of casein. Pages 67-75 in: Food Protein—Structure and Functionality. K. D. Schwenke and R. Mothes, eds. Academic Press: New York, NY.

Meisel, H., Walsh, D. J., Murray, B., and FitzGerald, R. J. 2006. ACE inhibitory peptides. Pages 269-315 in: Nutraceutical Proteins and Peptides in Health and Disease, 4th Ed. Y. Mine and F. Shahidi, eds. Academic Press: Boca Raton, FL.

Mojica, L., Chen, K., and González de Mejía, E. 2015. Impact of commercial precooking of common bean (*Phaseolus vulgaris*) on the generation of peptides, after pepsin-pancreatin hydrolysis, capable to inhibit dipeptidyl peptidase-IV. J. Food Sci. 80:H188-H198.

National Research Council. 1996. Guide for the Care and Use of Laboratory Animals. National Academy Press: Washington, DC.

Ondetti, M. A. 1977. Design of specific inhibitors of angiotensin-converting enzyme: New class of orally active antihypertensive agents. Science 196:441-444.

Oseguera-Toledo, M. E., González de Mejía, E., and Amaya-Llano, S. L. 2015. Hard-to-cook bean (*Phaseolus vulgaris* L.) proteins hydrolyzed by Alcalase and bromelain produced bioactive peptide fractions that inhibit targets of type-2 diabetes and oxidative stress. 76:839-851.

Patchett, A. A., Harris, E., Tristram, E. W., Wyvratt, M. J., Wu, M. T., and Taub, D. 1980. A new class of angiotensin-converting enzyme inhibitors. Nature 288:280-283.

Pihlanto-Leppälä, A. 2000. Bioactive peptides derived from bovine whey proteins: Opioid and ACE-inhibitory. Trends Food Sci. Technol. 11: 347-356.

Rui, X., Boye, J. I., Simpson, B. K., and Prasher, S. O. 2013. Purification and characterization of angiotensin I-converting enzyme inhibitory peptides of small red bean. J. Funct. Foods 5:1116-1124.

Saito, Y., Wanezaki, K., Kawato, A., and Imayasu, S. 1994. Structure and activity of angiotensin I converting enzyme inhibitory peptides from sake and sake lees. Biosci. Biotechnol. Biochem. 58:1767-1771.

Tsai, J. S., Chen, J. L., and Pan, B. S. 2008. ACE-inhibitory peptides identified from the muscle protein hydrolysate of hard clam (*Meretrix lusoria*). Process Biochem. 43:743-747.

Udenigwe, C. C., and Aluko, R. E. 2012. Food protein-derived bioactive peptides: Production, processing and potential health benefits. J. Food Sci. 77:R11-R24.

Valdez-Ortiz, A., Fuentes-Gutiérrez, C. I., Germán-Báez, L. J., Gutiérrez-Dorado, R., and Medina-Godoy, S. 2012. Protein hydrolysates obtained from Azufrado (sulphur yellow) beans (*Phaseolus vulgaris*): Nutritional, ACE-inhibitory and antioxidative characterization. J. Food Sci. Technol. 46:91-96.

Wu, G., Davis, P. K., Flynn, N. E., Knabe, D. A., and Davidson, J. T. 1997. Endogenous synthesis of arginine plays an important role in maintaining arginine homeostasis in postweaning growing pigs. J. Nutr. 127:2342-2349.

Wu, J., and Ding, X. 2001. Hypotensive and physiological effect of angiotensin converting enzyme inhibitory peptides derived from soy protein on spontaneously hypertensive rats. J. Agric. Food Chem. 49: 501-506.

Yamamoto, N., Akino, A., and Takano, T. 1994. Antihypertensive effects of different kinds of fermented milk in spontaneously hypertension rats. Biosci. Biotechnol. Biochem. 58:776-778.

[Received April 14, 2016. Accepted September 27, 2016.]