Advances in Bioresearch
Adv. Biores., Vol 5 (4) December 2014:54-60
©2014 Society of Education, India
Print ISSN 0976-4585; Online ISSN 2277-1573
Journal's URL:http://www.soeagra.com/abr.html
CODEN: ABRDC3
ICV 7.20 [Poland]

Advances in Bioresearch

ORIGINAL ARTICLE

Obtaining of Antioxidant Peptides from Protein Concentrate of Three Bean Varieties (*Phaseolus vulgaris* L.)

María Carmen Oliver-Salvador[a] , Teresita de Jesús Ariza-Ortega[a], María Guadalupe Ramírez-Sotelo[a], Gustavo Valencia-del-Toro[a], Carlos Edmundo Salas[b], Luciano Capettini[b] and Jorge Yáñez-Fernández*

[a] National Polytechnic Institute. Professional Interdisciplinary Biotechnology Unit. Av. Aqueduct s / n, Col. Barrio La Laguna Ticomán, México DF, CP 07340. México.
[b]Minas Gerais Federal University. BiologicalSciencesInstitute. Av. Antônio Carlos 6627. Pampulha. Belo Horizonte. MG. CEP 31270-901. Brazil.
E-mail: jyanezfe@ipn.mx

ABSTRACT

Proteins are an important component of food as an energy source and for the growth of living organisms. The common bean (Phaseolus vulgaris L.) has for many years been an important source of protein due to its high protein content and now peptides from some of these species have been attributed with a wide range of biological properties, with potential applications in food and pharma. The objective of this work, then, is to obtain peptides from three varieties of bean (black plus, Azufrado Higuera and Pinto Saltillo) using the enzyme Alcalasa® and to determine their antioxidant activity. The peptides obtained were separated by ultrafiltration and HPLC, and the antioxidant activity of the peptide fractions were assessed by DPPH+ and chemiluminescence methods. The peptide fractions with molecular weights from 1 to 3kDa presented the highest antioxidant activity in both methods ($p \leq 0.05$). The black plus bean was observed to have the highest DPPH+ free radical scavenging activity (40.19 ± 3.83%) and the azufrado higuera variety the greatest inhibition of luminol radical (94.02 ± 1.09%). In addition, a peptide was separated with molecular weight of 656.26 Da,96.28%antioxidant activity and an amino acid sequence, NEGEAH.

Keywords: *Phaseolus vulgaris, peptide, antioxidant, protein, hydrolysates, chemiluminescence, biological activity.*

Received 07/10/2014 Accepted 29/11/2014

How to cite this article:
María Carmen Salvador -O , Teresita de Jesús Ariza-O, María G R S, Gustavo Valencia-del-T, Carlos E S, Luciano C and Jorge Yáñez-F .Obtaining of Antioxidant Peptides from Protein Concentrate of Three Bean Varieties (*Phaseolus vulgaris* L.). Adv. Biores., Vol 5 [4] December 2014: 54-60: DOI: 10.15515/abr.0976-4585.5.4.5460

INTRODUCTION

Cellular aging is a hot topic all over the world because of the many causes of cellular oxidation; free radicals can be environmental or a result of the stress of everyday life. In light of this, some natural or synthetic peptides are used to counter the harmful effect of free radicals [1]. Recent studies have shown that it is possible to obtain bioactive peptide fractions from protein hydrolysates, especially if the peptides contain from 2 to 15 amino acid residues [2]. Various peptides of plant and animal origin have been reported with relevant bioactive potential, such as the animal peptides obtained from milk, egg, blood plasma and fish muscle which have been identified, and in some cases chemically synthesized, with proven antihypertensive, antimicrobial, anti-cariogenic, antiulcerant, antitumor, anticoagulant, antithrombotic or antioxidant activity [3,4] It is also documented that vegetable peptides such as those obtained from soy, chick peas, sunflowers, canola, peas and amaranth can have an important effect on metabolic regulation and modulation similar to animal peptides, suggesting their potential as functional food ingredients.

