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# Bioactive Peptides by *in vitro* Digestion of Germinated Bean Cotyledons Extrudates

Lidia López-Barrios[1], Erick Heredia-Olea[1], Daniel Guajardo-Flores[1], Esther Pérez-Carrillo[1] & Janet A Gutiérrez Uribe[1]

[1]Tecnológico de Monterrey, Centro de Biotecnología FEMSA, Monterrey, Nuevo León, México

Correspondence: Erick Heredia Olea, Tecnológico de Monterrey, Centro de Biotecnología FEMSA, Av. Eugenio Garza Sada 2501 Sur, Monterrey, Nuevo León, C.P. 64849, México. E-mail: erickho@itesm.mx

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**Abstract**

Germinated black bean cotyledons were extruded at two different screw speeds (350 or 400 rpm). Extrudates were digested with pepsin and pancreatin to evaluate the antioxidant and anti-inflammatory activities of hydrolysates collected at 0, 60, 90, 120 and, 180 min. Soluble protein recovered before the enzymatic digestion of extrudates obtained at 350 rpm (E1), or 400 rpm (E2) showed the highest antioxidant (AOX) capacity, with 2,790 or 2,335 µmol eq Trolox/g, respectively and the best nitrogen oxide inhibitory. Even though extrudates presented different peptides profiles, the enzymatic digestion of the storage proteins released similar peptides. RLL and YAL were among the identified peptides obtained after 180 min of enzymatic digestion. Extrusion can be a useful process to produce novel functional ingredients from legume proteins for the food industry.

**Keywords:** anti-inflammatory activity, antioxidant activity, black beans, extrusion, legumin, peptides, protein hydrolysates

## 1. Introduction

Diverse studies had shown the health benefits of legumes in daily diet like reduction of atherosclerosis, diabetes, hypertension, osteoporosis, cancer, etc. The high protein content, around 18-32%, and good amino acid profile give to legumes the potential to be the best alternative to eradicate diseases produced caused by inadequate nutrition. However, legumes had a lack of S-c containing amino acids (cysteine, cystine, and methionine), for this reason the legumes need to be complemented with cereals to compensate the lack of amino acids (Iqbal et al., 2006). Despite this amino acid deficiency, legumes had comparable amino acid profile (~80-90) compared with daily products as milk, cheese, eggs, fish, or meat (Sosulski & Imafidon 1990). Common bean (*Phaseolus vulgaris* L.) is a highly consumed legume worldwide that contains bioactive compounds including peptides. Activities described for common bean bioactive peptides (BP) include antioxidant, antihypertensive, anti-inflammatory, anticancer, antifungal, and antidiabetic (Luna-Vital et al., 2014; Rocha et al., 2015, Marques et al., 2015). The peptides can be obtained by enzymatic hydrolysis, fermentation, or food processes that hydrolyze or disrupt massive proteins groups like globulins. Isolated BP can imply high production costs and difficulties on their incorporation into new foods. Functional foods with BP obtained during the production process are an excellent alternative, particularly to eliminate an additional enzymatic hydrolysis step (López-Barrios et al., 2014).

Usually, bean flours are not used as food ingredients due to intrinsic antinutritional factors and the low functionality of starch (Rocha-Guzmán et al., 2006), but these problems can be solved pretreating the beans or bean flours. Germination is an alternative to reduce the antinutritional compounds found in raw bean, such as phytic acid, trypsin inhibitors, and alpha-amylase inhibitors (Mendoza-Sánchez et al., 2016). The antioxidant and anti-inflammatory activity of black bean hydrolysates were reported to increase with germination (de Souza Rocha et al., 2015; López-Barrios et al., 2016).

