PAPER

ISOLATION OF BABY LIMA BEAN (*PHASEOLUS LUNATUS*) PROTEINS FRACTIONS AND EVALUATION OF THEIR ANTIOXIDANT ACTIVITY

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ABSTRACT

The aim of this work was to obtain fractions of baby Lima Bean Protein Concentrate (LBPC) from (*Phaseolus lunatus*) and to evaluate their antioxidant activity. LBPC was prepared by alkaline extraction and isoelectric precipitation at pH 4.5. LBPC was subject to gastrointestinal digestion simulation. LBPC was fractionated using a DEAE Affi-Gel Blue Gel column. LBPC presented a protein content of 77.20% with a protein solubility capacity ranging between 37.34% to 99.98%. The antioxidant activity was evaluated using the FRAP, DPPH and ORAC methods. LBPC cationic fractions presented a high antioxidant activity when using the ORAC method with values ranging between 0.47 to 3.37 μmol TE/μmol of sample.

Keywords: baby lima bean, *Phaseolus lunatus*, simulated gastrointestinal digestion, protein concentrate, fractions, antioxidant activity

1. INTRODUCTION

Soybean Protein Isolate (SPI) are produced with defatted soybean flour. SPI is used in the food industry as a food ingredient for different purposes due to its functional properties such as a high solubility, a high foaming capacity and a high protein content. In Ecuador, soybeans and SPI imports for food purposes are high. The Government has implemented measures to promote the search for new native matrices that allow obtaining protein concentrates and isolates. Baby lima bean (*Phaseolus lunatus*) is a legume belonging to the Fabaceae family. This crop has been described for the exceptional potential in adaptation to lowland tropical conditions and potentially important as a food legume (DRAGO *et al.*, 2016; WU *et al*., 2016). Animal proteins, such as meat, milk, fish and eggs, are generally expensive and relatively difficult to acquire, which has led to a worldwide increase in research into vegetable protein sources. For their high protein content, legumes have formed an important part of this search being a cheaper, an alternative protein source and an important crop nitrogen fixative (SATHE and SALUNKHE 1981). Legumes are a source of quality protein in developing countries of South America such as Ecuador, Colombia, Peru and Venezuela. *Phaseolus lunatus* seeds have a high protein content with 26% of protein content (BETANCUR-ARCONA *et al., 2009).* The protein isolate obtained from *Phaseolus lunatus* presents a protein content of 71.13%-73.75%. This protein content in lima bean makes this plant to be an excellent protein source for food industry applications (CHEL-GUERRERO *et al.,* 2002). *Phaseolus lunatus* can be used to manufacture concentrate and isolate protein with excellent functionals properties.

The easiest way to obtain proteins from animal and plant sources is through alkaline extraction followed by isoelectric precipitation. For the characterization of the proteins present in protein isolates, different analytical techniques such as chromatography methods have been used. Many ion exchange columns (IEC) have been used to fraction different protein concentrates and isolate them to evaluate their biological activities and their possible use as functional ingredients in the food industry (CARRILLO *et al*., 2016a; 2016b; 2018; DE CASTRO and CASON 2017; GASPARD, 2017; TABTABAEI *et al*., 2017). *Phaseolus angularis* (red bean) globulins have been fractionated using an IEC of DEAE-Sepharose. Native and heated fractions were characterized using the electrophoresis technique. *Phaseolus vulgaris, Phaseolus lunatus, Canavalia ensiformis* and *Mucuna pruriens* legume plants belonging to the Fabaceae family have been hydrolyzed and fractionated with different methods such as ultrafiltration with membrane. Total hydrolysates and fractions have been described with different biological activities such as antioxidant, antibacterial, antihypertensive and antihyperglycemic activities using *in vivo* and *in vitro* models for their evaluation (CÁRDENAS *et al*., 2018; CUNSOLO *et al*., 2007; LUNA-VITAL *et al*., 2015; MAGAÑA *et al*., 2015; MAMILLA and MISHRA 2017; YOSHIDA 1989).

Different antioxidant activity methods are used to evaluate the potential of different compounds to determine the quality of foods of animal and vegetal sources. These antioxidant activity methods allow to enhance the nutritional and biological value of these components. These methods allow a biological characterization of bioactive compounds as polyphenols, proteins and peptides. Among the most used methods to evaluate antioxidant activity are the ORAC and FRAP methods. There is a high interest in finding new natural antioxidant compounds. Vegetal proteins can be a source of compounds with antioxidant abilities such as polyphenols, lipids, and proteins. Proteins can be hydrolyzed with different enzymes, being possible to simulate human condition of gastric digestion and duodenal digestion (HERNÁNDEZ-LEDESMA *et al*., 2004; ORSINI DELGADO *et al*., 2011; VILCACUNDO *et al*., 2018a;).

Protein concentrates and protein isolates with antioxidant activities have been reported by different authors (JE *et al*., 2005; OLAGUNJU *et al*., 2018; ORSINI-DELGADO *et al*., 2016; POWNALL *et al*., 2010). For example, quinoa protein concentrate (QPC) Chenopodium quinoa and amaranth protein concentrate (APC) Amaranthus caudatus have been described with antioxidant activity and the capacity to inhibit lipid peroxidation in the zebrafish model (VILCACUNDO *et al*., 2017; 2018b).
The aim of this study was to obtain baby lima bean (*Phaseolus lunatus*), protein concentrate, and determine its *in vitro* digestibility. Protein concentrate was fractionated using an exchange column, and the antioxidant activity was evaluated using the ORAC, FRAP and DPPH methods.

2. MATERIALS AND METHODS

2.1. Reagents

Porcine gastric mucosa pepsin (4500 U/mg), porcine pancreas pancreatin (10000 U/mg), 2,2'-azobis (methylpropionamide)-dihidrochloride (AAPH), 2, 4, 6-tripyridyl-s-triazine (TPTZ), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2-diphenyl-1-picrylhydrazyl (DPPH), fluorescein disodium (FL) and trifluoracetic acid (TFA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The other reagents used in this study were of analytical grade.