Furthermore, the bean (*Phaseolusvulgaris* L.),which belongs to the legume family, is grown and consumed as part of the human diet for its high protein content primarily in Latin American countries and also in regions of Europe, Asia and Africa[5], [6]. Since the bean is a common food, it is even more important to

study and identify its bioactive peptides, suggesting its inclusion in the human diet to promote health and the prevention of diseases caused by free radicals and stress[1].
Thus, the aim of this study was to obtain peptide fractions of different molecular sizes from the black plus bean (BPB), Azufrado Higuera bean (AHB) and Pinto Saltillo bean (PSB), and identify the peptide with highest antioxidant activity and its possible amino acid sequence.

MATERIALS AND METHODS

Materials

The bean seeds were provided by the National Institute of Forestal, Agricultural and Livestock Research (Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, INIFAP, Mexico). The varieties analyzed were: black plus bean (BPB) FRI-064-2109993, Azufrado Higuera bean (AHB) FRI-001-220995 and Pinto Saltillo bean (PSB) 1424-FRI-026-120901/C. The reagents used were analytic grade from J. T. Baker (Mexico), Sigma-Aldrich (Sigma Chemical Co., USA), or Bio-Rad (Bio-Rad Laboratories Inc., USA).

Collection and preparation of bean flours

Impurities from the bean seeds (BPB, AHB and PSB) were eliminated manually. Subsequently, the seeds were reduced in size using a grain mill and passed through a 40-mesh testing sieve (Montinox, Mexico). The flour was defatted with hexane (1:5 w/v), and kept in constant agitation at 4 °C for 8 h. The defatted flour was sieved until reaching a particle size corresponding to an 80-mesh sieve and stored in hermetically sealed bags[2].

Proximate chemical characteristics of the bean flours

The bean flours were analyzed to determine the content of humidity, protein(N x 6.25),crude fiberand ash were performed according to the methodology proposed in the Official Methods of Analysis [7]. The total carbohydrates were determined by difference.

Extraction of protein concentrates from the beans

Protein extraction was performed by isoelectric precipitation according to Carrasco *et al* [8].The flour samples were suspended in distilled water (1:10 w/v) adjusting the pH to 8.0 and kept under agitation for 1 h. The sample was centrifuged at 12,400×*g* and 4°C for 30 min in a J2-MC centrifuge (Beckman Coulter, USA). The pH of the supernatant was adjusted to 4.5 to achieve protein precipitation, maintaining constant agitation at 4°C for 1h. The sample was then centrifuged at 12,400×*g* and 4°C for 30 min. The retained solids were lyophilized in a Labconco Free Zone 6 Plus (LABCONCO, USA)[8]. The protein concentrate content of the beans was determined by AOAC 954.01 method [7].

Characterization of protein concentrates

The bean concentrates were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a Mini PROTEAN 3 cell (BIO-RAD Hercules, USA) as described by Laemmli [9].The soluble protein from each sample was determined using a standard curve of bovine serum albumin[10].The resolving gel was prepared at 15% and the stacking gel at 6% (w/v). After electrophoresis at 100 mVfor 90min, the protein bands were stained with 0.1% (w/v) Coomassie blue. The molecular weight marker was RPN 2107 (ECL, Amersham Biosciences).

Enzymatic hydrolysis

The enzymatic hydrolysis process was done with the method described by Torruco*et al*. [2].The lyophilized protein concentrates at 4% (w/v) were suspended in water in a glass reactor at a temperature of 55°C, kept in constant agitation for 120 min and the pH maintained at 8.0 with the addition of 0.1 mol·L^{-1}NaOH using a pH-STAT702 Titrino (Metrohm, Germany). Hydrolysis was performed with Alcalasa® (EC 3.4.21.62) with an enzyme/substrate ratio of 0.3 AU·g^{-1}.The degree of hydrolysis during the enzymatic reaction was determined by the equation proposed by Adler-Nissen [11].

Collection of peptide fractions

The hydrolysates were fractionated on an ultrafiltration Amicon Labscale™ system (Millipore, Germany) with molecular weight cut-off membranes of 10 kDa (NMWL: 10,000), 3 kDa (NMWL: 3000) and 1 kDa (NMWL 1000). Following this process, four samples from each bean variety were obtained with different molecular weights: fraction of less than1 kDa (f<1), fraction of 1-3 kDa (f 1-3), fraction of 3-10 kDa (f 3-10) and fraction of more than 10 kDa (f>10). The soluble protein content of each sample was determined[10].