Extrusion is an interesting technology applied to legume flours to modify its functional properties and reduce the antinutritional factors due to the combined effect of shear stress, specific mechanical and thermic energies that transform legumes flours in high-value products at a low cost. Usually, extrusion has been applied exclusively to soybean defatted flours, but in the latest years, this technology has been used to legumes (Rocha-Guzmán et al. 2006). Extrusion can process in a few seconds plenty of bean flours inactivating some antinutritional factors

within the beans like the trypsin inhibitors and lipoxygenases (Atienzo Lazos et al., 2011). Extrusion enhanced the anti-inflammatory effect of amaranth (*Amaranthus hyochondriacus*) flour soluble protein hydrolysates (Montoya-Rodríguez et al., 2014a; Montoya-Rodríguez et al., 2014b; Montoya-Rodríguez et al., 2015). Extrusion and germination have already been successfully used to improve common beans nutritional properties and the development of new food products (Kelkar et al., 2012; López et al., 2013; Nyombaire et al., 2011; Simons et al., 2014; Vidal-Valverde et al., 2002). Temperature and pressure affect protein bioaccessibility and therefore have a significant effect on the release of bioactive peptides (Garcia-Mora et al., 2016). Hence, extrusion conditions could have an impact on the proteins subjected to digestion and generation of bioactive peptides.

It is imperative to point out that bioactive peptides must retain or enhance their beneficial effect on health after digestion to be used as a functional food ingredient (García-Mora et al., 2017). Therefore, the aim of this research was to evaluate the effect of thermoplastic extrusion on the antioxidant and anti-inflammatory activities of the soluble protein from germinated black bean cotyledons and the changes after simulated gastrointestinal digestion.

## 2. Materials and Methods

### *2.1 Chemicals*

Germinated cotyledon black bean was produced according to Guajardo-Flores et al. (2016). Solutions like 1 M HCl, 0.9 M $NaHCO_3$, 1 M NaOH were from DEQ (Monterrey, México). Pepsin from porcine gastric mucose (P7000), pancreatin from porcine pancreas (P7545), trolox standard, fluorescein, 2,2'azobis (2-amidinopropane) dihydrochloride (AAPH) and BSA were pushing from Sigma-Aldrich, St. Louis, MO, USA. Electrophoresis buffers, reagents, Mini-protean Tetra Cell, molecular weight marker and, sample buffer were purchased from BioRad Laboratories Inc. (Hercules, CA, USA). Human (*Homo sapiens*) colon epithelial adenocarcinoma cells, Caco-2 and mouse (*Mus musculus*) macrophage cell line RAW 264.7 were from ATCC, Manassas, VA, USA. DMEM medium, fetal bovine serum, PBS (phosphate buffer solution), and penicillin/streptomycin were purchased from GIBCO (Grand Island, NY, USA); while dichlorofluorescein diacetate (DCFH-DA) and lipopolysaccharide (LPS) from Sigma-Aldrich (St. Louis, MO, USA). Griess reagent system kit G2930 was obtained from Promega (Madison, WI, USA) and high performance chromatography (HPLC) grade water, trifluoroacetic acid and acetonitrile from CTR Scientific (Monterrey, México).

Black beans (*Phaseolus vulgaris)* var. *San Luis was* obtained from INIFAP Nicolás Bravo, Durango, México. Germination was carried on according to Guajardo-Flores et al., (2012), in a dark chamber at 24 ℃ and constant distilled water nebulization. After 24 h, black beans were dried in a convection oven (Electrolux EOG Gas single oven X 601, Stockholm, Sweden) at 60 ℃ for 4 h. Beans were dehulled in a decorticator (Nutana Machine, Saskatoon, SK, Canada) equipped with a set of five 30 cm diameter carborundum (60 grit) disks. Cotyledons were then separated from coats with a 4 mm mesh, and finally, cotyledons were ground in a knife mill (Wiley Mill®, Swedesboro, NJ, USA) to pass a 1 mm screen.

### *2.2 Extrudate Production*

A twin-screw co-rotating extruder (BCTM-30, Bühler AG, Uzwil, Switzerland), with a shaft of 800 mm total length and a ratio L/D = 20 was used. The barrel of the extruder was composed of five blocks, and the last block was preheated at 90 ℃ by a heat exchanger device (Tool Temp, Bühler AG, Uzwil, Switzerland). Cotyledons flour was placed on the solids feeder and water on the liquid feeder; fluxes were set at 24.7 kg/h and 4.0 kg/h respectively. A die with a single circular 4 mm hole was used. Two different screws speeds, 350 and 400 rpm, were used and in consequence, the temperature in the last zone of the barrel was 135 ℃ and 149 ℃, respectively. Round extrudates was obtain setting cutter at 600 rpm using three knives. The expanded products with expansion index of 4.42 were dried in a convection oven (Electrolux EOG Gas single oven X 601, Stockholm, Sweden) at 100 ℃ per 8 min.