2.2. Material vegetal

Baby lima bean (*Phaseolus lunatus*) samples were obtained from a native crop of the Recinto la Sequita of Manabí region (Manabí-Ecuador). The seeds of baby lima bean were collected in July,2016. The samples are registered in the ESPOL sample collection. The humidity of beans was determined with a value <10%. Then, the beans were ground using a Perten Laboratory mill 3100 and sifted in a sieve Advantech DuraTapTM DT168 with mesh # 70 (0.210mm). The obtained flour bean was vacuum packed and stored at laboratory temperature.

2.3. Proximate analysis

Proximal flour bean analysis and LBPC was performed, according to the official methods of the Official Association of Analytical Chemists (AOAC 1997). Moisture content was determined by the AOAC 925.10 method (AOAC 1997), protein by the AOAC 2001.11 Kjeldhal method (factor 6.25) (AOAC 2001), fat by the AOAC 922.06 method (AOAC 1997), and ash by the AOAC 923.03 method(AOAC 1997).

2.4. Preparation of LBPC

LBPC was prepared according to POVEDA *et al. (*2016). The bean flour was defatted with hexane (1:10 w/v) for 24 h. Then, 100 g of defatted flour was resuspended in Milli-Q water (1:10, *w:v*) at pH 9.5 for 15, 30, 45 and 60 min at 40°C. Then, the solution obtained was centrifuged at 5000 x g for 30 min at 25°C. The supernatant solution was adjusted to pH 4.5 using 1 N HCl and centrifuged for 20 min at 8000 x g at 25°C. The precipitated obtained was stored and then adjusted at pH 7.0 with 0.1 M NaOH. Then, the neutralized precipitated was lyophilized and frozen at -80°C.

2.5. Protein solubility capacity

LBPC were dissolved in distilled water at a concentration of 0.2% (*w:v*) and the pH of the suspension was adjusted to pH 2.0 – pH 10.0 using solutions of 0.001N HCl and 2N NaOH. The suspensions were shaken for 1 h and centrifuged at 10000 rpm for 10 min in a Sorvall Legend Micro 17 centrifuge (ThermoFhiser Scientific, Germany). The content of protein in the supernatant was analyzed with the BCA protein method. The content of soluble protein was expressed as the percentage of the content of protein present in the sample (PAZMIÑO *et al.*, 2018).

2.6. Fractionation of LBPC with an anion-exchange column

20 mg/mL of LBPC was centrifuged at 10000 x g for 20 min and 1 mL of supernatant was injected in the column of anionic exchange chromatography DEAE Affi-Gel® Blue Gel (Bio-Rad, Hercules, CA, USA). Then, 10 mL of buffer Tris-HCl 50 mM at pH 7.0 was loaded in the column to elute proteins not adhered in the column as proteins with charge positive. Then, 10 fractions of 1 mL each, were collected, then 10 mL of NaCl 500 mM were loaded in the column to elute the retained proteins (negative proteins) (QI *et al.*, 2001). 10 fractions of 1 mL each, were collected to be analyzed using the SDS-PAGE electrophoresis method. Protein contents of fractions were determined using the BCA method.

2.7. Gastric and duodenal digestion of LBPC

LBPC (10 mg/mL) was subject to simulated gastric digestion using pepsin enzyme (2000 U/mL) at pH 3.0 for 2 h at 37°C with agitation. The pepsin enzyme was inactivated by heating at 80°C for 10 min. One milliliter of gastric digestion was mixed with one milliliter of pancreatin enzymes (100 U/mL) at pH 7.0 for 2 h at 37°C. The enzymatic reaction was stopped by heating at 90°C for 10 min (PIÑUEL *et al.*, 2019).

2.8. % degree of hydrolysis by the Orthophthalaldehyde (OPA) assay

The hydrolysis degree (%DH) of gastric and duodenal digest from LBPC was determined using the OPA method described by PIÑUEL *et al.* (2019).
OPA solution: 25 mL of sodium tetraborate (100 mmoL/L) was mixed with 2.5 mL of 20% (w/v) sodium dodecyl sulfate, 40 mg of OPA was dissolved in 1.0 mL of methanol and 100 μL of 2-mercaptoethanol. The final volume was of 50 mL.
Derivatization OPA: 10 μL of the sample was mixed with 3.4 mL of the OPA solution and the mixture was stored at 25°C for 2 min. The absorbance was measured at 340 nm. %DH was determinate with the equation:

$$\%DH = Abs \times 1{,}934 \times d/c$$

where Abs is the sample absorbance, d is the factor of dilution and c is the concentration of protein in LBPC (mg/mL).

2.9. SDS-PAGE electrophoresis analysis of LBPC and their fractions

LBPC and LBPC fractions were analyzed using the SDS-PAGE electrophoresis method. The samples were analyzed using 15% polyacrylamide gels in a Mini-Protean

electrophoresis system (Bio-Rad, Hercules, CA, USA). The standard protein (Precision Plus ProteinTM Unstained standard, Bio-Rad) with a range of 10 kDa-250 kDa was used to determine the molecular weight of proteins present in the samples (QUINTEROS *et al.*, 2016; TOAPANTA *et al*., 2016).

2.10. RP-UHPLC analysis of LBPC fractions

LPBC and LBPC fractions were analyzed by RP-UHPLC technique using the Agilent 1200 infinity series UHPLC (Agilent Technologies, Waldbron, Germany). The signal of the chromatograms was registered at the wavelength of 280 nm. The separation of the samples was made with the help of a Zorbax EC C18 Agilent Poroshell 120 (4.6 mm x 50 mm x 2.7 μm) analytical column. The solvents used were solvent A [Milli-Q water + TFA 0.37%] and solvent B [acetonitrile + TFA, 0.270%]. Samples were eluted at 1.0 mL/min with a lineal gradient from 0% to 70% of solvent B for 15 min. Before analysis all samples were filtered with a membrane filter of 0.45 μm and then were centrifuged for 2 min at 12000 rpm at 4°C. Samples were injected for three times (LARA *et al.*, 2017).