Antioxidant activity

$DPPH^+$ free radical scavenging activity

The scavenging capacity of the free radical $DPPH^+$ (2,2-diphenyl-1-picrylhydrazylin methanolic solution 0.1 mmol·L^{-1}) was determined by the method described by Valdés *et al*[12].An aliquot of 0.1 mL of the sample to be analyzed (fractions of hydrolyzed bean proteins at a concentration of 2 g·L^{-1}) or of the reference antioxidant (trolox 0 - 1.2 mmol·L^{-1}) was mixed with 2.9 mL of the $DPPH^+$ solution. The

absorbance (Abs) was read at 30 and 60 min of reaction at a wavelength of 515 nm on a Lambda XLS spectrophotometer (Perkin Elmer, USA) using methanol as the reference sample. Antioxidant activity (%AA) in terms of the free radical scavenging of the samples was determined with the following equation:

$$\%\ AA = \frac{Initial\ Abs\ of the\ DPPH - Abs\ of\ the\ sample\ after\ 30\ or\ 60\ min}{Initial\ Abs\ of\ the\ DPPH} \times 100$$

Inhibition reaction of free radical luminol

The inhibition of free radical 3-aminophthalhydrazide (luminol) was determined following the protocol of Georgetti *et al*. [13].A reaction cell containing 10 µL of the sample (peptide fractions of bean or the standard antioxidants), 960 µL of phosphate buffer (PBS 0.1 mol L^{-1}, pH 7.4), 10 µL of the luminol solution (2 g L^{-1} dissolved in dimethyl sulfoxide) and 10 µL of H_2O_2 (5×10^{-5} mol·L^{-1} dissolved in PBS) was placed inside the adapter of the luminometer 1250 (BioOrbit, Finland). PBS was used as the reference to calibrate the equipment. The oxidation reaction initiated when 10 µL of the peroxidase enzyme solution (HRP 0.2 IU·mL^{-1}dissolved in water) was added. The reaction was followed-up with theWinDaq (DataQ Instruments Inc., USA) program for 10 min to obtain the luminescence curves. The results were expressed as antioxidant activity (%AA) in terms of luminometric inhibition with the equation:

$$\%AA = \frac{Area\ under\ the\ curve\ of\ the\ sample}{Area\ under\ the\ curve\ of\ the\ reference} \times 100$$

Peptide separation by HPLC

The peptide separation was performed according to the method described by Srihongthong *et al* [14].The peptide fractions of 1 - 3 kDa (1 g·L^{-1}) were eluted at a wavelength of 218nm in a C-18 column (Grace Vydac, USA) adapted to an HPLC LC-20AT (Shimadzu, Japan) with a gradient program (0 – 35%) of acetonitrile-trifluoroacetic acid 0.1% (v/v) and a flow of 1 mL·min^{-1}. Peptide groups were collected initially and the capacity to inhibit luminol radical was determined for each group [13]. Subsequently, the peptides were collected individually and their capacity to inhibit luminol radical was determined. The peptide with the highest degree of antioxidant activity was subjected to mass spectrometry analysis to determine its molecular weight and elucidate its amino acid sequence.

Determination of molecular weight and identification of the peptide

The molecular weight of the peptide with highest antioxidant activity was determined with mass spectrometry using a 4800 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, USA) following the protocol of Alemán *et al* [15] with 1 µL of the purified peptide (1 g·L^{-1}).The identification of the purified peptide was performed with the ExPASy (Bioinformatics Resource Portal, Switzerland) database system, using as a reference the phaseolin sequence (UniProt: P02853), the main protein in *P. vulgaris* L.seeds.

Statistical analysis

A one-way variance analysis and Tukey's honestly significant difference test ($p \leq 0.05$) were performed to compare means using SAS 9.0 software for Windows (SAS Institute Inc., USA).