### *2.3 Enzymatic Digestion and Soluble Protein Quantification*

Extrudates were ground in a coffee mill (Krops GX 4100 México) before protein hydrolysis according to Lo and Li-Chan (2005). Extrudates flour was suspended in distilled water (1:20 w/v) containing 0.02% sodium azide, pH was adjusted to 2.0 with 1M HCl, and then pepsin from porcine gastric mucosa was added (1:20 w/w protein basis). The solution was incubated for 1 h under agitation (250 rpm) at 37 ℃, and the pH was adjusted to 5.3 with 0.9 M $NaHCO_3$ to add pancreatin from porcine pancreas (1:20 w/w protein basis). pH was adjusted again to 7.5 with 1 M NaOH, and the suspension was incubated for 180 min under agitation at 37 ℃. Aliquots of hydrolysates suspensions were collected during digestion at 0, 60, 90, 120 and, 180 min. Samples were

submerged in boiling water for 10 min to stop the digestion. After cooling, hydrolysates were neutralized, clarified by centrifugation at 10,000 g for 15 min and frozen (-20 ℃). A bicinchoninic acid assay (BCA) assay determined the soluble protein content of hydrolysates. Briefly, samples were diluted 1:4 and 20 µL of each hydrolysate reacted with 200 µL of BCA reactive in a 96 well microplate, along with a BSA calibration curve (0-10 mg/mL). After 20 min, absorbance at 562 nm was recorded in a microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA) with KC4 v3.4 software.

*2.4 Electrophoresis*

SDS-PAGE electrophoresis of samples was executed using a Mini-Protean Tetra Cell. Gels consisted of a 10% polyacrylamide resolving gel (pH 8.8) and a 5% stacking gel (pH 6.8). Samples were dissolved in 2X Laemmli sample buffer and 15 µl loaded onto gels (26 µg protein/well based on the soluble protein concentration determined by BSA assay), including a molecular weight marker (Precision Plus Protein Kaleidoscope). After running at 200 V, 10 mA, 3.0 W, gels were stained with silver according to the Chevallet et al. (2006) protocol.

*2.5 Cellular Antioxidant Activity*

The optimized method by Wan, Liu, Yu, Sun, and Li (2015) with Caco-2 cells was used to assess cellular antioxidant activity (CAA) of protein hydrolysates. Cells were seeded in a black 96-well plate (100 µL at $1 \times 10^5$ cells/well), with Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 5% fetal bovine serum, and maintained at 37 ℃ in a humidified 5% $CO_2$ incubator. After 24 h, cells were washed with PBS (phosphate buffer solution) and incubated for 20 min with 100 µL of hydrolysates at 0.01 mg/mL of soluble protein and 60 µM of dichlorofluorescein diacetate (DCFH-DA). After rewashing the cells, 100 µL of 2,2'-Azobis(2-methylpropionamidine) dihydrochloride (AAPH) (100 mM) was added to induce oxidative stress, and fluorescence emitted at 538 nm with excitation at 485 nm was measured every 2 min for 120 min at 37 ℃. CAA was calculated as a percentage using the following formula:

$$CAA = 100 - \left(\frac{NET\ AUC\ sample}{NET\ AUC\ positive\ control}\right) \times 100$$

Positive control was cells incubated with dichloro-dihydro-fluorescein diacetate (DCFH-DA) (wit out hydrolysates) and induced with AAPH.

*2.6 Nitric Oxide Inhibitory Activity*

Nitric oxide (NO) inhibitory activity was measured as an indirect effect of anti-inflammatory activity. NO production induced by lipopolysaccharide (LPS) in mouse macrophage cell line RAW 264.7 (ATCC, Manassas, VA, USA) was used. Cells were cultured in 96-well plates (50 µL, $1 \times 10^5$ cells/well) with 100 µL of DMEM medium containing 10% fetal bovine serum, 1% penicillin/streptomycin (GIBCO Grand Island, NY, USA), and incubated at 37 ℃ in 5% $CO_2$. After 4 h, hydrolysates were added to cells (50 µL/well at 0.04 mg/mL) and left 24 h in incubation. Cells were activated with LPS (50 µL, 10 µg/mL) during 24 h. For nitrite determination, 100 µL of supernatant from each well was transferred to a new plate, and the assay was made according to Griess reagent system kit (Promega, Madison, WI, USA). The original plate where cells remained was used to evaluate cell viability. Cell titer 96 aqueous one solution cell proliferation assay (Promega, Madison, WI, USA) was used. Briefly, 20 µL of cell titer reagent was added to each well and cells were incubated for 45 min (37 ℃ in the dark). Absorbance at 490 nm was measured in microplate reader (Bio-Tek Instruments Inc., Winooski, VT, USA). The NO production was esteemed as percentage considering cells with LPS stimuli and no treatment as 100%. Mouse macrophage cell line was acquired from American Type Culture Collection (ATCC, Manassas, VA, USA).