2.11. Antioxidant activity of LBPC and their fractions

The colorimetric assays ferric ion reducing antioxidant power (FRAP) and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was performed following the procedure described by BENZIE and STRAIN (1996) and PIÑUEL *et al*. (2019) respectively. The antioxidant capacity of LBPC and LBPC fractions was evaluated using an FRAP assay. The solutions used were 300 mM acetate buffer at pH 3.6, 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3$ $6H_2O$ solution. A new working solution was prepared by a mixture of 25 mL acetate buffer with a 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3$ $6H_2O$ solution and then incubated at 37°C before use. LBPC and fractions (150 mL) were mixed with 2850 mL of the FRAP solution for 30 min in darkness. Readings of the colored product were measured at a wavelength at 593 nm. The standard Trolox linear curve was used as control (20 to 1200 mM). All results were expressed as mM of Trolox equivalents (TE) per g sample. All experiments were made in triplicate. LBPC and fractions were used to evaluate their antioxidant activity using the DPPH method. The ability to capture free radicals by antioxidants was analyzed using the radical species DPPH, measuring the decrease of the absorbance at 517 nm spectrophotometrically (spectrum SP-2100UV/SP spectrophotometer, China). Each assay was made five times, the value of antioxidant activity being expressed as mg of TE/100 g sample.

The oxygen radical absorbance capacity fluorescein (ORAC-FL) assay was made according to MOORE *et al* (2005). A synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA) was used. Samples and Trolox standards were prepared with 50% acetone. All other reagents were prepared in a 75 mmol/L phosphate buffer (pH 7.4). Each well in a 96-well plate contained 30 µL of 20 µmoL/mg sample or 50% acetone for blank and 225 µL fluorescein (81.63 nmol/L). The plate with a cover was incubated for 20 min in 37°C, and then 25 µL AAPH (0.36 mol/L) were added to each well to start reaction, resulting in a final total volume of 280 µL. The fluorescence was recorded every minute for 2 h at 37°C, where excitation and emission of wavelengths were 485 and 528 nm. Trolox was used as standard (0-200 μmoL) with a standard curve ($y=0.034x+0.6068$), $R^2=0.999$. Standards and samples were performed in triplicate. Results were expressed as μmol TE/μmol of sample.

2.12. Statistical analysis

Results are presented as means±standard deviation from three replicates of each experiment. Differences between mean values were determined by the analysis of variance (ANOVA). The post hoc analysis was made by the Tukey and Dunnet test. All tests were considered significant at $P < 0.05$ using the software GraphPad Prism 4.

3. RESULTS AND DISCUSSION

3.1. Analysis proximal of flour bean and LBPC

The table 1 shows the proximate analysis of flour bean and LBPC. Flour bean present moisture with a value of 8.95% and LBPC present a value of 9.37%. Flour bean present a protein content of 20.48% and LBPC present a protein content of 77.20%. LBPC present an increase of protein content. The carbohydrates content of flour bean was 65.42%. LBPC present a decrease with a value of 10.63%. CHEL-GUERRERO *et al*. (2002); GALLEGOS-TINTORÉ, *et al*. (2004); TORRUCO-UCO *et al*. (2009); BETANCUR-ANCONA *et al*. (2009); GUZMÁN-MÉNDEZ *et al*. (2014) and DRAGO *et al*. (2016) report protein contents from lima bean with values of 71.13%, 71.13%, 71.80%, 69.90%, 71.88% y 74.06%. The protein content in this study is higher compared to the values reported. These differences are may be due to the varieties used in each study.

Table 1. Proximate analysis of flour bean and LBPC.

Analysis	Flour bean (%)	LBPC (%)
Moisture	8.95%±0.14	9.37%±0.26
Protein	20.48%±1.17	77.20%±0.02
Fat	1.57%±0.10	0.56%±0.03
Ashes	3.58%±1.23	2.24%±0.30
Carbohydrates	65.42±2.25	10.63±3.10

Results are expressed as mean±standard deviation (n=3)

3.2. Protein solubility capacity

Protein solubility is considered one of the most important functional properties in proteins. When a food protein has high solubility, this ingredient can be used for many industrial proposes, a food protein with low solubility decreases the industrial possibilities when used as an ingredient. Solubility capacity can affect other protein functional properties.

The flour bean solubility curve is shown in Fig. 1A. Proteins are more soluble in acidic regions (pH 2.0) and in alkaline regions (pH 12) with a value of 100%. Between pH 3.0 to pH 5.0 the solubility is reduced. Between pH 6.0 to pH 10.0 the solubility of proteins is relatively good. LBPC proteins solubility profile presents the typical U-shape of the legume extracts, with a minimum solubility at the isoelectric point and a greater solubility at low acidic pH and high alkaline pH (PAZMIÑO *et al.,* 2018). At pH 2.0 and pH 12.0,

LBPC present the highest percentage of solubility with values of 99.98±0.04% and 98.82±0.05%. At pH 6.0, these proteins present solubility values of 45.71±0.02% and at pH 8.0 these proteins present solubility values 57.95%. The lowest solubility was reached at pH 4.0 with a value of 37.34% (Fig. 1B). SEIDU *et al*. (2015) reported protein solubility of protein from skin lima bean with percentage between 25% to 90%. BETANCUR-ANCONA *et al*., 2009 reported protein solubility capacity of protein isolate obtained of lima bean with values of 15% to 70%. CHEL-GUERRERO *et al*., 2002 reported protein solubility of protein isolate from lima bean with values between 5% to 70%.