RESULTS AND DISCUSSION

Proximate chemical characteristics of the flours

The results of the proximate chemical analysis of the flours obtained from BPB, AHB and PSB show significant differences ($p \leq 0.05$) in the chemical composition of the three bean varieties (Table 1). Significant differences ($p \leq 0.05$) were observed in the protein content between the bean varieties; AHB had the highest protein content, followed by PSB and BPB. The results obtained are within the protein range of 23.24 - 27.06% reported by other authors [2,4,6,7]. In addition, the AHB variety presented a higher protein content than that reported by Valdéz *et al* [18] and the PSB showed similar results to those published byWang *et al*. [17] in the same variety. Some authors report that the variation in proteins and the proximate chemical composition in general may be due to factors controlling nutrient acquisition, maturation and accumulation of starch in the seeds, soil type and other crop conditions [19,20].

Characterization of protein concentrates

Protein content

The protein content obtained for the BPB, AHB and PSB concentrates was 79.49 ± 2.30, 82.34 ± 0.82 and 78.41 ± 1.77%, respectively. Makri and Doxastakis [6] reported a protein content of 22.5% in black bean flour with an increase in protein concentration of 72.3% after the extraction process. Valdéz *et al* [18] also reported 61% protein in AHB concentrates and Wang *et al* [16] 62.55% for PSB; in both cases the protein contents obtained in this work are higher than those reported for the same bean varieties. Nevertheless, it must be considered that the differences in protein content of the different bean varieties

may be caused by factors such as harvest period and crop site, as well as other factors specific to the seed type.

Table 1. Chemical composition from flours in different beans varieties

Component	FNP (%)	FAH (%)	FPS (%)
Humedad	7.36 ± 0.04^{c}	7.57 ± 0.08^{b}	8.02 ± 0.06^{a}
Proteína	16.49 ± 1.45^{c}	24.21 ± 0.14^{a}	21.55 ± 0.04^{b}
Grasa	1.68 ± 0.05^{a}	1.44 ± 0.17^{a}	1.43 ± 0.04^{a}
Fibra	5.12 ± 0.55^{a}	3.28 ± 0.18^{b}	5.42 ± 0.28^{a}
Cenizas	5.03 ± 0.06^{a}	4.60 ± 0.03^{b}	4.60 ± 0.07^{b}
Carbohidratos	64.32 ± 0.92^{a}	58.91 ± 0.31^{b}	58.98 ± 0.16^{b}

†Values followed by de same letter, in the same row are not significantly different (P<0.05) by Tukey´s test. Average values of three determinations ± standard deviation of the series. FNP= Black Plus bean, FAH= Azufrado Higuera bean, FPS= Pinto Saltillo bean.

Table 2. DPPH radical –scavenging activity of peptides from bean varieties

	% Antioxidant activities of peptides fractions from bean hydrolysates					
Variety	Concentrated^{D}	Hidrolizado ^{C}	f>10 kDa^{CD}	3-10 kDa^{B}	1-3 kDa^{A}	f<1 kDa^{B}
FNP ^{a}	21.16 ± 1.83	26.15 ± 1.46	27.68 ± 1.30	34.46 ± 4.65	40.19 ± 3.83	26.09 ± 0.97
FAH ^{a}	21.59 ± 0.86	24.16 ± 0.70	18.02 ± 1.92	35.50 ± 0.63	36.91 ± 0.94	35.31 ± 0.71
FPS ^{b}	12.70 ± 0.74	17.59 ± 3.94	20.31 ± 3.18	30.18 ± 7.97	32.30 ± 1.80	37.71 ± 1.63

†Average values of three determinations ± standard deviation of the series. Different letters in rows indicate significant differences (Tukey, p ≤ 0.05). Different letters in column indicate significant differences (Tukey, p ≤ 0.05).FNP= Black Plus bean, FAH= Azufrado Higuera bean, FPS= Pinto Saltillo bean.

Table 3. Antioxidant activity of the hydrolysates from different beans varieties by the *OH/luminal Chemiluminescence method.