*2.7 HPLC-MS-TOF Analysis*

Hydrolysates were analyzed by HPLC-MS-TOF (Model G1969A Agilent 1100, Santa Clara, CA, USA) as previously reported (Torres-Fuentes et al., 2015). Samples adjusted to a concentration of 0.15 mg/mL were injected into a XBridge BEH300 $C_{18}$ column (250 × 4.6 mm, 5 µm of particle size, Waters Corporation, Milford, MA, USA), injection volume was 5 µL. Solvent A consisted of HPLC grade water with trifluoroacetic acid (TFA, 1000:0.37, v/v) and solvent B was acetonitrile and TFA (1000:0.27, v/v). Elution was conducted with a linear gradient of solvent B in A from 0% to 70% over 90 min, at a flow rate of 0.75 mL/min.

Positive ions were recorded over the mass/charge (m/z) range of 100-2800. Nitrogen was used as nebulizing and drying gas (35 psi, 12 L/min, 350 ℃). The capillary was held at 3 kV, and fragment voltage at 225 V. Total ion chromatogram (TIC) of germinated cotyledon was subtracted from TIC of germinated cotyledons extrudates to obtain the accurate mass of peptides produced by the extrusion process. On the other hand, TIC from undigested extrudates was subtracted from TIC of extrudates hydrolysates achieved after 180 min to obtain the accurate

mass of peptides produced by digestion. Mass spectra were extracted with MassHunter workstation software (Agilent Technologies, Santa Clara, CA, USA). Peptides were identified using MASCOT Peptide Mass Fingerprint server with the following parameters: SwissProt and NCBInr database, peptide tolerance 1.2 Da, no enzyme and variable modification, taxonomy *Viridiplantae*. Sequences identified from *Phaseolus vulgaris* were searched in Biopep database for their potential biological activity.

*2.8 Statistics*

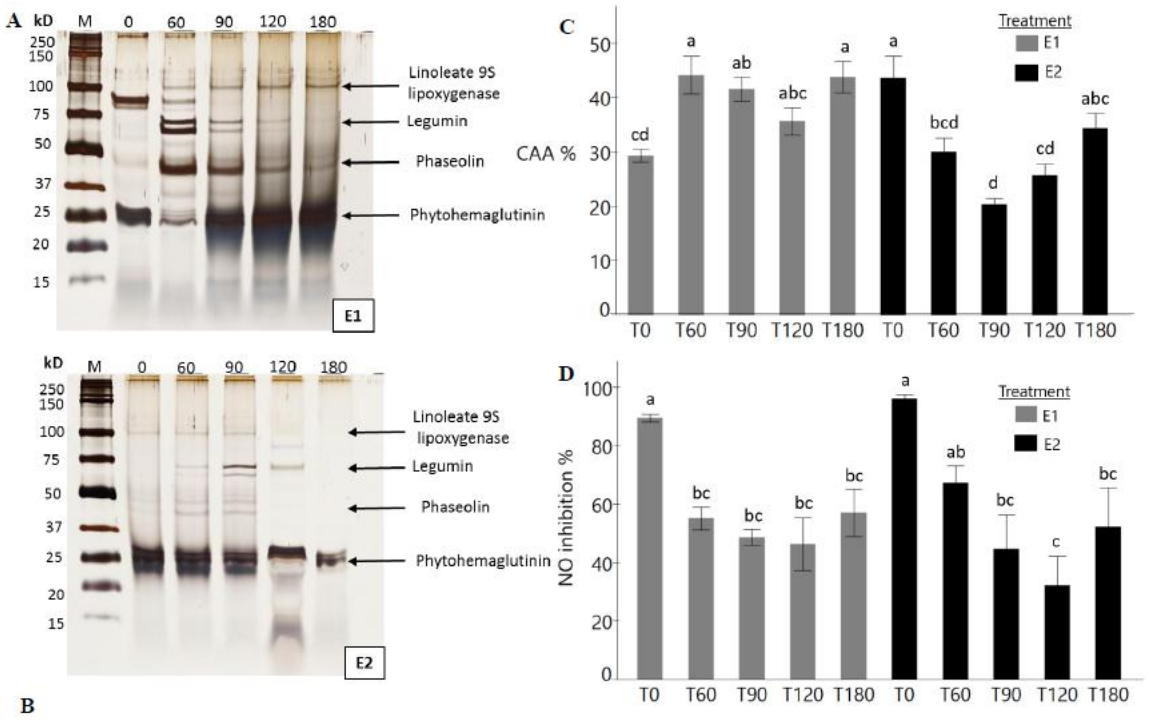
Results were expressed as mean ± standard error; all results are derived from at least 3 replicates. The software JMP 11.2.0 (SAS Institute Inc., Cary, NC, USA) was used to determine significant differences by ANOVA and Tukey test to compare treatments with a significance level of $\alpha=0.05$ ($p\leq0.05$).