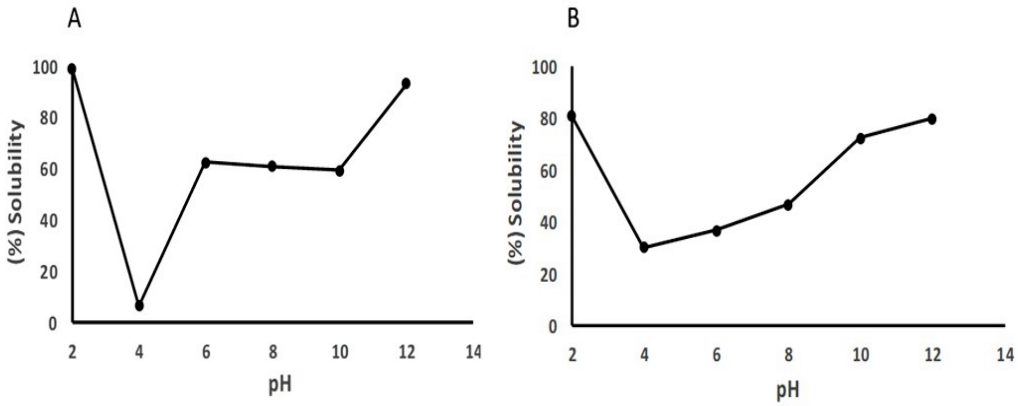

Figure 1. Percentage of solubility of flour and LBPC at different pHs.
A) flour bean B) LBPC.

3.3. LBPC protein profile and their digestibility

LBPC was obtained by alkaline extraction at pH 9.5 for 15-30-45 and 60 min of agitation at 40°C, followed by isoelectric precipitation at pH 4.5 and then analyzed by SDS-PAGE electrophoresis. Fig. 2A shows the LBPC proteins profile with bands between 10 kDa to 100 kDa. The bands with the higher intensity present molecular weights of 22 kDa, 25 kDa and 30 kDa. The bands with the lower intensity were the bands with low molecular weights with 10 kDa, 15 kDa, 20 kDa, 55 kDa and 60 kDa. The protein profile is composed as follows: one band of 100 kDa, one band of 70 kDa, doublet with 55 kDa and 60 kDa, triplet with 40 kDa, 35 kDa and 30 kDa, triplet of 20 kDa, 22 kDa and 25 kDa.
SPARVOLI *et al*. (1996) reported a similar protein profile identified as phaseolin from lima bean (*Phaseolus lunatus*) with molecular weights of one band (70 kDa), doublet (54 kDa - 58 kDa), triplet (32 kDa, 35 kDa and 38.5 kDa) and doublet (21 kDa - 25 kDa). These samples were analyzed for reaction of Western blot against phaseolin (*Phaseolus vulgaris*). The LBPC protein profile was the same at the different times alkaline extraction was assayed (15-30-45-60 min). Flour of *Phaseolus lunatus* presents a 20.48% of protein content. LBPC present a higher protein content with a value of 77.20% (Table 1).

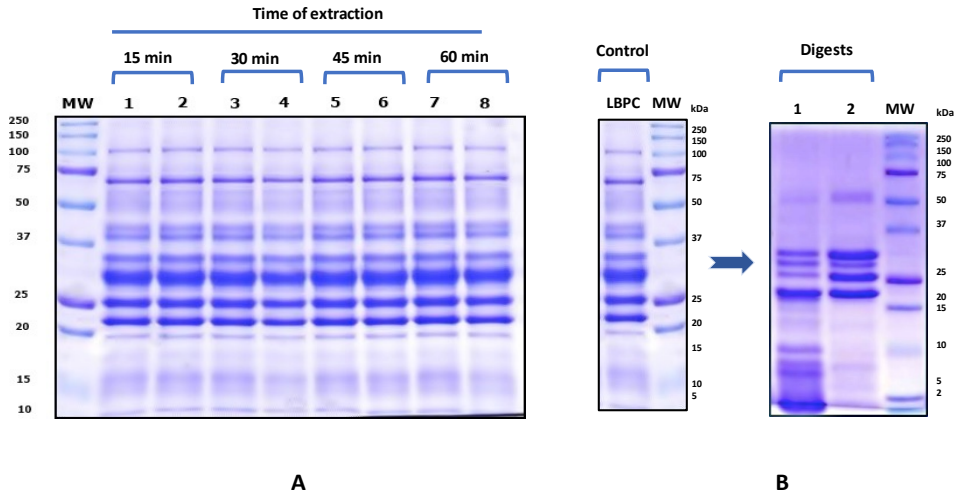

Figure 2. SDS-PAGE electrophoresis analysis of LBPC and gastric and duodenal digest of LBPC.
A) Profile protein of LBPC obtained at different times at 40°C and pH 4.5. MW (molecular weight).
B) LBPC control (without hydrolysis).
Lane 1: gastric digest of LBPC, lane 2: duodenal digest of LBPC.

In common bean (*Phaseolus vulgaris*) phaseolin protein content represents around 50% of the total seed protein (BOSCHIN *et al.*, 2014). It is made up of a small number of polypeptides and presents an extensive variation in the electrophoretic pattern, mostly observed among wild-growing beans. Comparison of electrophoretic patterns of total seed proteins of cultivated species belonging to the genus *Phaseolus* showed that the *Phaseolus lunatus* pattern is quite different from the others, lacking major polypeptides with molecular mass similar to those of phaseolin (LIOI, 1987).
Different authors have reported 7S globulin (vicilin) from *Phaseolus vulgaris*. with molecular weights between 40 kDa to 54 kDa. MONTOYA *et al.* (2008; 2010) reported the protein profile of a different variety of Phaseolus. They reported a high content of globulins and reported 2 to 6 bands between 40 kDa to 54 kDa. They identified these bands as Vicilin (7S globulin). CARRASCO-CASTILLA *et al.* (2012) reported *Phaseolus vulgaris* protein profiles. They identified ten protein bands with molecular weights (MW) ranging from 15 to 200 kDa in the protein isolate. The 41 kDa and 46 kDa bands, correspond to the phaseolin subunits and the most abundant proteins. The 15 kDa, 18 kDa, 25 kDa and 32 kDa, bands correspond to proteins belonging to the lectin-family.
GARCÍA-MORA *et al.* (2015) reported a protein profile from *Phaseolus vulgaris*. var. pinto, with bands between 10 kDa to 97 kDa. Bands with molecular weights of 25 kDa, 45 kDa and 50 kDa were identified as phaseolin. Phytohemagglutinins (32 kDa), α-amylase inhibitor (18 kDa) and α-amylase β subunit (15 kDa) were identified in the pinto bean protein concentrate. Phaseolin band in *Phaseolus vulgaris* present high intensity and represent about 50% of total protein content. *Phaseolus lunatus* presents an absence of this high intensity band. Phaseolin protein of *Phaseolus lunatus* corresponds to another band with a different molecular weight.
LBPC and their fractions were subject to an *in vitro* gastrointestinal digestion simulation using pepsin enzyme for the gastric phase and a pepsin/pancreatin enzymes mix for the duodenal phase. In the gastric phase, bands with molecular weights of 20 kDa, 22 kDa and 25 kDa present resistance to hydrolysis with pepsin. We found bands with molecular