	% antioxidant activity of bean peptides from beans					
Variety	Concentrated^{F}	Hydrolyzates^{D}	f >10 kDa^{E}	3-10 kDa^{C}	1-3 kDa^{A}	f <1 kDa^{B}
FNP ^{b}	36.07 ± 1.77	77.49 ± 1.43	83.72 ± 2.48	87.33 ± 0.73	92.68 ± 3.07	76.32 ± 0.91
FAH ^{a}	72.84 ± 2.42	74.14 ± 2.51	47.06 ± 3.11	85.57 ± 1.07	94.02 ± 1.09	91.49 ± 1.15
FPS ^{c}	42.77 ± 2.91	74.21 ± 1.58	41.97 ± 1.92	74.84 ± 2.42	93.85 ± 0.83	90.60 ± 1.32

†Average values of three determinations ± standard deviation of the series. Different letters in rows indicate significant differences (Tukey, p ≤ 0.05). Different letters in column indicate significant differences (Tukey, p ≤ 0.05).FNP= Black Plus bean, FAH= Azufrado Higuera bean, FPS= Pinto Saltillo bean

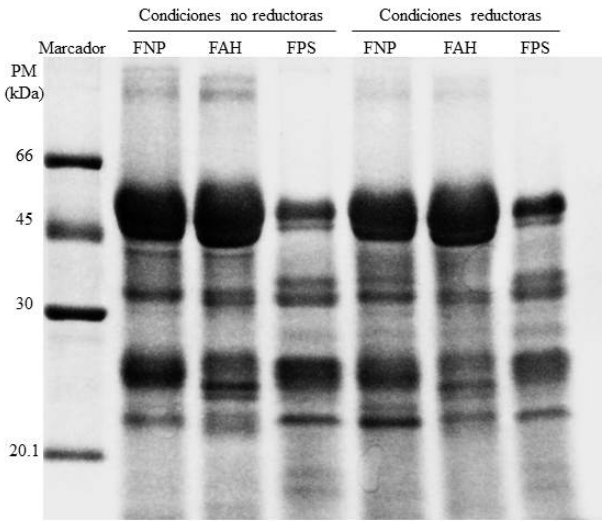

Figure 1. Polyacrylamide gel electrophoresis (SDS-PAGE) profiles of protein concentrates from Black plus(FNP), Azufrado Higuera (FAH) and Pinto Saltillo (FPS) beans

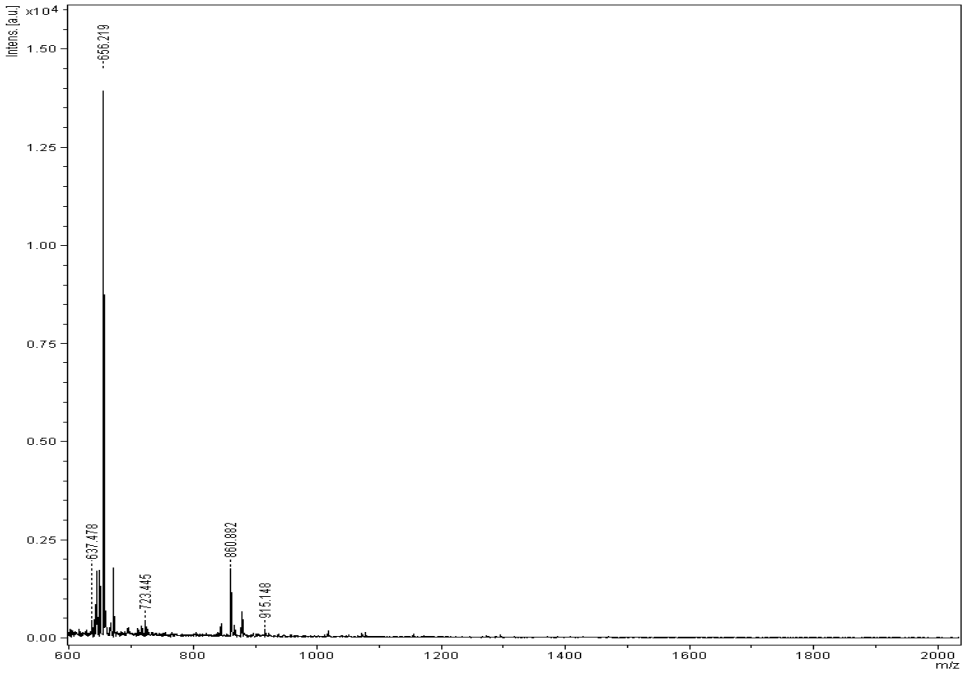

Figure 2. Mass spectra of a FAH peptide (< 3 kDa) with antioxidant activity

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

The electrophoretic profile with and without reducing agent (β-mercaptoethanol) of the protein content of the bean flour of the three varieties used (BPB, AHB, PSB), showed mainly bands of 50 and 30 kDa (Figure 1). The proteins with molecular weights close to 50 kDaand 30 kDa correspond to the *phaseolus* family and lectins, respectively, which coincides with the findings of Camacho *et al* [20] for other bean varieties. Phaseolin is the largest protein reserve of bean seeds; Emaniand Hall[22]reported that 50% of the protein content of beans belongs to the phaseolin group, which in turn differ in asparagine causing three structures of different molecular weights to form: α (51,000-53,000 Da), β (47,000-48,000 Da) andγ (43,000-46,000 Da).