## 3. Results and Discussions

*3.1 Soluble Protein Profile and of Germinated Extruded Cotyledon Black Bean*

The SDS-PAGE patterns showed the difference in protein size due to extrusion treatment (E1 and E2) and time of enzymatic treatment (Figure 1A). Before enzymatic digestion, germinated cotyledons extrudates (E1 and E2) presented a strong signal around 25 kDa, which according to bibliography corresponds to the protein phytohemagglutinin (Figure 1A) (Garcia-Mora et al., 2016). The irregular definition of the 25 kDa signal also indicates contamination with protein fragments produced during extrusion, as it was observed in previous reports using high temperature or pressure (Garcia-Mora et al., 2016; Mojica & de Mej á, 2015). Particularly for E2, the band around 25 kDa was more intense and irregular than the observed in E1 because of screw speed, specific mechanical energy (SME) and temperature were more intense for the E2 treatment compared with E1. Alonso et al. (2001) studied the nutritional effect of extrusion of *P. sativum* L. cv Ballet and *P. vulgaris* L. var. Pinto with *in vivo* assay. The extrusion was carried out at 150 and 155 ℃. They prove the enhanced bioavailability of mineral nutrients without changes in the chemical composition between raw material and extrudates. They demonstrated the destruction of antinutritional factors as phytates due to extrusion temperature. Batista and collaborators (2010) extruded common beans (*Phaseolus vulgaris*, *L.*) at 150 ℃ decreasing antinutrients factors as phytic acid, lectin, α-amylase, and trypsin inhibitors, enhancing the starch and protein *in vitro* digestibilities. In this research similar conditions were use. The reduction of protein bean molecular weight indicates the effect of temperature and shear stress inside the extruder barrel, leading to the reduction of antinutritional factors and enhancing the protein digestibility as the referenced works.

Also, before enzymatic digestion, an unidentified band around 85 kDa was present only in E1. This band remained in the 60 min enzymatic hydrolysate but at a much lower intensity and finally disappeared at 90 min. Small fragments of protein (<15 kDa) were most noticeable in E1, even before enzymatic hydrolysis, due to the higher temperature generated during extrusion. Furthermore, those small bands increased their intensity and definition after 90 min of enzymatic digestion as consequence of the generation of small peptides, as it has been reported for extruded amaranth (Montoya-Rodr ǵuez et al., 2015). Extrusion did not eliminate proteins as previously suggested (Kelkar et al., 2012). Extrusion affected the solubility of germinated cotyledons proteins due to their aggregation or interactions with non-protein macromolecules (Chen, Wei, & Zhang, 2011, Garcia-Mora et al., 2016). During enzymatic digestion of the extrudates, the protein aggregation was reduced, and therefore proteins were solubilized (Figure 2B). Hence, 60 or 90 min of enzymatic digestion were required to observe the characteristic bands from phaseolin subunits (40 and 53 kDa) and legumin (70 kDa) for E1 or E2, respectively (Figure 2A). In both extrudates, as enzymatic digestion continued, these protein bands lost their intensity as an indicator of the improvement of protein digestion due to extrusion. The extrusion temperature denatured the protein structure; nevertheless, the SME (the mainly result of shear stress) is the most critical variable that affects the denaturation and disruption of proteins (Riaz, 2000).