weights of 50 kDa and under 10 KDa. Phaseolin protein presents resistance to hydrolysis with pepsin/pancreatin (Fig. 2B).
In the duodenal phase, the protein profile of hydrolysate present bands with molecular weights between 20 kDa to 50 KDa. Bands with low molecular weight were partially hydrolyzed with pepsin/pancreatin. Bands of 20 kDa, 22 KDa and 25 KDa present resistance to duodenal hydrolysis. *Phaseolus lunatus* phaseolin protein presents resistance to hydrolysis with pepsin and pepsin/pancreatin. Phaseolin from different *Phaseolus* species has been reported to be resistant to enzymatic hydrolysis with pepsin and pepsin/pancreatin.
LBPC gastric digest present 9.3% DH and LBPC duodenal digest present 16.2% DH. These results are in accordance with the resistance at the hydrolysis observed in the electrophoresis analysis. BETANCUR-ANCONA *et al. (*2009) reported protein isolate (*Phaseolus lunatus*) hydrolysis using an enzymatic (trypsin, chymotrypsin and peptidase) mix, with a hydrolysis degree of 79.8% of HD. Different *in vitro* hydrolysis methods have been used to evaluate *in vitro* hydrolysis of common bean seeds. There are differences in the %HD reported in these studies. These differences must be due to the type and variety of seeds, geographic position of the cultivar and the differences in the method of hydrolysis and enzymes used, time of incubation, pHs of simulation, temperature, proportion of enzymes and combination of enzymes. For example, MONTOYA *et al.* (2008; 2010) reported hydrolysis of isolated phaseolin, treated and not treated thermally, of 43 varieties hydrolyzed with pepsin at pH 2.0 and hydrolyzed with pepsin and pancreatin dissolved at pH 7.5. In the gastric phase, at 120 min of incubation with pepsin the %HD was 5.2% in the unheated sample and 7.5% in the heated sample. In the duodenal phase, at 360 min of incubation with pepsin and pancreatin, the %HD was 11% to 27% for the unheated sample and 57% to 96% for the heated sample. The gastrointestinal digest presented a high % HD but the results were different depending on the variety of *Phaseolus vulgaris* used.
TORRUCO-UCO *et al. (2009) reported* hydrolysates of *Phaseolus vulgaris* from Mexico obtained with Alcalase and Flavourzyme for 30 min. They found a %DH of 49.48% and 26.05% respectively.

3.4. Characterization of fractions of LBPC by SDS-PAGE electrophoresis

LBPC fractions were obtained using a DEAE Affi-Gel Blue Gel chromatographic column. Ten cationic and anionic fractions were obtained to be analyzed with RP-UHPLC and SDS-PAGE electrophoresis. The anionic fractions numbered as fraction 1, F1, fraction 2, F2, fraction 3, F3 and fraction 4, F4 were chosen for their high protein content. The cationic fractions numbered as fraction 5, F5, fraction 6, F6, fraction 7, F7 and fraction 8, F8 were chosen for their high protein content. Fig. 3 shows the protein profile of cationic and anionic LBPC fractions. All fractions present an identical profile of proteins with molecular weights between 20 kDa to 50 kDa. Triplet with bands of 20 kDa, 22 kDa and 25 KDa were the bands with higher intensity. These bands correspond to the phaseolin protein. In the Cationic fractions, F5 and F6 present a higher protein content than F3 and F4. In the anionic fractions, F3 and F4 present a higher protein content than F5 and F6. The cationic fractions present a higher protein content than the anionic protein content. SPARVOLI *et al.* (1996) reported cationic fractions from *Phaseolus lunatus* obtained by an ion exchange chromatography with a Mono-Q HR5/5column coupled to the FPLC system. They reported nine cationic fractions with presence of triplet bands with molecular weights of 32 kDa, 35 kDa and 38.5 kDa in all fractions. These bands correspond to the

phaseolin protein. In this study, all fractions present triplet of bands but with lower molecular weights (20 kDa, 22 kDa and 25 kDa). The protein content fractions were determined using the BCA method. The anionic fractions, F1 (10.4%), F2 (11.5%), F3 (17.4%) and F4 (49.4%) have the percentage of protein content in brackets. These previous results show a correlation of the protein content with the intensity of the band in the gels of polyacrylamide. Cationic fractions F5 and F6 present a higher protein content with values of 77.9% and 77.2% of protein respectively.