Enzyme hydrolysis

The degree of hydrolysis obtained for the AHB, BPB and PSB protein concentrates was 22.04 ± 2.33, 21.25 ± 1.61 and 21.18 ± 2.50%, respectively. Torruco *et al* [2] reported degrees of hydrolysis of 49.48% for the black Jamapa variety and Valdéz *et al* [12] 73% for the AHB variety. Figueroa *et al* [23], however, mentioned that the degree of hydrolysis depends on the initial substrate concentration, enzyme type and concentration, temperature and reaction time; the latter is consistent with the results obtained in this work due the lower concentration of substrate used.

Determination of antioxidant activity by the DPPH⁺ free radical scavenging method

The results show that the antioxidant activity of the total hydrolysates and their peptide fractions presented significant differences ($p \leq 0.05$) between 30 and 60 min of reaction, which agrees with that reported by Rocha *et al* [24]. The antioxidant effect was observed to be dependent on the variety of bean used, the BPB and AHB varieties having the highest antioxidant activity (Table 2). It was also observed that the peptide group between 1 and 3 kDa showed the highest antioxidant activity (DPPH⁺free radical method) with values of 40.19, 36.91 and 32.30% in BPB, AHB and PSB, respectively; followed by peptide fractions with molecular weights of 3-10 kDa and those of less than 1 kDa. Only a few published works with vegetable protein hydrolysates and their peptide fractions show results of antioxidant activity; such is the case of Torruco *et al* [2]who used ABTS⁺free radical [2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] to determine that the protein hydrolysate of black Jamapa bean with Alcalasa® had up to 60.59% inhibition. Furthermore, Wang *et al* [25] reported that they obtained higher values of antioxidant activity in peptides of 1-10 kDa from egg protein, up to 62.64%. These results agree with those obtained in this work indicating that in protein hydrolysates, the antioxidant effect is enhanced with regard to the reduced molecular size of the peptide.

Determination of antioxidant activity by chemiluminescence

The peptide fractions presented values of antioxidant activity in the range of 36.07 - 94.02% (Table 3). The fractions with highest antioxidant activity (determined as inhibition of luminol free radical) were those corresponding to molecular weights from 1-3 kDa with 92.68%, 94.02% and 93.85% in BPB, AHB

and PSB, respectively, followed by the peptide fractions with molecular weights less than 1 kDa. For its part, AHB presented antioxidant activity of 91.49% ($p \leq 0.05$), higher than the BPB and PSB varieties. The size of the peptides with highest antioxidant activity (peptides with MW < 3 kDa) agrees with that reported by Carrasco *et al*. [8]who found the maximum reduction of ABTS+ radical with black Jamapa bean with molecular weights less than or equal to 700 Da.

A comparison of the antioxidant activity presented by the total hydrolysates and their peptide fractions determined by both methods showed similar tendencies, however, an increase in antioxidant activity was seen with the luminescence method which may be associated with the nature of the radicals, the antioxidant compounds used as reference and the sensitivity of the method. Chemiluminescence has been reported as a method to determine antioxidant activity with the advantage of requiring small samples and being highly sensitive in monitoring the reaction [26]. This is important when working with small quantities from a separation and purification process as in the case of peptides.

Separation of peptides with antioxidant activity

Chemiluminescence was used to determine the antioxidant activity of the 13 peptide groups obtained from the peptide fraction processed by HPLC (f 1-3 kDa of AHB). Two groups eluted at concentrations of 7 - 8.75% and 8.75 - 10.5% (v/v) of acetonitrile/TFA presented percentages of antioxidant activity higher than 80%; these groups were therefore taken for a second separation and all the new fractions were equally analyzed. It was determined that the peptide fraction with the highest antioxidant activity (85.47%) was the one eluted at 9.7% (v/v) acetonitrile/TFA. In order to separate the fraction obtained, it was analyzed by HPLC and the antioxidant activity was seen to increase up to inhibition values of 96.28 ± 0.77%.