| | Digestion time (min) | | | | |
|---|---|---|---|---|---|
| | 0 | 60 | 90 | 120 | 180 |
| Extrudate sample | mg of soluble protein/g of extrudate | | | | |
| E1 | 6.8 ± 3.4c | 25.3 ± 4.6bc | 33.1 ± 4.9ab | 45.8 ± 6.5ab | 55.2 ± 9.6a |
| E2 | 7.9 ± 0.9b | 36.3 ± 7.9ab | 65.8 ± 4.4a | 43.6 ± 9.3ab | 49.2 ± 10.7ab |

Figure 1. Protein profile (A), soluble protein content (B), antioxidant activity (C), and anti-inflammatory activity (D) of soluble protein hydrolysates (0-180 min) from germinated cotyledons extrudates obtained at 350 rpm (E1) and 400 rpm (E2). Treatment comparisons were made and different letters ([a]) indicate significant differences among hydrolysates of the same extrudate ($\alpha$<0.05). T0, T60, T90, T120 and T180 refer to hydrolysis time at 0, 60, 90, 120 and 180 min, respectively

*3.2 Cellular Antioxidant Activity*

The AOX compounds must get inside the Caco-2 cells to exert their activity, and therefore membrane permeability is determinant to the compounds assayed (Wan et al., 2015). In consequence, bioactive compounds can have different activity in both chemical and cells assays. When tested at 0.01 mg/mL, E2 without enzymatic digestion presented higher CAA compared to E1 (Figure 1C), probably due to the intensity of the unidentified signal of around 85 kDa observed in the electrophoretic profile (Figure 1A).

In a cellular environment, peptides with lower molecular weight have stronger ability to scavenge free radicals than large molecules (Shi, Kovacs-Nolan, Jiang, Tsao, & Mine, 2014). Enzymatic digestion of E1 increased the content of small peptides as it can be noticed in the electrophoretic profile (Figure 1A), which in turn was reflected in the cellular antioxidant activity (Figure 1C).

The different case occurred with E2, which decreased its cellular antioxidant activity with enzymatic digestion, from 60 to 120 min. E2 hydrolyzed 90 min presented a particular decrease in AOX activity in CAA assays. This hydrolysate showed the most intense legumin band (Figure 1A). Interestingly, previous reports indicate that legumin must be converted into peptides to increase the AOX capacity (Garcia-Mora et al., 2014). AOX capacity increased in the hydrolysates digested for 120 and 180 min.

*3.3 NO Inhibitory Activity*

E1 and E2 exhibited the highest NO inhibitory activity before enzymatic digestion, being 89.5 and 96.1% respectively (Figure 1D). As observed with amaranth, extrusion improved the anti-inflammatory activity tested in macrophages (Montoya-Rodr ǵuez et al., 2014a). After 60 min digestion, only E1 hydrolysates reduced their

NO inhibitory activity to 55.2%. Protein profile of E2 at 60 min of enzymatic digestion was very similar, as well as their anti-inflammatory activities. By 90 min of E2 enzymatic digestion, legumin and phaseolin regained solubility, and the anti-inflammatory activity was reduced. Therefore, peptides generated by the extrusion process (E2 T0) were at higher concentrations and conferring higher activity before legumin regained solubility. Enzymatic digestion of legumin produced bioactive peptides as it can be noticed on the increase of activity at 180 min that did not reach the NO inhibition observed for E2 T0. Rocha et al. (2014) shown the enhanced effect of AOX capacity of germination and the adverse effect of enzymatic digestion over the AOX capacity. The addition of alcalase in the germinated cowpea beans did not increase the AOX capacity, but the hydrolysis with alcalase of regular cowpea bean generated a good AOX capacity. Despite the decreased activity of E1 and E2 when digested, extrusion improved the anti-inflammatory activity of germinated cotyledon previously reported (López-Barrios et al., 2016).