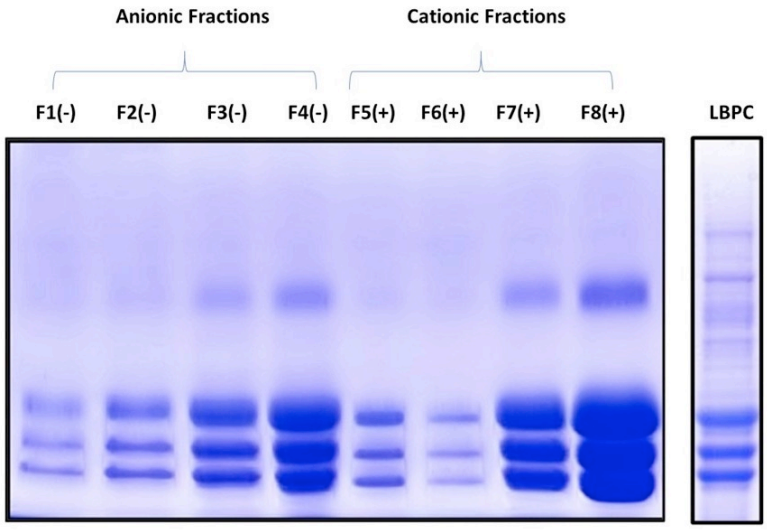

Figure 3. Protein profile of LBPC and cationic and anionic fractions using SDS-PAGE electrophoresis analysis.

3.5. Characterization of LBPC fractions by UHPLC analysis

Cationic and anionic fractions of LBPC were characterized using the UHPLC technique. The cationic fractions F5, F6, F7 and F8 have the same profile of proteins with four peaks in the chromatogram. F8 present higher intensity in the absorbance at 280 nm. Peak number one shows the highest intensity with a value of 20 AU. These results show a correlation with the intensity of the bands in the gel SDS-PAGE and the percentage of protein determined by the BCA method. These results suggest that this fraction present a higher protein content. These proteins are rich in tryptophan, this amino acid is absorbed at 280 nm (Fig 4).

LBPC anionic fractions show three peaks. F1 and F2 present minor absorbance at 280 nm. These results show a correlation with the SDS-PAGE electrophoresis results of these fractions. F3 and F4 present major absorbance at 280 nm and in the gel SDS-PAGE these fractions present a higher protein content because the staining is strong. Peak number one of fractions F3 and F4 shows a high content of tryptophan amino acid in these sequences, if we look at the polyacrylamide gel and the percentage of protein calculated by the BCA method (Fig. 5).

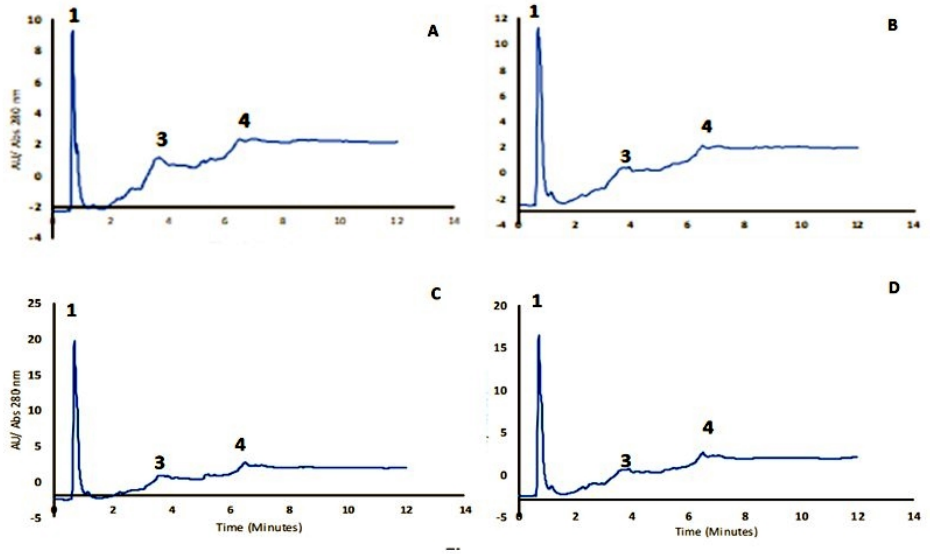

Figure 4. RP-UHPLC analysis of anionic fractions of LBPC. A) anionic fraction F1, B) anionic fraction F2, C) anionic fraction F3 (and D) anionic fraction F4.

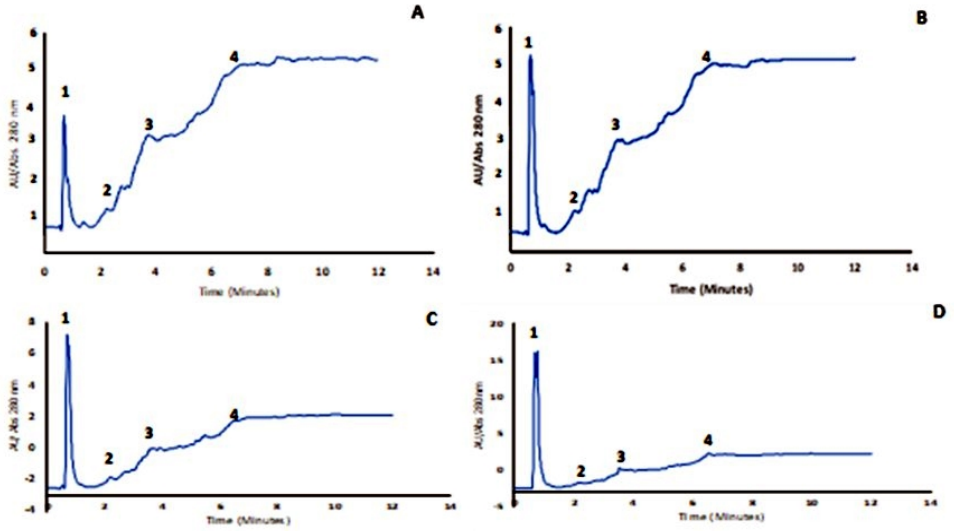

Figure 5. RP-UHPLC analysis of cationic fractions of LBPC. A) cationic fraction F5, B) cationic fraction F6, C) cationic fraction F7 and D) cationic fraction F8.