Identification of the peptide with antioxidant activity

The peptide fraction with highest antioxidant activity was analyzed by mass spectrometry and was found to have a molecular weight of around 656.263 Da (Figure 2). Using the Expert Protein Analysis System and taking as reference the predominant protein in beans (phaseolin, UniProt: P02853), the amino acid sequence of the peptide with highest antioxidant activity of the AHB protein hydrolysate was determined to be a hexapeptide corresponding to residues Asn - Glu - Gly - Glu - Ala - His (NEGEAH). The size of this peptide is close to that of other peptides with antioxidant activity such as PYFNK of 667.80 Da reported by Wang *et al* [25]and other hexapeptides with biological activity ascertained by Yamada *et al* [3] with novokinin (RPLKPW) and ovokinin (RADHPF). Furthermore, Srihongthong *et al* [14] attributed antioxidant activity to the His and Tyr residue contenting the peptide and determined an activity of72 ± 0.99% in an animal peptide. Alemán *et al* [15] have also reported that a single peptide can possess more than one biological activity and therefore the peptide obtained in the laboratory may present more biological activities.

CONCLUSIONS

In both of the methods used it was observed that on reducing the molecular size of the peptide fractions, the antioxidant activity of the peptides increased. The peptides with highest antioxidant activity were those corresponding to the peptide fractions of bean hydrolysates with molecular weights of 1-3 kDa and less than 1 kDa, especially from the Azufrado Higuera variety, with values of up to94.02%. A peptide was separated from AHB with antioxidant activity of 96.28% and molecular weight of 656.263 Da, whose amino acid sequence corresponds to NEGEAH.

REFERENCES

1. Jiménez A., M.A., Román, C.N.R., & García, I. (2011). Actividad antioxidante y antimicrobiana del extracto hexánoico y compuestos puros del rizoma de *Aristolochiataliscana*. Rev. Mex. Cienc. Farm., 42(3):35-41.
2. Torruco, U.J., Chel, G.L., Martínez, A.A., Dávila, O.G., & Betancour, A.D. (2009). Angiotensin-I converting enzyme inhibitory and antioxidant activities of protein hydrolysates from *Phaseolus lunatus* and *Phaseolus vulgaris* seeds.LWT Food Sci. Technol., 42:1597-1604.
3. Yamada, Y., Nishizawa, K.,Yokoo, M., Zhao, H., Onishi, K., Teraishi, M., Utsumi, S., Ishimoto, M., &Yoshikawa, M.(2008). Anti-hypertensive activity of genetically modified soybean seeds accumulating novokinin. Peptides, 29(3):331-337.
4. Siddiq, M., Ravi, R., Harte, J.B., & Dolan, K.D. (2010). Physical and functional characteristics of selected dry bean (*Phaseolus vulgaris* L.) flours.LWT Food Sci. Technol., 43(2):232-237.
5. Boye, J., Zare, F., & Pletch, A. (2010). Pulse proteins: Processing, characterization, functional properties and applications in food and feed. Food Res. Int., 43:414–431.
6. Makri, E.A., & Doxastakis, G.I.(2006). Emulsifying and foaming properties of *Phaseolus vulgaris* and *coccineus* proteins. Food Chem., 98(3):558-568.
7. AOAC. (1997). In: William Horwitz (Ed.). Official Methods of Analysis(17th ed.). Washington.