### *3.4 Qualitative Characterization of Hydrolysates by HPLC-MS-TOF*

Protein hydrolysates were analyzed, and the HPLC-MS-TOF profiles are shown in Fig. 2. To approximate the identity of the peptides generated by the extrusion (E1 and E2) of germinated black bean cotyledons, the signal from unextruded total ion chromatogram (TIC) was subtracted from their TIC. In E2 there were more peptide signals than in E1 confirming that extrusion conditions affected the release of peptides. Besides small m/z signals, ions of m/z 705 and 543 were found in both extrudates samples (Table 1). Additionally, un-extruded germinated cotyledon also presented an m/z signal at 705, but the difference in the mass spectrum indicates that it was not the same peptide than the one detected in E1 and E2.

Amino acid sequences from *P. vulgaris* peptides with molecular weight of 704 Da were obtained from databases, but none has been previously reported with biological activity. In contrast, the peptides related to m/z of 689 or 543 were found in Biopep database as *P. vulgaris* peptides with angiotensin converting enzyme (ACE) inhibitor and AOX activity or dipeptidyl peptidase IV (DPP IV) inhibitory activity, respectively (Table 1). The results of the weight of bioactive peptides match with those obtained for Rocha et al., (2014), detecting a variety of peptides from 259.2 to 2010.0 Da.

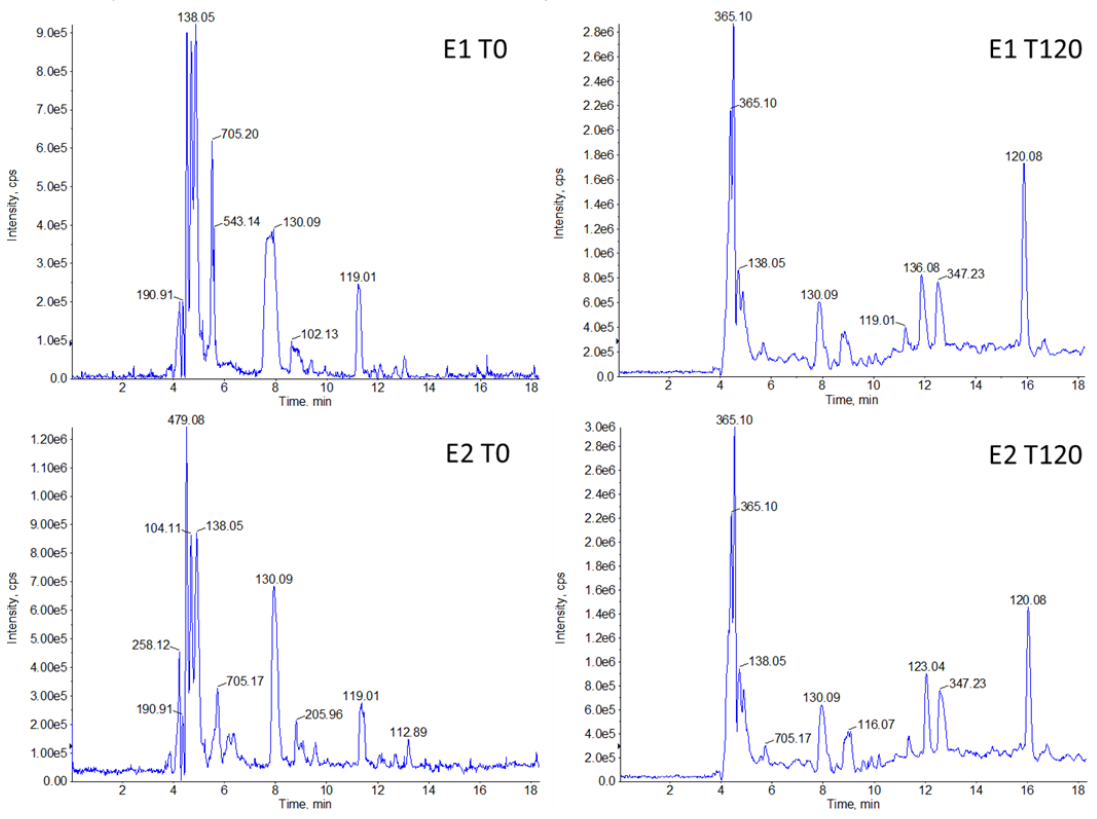



Figure 2. Total ion chromatogram of extrudated germinated cotyledons at 39 ℃ (E1 T0), 145 °C (E2 T0) and their 120 min hydrolysates (E1 T120, E2 T120)

On the other hand, the differences between the TIC of extrudates and their correspondent 120 min hydrolysates demonstrate that extrusion generated different peptides than those obtained after enzymatic digestion (Figure 1). Interestingly, TIC from E1 and E2 digested 120 min were very similar. Even though extrudates presented different peptides profiles as consequence of the extrusion process, the enzymatic digestion of the storage proteins released similar peptides.