3.6. Antioxidant activity of LBPC and fractions of LBPC

Fig. 6 shows the results of LBPC and their fractions antioxidant activity, using the FRAP method. All samples assayed present antioxidant activity. LBPC present a value of 1.67±0.14 mg TE/g of sample. This is the highest value. The positive fractions present the

higher activities, F5 with value of 1.26±0.06 mg TE/g of sample, F6 present a value of 1.61±0.37 mg TE/g of sample, F7 present a value of 1.15±0.05 mg TE/g of sample, F8 present a value of 1.53±0.26 mg TE/g of sample. LBPC negative fractions were active with values between 1.15±0.05 to 1.23±0.18 mg TE/g of sample. F8 present the highest value with 1.23±0.18 mg TE/g of sample. Positive fractions were more active than negative fractions but the LBPC sample was more active than the negative fractions. This situation shows the correlation with the ORAC activity. In the ORAC activity, positive fractions were more active than negative fractions.

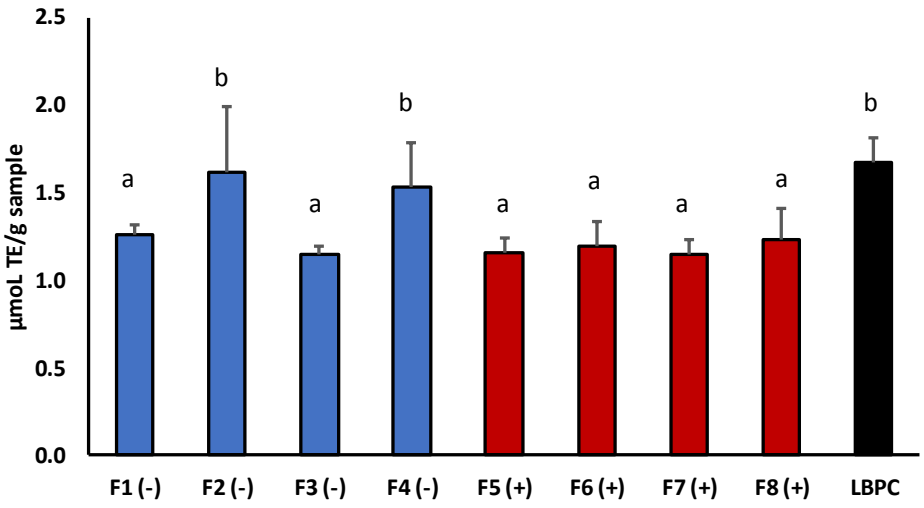

Figure 6. Antioxidant activity of LBPC and their fractions using the FRAP method. Different letters represent significant differences between LBPC vs sample as $P < 0.05$ $(n=3)$.

Antioxidant activity of LBPC fractions also was evaluated using the DPPH method. LBPC cationic fractions were more active than LBPC negative fractions. Fig. 7 shows the antioxidant activity of LBCP fractions. F5 present a value of DPPH of 189.38 µmoL TE/g of fraction, F8 present a value of DPPH of 191.33 µmoL TE/g of fraction, F7 show a value antioxidant of 196.17 µmoL TE/g of fraction and F8 show a value of 205.19 µmoL TE/g of fraction. This sample present higher antioxidant activity. LBPC negative fractions present value of DPPH between 36.79 to 48.96 µmoL TE/g of fraction. LBPC control present a value of 84.08 µmoL TE/g of LBPC. Different works have been reported fractions obtained of food proteins using different methods of isolation or separation with biological properties. For example, RODRIGUEZ SAINT-JEAN *et al.* (2013) have described fractions isolated of αS_2 casein bovine with strong antiviral activity against the infectious hematopoietic necrosis virus of salmonid fish. The fractions were isolated using ion-exchange chromatography.
Fig. 8 shows the LBPC antioxidant activity results and their fractions using the ORAC method.

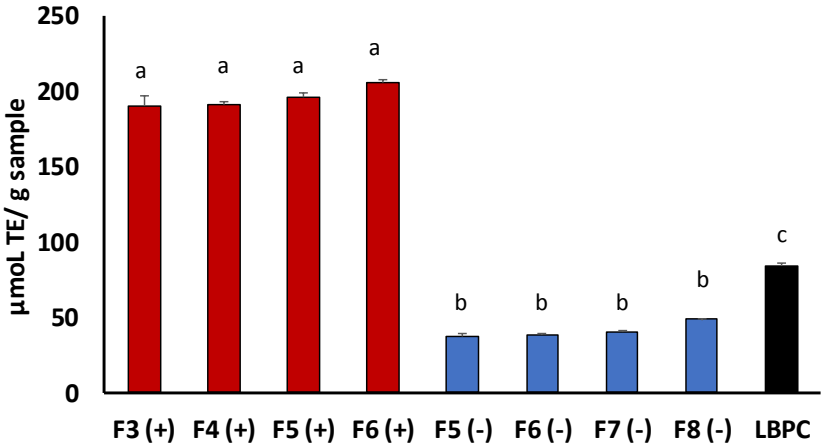

Figure 7. Antioxidant activity of LBPC and their fractions using the DPPH method. Different letters represent significant differences between LBPC vs sample as P <0.05 (n=3).