8. Carrasco, C.J., Hernández, Á.A.J., Jiménez, M.C., Jacinto, H.C., Alaiz, J.M., Girón, C., Vioque, J., & Dávila O.G. (2012). Antioxidant and metal chelating activities of peptide fractions from phaseolin and bean protein hydrolysates. Food Chem., 135:1789–1795.
9. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4.Nature, 227:680-685.
10. Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal.Biochem.,* ***72:***248-254.
11. Adler-Nissen, J. (1986). Enzymatic hydrolysis of food proteins. Elsevier Applied Science Publication. New York.
12. Valdés, S.T., Medeiros, C.C.M., Michelluti, D.J., & García, T.V.L.C.(2011). Association of genotype and preparation methods on the antioxidant activity, and antinutrients in common beans (*Phaseolus vulgaris* L.).LWT Food Sci. Technol., 44(10):2104-2111.
13. Georgetti, S.R., Casagrande, R., Di-Mambro, V.M., Azzolini, A.E.C.S.,& Fonseca, M.J.V. (2003). Evaluation of the antioxidant activity of different flavonoids by the chemiluminiscence method.APPS Pharm. Sci., 5(2):111-115.
14. Srihongthong, S., Pakdeesuwan, A., Daduang, S., Araki, T., Dhiravisit, A., &Thammasirirak, S. (2012). Complete amino acid sequence of globin chains and biological activity of fragmented crocodile hemoglobin (*Crocodylussiamensis*). Protein J., 31(6):466-76.
15. Alemán, A., Giménez, B., Pérez, S. E., Gómez, G. M. C., & Montero, P.(2011). Contribution of Leu and Hyp residues to antioxidant and ACE-inhibitory activities of peptide sequences isolated from squid gelatin hydrolysate. Food Chem., 125:334–341.
16. Wang, N., Hatcher, D.W., Tyler, R.T., Toews, R., &Gawalko, E.J. (2010). Effect of cooking on the composition of beans (*Phaseolus vulgaris* L.) and chickpeas (*Cicerarietinum* L.).Food Res. Int., 43(2):589–594.
17. Chung, H.J., Liu, K.Q., Pauls, P., Fan, M.Z., & Yada, R.(2008). *In vitro* starch digestibility, expected glycemic index and some physicochemical properties of starch and flour from common bean (*Phaseolus vulgaris* L.) varieties grown in Canada. Food Res. Int., 41(9):869-875.
18. Valdéz, O.A., Fuentes, G.C.I., Germán, B.L.J., Gutiérrez, D.R., &Medina, G.S.(2012). Protein hydrolysates obtained from Azufrado (sulphur yellow) beans (*Phaseolus vulgaris*): Nutritional, ACE-inhibitory and antioxidative characterization. LWT Food Sci.Technol., 46:91–96.
19. [Osborn, T.C., & Brown, J.W.S. (1988). Genetic control of bean seed protein. Crit. Rev. Plant Sci., 7(1):93-116.
20. Paredes, C.M., Becerra, V.V., &Tay, U.J. (2009). Inorganic nutritional composition of common bean (*Phaseolus vulgaris* L.) genotypes race Chile. Chil. J. Agr. Res., 69(4):486-495.
21. Camacho, E.M.K., Peinado, G.L.I., López, V.J.A., Valdéz, O.A., Salinas, P.R.A., Moreno, H.C.G., & Medina,G.S. (2010). Caracterización proteómica de granos de frijol azufrado (*Phaseolusvulgaris*) cultivados en el Estado de Sinaloa. Ra Ximhai, 6(1):23-36.
22. Emani, C., & Hall, T.C. (2008). Phaseolin: Structure and Evolution. The Open Evolution Journal, 2:66-74.
23. Figueroa, O.A., Zapata, J.E., & Gutiérrez, G.A. (2012). Modelamiento de la cinética de hidrólisis enzimática de proteínas del plasma bovino. Revista de Ingeniería de Antioquia, 17:71-84.
24. Rocha G., N.E., Herzog, A., González, L.R.F., Ibarra, P.F.J., Zambrano, G.G., &three different color groups of common bean cultivars (*Phaseolusvulgaris*). Food Chem., 103:521-527.
25. Wang, B., Li, Z.R., Chi, C.F., Zhang, Q.H., & Luo, H.Y. (2012a). Preparation and evaluation of antioxidant peptides from ethanol-soluble proteins hydrolysate of *Sphyrnalewini* muscle. Peptides, 36(2):240-250.
26. Jakubowski, W., Ertel, D., Bilinski, T., Kedziora, J., &Bartosz G. (1998). Luminol luminescence induced by oxidants in antioxidant deficient yeast *Saccharomyces cerevisiae*. Biochem.Mol. Biol. Int., 45(1):191-203.