TIC of extrudates were subtracted to each of their hydrolysates to approximate the identity of peptides generated by enzymatic digestion. Overall, E1 presented more peptide signals and increased with hydrolysis time (data not shown). Among the identified peptides obtained after 180 min of enzymatic digestion, RLL and YAL have been previously described as part of bioactive peptides (Table 1). YAL belongs to a peptide sequence with ACE inhibitory activity from marine products waste (Wako et al., 1996). RLL was found in two peptides from precooked and digested *P. vulgaris,* with ACE and DPP IV inhibitory activities (Mojica et al., 2015).

Table 1. Peptide sequences identified according the differential m/z signal between un-extruded and extruded germinated cotyledons

| *m/z* | Mass in E1 | Mass in E2 | Mass (calc.) | Sequence | Enzyme / Database | Biological activities | *P. vulgaris* source protein |
|---|---|---|---|---|---|---|---|
| 705 | 704.20 | 704.17 | 703.39 | CEVIIK | Trypsin / NCBInr | NF | Hypothetical protein PHAVU_004G1381001g |
| | | | 705.34 | GGSSDKR | TrypChymo / SwissProt | NF | Wound-induced basic protein |
| | | | 704.42 | FQIGKL | Chymotrypsin / NCBInr | NF | Hypothetical protein PHAVU_011G1771001g |
| | | | 703.28 | PHGCQY | Pepsin / NCBInr | NF | Hypothetical protein PHAVU_002G2817001g |
| 479 | NP | 478.08 | 477.28 | RFR | Trypsin / SwissProt | NF | Chloroplast inner envelope protein |
| | | | 477.17 | SEEN | Trypsin / NCBInr | NF | Hypothetical protein PHAVU_009G2231001g |
| | | | 478.28 | VYVV | Trypsin / NCBInr | NF | Hypothetical protein PHAVU_006G1949001g |
| 689 | NP | 689.19 | 688.77 | RWAEK | BIOPEP | ACE inhibitor and AOX | Hypothetical protein PHAVU_005G180600g |
| | | | 688.35 | LDRGQT | Trypsin / SwissProt | NF | Thaumatin-like protein |
| 543 | 542.14 | 542.14 | 542.62 | WIQP | BIOPEP | DPP IV inhibitor | Hypothetical protein PHAVU_L009200g |
| 365 | 365.1 | 365.1 | 365.19 | YAL | Chymotrypsin, pepsin, Trypsin / SwissProt | Part of ACE inhibitor peptides | 35 kDa cell wall protein |
| 400 | 400.25 | NP | 400.27 | RLL | Pepsin / NCBInr | NF | Hypothetical protein PHAVU_009G0280000g |

E1, extrudate generated at 350 rpm; E2, extrudate generated at 400 rpm; NP, Not present; NF, Not found

ACE inhibitor, angiotensin converting enzyme inhibitory activity (related to hypertension); AOX, antioxidant activity; DPP IV inhibitor, dipeptidyl peptidase IV inhibiting activity (related to antidiabetic activity)

## 4. Conclusions

Extrusion of germinated black bean cotyledons improved the release of bioactive peptides with antioxidant and anti-inflammatory activities. Peptides generated by the extrusion process without enzymatic treatment were at higher concentrations and conferring higher cellular antioxidant activity. Protein aggregates generated by extrusion affected the release of proteins that do not have AOX activity before being digested, this was particularly observed for legumin. Even though extrudates presented different peptides profiles, the enzymatic

digestion of the storage proteins released similar peptides. Extrusion of germinated black bean cotyledons can be a useful process to produce novel functional ingredients from legume proteins for the food industry.

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