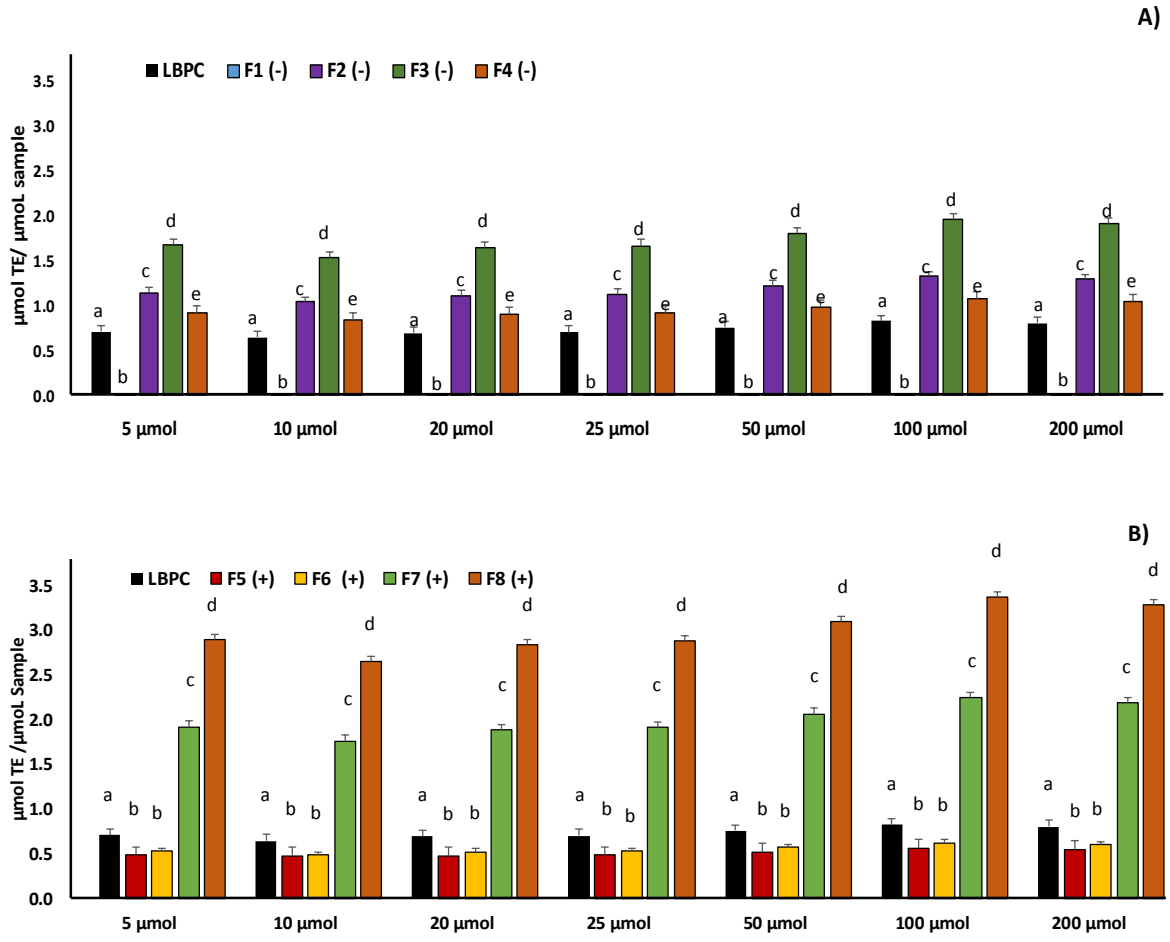

Figure 8. Antioxidant activity of LBPC and their fractions using the ORAC method. A) Anionic fractions, B) Cationic fractions. Different letters represent significant differences between LBPC vs sample as P <0.05 (n=3).

Non-digested LBPC showed an ORAC value between 0.64±0.06 to 0.81±0.06 μmoL TE/ μmoL of sample. Vilcacundo *et al.* (2018) reported quinoa protein concentrates with values of 0.42±0.03 μmoL TE/μmoL of protein. NONGONIERMA *et al. (*2015) reported quinoa proteins with ORAC values of 0.26±0.07 μmoL TE/mg of sample. LBPC cationic fractions (F5 and F6) present lower antioxidant activity than LBPC with ORAC values between 0.47±0.09 to 0.56±0.09 μmoL TE/μmoL of sample and 0.48±0.03 to 0.61±0.03 μmoL TE/μmoL of sample respectively. The cationic fractions F7 and F8 present higher antioxidant activity than the F5 and F6 and anionic fractions (F1, F2, F3 and F4) with ORAC values between 1.75 to 2.24±0.03 μmoL TE/μmoL of sample and 2.64±0.05 to 3.37±0.05 μmol TE/μmoL of sample respectively. The anionic fraction F1 present an absence of antioxidant activity using the ORAC method, F3 and F4 present higher ORAC values (1.03±0.06 to 1.31±0.06 μmoL TE/μmoL of sample) and (1.52±0.06 to 1.94±0.06 μmoL TE/μmoL of sample). Fraction F4 present ORAC values between 0.83±0.08 to 1.06±0.08 μmoL TE/μmoL of sample.

CARRILLO *et al. (*2016a; 2016b) have described hydrolysates and fractions isolate of lysozyme protein with antioxidant activity and capacity to inhibit lipid peroxidation in zebrafish larvae, the fractions from lysozyme were isolated using the IEC technique. Peptides were identified by HPLC-ESI-MS-MS. Peptides presented antioxidant activity using the ORAC method. CARRILLO *et al*. (2018) have described fractions isolated of lysozyme protein with antibacterial activity against *Escherichia coli* and *Staphylococcus carnosus*. Fractions were separated using a membrane of cationic exchange chromatography technique. PIÑUEL *et al*. (2019) have reported antioxidant fractions obtained of digest of *Phaseolus vulgaris* separated using ultrafiltration membrane of 3 kDa and 10 kDa. Fractions with biological activities can be isolated of food proteins of animal or vegetal sources and can be used for different proposes. When the proteins are subject to enzymatic hydrolysis the peptides can be identified and synthetized to be studied.

4. CONCLUSIONS

Baby lima bean (*Phaseolus lunatus*) seeds from Ecuador used in this study present a high protein content. These seeds can be a new source of vegetable protein and used for different purposes in the food industry. In this study, LBPC was obtained using an alkaline extraction, followed by an isoelectric precipitation, LBPC present a complex proteins profile. Phaseolin is the most abundant protein in the *Phaseolus lunatus* protein concentrate. Phaseolin protein presents resistance to hydrolysis with pepsin in the gastric phase and to pepsin/pancreatin in the duodenal phase. LBPC present a high solubility and their fractions present a high antioxidant activity. For the previous reasons, *Phaseolus lunatus* proteins can have a potential use in the formulation and development of new functional ingredients food.

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